

Structural Studies on Photosystem II

by

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Abstract

The main aim of the work presented is to elucidate the structure of photosystem II (PSII). With this in mind, a number of highly purified PSII core preparations were isolated. These preparations were characterised with respect to their subunit and pigment composition, lipid content, oxygen evolution capability, molecular weight, purity and stability. The following were of particular interest:

- (1) Monomeric (240kDa) and dimeric (445kDa) PSII complexes, consisting of the subunits CP47, CP43, D2, D1, cytochrome b559 (cytb559) and the 33kDa extrinsic polypeptide, as well as some low molecular weight components.
- (2) A dimeric complex, as in (1), but lacking the 33kDa subunit (426kDa).
- (3) A dimeric PSII-LHCII (photosystem II-light harvesting complex II) supercomplex (725kDa) consisting of the subunits listed in (1), as well as the polypeptides CP24, CP26, CP29 and LHCII.

Single particle analysis of these complexes yielded electron density images, having a resolution of 25Å. Comparing these images showed the PSII-LHCII supercomplex to consist of a central PSII dimer, flanked by two sets of chlorophyll-a/b-binding proteins (CP24, CP26 and CP29 and two LHCII trimers). The finding that the dimeric PSII core is more stable and active than its monomeric counterpart, suggests that PSII may exist as a dimer *in vivo*, a conclusion also reached from image analysis of the PSII-LHCII supercomplexes. Comparison of the electron density images of dimeric PSII cores before and after the removal of the 33kDa extrinsic polypeptide allowed the localisation of this subunit. Also presented are models of the possible locations of other subunits, based on a variety of published data.

The purity and stability of the 445kDa O₂-evolving dimers makes them useful for crystallisation trials. The conditions used to obtain initial two- and three-dimensional crystals of various PSII complexes are presented and provide a basis for crystallisation trials aimed at obtaining a high resolution structure of PSII.

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Table of Contents

Abstract.....	2
Acknowledgements.....	3
Table of Contents	4
List of Tables.....	11
List of Figures	12
Glossary	16
Chapter 1 Introduction	18
1.1 The importance of PSII structural studies	18
1.2 Aims and thesis outline	18
1.3 Photosynthetic electron transport.....	19
1.4 The structure, function and location of the subunits associated with PSII	24
1.4.1 The structure of the D1 subunit.....	27
1.4.2 The structure of D2	32
1.4.3 The structure of cytochrome b559	35
1.4.4 The structure of CP47	39
1.4.5 The structure of CP43	44
1.4.6 The structure of the 33kDa subunit.....	47
1.4.7 The 23kDa extrinsic subunit encoded by the <i>psbP</i> gene	49
1.4.8 The 16kDa extrinsic subunit encoded by <i>psbQ</i>	50
1.4.9 The 22kDa intrinsic polypeptide encoded by <i>psbS</i>	50
1.4.10 The <i>psbH</i> gene product.....	51
1.4.11 The <i>psbI</i> gene product.....	51
1.4.12 The <i>psbJ</i> gene product.....	52
1.4.13 The <i>psbK</i> gene product.....	52
1.4.14 The <i>psbL</i> gene product	53
1.4.15 The <i>psbM</i> gene product	53
1.4.16 The <i>psbN</i> gene product.....	54
1.4.17 The 4.1kDa polypeptide.....	54
1.4.18 The 5kDa extrinsic polypeptide.....	54
1.4.19 The 6.1kDa polypeptide.....	55
1.5 The oligomeric nature of PSII.....	55
1.6 Organisation of the major PSII and chlorophyll a/b binding proteins composing the PSII-LHCII supercomplex.....	57
1.7 Summary.....	61
Chapter 2 Methods	63
2.1 PSII particle isolation.....	63

2.1.1 Isolation of sub-chloroplast PSII enriched membranes (BBYs)	63
2.1.2 Isolation of oxygen-evolving PSII cores from peas	63
2.1.3 Isolation of PSII reaction centres from peas	64
2.1.4 Isolation of the CP47-D1-D2-cytb559 complex and CP43 from peas	64
2.1.5 Isolation of oxygen-evolving cores from spinach (<i>Spinacea oleracea</i>) leaves using HTGP or OG	66
2.1.6 Isolation of monomeric and dimeric oxygen-evolving PSII cores from spinach	67
2.1.7 Isolation of dimeric PSII particles lacking the 33kDa extrinsic polypeptide	67
2.1.8 The isolation of a dimeric PSII/LHCII complex from spinach	67
2.2 Subunit analysis	68
2.2.1 Preparing gels for SDS polyacrylamide gel electrophoresis	68
2.2.2 Sample solubilisation and loading	69
2.2.3 Gel running conditions	70
2.2.4 Staining gels with Coomassie R250	70
2.2.5 Silver Staining	70
2.3 Western blotting	71
2.3.1 Transfer and detection of SDS-PAGE polypeptides	71
2.3.2 Primary antisera	72
2.4 Oxygen evolution	72
2.5 HPLC size exclusion analysis	73
2.6 Pigment analysis	74
2.7 Stability trials	75
2.7.1 The thermostability of oxygen-evolving PSII cores in the presence of 0.3 and 2.4M sucrose	75
2.7.2 Method of determining the effect of freezing PSII cores, on D1 and D2 degradation	75
2.8 Image enhanced single particle analysis	76
2.9 Two-dimensional crystallisation	78
2.9.1 Method of studying dimyristoyl phosphatidyl choline:PSII core bilayer formation	78
2.9.2 Crude estimation of the amount of thylakoid lipid associated with various PSII core preparations	79
2.10 Three-dimensional crystallisation trials	80
2.10.1 Preparation of concentrated protein stock solutions	80
2.10.2 Setting up crystallisation trials	81

Chapter 3 The characterisation of oxygen-evolving PSII cores isolated from BBYs

using HTGP	85
3.1 Introduction.....	85
3.1.1 Chloroplast and thylakoid isolation	85
3.1.2 The purification of BBYs from thylakoids	86
3.1.3 Isolation of detergent solubilised PSII core complexes from BBYs.....	86
3.1.4 Characterisation of the purity, activity and stability of isolated PSII core preparations.....	87
3.2 Results and discussion	96
3.2.1 SDS/gel electrophoresis.....	96
3.2.1.1 Identification of the subunits associated with HTGP cores.....	96
3.2.1.2 Determining the variation of subunit composition between triplicate thylakoid, BBY and HTGP core preparations	98
3.2.2 Western blot analysis of thylakoids, BBYs and isolated core particles with antibodies raised against CP47, CP43, D2, D1 and the 33kDa extrinsic subunit.	99
3.2.3 Pigment analysis.....	106
3.2.3.1 Spectra of isolated thylakoids, BBYs, HTGP cores and the LHCII fraction	106
3.2.3.2 Triplicate spectra.....	108
3.2.3.3 Determining the number of chlorophyll a, chlorophyll b and β -carotene molecules associated with each of the triplicate HTGP core complexes.	109
3.2.4 Oxygen evolution rates driven by triplicate core preparations.....	110
3.2.5 HPLC size exclusion analysis of HTGP core preparations	112
3.2.6 Lipid analysis.....	114
3.3 Conclusion	114

Chapter 4.0 The characterisation of an oxygen-evolving PSII core preparation isolated

using octyl glucoside	115
4.1 Introduction.....	115
4.2 Results and discussion	116
4.2.1 Subunit composition.....	116
4.2.1.1 Determining subunit composition of triplicate OG core preparations.	116
4.2.2 Western blot analysis of OG cores.....	118
4.2.2.1 Western blot analysis of isolated OG cores using antibodies raised against CP47, CP43, D2 D1 and the 33kDa extrinsic subunit	118

4.2.2.2 D1 and D2 specific Western blot analysis of freshly prepared OG cores	121
4.2.3 Oxygen evolution rates driven by triplicate OG core preparations	122
4.2.4 Pigment analysis of OG cores	124
4.2.4.1 Triplicate OG core spectra	124
4.2.4.2 Comparing the average HTGP and OG core spectra	125
4.2.4.3 Determining the number of chlorophyll a, chlorophyll b and β -carotene molecules associated with each of the OG core preparations 1,2 and 3.	126
4.2.5 HPLC size exclusion analysis of the OG core preparation.....	128
4.2.6 Lipid analysis.....	129
4.3 Conclusions.....	129
Chapter 5 The use of sucrose gradient centrifugation to purify discrete monomeric and dimeric PSII complexes.....	130
5.1 Introduction.....	130
5.2 Results and discussion	131
5.2.1 Experiment to optimise the detergent concentration used to resolve oligomeric core complexes from OG core preparations	131
5.2.2 The subunit composition of the chlorophyll binding fractions resolved from OG cores by sucrose density gradient centrifugation.....	133
5.2.3 Molecular weight determination of the three sucrose gradient purified chlorophyll binding fractions.	135
5.2.4 Comparison of the spectra of OG cores, sucrose gradient purified PSII core monomers and dimers.....	138
5.2.5 Determining the number of chlorophyll a, chlorophyll b and β -carotene molecules associated with each of the triplicate PSII core complexes.....	142
5.2.6 Oxygen evolution rates driven by sucrose gradient purified PSII monomers and dimers	143
5.2.7 Study of the PSII subunits breakdown fragments associated with monomeric and dimeric sucrose gradient purified PSII core complexes	145
5.2.7.1 Western blot analysis of sucrose gradient purified PSII monomers and dimers using antibodies raised against CP47, CP43, D2, D1 and the 33kDa extrinsic subunit.....	145
5.2.7.2 Comparison of the levels of D1 and D2 subunit breakdown fragments associated with freshly prepared and frozen OG core, PSII monomer and PSII dimer preparations	150

5.3 The isolation of a dimeric PSII core complex lacking the 33kDa extrinsic polypeptide.....	153
5.3.1 Subunit composition of Tris-washed dimers of PSII.....	153
5.3.2 HPLC size exclusion analysis to compare the molecular weights of isolated PSII monomers and dimers (CP47, CP43, D2, D1, cytb559 and 33kDa extrinsic polypeptide) with Tris-washed dimers (CP47, CP43, D2, D1 and cytb559).....	155
5.4 Isolation and characterisation of dimeric PSII-LHCII dimers from BBYs.....	158
5.4.1 Isolation of dimeric PSII-LHCII dimers from BBYs	158
5.4.2 Subunit composition of chlorophyll binding complexes resolved by sucrose density gradient centrifugation after solubilising BBYs with 30 mM DM	159
5.4.3 HPLC size exclusion analysis of Fraction 4.....	161
5.4.3 Pigment analysis of Fraction 4.....	163
5.4.3.1 Spectral analysis of Fraction 4.....	163
5.4.3.2 Analysis of the pigment composition of Fraction 4 using reverse phase HPLC analysis	164
5.4 Conclusion.....	165
Chapter 6 Single particle analysis of dimeric PSII complexes differing in subunit composition.....	167
6.1 Introduction.....	167
6.2 Results.....	168
6.2.1 Electron micrographs of the spinach and <i>Synechococcus</i> particles analysed in this chapter.....	168
6.2.2 Image Enhancement analysis of sucrose density gradient purified core monomers and dimers	170
6.2.3 Classification of side-views of dimeric <i>Synechococcus</i> (CP47, CP43, D2, D1, cytb559 and 33kDa extrinsic polypeptide) complexes	172
6.2.4 Location of the 33kDa subunits in dimeric PSII core complexes	173
6.2.5 Comparison of PSII cores images with those obtained from the particles identified in PSII-LHCII complex preparations	175
6.3 Discussion.....	177
6.3.1 The dimeric nature of isolated PSII core complexes.....	177
6.3.2 Structure of the PSII-LHCII supercomplex	177
6.3.3 Location of the 33kDa subunit.....	185
6.3.4 A model of the subunit composition of the PSII-LHCII supercomplex.	186
6.4 Conclusion	190

Chapter 7 Initial two-dimensional crystallisation trials of PSII	191
7.1 Introduction to two-dimensional membrane protein crystallisation.....	191
7.1.1 Two-dimensional crystallisation of detergent solubilised membrane proteins	192
7.1.2 Protein.....	194
7.1.2.1 Protein purity	194
7.1.2.2 Protein concentration	194
7.1.2.3 Electrostatic interactions of proteins	195
7.1.3 Lipids	196
7.1.3.1 Lipid head groups.....	198
7.1.3.2 The hydrophobic tail	199
7.1.3.3 Thylakoid lipid composition.....	201
7.1.3.3.1 MGDG and DGDG	203
7.1.3.3.2 Sulphoquinovosyldiacylglycerol	204
7.1.3.3.3 Phosphatidyl glycerol.....	204
7.1.3.3.4 Phosphatidyl choline	204
7.1.4 Detergents.....	205
7.1.5 Ionic strength and pH	205
7.2 Results	206
7.2.1 The effect of lipid:protein ratio on the formation of pea HTGP core/DMPC sheets during initial two-dimensional crystallisation trials	206
7.2.2 Stability trials.....	211
7.2.3 Crude estimation of the amount of thylakoid lipid still associated with spinach HTGP and OG core preparations	216
7.3 Discussion	221
7.4 Conclusion	224
Chapter 8 Three-dimensional crystallisation trials of PSII	225
8.1 Introduction.....	225
8.1.1 General introduction	225
8.1.2 Membrane Protein solubility	226
8.1.3 Attracting Forces	227
8.1.4 Solubilising forces	227
8.2 Results and Discussion.....	228
8.2.1 Initial crystallisation screens of a number of different PSII particle preparations.	228
8.2.2 Crystallisation trials of oxygen-evolving core complexes using PEG4000	230
8.2.3 Identification of alternative crystallisation conditions	233

8.3 Conclusion 234

Chapter 9 Final conclusion and future work..... 235

Chapter 10 References..... 238

List of Tables

Table 1.1 The subunits of PSII	25,26
Table 1.2 A conversion table relating the nomenclature of Janssen, (1994), Green <i>et al.</i> , (1991), Peter & Thornber, (1991b), Bassi <i>et al.</i> , (1990) and Harrison & Melis, (1992)	59
Table 2.1 The composition of solutions used to prepare two types of 10-17% linear gradient resolving gel	68
Table 2.2 The composition of two types of stacking gel	69
Table 2.3 Table showing sample loading for gels which were Coomassie stained and Western blotted	70
Table 2.4 Procedure used to silver stain PSII polypeptides resolved by SDS-PAGE	71
Table 2.5 Conditions used to measure the oxygen evolution rates of thylakoids, BBYs and PSII cores	73
Table 2.6 Experimental design to test the effect of temperature and sucrose concentration on PSII core stability	75
Table 2.7 Table of crystallisation stocks used to screen PSII core complexes for possible crystallisation conditions	83
Table 3.1 Characteristics of published PSII core preparations	89,90
Table 3.2 The pigment composition of the chlorophyll a/b binding proteins associated with PSII	92
Table 3.3 Estimating the theoretical molecular weight of a PSII core (CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic polypeptide) from its polypeptide, chlorophyll a and pheophytin composition.	93
Table 3.4 The pigment composition of three HTGP core preparations	109
Table 3.5 Oxygen evolution rates of thylakoids, BBYs and HTGP cores using DCBQ and DMBQ as electron acceptors	110
Table 4.1 Oxygen evolution rates of three OG core preparations using DCBQ and DMBQ as electron acceptors	122
Table 4.2 The pigment composition of three OG core preparations	126
Table 5.1 The pigment stoichiometry of sucrose gradient purified PSII monomer and dimer preparations containing CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic polypeptide.	142
Table 5.2 Oxygen evolution rates of isolated PSII monomers and dimers, in the presence of DCBQ and DMBQ as electron acceptors	143
Table 5.3 The pigment composition of the PSII-LHCII complex (Fraction 4, Figure 5.18)	164
Table 6.1 A review of the ionic strength contributions of the ions present in the buffers used to solubilise PSII from the thylakoid membrane	185
Table 7.1 The structure and charge properties of some common polar lipids	199
Table 7.2 The effect of chain length and hydrocarbon chain saturation on the crystal/liquid-crystalline phase transition of some phospholipid bilayers at pH7 and an ionic strength of 0.1.	200
Table 7.3 Thylakoid polar lipid head group structure and properties	202
Table 7.4 Fatty acid composition of polar thylakoid lipid classes of broad bean (<i>Vicia faba</i>) thylakoid membranes	203

List of Figures

Figure 1.1 Photosynthetic electron transport and ATP synthesis.	20
Figure 1.2 The subunits associated with PSII	21
Figure 1.3 The association of the redox components with the PSII reaction centre.	21
Figure 1.4 Electron transfer in PSII.	22
Figure 1.5 Amino acid sequence of the L subunits of the purple bacterial reaction centre from <i>Rhodospseudomonas viridis</i> and <i>Rhodobacter sphaeroides</i> .	27
Figure 1.6 Folding model of D1.	29
Figure 1.7 Homology between the M subunit amino acid sequence of <i>Rhodospseudomonas viridis</i> (Mv) and <i>Rhodobacter sphaeroides</i> (Ms) and that of spinach D2.	32
Figure 1.8 Folding model of the D2 subunit.	34
Figure 1.9 Aligned amino acid sequences of the α and β subunits of cytb559.	36
Figure 1.10 Model of cytb559 structure.	37
Figure 1.11 Donor and acceptor side photoinhibition diagram.	38
Figure 1.12 The cytb559 driven electron transport scheme proposed by Barber & De Las Rivas, (1993).	39
Figure 1.13 Folding model of spinach CP47	41
Figure 1.14 Folding model of spinach CP43.	46
Figure 1.15 Alignment of the mature 33kDa subunit (<i>psbO</i>) protein sequences.	48
Figure 1.16 Models depicting the relative organisation of subunits within an PSII-LHCII supercomplex.	60
Figure 2.1 The automated Micro-Batch Protein Crystallisation System (Chayen <i>et al.</i> , 1990).	81
Figure 2.2 The four bore tip from which the stock solutions are dispensed.	82
Figure 2.3 A Terazaki plate in which wells in lanes 1-4 have been filled with paraffin oil.	82
Figure 3.1 Identification of subunits of SDS-PAGE resolved thylakoid, BBY, PSII core and LHCII samples.	97
Figure 3.2 Variation in subunit composition between triplicate PSII preparations at the thylakoid, BBY and isolated core stage.	98
Figure 3.3 Standard curve of molecular weight markers used to calculate the molecular weights of the bands shown in Figures 3.4-3.6.	101
Figure 3.4 The detection of CP47, CP43, 33kDa extrinsic, D2 and D1 subunit aggregation and breakdown products in thylakoids using Western blot analysis.	102
Figure 3.5 The detection of CP47, CP43, 33kDa extrinsic, D2 and D1 subunit aggregation and breakdown products in BBYs using Western blot analysis.	103

Figure 3.6 The detection of CP47, CP43, 33kDa extrinsic, D2 and D1 subunit aggregation and breakdown products in isolated HTGP cores, using Western blot analysis.	104
Figure 3.7 Spectra of isolated thylakoids, BBYs, HTGP cores and the LHCII fraction spectra.	107
Figure 3.8 Spectra of three HTGP PSII core preparations.	108
Figure 3.9 Standard curve for HPLC size exclusion analysis	112
Figure 3.10 Elution profile of two HTGP core preparations.	113
Figure 4.1 A comparison of the subunit composition of a HTGP core sample with three OG core preparations.	116
Figure 4.2 The detection of CP47, CP43, 33kDa extrinsic, D2 and D1 aggregation and breakdown products in isolated OG cores using Western blot analysis.	118
Figure 4.3 D1 and D2 specific Western blots of a freshly prepared OG core preparation.	121
Figure 4.4 Spectral analysis of triplicate OG cores	124
Figure 4.5 Comparison of average OG and HTGP core spectra.	125
Figure 4.6 Elution profile of two OG core preparations subjected to HPLC size exclusion analysis.	128
Figure 5.1 The effect of detergent concentration on the separation of three chlorophyll binding complexes from OG cores using sucrose density centrifugation.	132
Figure 5.2 The subunit composition of the three chlorophyll binding fractions resolved by sucrose density gradient centrifugation.	134
Figure 5.3 Molecular weight calibration curve.	135
Figure 5.4 HPLC elution profile of the two sucrose gradient purified core fractions.	137
Figure 5.5 HPLC size exclusion analysis of the upper sucrose gradient fraction	137
Figure 5.6 Spectral comparison of two sucrose gradient purified PSII monomer preparations.	138
Figure 5.7 Spectral comparison of three sucrose gradient purified PSII dimer preparations.	139
Figure 5.8 Comparison of average OG core, monomer and dimer spectra.	140
Figure 5.9 Comparison of OG core and upper sucrose gradient fraction spectra.	141
Figure 5.10 The detection of CP47, CP43, D2 and D1 and the 33kDa extrinsic subunit aggregation and breakdown products, in isolated PSII monomer preparations.	146
Figure 5.11 The detection of CP47, CP43, D2 and D1 and the 33kDa extrinsic subunit aggregation and breakdown products, in isolated PSII dimer preparations.	147
Figure 5.12 The effect of freezing on D1 damage of OG cores and sucrose gradient purified monomers and dimers.	150
Figure 5.13 The effect of freezing on D2 damage of OG cores and sucrose gradient purified monomers and dimers.	151
Figure 5.14 SDS-PAGE and 33kDa specific Western blot analysis of PSII dimers before and after washing with 980mM Tris pH8.0, 2mM DM.	154
Figure 5.15 Standard curve for HPLC analysis of Tris-washed and control samples.	155

Figure 5.16 HPLC size exclusion elution profiles of PSII monomers, dimers and Tris-washed PSII complexes.	156
Figure 5.17 Sucrose density gradient centrifugation of DM solubilised BBYs.	158
Figure 5.18 Subunits composition of the chlorophyll binding fractions resolved from BBYs by sucrose density gradient centrifugation (Figure 5.17).	159
Figure 5.19 HPLC size exclusion profile of Fraction 4.	161
Figure 5.20 Spectral comparison of isolated (CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic polypeptide) monomers and dimers with that of Fraction 4.	163
Figure 6.1 Electron micrographs of PSII preparations.	169,170
Figure 6.2 Image Enhancement analysis of sucrose density gradient purified core monomers and dimers.	171
Figure 6.3 A classification of 443 side-view projections of the dimeric <i>Synechococcus</i> PSII core complexes shown in Figure 6.1B.	172
Figure 6.4. Single particle analysis of top and side-view projections of dimeric spinach and <i>Synechococcus</i> PSII complexes before and after Tris-washing.	174
Figure 6.5 Single particle analysis of top- and side-view projections of the complexes detected in Figure 6.1A.	176
Figure 6.6 The position of two LHCII trimers within the PSII-LHCII supercomplex.	179
Figure 6.7 Photoinhibition and repair cycle of Barbato <i>et al.</i> , (1992a).	182
Figure 6.8 Subunit topology of the PSII-LHCII supercomplex.	187
Figure 6.9 Topology of CP47-D1-D2-cytb559 complexes.	188
Figure 7.1 Diagram showing the driving force of bilayer aggregation at high protein concentrations	195
Figure 7.2 The effect of lipid head and tail volume ratio on aggregation.	198
Figure 7.3 The crystal/liquid-crystal transition of bilayer forming lipids	198
Figure 7.4 DMPC vesicles and bilayers containing PSII cores obtained after dialysing for 24h at 27°C and 1h at 37°C.	207
Figures 7.5 DMPC vesicles and bilayers in a sample containing a DMPC:pea HTGP core ratio of 5000:1, after dialysing for 72h at 27°C and 3h at 37°C	208
Figures 7.6 DMPC vesicles and bilayers in a sample containing a DMPC:pea HTGP core ratio of 1000:1, after dialysing for 72h at 27°C and 3h at 37°C.	209
Figures 7.7 DMPC vesicles and bilayers in a sample containing a DMPC:pea HTGP core ratio of 500:1, after dialysing for 72h at 27°C and 3h at 37°C.	210
Figure 7.8 Spectrum of intact PSII core isolated from pea BBYs using HTGP.	211
Figure 7.9 Changes in absorption spectra of pea HTGP cores during an 11 day incubation period, in the presence of 0.3M sucrose at 4, 18 and 27°C.	213

Figure 7.10 Spectra of pea HTGP cores after an 11 day incubation at 27, 18 and 4°C, in the presence of either 0.3 or 2.4M sucrose	214
Figure 7.11 The change in 419:437nm absorption ratio of pea HTGP cores with time, in the presence of 0.3 and 2.4M sucrose	215
Figure 7.12 Electron micrograph of negatively stained HTGP cores	217
Figure 7.13 Electron micrograph of negatively stained detergent depleted HTGP cores	218
Figure 7.14 Electron micrograph of negatively stained OG cores	219
Figure 7.15 Electron micrograph of negatively stained detergent depleted OG cores	220
Figure 8.1 Crystals of CP43	229
Figure 8.2 Spinach cores prepared by HTGP solubilisation	231
Figure 8.3 HTGP core crystals grown in the presence of MgCl ₂	232
Figure 8.4 Aggregates obtained using a method modified from Adir <i>et al.</i> , (1992)	234

Glossary

AA	Amino acids
AMPS	Ammonium persulphate
AP buffer	100mM Tris pH9.5, 100mM NaCl, 50mM MgCl ₂
BBYs	Granal stacks isolated according to Berthold <i>et al.</i> , (1981)
BCIP	5-bromo-4-chloro-3-indoylphosphate p-toluene salt
C-Terminal	Carboxy-terminal
CAB proteins	Chlorophyll a/b binding proteins
Chl	Chlorophyll
CF ₀ /CF ₁	ATP synthase
CP proteins	Chlorophyll proteins
Cytb	Cytochrome b
Cytb _{LP}	A low potential cytochrome b, which is part of the cytb _{6f} complex
Cytb _{HP}	A high potential cytochrome b, which is part of the cytb _{6f} complex
Cytb559	Cytochrome b559
Cytb _{6f}	Cytochrome b _{6f}
DCBQ	2,6-dichloro-p-benzoquinone
DCIP	2,6-dichloro-indophenol
DCMU	3-(3,4-dichloro phenyl)-1,1-dimethyl urea
DEPC	Diethyl pyrocarbonate
DGDG	1,2-diacyl- α -D-galactopyranosyl-(1'-6')-(β -D-galactopyranosyl (1'-3))-sn-glycerol
DM	n-dodecyl- β -D-maltoside
DMBQ	2,5-dimethyl-benzoquinone
DMPC	Dimeristoyl phosphatidyl choline
EDTA	(Ethylene dinitrilo) tetraacetic acid disodium salt
EM	Electron microscope
Ext.	Extrinsic
Fab	Antigen specific (light chain) region of antibody molecule
FPLC	Fast protein liquid chromatography
HPLC	High pressure liquid chromatography
HSB buffer	5mM MgCl ₂ , 15mM NaCl and 2mM Mes pH6.5
HTGP	n-heptyl-thioglucopyranoside
Int.	Intrinsic
LHCII	Light harvesting complex II
MAGIC buffer	20mM Mes pH6.3, 5mM MgCl ₂ , 15mM NaCl and 10% w/v glycerol
Mes	(2-[N-morpholino]ethane-sulphonic acid)
MGDG	1,2-diacyl-(β -D-galactopyranosyl (1'-3))-sn-glycerol
MNCB	25mM Mes pH6.5, 10mM NaCl, 5mM CaCl ₂ and 10mM NaHCO ₃

MNCD	25mM Mes pH6.5, 10mM NaCl, 5mM CaCl ₂ and 2mM DM
MPD	2-methyl-2,4-pentenediol
Mr	Relative molecular mass
N-Terminal	Amino-terminal
NBT	Nitroblue tetrazolium chloride
OEC	Oxygen-evolving complex
OER	Oxygen evolution rate
OG	Octyl-glucoside
PAGE	Polyacrylamide gel electrophoresis
PBQ	p-benzoquinone
PEG 4000	Polyethylene glycol (Mr=4000)
Pheo	Pheophytin
PIPES	(Piperazine-N,N'-bis[2-ethane-sulphonic acid])-disodium salt
PQ	Plastoquinone
PSI	Photosystem I
PSII	Photosystem II
P680	Primary oxidant of PSII
Q _A	Plastoquinone molecule associated with Q _A site.
Q _B	Plastoquinone molecule associated with Q _B site.
RC	Reaction Centre
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SMNCB	0.3M sucrose, 25mM Mes pH6.5, 10mM NaCl, 5mM CaCl ₂ and 10mM NaHCO ₃
SNT buffer	0.2M sucrose, 0.1M NaCl, 50mM tricine pH8.0, 5mM MgCl ₂
TBS	Tris-buffered saline (50mM Tris-HCl pH7.4, 150mM NaCl)
TEMED	NNN'N' Tetramethylethylenediamine
Tris	2-amino-2-hydroxy-methylpropane-1,3-diol
TSB-30 buffer	20mM Mes pH6.5, 10mM MgCl ₂ , 30mM CaCl ₂ , 0.5M mannitol, 0.03% DM
Tween 20	Polyoxyethylene sorbitan monolaurate
UVCD	Ultra-violet circular-dichroism
Y _D	D2-Tyr160
Y _Z	D1-Tyr161

Chapter 1 Introduction

1.1 The importance of PSII structural studies

PSII is a membrane protein complex, found in the thylakoid membranes of cyanobacteria and green plants. It is that part of the photosynthetic apparatus that splits water into its elemental constituents. Oxygen is released into the atmosphere while "hydrogen", in the form of protons and electrons, is used mainly to reduce carbon dioxide to organic molecules. The evolutionary significance of PSII cannot be overstated. It allowed our planet to sustain an oxygenic atmosphere and thus paved the way for development of animal and plant life as we know it today. Moreover, because it used the most plentiful substrates, solar energy and water, it gave rise to the production of biomass on an enormous scale, reflected in both reserves of fossil fuels and by the immense quantity and diversity of life which surrounds us daily. For these reasons alone the studies of PSII structure are justified. But, in addition, the elucidation of the structure of PSII may unlock the blueprint for the design of a synthetic catalyst, able to drive water photolysis. The incorporation of such a catalyst into a solar cell could ultimately provide a way of producing clean fuel (O_2 and H_2), utilising the sun's energy. As combustion of these gases generates water, the substrate of the reaction, this process allows the conversion of light to chemical energy without the production of harmful bi-products. As part of a clean energy strategy, such a cyclical system might help in the reduction of the ever increasing atmospheric CO_2 levels, produced as a result excessive use of fossil fuels.

Studying the structure of PSII is not only valuable in its own right, but as part of a strategy to understand the unique properties of membrane proteins in general. Membrane proteins are a class of proteins of intense interest to both the medical community and pharmaceutical industry. They include hormones and other signal receptors, ion channels and energy transducers all of which are potential drug target sights. Understanding their structures would allow the design of highly specific drugs, which should result in a reduction in side effects seen with less specific compounds. Despite their importance, membrane proteins can often only be purified in small quantities. Photosynthetic proteins differ in this respect. Their abundance makes them relatively easy to isolate in large quantities and several have already yielded high resolution structures. The intrinsic interest and abundance of PSII make it a model, with which two- and three-dimensional membrane protein crystallisation strategies can be developed, thus reducing the requirements for large amounts of rarer proteins in future studies.

1.2 Aims and thesis outline

The ultimate goal of the work described in this thesis was to elucidate the high resolution structure of PSII. This thesis describes the steps taken towards achieving this aim. A number of PSII core preparations were subjected to detailed biochemical analysis, so as to determine whether they were homogeneous, intact, active and therefore suitable for large scale crystallisation trials (Chapters 3-5). Several of the characterised particles were then subjected to electron microscopy, coupled with image enhancement studies, so as to gain structural detail at about 15-25Å resolution (Chapter 6),

before embarking on initial two-dimensional (Chapter 7) and three-dimensional (Chapter 8) crystallisation screens to identify potential conditions for future experiments.

This introduction tries to provide the background for the structural studies presented in later chapters. Section 1.3 describes PSII involvement in the photosynthetic electron transport chain. As PSII is a complex, consisting of many subunits, their structure, function and location within the complex is reviewed in Section 1.4. This information is drawn together in Sections 1.5 and 1.6, by presenting, published models of the organisation of the subunits of PSII as part of a PSII-LHCII supercomplex, and the current controversy over the oligomeric state of PSII *in vivo*.

1.3 Photosynthetic electron transport

Thylakoid membranes of higher plants, algal chloroplasts and cyanobacteria contain all the membrane protein complexes needed for trapping light energy and converting it to chemical energy (ATP and NADPH). The ATP and NADPH formed by this process provide plant cells with the chemical energy and reducing equivalents required for CO₂ fixation. The reactions, which lead to photosynthetic ATP and NADPH synthesis, are carried out by five membrane protein complexes. They are PSII, photosystem I (PSI), light harvesting complex (LHCII), the cytochrome b₆f complex (cytb₆f) and the ATP synthase complex (CF₀/CF₁). Biochemical fractionation, immunolabelling and freeze fracture techniques have been used to examine the distribution of these complexes in the thylakoids. 85% of the PSII complexes are found in the granal stacks, while a similar percentage of PSI particles are located in the stroma regions (Andersson & Anderson, 1980; Staehelin & Arntzen, 1983). ATP synthase is located exclusively in the stroma region (Miller & Staehelin, 1976), whereas the cytb₆f complex is divided equally between the stacked and unstacked regions (Allred & Staehelin, 1985). The trimeric LHCII complexes are mostly found in the stacked regions, but during light-induced State 1-State 2 transitions a sub-fraction of such complexes has been shown to migrate reversibly between grana and stroma regions (Barber, 1982a; Kyle *et al.*, 1983; Larsson *et al.*, 1987).

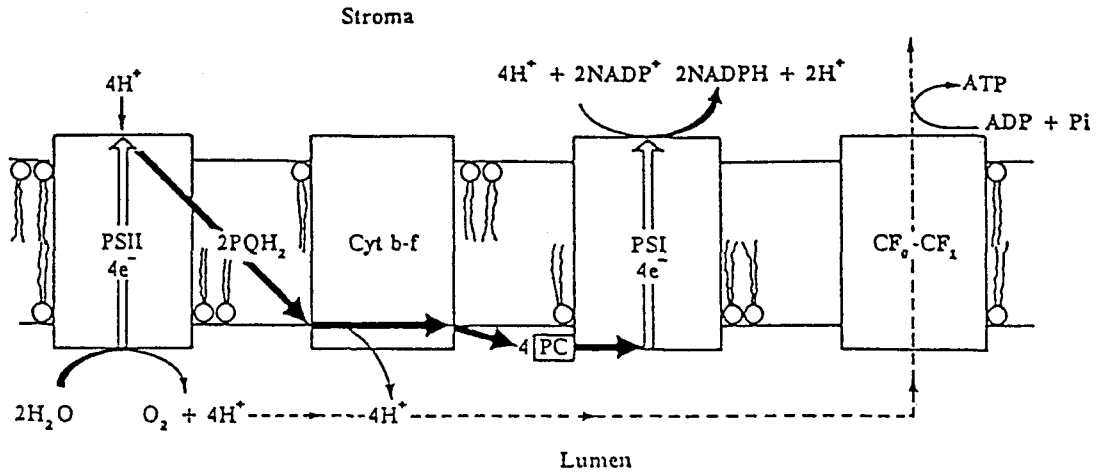
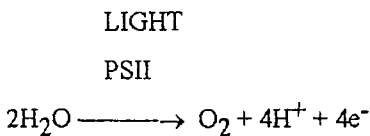


Figure 1.1 Photosynthetic electron transport and ATP synthesis. Figure taken from Barber, (1989).

Figure 1.1 illustrates the reactions driven by each of the above complexes. This section outlines how PSII drives the first stage of the photosynthetic electron chain and the subsequent electron transfer reactions of the cyt_{b_6f} , PSI and the CF_0/CF_1 complexes.



Equation 1.1

Equation 1.1 summarises the reaction catalysed by PSII. Although, PSII is thought to consist of the subunits shown in Figure 1.2, only relatively few of these are required to drive the electron transport reactions which facilitate water photolysis (Figure 1.3).

P680 is thought to consist of two closely associated chlorophyll molecules and is the most oxidising redox component of PSII. Figure 1.4 illustrates how illumination of PSII facilitates water oxidation. On illumination, P680 is converted to its excited state, P680* (Figure 1.4, Step 1). P680* contains a single high energy unpaired electron, which is donated to pheophytin (Pheo) (Figure 1.4, Step 2), thus forming the radical pair, $\text{P680}^+ \text{Pheo}^-$.

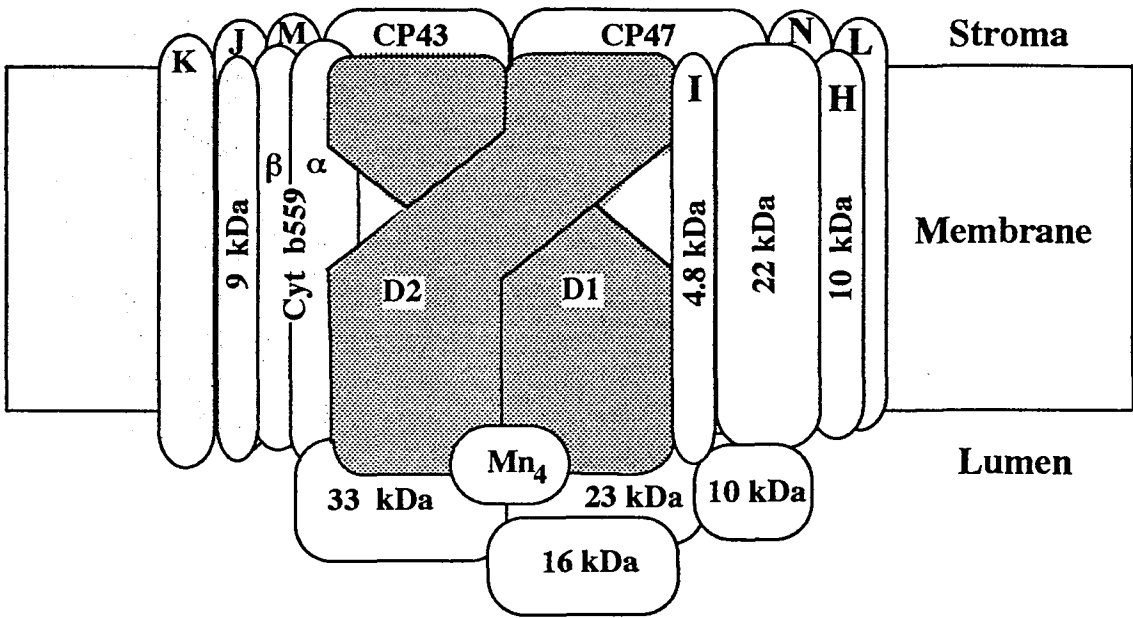


Figure 1.2 The subunits associated with PSII

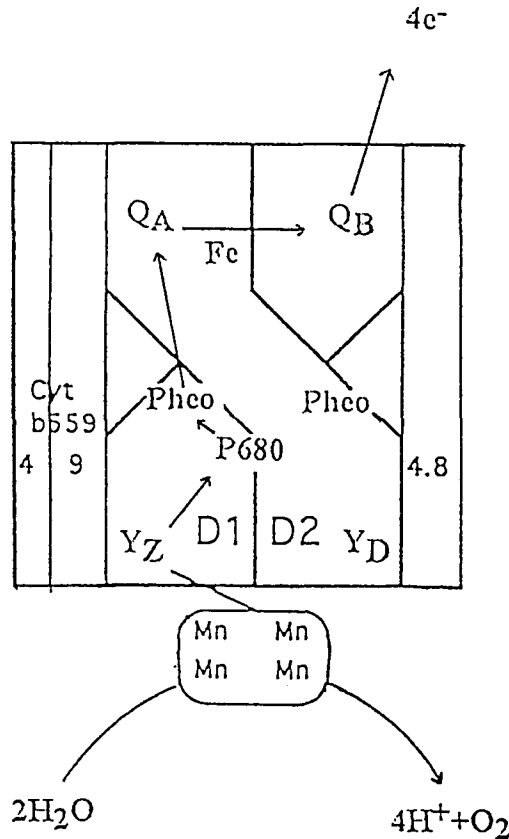


Figure 1.3 The association of the redox components with the PSII reaction centre. The D1 and D2 polypeptides bind all the components directly involved in electron transport. The arrows indicate the electron transport pathway from water via the Mn cluster, Y_Z (D1-Tyr161), P680, Pheo, Q_A and Q_B . The substrate water, binds to a Mn cluster, attached to the luminal surface of PSII.

Each time $P680^+$ is formed, it accepts an electron from Tyr161 of D1 (Z in Figure 1.4), and so forms P680 (Figure 1.4, Step 3). Illumination of PSII allows the $P680$, $P680^*$, $P680^+$ cycle to be repeated thereby enabling the sequential extraction of electrons from Tyr161 (Z in Figure 1.4). Each time Tyr161 donates an electron to $P680^+$, it accepts another from water via the Mn cluster, depicted in Figure 1.4 (Steps 4-9). Joliot *et al.*, (1969) and Kok *et al.*, (1970) showed that short light flashes induced a single turnover of P680. By subjecting PSII to sequential light flashes of this type, the Mn cluster was shown to pass through a series of oxidation states referred to as S_0 - S_4 (Figure 1.4, Steps 5-9). The half-lifetimes for these S-state transitions have been calculated using UV measurements (Dekker *et al.*, 1984; Renger & Weiss, 1986; Saygin & Witt, 1987). Their transition rates are as follows: S_0 to S_1 , 30-50 μ s; S_1 to S_2 , 40-110 μ s; S_2 to S_3 , 100-350 μ s; S_3 to S_4 to S_0 , 1.0-1.5ms. The kinetic parameters for Y_Z^+ reduction are: S_0 to S_1 , $\approx$ 50 μ s; S_1 to S_2 , $\approx$ 50 μ s; S_2 to S_3 , 400-600 μ s; S_3 to S_4 to S_0 , 1-1.3ms (Boska & Sauer, 1984; Cole & Sauer, 1987). Thus, it was concluded that Y_Z^+ accepts electrons directly from the Mn cluster. Babcock (1987) suggested that the S_3 - S_4 - S_0 transition is the rate limiting step of this cycle. This was expanded on by Plijter *et al.*, (1988) who suggested that O_2 release was the overall rate limiting step of the S-state cycle.

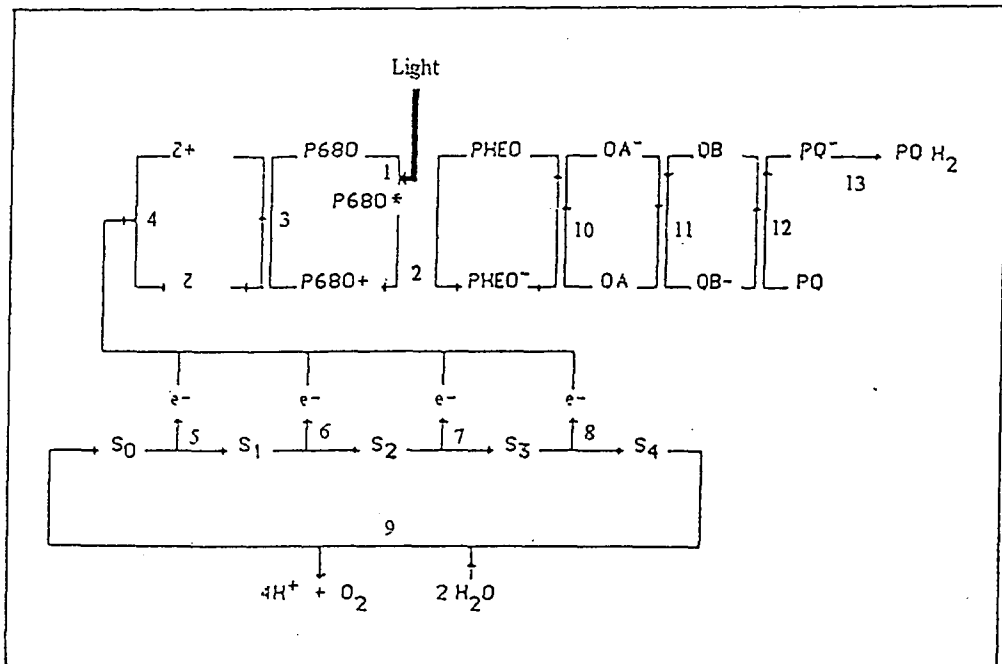


Figure 1.4 Electron transfer in PSII. The numbered electron transport steps are described in the text.

After the extraction of four electrons from water by this process, an oxygen molecule is released and two water molecules are thought to bind to the Mn cluster. Thus, the Mn cluster appears to act as a charge storage device during the water oxidation process. The electrons, which are accepted by $P680^+$, are passed down the electron transport chain, depicted by reaction steps 2, 10, 11, 12 and 13

in Figure 1.4. In this way, pheophytin accepts electrons from P680 (Figure 1.4, Step 2) and passes them on to a plastoquinone molecule (Q_A), tightly associated with the Q_A site (Figure 1.4, Step 10). Q_A^- passes its electron on to a second plastoquinone molecule (PQ), associated with the Q_B site of PSII (Figure 1.4, Step 11). Each plastoquinone associated with the Q_B site is able to accept two electrons derived from water and two protons from the stroma before being released into the lipid matrix as PQH_2 (Figure 1.4, Step 13).

The non-polar nature of PQH_2 allows it to migrate to the luminal side of the membrane where it binds to the $cytb_6f$ complex (Figure 1.1). This complex acts as a plastoquinol-plastocyanin oxidoreductase and drives the reaction summarised in Equation 1.2 (reviewed in Hauska, 1986). $Cytb_6f$ accepts two electrons from each PQH_2 molecule, releasing two protons into the lumen and PQ back into the lipid matrix. The electrons accepted from PQH_2 are passed onto plastocyanin (PC) (Equation 1.2). Figure 1.1 shows the release of $4H^+$ from the $cytb_6f$ complex, as four electrons were released for every two water molecules oxidised according to Equation 1.1.



Having accepted an electron, PC^- is released from the $cytb_6f$ complex, and is then able to act as an electron donor to PSI (Figure 1.1). Using light energy, PSI accepts electrons from PC to drive the NADPH synthesis reaction described in Equation 1.3.

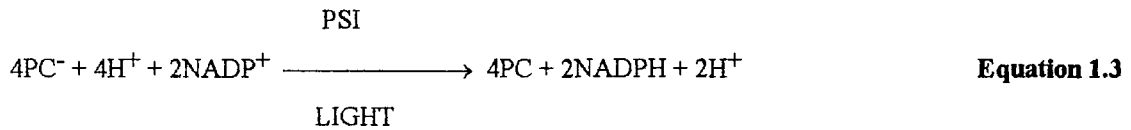


Figure 1.1 suggests that, for every molecule of oxygen released by PSII, the PSII- $cytb_6f$ -PSI electron transport chain releases $8H^+$ into the luminal space (Figure 1.1). However, this value may be higher if as suggested by Mitchell, (1976) the Q-cycle is in operation. The Q-cycle is a mechanism, by which the oxidation of PQH_2 involves electron donation to PC (as indicated above) via the low potential of the $cytb_6f$ complex. The reduced $cytb_{LP}$ passes the electron to a high potential $cytb$ ($cytb_{HP}$) and then to a PQ molecule. As a result, two turnovers of the Q-cycle yield a PQH_2 molecule. The resultant proton gradient is utilised by the CF_0/CF_1 complex to drive ATP synthesis (Figure 1.1).

1.4 The structure, function and location of the subunits associated with PSII

A large number of subunits are associated with PSII (Figure 1.2 and Table 1.1). The literature regarding the structure, function and location of each of these is reviewed in this section with special emphasis placed on the properties of CP47, CP43, D2, D1, the α and β subunits of cytb559 and the 33kDa extrinsic polypeptide, which together constitutes the "core" of the complex.

These seven polypeptides are present in all PSII core particles, capable of driving high rates of oxygen evolution *in vitro*, and must be present in any PSII complexes subjected to crystallisation trials, if the structural arrangements of the components driving the water splitting reaction are to be elucidated.

Table 1.1 The subunits of PSII

Gene	Gene product identified in higher plants	Protein	Number of AA before/ after processing	Actual (apparent) Mr of mature polypeptide (kDa)	Intrinsic/extrinsic protein (Number of predicted transmembrane helices)	Phospho-related	Function
<i>psbA</i> (C)	+	D1	353/343	38.0 (30-34)	Intrinsic (5)	+	RC: binds Y ₇ , P680, Pheo, Q _B , Mn
<i>psbB</i> (C)	+	CP47 (CPa-1)	508	55.6 (47)	Intrinsic (6-7)	?	Inner antenna: binds chl a
<i>psbC</i> (C)	+	CP43 (CPa-2)	461/459	50.1 (43)	Intrinsic (6-7)	+	Inner antenna: binds chl a
<i>psbD</i> (C)	+	D2	353/352	39.4 (30-34)	Intrinsic (5)	+	RC binds Y _D , P680, Q _A
<i>psbE</i> (C)	+	α-subunit cytb559	83/82	9.3 (9)	Intrinsic (1)	?	RC:haem-binding, side path electron transport donor
<i>psbF</i> (C)	+	β-subunit cytb559	39/38	4.4 (4)	Intrinsic (1)	?	RC:haem-binding, side path electron transport donor
<i>psbH</i> (C)	+	8-10kDa phospho-protein	73/72	7.6 (9)	Intrinsic (1)	+	Protection against high light
<i>psbI</i> (C)	+	-	36/36	4.2 (5)	Intrinsic (1)	?	RC unknown function
<i>psbJ</i> (C)	-	-	?	?	?	?	?
<i>psbK</i> (C)	+	-	61(3*)/37	4.3 (4)	Intrinsic (1)	?	Regulatory
<i>psbL</i> (C)	+	-	38/37	4.4 (5)	Intrinsic (1)	?	Acceptor side function
<i>psbM</i> (C)	?	-	?	?	?	?	?
<i>psbN</i> (C)	?	-	?	?	?	?	?
<i>psbO</i> (N)	+	33kDa extrinsic (MSP, OEE1)	331/247	26.5 (33)	extrinsic	?	Donor side function: protects and stabilises Mn, promotes Cl ⁻ /Ca ²⁺ binding

<i>psbP</i> (N)	+	23kDa extrinsic (OEE2)	267/186	20.2 (23)	extrinsic	?	Donor side function promotes Cl ⁻ /Ca ²⁺ binding
<i>psbQ</i> (N)	+	17kDa extrinsic (OEE3)	232/149	16.5 (17)	extrinsic	?	Donor side function promotes Cl ⁻ /Ca ²⁺ binding
<i>psbR</i> (N)	+	10kDa intrinsic	140/99	10.8 (10)	intrinsic	?	Interacts with <i>psbO</i> , <i>PsbP</i> (?) Acceptor side function (?)
<i>psbS</i> (?)	+	22 kDa intrinsic	274/205	?(22)	intrinsic (4)	?	Interacts with <i>psbO</i> , <i>PsbP</i> (?) regulating Q _A -Fe ²⁺ -Q _B (?)
not identified (N)	+(1*)	7/6.1 kDa protein (4*)	?	?(7-6.1)	?	?	?
not identified (N)	+(2*)	5kDa extrinsic (5*)	?	?(5)	extrinsic	?	?
not identified (N)	+(1*)	4.1/3.2kDa (6*)	?	?(4)	?	?	?

References for Table 1.1:

Genes were designated according to the nomenclature revision by Hallick, (1989).

The protein size data was taken from Nixon *et al.*, (1992a). Nuclear (N) or chloroplast (C) gene locations are indicated according to Erickson & Rochaix, (1992).

1* Schröder *et al.*, (1988); Ikeuchi *et al.*, (1989a); also identified in *Chlamydomonas reinhardtii* (de Vitry *et al.*, 1991).

2* Ljungberg *et al.*, (1986b); Ljungberg, (1987); Murata *et al.*, (1987); Ikeuchi *et al.*, (1989a).

3* *psbK* precursor length based on comparison with rice sequence (Hiratsuka *et al.*, 1989).

4* N-terminal sequence data reported from spinach and wheat (Schröder *et al.*, 1988; Ikeuchi *et al.*, 1989a).

5* N-terminal sequence data reported from spinach and wheat (Murata *et al.*, 1987; Ikeuchi *et al.*, 1989a).

6* N-terminal sequence data reported from spinach and wheat (Ikeuchi *et al.*, 1989a; Ikeuchi *et al.*, 1989b).

1.4.1 The structure of the D1 subunit

The D1 polypeptide is found in the reaction centre of PSII and is closely associated with D2, the α and β subunits of cytb559 and the *psbI* gene product (Nanba & Satoh, 1987; Barber *et al.*, 1987). The reaction centre of PSII almost certainly will show a structural as well as a functional homology to the purple bacteria reaction centres of *Rhodospseudomonas viridis* and *Rhodobacter sphaeroides* (Deisenhofer *et al.*, 1984; Deisenhofer *et al.*, 1985; Michel *et al.*, 1986; Allen *et al.*, 1987). Both the purple bacteria and PSII reaction centres drive light induced electron transport reactions. However, whereas PSII is able to extract electrons from water, purple bacteria cannot. Amino acid sequence homology studies suggest that the two photosystems have many similarities in the way that they arrange the redox active components involved in electron transport, but differ considerably on the donor side of PSII.

This section utilises the similarity between the primary structures of the D1 subunit and the L subunits of *Rhodospseudomonas viridis* and *Rhodobacter sphaeroides* (Figure 1.5) to produce the folding model shown in Figure 1.6.

		I			
Lv	ALLSFER-KYRVRGGTLIGGDL-FDFWV--GPYFVGFFGVSAIFFIFLGVSLIGYAASQ		55		
Ls	ALLSFER-KYRVPGGTLVGGNL-FDFWV--GPYFVGFFGVATFFFAALGIILIAWSAVL		55		
D1	TAILERRESESLWGRF-CNWTSTENRLYI-GWFGVLMIPTLLTATSVFIIAFIAAPPVDI		60		
		II	Bph Bph		
Lv	GPTWDP-----FAISINPPDLKYGL-GAAP-----LLEGGFWQAITVCALGAFISWMLREVEISRKL		111		
Ls	OQTWNP-----QLISVYPPALEYGL-GGAP-----LAKGGLWQIITICATGAFVSWALREVEICRKL		111		
D1	DGI-REPVSQS-LLYGNNIISGAIPTSAAGLHFYPIWEA-ASVDEWLYNGGPYELIVLHFLLCVACVYMGREWELSFRL		137		
	III	Yz	Bch	IV ^P	Fe, Q _B
Lv	GIGWHVPLAFCVPIPFHFCVLOVFRPLLLGSWGHAFPYGILSHLDWVNNGFYQYLNWHYNFGHMSSVSFLFVNAMALGLHG				191
Ls	GIGYHIPFAFAFAILAYLTLVLFPRVMMGAWGYAFPYGIWTHLDWVSNTGYTYGNFHYNPAHMAISFFFTNALALALHG				191
D1	GMRPWIAVAYSAPVAAATAVFLIYPICQGSFSDGMPLGISGTFNFHIVFQAEH-NILMIIPFRLMGVAGVFGGSLFSAMIIG				216
		Q _B	Q _B	V	Fe
Lv	GLILSVANPGDG-----DKVKTAEH-----ENQYFRD--VVGYS-IGALSIIHRLGLFLASNIFLTGAFTIASG				252
Ls	ALVLSAANPEKG-----KEMRTPDH-----EDTFFRD--LVGYS-IGTLGIHRLGLLLSLASVFFSALCMIITG				252
D1	SLVrSSLIRETTENESA--NEGVRFGQEEETYNIVAAGHYFGRILIFOYASFNNRSRLHFFLAAMPVVGWIFLTALGISTMA				294
Lv	PFWTRGWPEWNGWMLDIPFWS				273
Ls	TIWFDQWVDWQWVKLPWVANIPGGING				281
D1	FNLNGFNFN-QSVVDSQGRVINTWADIINRANLGMEV-MHE-RNAHNFPLDLA				344

Figure 1.5 Amino acid sequence of the L subunits of the purple bacterial reaction centre from *Rhodospseudomonas viridis* and *Rhodobacter sphaeroides*. Figure taken from Satoh, (1993). The regions labelled I-V represent the positions of the predicted transmembrane helices. Liganding positions of cofactors are marked as follows: Bph = bacteriopheophytin, Yz = Tyr161 of D1, Bch = bacteriochlorophyll, p = primary reactant (eg. P680 in higher plants), Fe = non-haem iron.

Comparison of 43 higher plant and cyanobacterial amino acid sequences of the D1 protein, shows this subunit to be highly conserved (reviewed in Svensson *et al.*, 1991; Satoh, 1993). Furthermore, the L subunits of *Rhodospseudomonas viridis* and *Rhodobacter sphaeroides* and the D1 subunit of PSII show a homology of about 15% (reviewed in Barber, 1987a; Michel & Deisenhofer, 1988; Satoh, 1993)(Figure 1.6). Although this is a relatively low degree of homology, the conserved regions are those which are involved in binding the purple bacterial electron transport chain cofactors. Such homology, observed in such widely divergent species, identifies regions which cannot be mutated without a resultant inhibitory or lethal effect. Thus, it is reasonable to assume that these sites are involved in binding similar redox active components identified in PSII reaction centres (Nanba & Satoh, 1987; Barber *et al.*, 1987). Figure 1.5 marks the predicted membrane spanning regions of D1. This information was used to produce the folding model shown in Figure 1.6. The results presented in the rest of this section not only provide considerable evidence to support this model, but summarise our current knowledge of D1 structure.

The N-terminus of the D1 precursor undergoes three post-translational modifications. First, the N-formylmethionyl residue (M) is cleaved from the N-terminus, thereby exposing threonine residue (Thr2), which is subsequently, acetylated and reversibly phosphorylated (Michel *et al.*, 1988). As D1 is phosphorylated at the stroma surface of the membrane (Michel *et al.*, 1988), the N-terminus is positioned on this side of the membrane in Figure 1.6. It has also been suggested that D1 can be palmitoylated and that this post-translational modification step may control the migration of D1 between the stroma and grana lamellae (Mattoo & Edelman, 1987). The site of palmitoylation was not identified.

The N-terminus of D1 is poorly conserved, until just prior to the point where the polypeptide starts to form transmembrane helix I. However, the amino acid sequences flanking each of the five transmembrane helices are highly conserved in D1 of the species analysed and to a lesser extent between D1 and the purple bacterial L subunit (Satoh, 1993) and so lend support for the 5 transmembrane helix models presented (see also Sayre *et al.*, 1986). Of all the transmembrane helices, helix I is the least conserved and no links between it and the D1 associated electron transport chain components have been made.

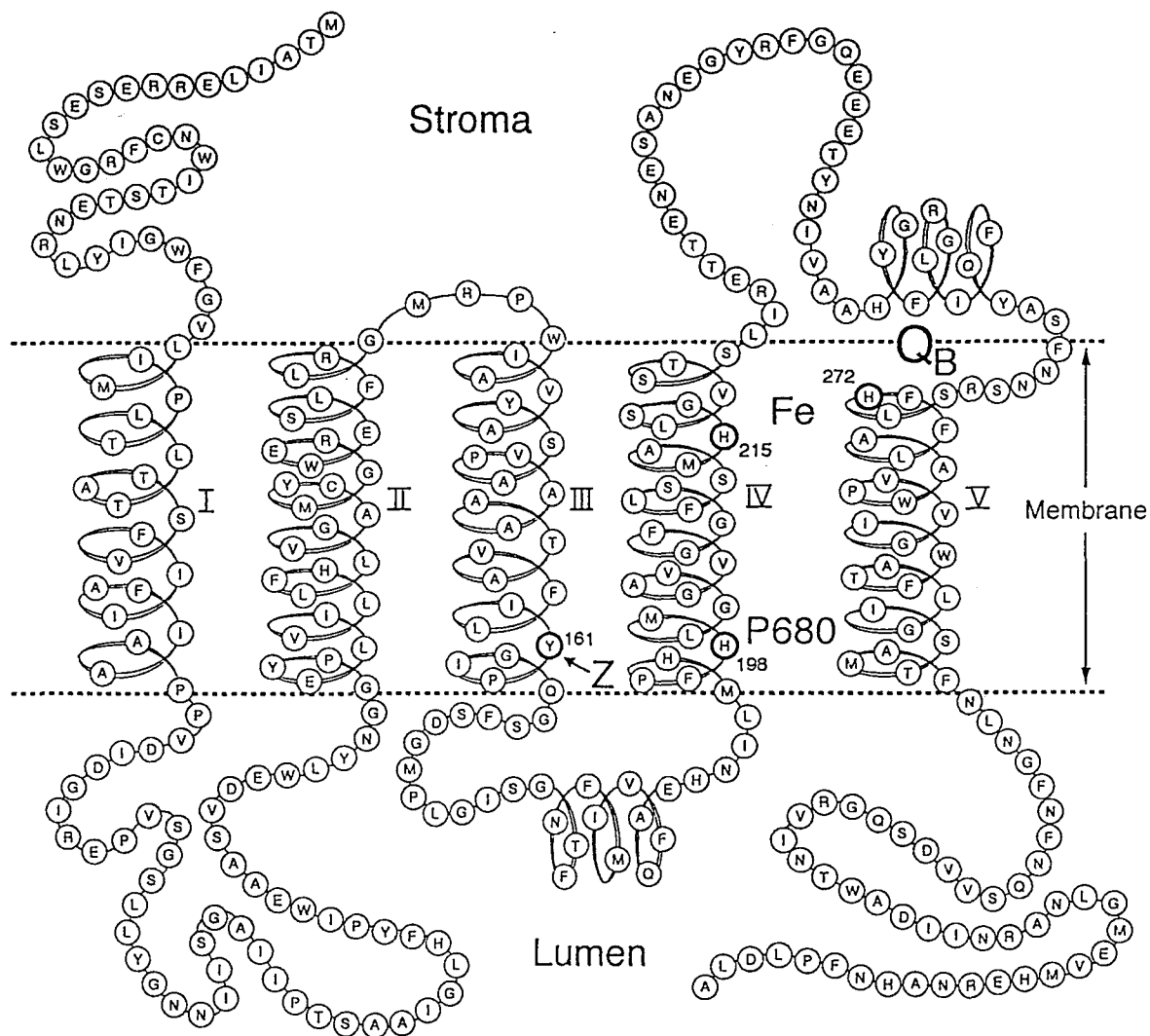


Figure 1.6 Folding model of D1.

The extrinsic luminal loops, are very highly conserved considering that the electron transport chain components Tyr161, P680, pheophytin, Q_A and Q_B are thought to be embedded in the membrane or associated with the stroma side of the PSII complex. This conservation and the luminal location of the Mn cluster, suggests that the loops connecting helices I and II, III and IV and the C-terminus may be involved in the assembly and integrity of the water splitting system.

Although the Mn cluster has been under intense investigation, its structure is far from clear (reviewed in Hansson & Wydrzynski, 1990; Sauer *et al.*, 1988; Rutherford, 1992). The currently favoured models suggest the existence of two distinct binuclear Mn centres (Guiles *et al.*, 1990) or a trinuclear Mn cluster associated with a mononuclear Mn centre (Hansson *et al.*, 1987; George *et al.*, 1989; Penner-Hahn *et al.*, 1990).

Spectroscopy data supports the view that Mn ions are oxidised during the S_0 to S_1 and S_1 to S_2 transitions (reviewed in Hansson & Wydrzynski, 1990). It has been proposed that Mn ions, in highest oxidation states are most tightly bound to PSII, as they are more easily dissociated from complexes in the S_0 and S_1 states, than in the S_2 or S_3 states when exposed to reducing agents (Beck *et al.*, 1986). Chloride ions may be involved in binding the Mn cluster to PSII, as the presence of high concentrations of these ions prevents the dissociation of the Mn cluster in the presence of reducing agents (Beck *et al.*, 1986).

There is also considerable evidence to suggest that Ca^{2+} ions are integrally involved in Mn cluster formation and stability (reviewed in Rutherford, 1992). The release of Ca^{2+} from reaction centres at pH 3, indicated that these ions may directly interact with either aspartate or glutamate residues, both of which have apparent pKa values of about 4 (Ono & Inoue, 1988). The finding that Ca^{2+} is required for photoactivation, the process by which the Mn cluster is assembled, is further evidence of its importance in maintaining the structural integrity of the latter (reviewed in Rutherford, 1992). Ca^{2+} is most tightly associated with Mn clusters in the S_1 and S_2 state (Rutherford, 1992) and its absence results in an inhibition of the $S_3 \rightarrow S_0$ transition (Boussac & Rutherford, 1988). Mn-EPR studies have shown that replacing Ca^{2+} with Sr^{2+} results in a marked change and so indicates that Ca^{2+} is liganded with this metal complex (Boussac & Rutherford, 1988). It has also been suggested that Ca^{2+} may control the access of water to the active site (Sivaraja *et al.*, 1989).

A number of D1 residues have been proposed to be potential Mn cluster ligands. These residues include the highly conserved Asp170, Glu189 and Gln165 (Svensson *et al.*, 1991). Mutagenesis of Asp170 affected both the assembly and stability of the Mn cluster (Nixon *et al.*, 1992; Nixon & Diner, 1992; Boerner *et al.*, 1992). Histidine residues are also potential Mn cluster ligands. Modification of histidine residues using the cross-linking agent DEPC, resulted in a reduction in the Mn binding affinity of D1 (Tamura *et al.*, 1989). The D1 histidine residues expected to be exposed to the luminal space are His92, His190, His332 and His337. Interestingly, the C-Terminal end of D1 is highly conserved up to Ala344 and contains His332/337. Ala344 forms the C-terminus of the mature D1 protein. If the C-terminus of D1 is not processed, the Mn cluster is unable to assemble (Diner *et al.*, 1988; also see Taylor *et al.*, 1988). Taken together with the DEPC-histidine modification results of Tamura *et al.*, (1989) this result suggests that His332 and 337 may indeed be involved in Mn cluster formation.

Tyr161 (Z in Figure 1.4, Steps 3 and 4) has been identified as the residue responsible for electron transport between the Mn cluster and $P680^+$ (Debus *et al.*, 1988; Debus *et al.*, 1989; Metz *et al.*, 1989; Takahashi & Satoh, 1989). The presence of a functional Tyr161 can be detected by a characteristic EPR signal (signal ν_F). Given the putative folding model presented in Figure 1.6, Tyr161 is exposed to a hydrophilic region near the luminal surface of the membrane (Svensson *et al.*, 1991) and must be in close association with the Mn cluster if it is to accept electrons from the latter. Interestingly, Baumgarten *et al.*, (1990) calculated that the centre of the Tyr161 phenolic ring lies 7Å from an oxidisable side chain. Svensson *et al.*, (1990) suggest that this 7Å distance is the distance

between the Mn cluster and D1-His190, and that the latter may hydrogen bond with D1-Tyr161 (Svensson *et al.*, 1990).

The D1 polypeptide is most highly conserved between the luminal surface of helix II and the stromal end of helix V. It is this region of the polypeptide which is thought to provide binding sites for P680, pheophytin and Q_B. D1-His198 is conserved in both the D1 subunit of PSII and the L subunit of purple bacteria reaction centres and so is thought to provide a ligand for P680 (reviewed in Barber, 1987a; Michel & Deisenhofer, 1988; Satoh, 1993). Ala287 (Helix V) is also conserved between higher plant D1 and the purple bacteria L subunit, but although it is located close to P680, its function is not known.

During normal electron transport P680 donates electrons to pheophytin. Glu130 is thought to provide a ligand for the redox active pheophytin via its 9-keto group (Moënné-Loccoz *et al.*, 1989; Lubitz *et al.*, 1989; Navedryk *et al.*, 1990). Indeed such liganding is similar to that observed between Glu104 and bacteriopheophytin in the *Rhodospseudomonas viridis* reaction centre (Michel *et al.*, 1986). Svensson *et al.*, (1990) suggest that D1-Tyr126 may correspond to the *Rhodospseudomonas viridis* L-Trp100, which also forms a hydrogen bond with bacteriopheophytin. Taking the analogy a step further, D1-Tyr147 and D1-Ala150 are proposed to correspond to L-Pro124 and L-Phe121 which are in contact with the pheophytin ring plane in purple bacteria (Michel *et al.*, 1985; Svensson *et al.*, 1990).

The redox active pheophytin molecule bound to D1, donates electrons to the quinone bound at the Q_A site, during normal electron transport (Figure 1.3). Q_A is thought to be bound to the D2 subunit, in line with the close association of the D1 and D2 polypeptides. His215 and His272 are conserved between purple bacteria and higher plants, and are thought to ligand the non-haem iron which is involved in electron transport between Q_A and Q_B. The location of a phosphate group on Thr2, exposed to the stroma side of the membrane, may also interact with this non-haem iron, thus exerting an effect on Q_A and Q_B electron transport (Michel *et al.*, 1988). Mutational studies have provided considerable information about the amino acids involved in binding plastoquinone at the Q_B site. A number of mutations close to its binding site result in altered Q_B binding properties (Satoh, 1993). Tyr237, Tyr254, Phe255, Gly256 have all been shown to be involved in binding Q_B (Michel & Deisenhofer, 1988; Hirschberg & McIntosh, 1983; Rochaix & Erickson, 1988; Dostatni *et al.*, 1988). The fact that Ser264 is conserved in both the D1 subunit of PSII and the L subunit of purple bacteria, has led to the suggestion that it too acts as a ligand to plastoquinone, bound to the Q_B site (Satoh, 1993; Trebst, 1986). As thylakoids, subjected to a mild trypsin digestion, lost their Q_B binding activity, without affecting electron transport between the oxygen evolution apparatus and Q_A (Renger *et al.*, 1985; Völker *et al.*, 1985; Völker *et al.*, 1986), its position near the stroma surface of the membrane is confirmed.

To gain a better understanding of how the electron transport chain components are arranged within the reaction centre the information available on D2 structure will be reviewed in Section 1.4.2.

1.4.2 The structure of D2

All the redox groups participating in the electron transport process from D1-Tyr161 to Q_B via P680, Pheo and Q_A are attached to the D1-D2 heterodimer (Trebst, 1986; Barber, 1987a). The ligands provided to these redox active components by D1 were discussed in the previous section. This section analyses the structure of D2 and tries to identify additional ligands for these components.

The D2 polypeptide has a high degree of homology to the M subunits of both *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides*. As *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides* reaction centre structures have been determined to a resolution of 2.5 and 2.8Å, they are useful models for D2 structure. Figure 1.7, which was adapted from the review of Satoh, (1993), shows the homology between the amino acid sequences of the M subunits of *Rhodopseudomonas viridis* (Mv) and *Rhodobacter sphaeroides* (Ms) (Allen *et al.*, 1987) and spinach D2.

D2	TIAVGKFTKD-EKDLFDSMDDWLRRDRFVFGWSGLLLFPCAYFALGGWFTGTTFTVTSWYT	61
Ms	AEYQNIQSQVQVRGPADLGMTEDVNLANRSGVGPFSTL-LGWF-GNAQL--GPIYLGSLGVLSTLFSGLMWFFTTIGIWFY	76
Mv	ADYQTIYTQIQARGPHITVSGEWDNDRVGKPFYSYWL--GXI-GDAQI--GPIYLGASGIAAFAGSTAILIILFNMAA	75
I		
D2	HGLASSYLECCNFLTAAVSTP----ANSLAHSLLLLWGPEAQGDFTRWCOLGGLWAFVALHGAFALIGFMLROFELARSV	137
Ms	QAGWNP-----AVFLRLDFFFSLEPPAPEYGLSFAAP-----LKEGGLWLIASFFMFVAVWSWWGRTYLRQAAL	140
Mv	EVHFDP-----LQFFRQFFWLGLYPPKAQYGM-GIPP-----LHDGGWWLMAGLFMTLSLGSWWIRVYSRARAL	138
II Bph		
D2	QLRPYNAIAFSGPIAVFVSFLIYPLGQSGWFFAPSFCAAIIFRFFILFFQGFH-NWTLNPFHMMGVAGVLGAALLCAIHG	216
Ms	GMGKHTAWAFLSAIWLWMVLGFIIRPILMGSWSEAVPYGIFSHLDWTNNFSLVHGNTLFYNPFHGLSIAFLYGSALLFAMHG	220
Mv	GLGTHIAWNFAAAIFFVLCIGCIHPTLVGSWSEGVFPFGIWPIDWLTAFSIRYGNFYCPWHGFSIGFAYGCGLLFAAHG	138
III Y _D Bch P IV Fe, Q _A		
D2	ATVENTLF-EDGDCANT--FRAFNPTQAEEITYSMVTANRFWSQ-IFGVA-FSNKRWLHFFMLFVPVTGLWMSALGVVGLA	291
Ms	ATILAVSRFGGERELEQIADRGTAAR-----AALFWRW-TMGFNA--TMEGIHRWAIWMVAVLTLTGGIGILLSG	288
Mv	ATILAVARFGGDREIEQITDRGTAVER-----AALFWRW-TIGFNA--TIESVHRWGWFSLMVMVSASVGILLTG	286
Fe Q _A Q _A Fe V		
D2	LNLRAYDFVSQEIARAEDPEFETFYTKNILLNEGIRAWMAAQDQPHENLIFPEEVLPRGNAL	353
Ms	TVV-DNWFYVWGQNHGMAPLN	307
Mv	TFV-DNWFYLWCVKHGAAPDPYALPATPDPAASLPAPK	323

Figure 1.7 Homology between the M subunit amino acid sequence of *Rhodopseudomonas viridis* (Mv) and *Rhodobacter sphaeroides* (Ms) and that of spinach D2. The regions labelled I-V represent the positions of the predicted transmembrane helices. Liganding positions of cofactors are marked as follows: Bph = bacteriopheophytin, Y_D = Tyr161 of D2, Bch = bacteriochlorophyll, p = primary reactant (eg. P680 in higher plants), Fe = non-haem iron.

The N-terminal region of D2 (Figures 1.7 and 1.8) is the least conserved of the whole protein, but is subject to a number of post-translational modifications. Michel *et al.*, (1988) showed that the postranslational modification to the pre-D2 polypeptide followed a pattern similar to that observed for D1. The N-formylmethionyl residue was reported to be cleaved from the N-terminus prior to the acetylation and phosphorylation of the exposed D2-Thr2.

The mutagenesis studies of the lumenally exposed loops of D2 have suggested that they too may provide a ligand for the Mn cluster. Vermaas, (1993) has suggested that D2-Glu69 is one such ligand.

The D2 polypeptide has a Tyr residue at position 160 which, in contrast to D1-Tyr161 acts as a poor electron donor to P680⁺. Indeed, its ability to donate electrons to P680 is approximately 10 orders of magnitude less than that of D1-Tyr161 and is typified by a dark stable EPR signal (Vermaas *et al.*, 1988b; Babcock *et al.*, 1989; Vass & Styring, 1991). The findings that the cavity, surrounding Tyr160 (TyrD) is more hydrophobic in character than the equivalent D1-Tyr161 pocket and that no metal binding sites have been detected near the D1-Tyr160, seem to explain the relative photochemical inactivity of the former (Svensson *et al.*, 1991).

Transmembrane helix IV contains the highest number of amino acids conserved, both in the D2 subunit of PSII and in the M subunit of purple bacteria. D2-His197 is one of these conserved amino acids and is thought to provide a ligand to P680, in addition to those formed with the D1 complex. Another histidine (D1-His215), closer to the stroma surface of the membrane, but still part of helix IV, is thought to provide a ligand for the non-haem iron or Q_A. Gly228, Trp253, Ala260 and His214 are suggested Q_A ligands (Sato, 1993). The location of these amino acids near the stroma surface places them near pheophytin, from which Q_A accepts electrons. Kless *et al.*, (1992) showed that modification in the 224-240 region of D2 had little effect on its function. This result corresponds well with the prediction that Q_A is liganded to amino acids closer to helix V (i.e. Trp253 and Ala260). The result is however surprising, in that this region contains a small segment (238-240) of a stretch of amino acids (238-264) that shows a higher degree of conservation than is observed in the D1 D/E loop (Barber, 1987a; Michel & Deisenhofer, 1988; Sato, 1993; Styring *et al.*, 1991). Indeed, the highly conserved stretch of the D2 D/E-loop (238-264) contains D2-Trp254, which has been suggested to be involved in Q_A binding (Breton *et al.*, 1991; Vermaas *et al.*, 1990).

The involvement of both the D1 and D2 in binding the Mn cluster and P680, demonstrates how closely associated these two subunits are. The fact that the D2 polypeptide forms the Q_A site, while the Q_B site is formed by D1, further demonstrates their close contact.

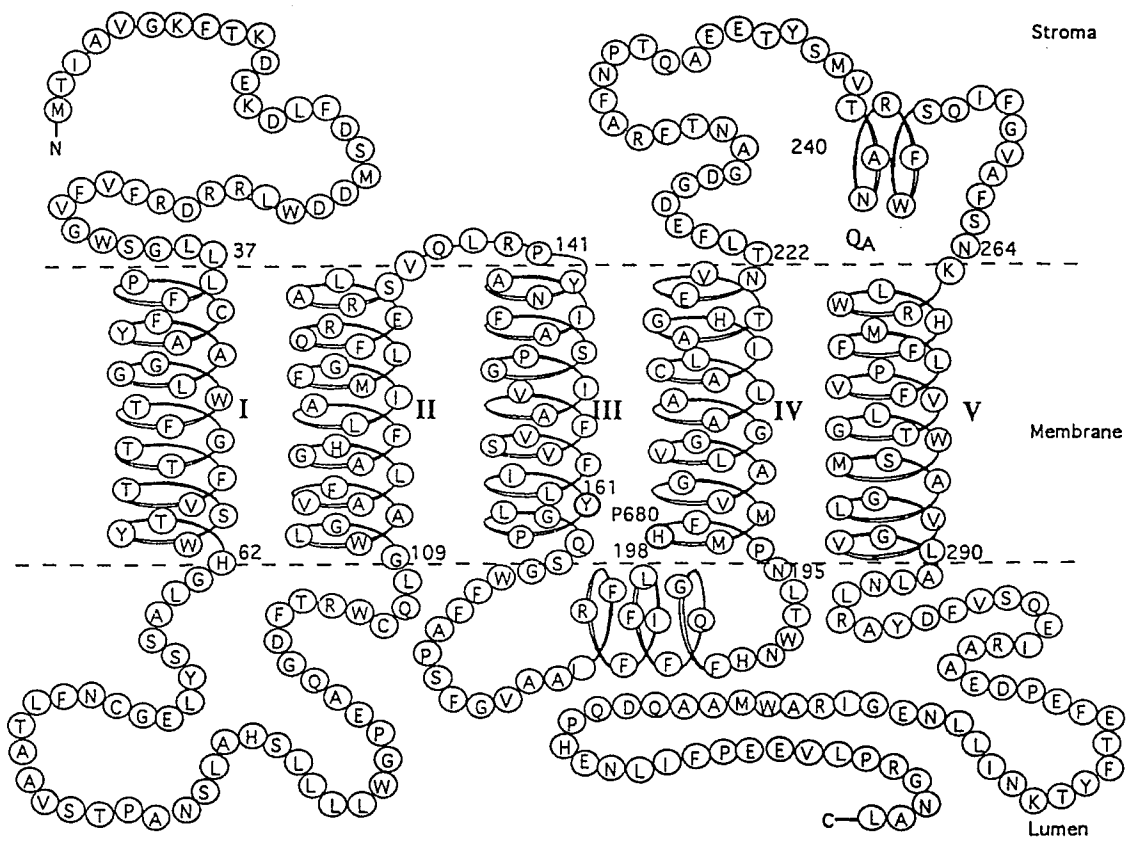


Figure 1.8 Folding model of the D2 subunit.

1.4.3 The structure of cytochrome b559

Cytb559 consists of two subunits α and β , having molecular weights of 9.2 and 4.3kDa respectively (Widger *et al.*, 1985). The *psbE* and *psbF* genes localised on the chloroplast genome (Westhoff *et al.*, 1985) encode the α and β subunits of cytb559. Their nucleotide sequences have been identified in spinach (Herrmann *et al.*, 1984), barley (Kruipinska & Berry-Lowe, 1988), wheat (Hird *et al.*, 1989; Webber *et al.*, 1989a), primrose and tobacco (Carrillo *et al.*, 1986), pea (Willey & Gray, 1989), rye (Kolosov *et al.*, 1989), *Euglena gracilis* (Cushman *et al.*, 1988) and in cyanobacteria (Pakrasi *et al.*, 1988). Both subunits are part of the reaction centre of PSII (Nanba & Satoh, 1987; Barber *et al.*, 1987). Cross-linking studies have shown the cytb559 subunits to be in contact with both D1 and D2 (Moskalenko *et al.*, 1992). It has also been proposed that they are in contact with the 33kDa extrinsic subunit (Tae *et al.*, 1988; Takahashi & Asada, 1991). Furthermore, the C-terminus of the α -subunit is involved in PSII assembly (Tae & Cramer, 1992). The genes coding for the α - (*psbE*) and β - (*psbF*) subunits have been isolated from a large number of higher plants and cyanobacteria, and sequenced. The amino acid sequences were aligned by Satoh, (1993) and are shown in Figure 1.9.

The α - and β -subunits of cytb559 are present in PSII in a ratio of 1:1 (Widger *et al.*, 1985). Each has a single hydrophobic domain, 26 and 25 amino acids in length, respectively (Herrmann *et al.*, 1984). A haem group has been shown to be liganded to the complex, via two histidine residues (Babcock *et al.*, 1985). Since the α - and β - subunits of cytochrome each only contain a single histidine residue, in their respective membrane spanning domains, the haem must cross-link two polypeptides into a dimeric complex (Widger *et al.*, 1985; Cramer *et al.*, 1986). If only one cytb559 dimer were present per reaction centre, as has been reported by Kobayashi *et al.*, (1990) and Gounaris *et al.*, (1990), then the cytochrome complex could only be a heterodimer. However, several reports of a stoichiometry of two haems per reaction centre PSII core complexes (Lam *et al.*, 1983; Murata *et al.*, 1984; Yamamoto *et al.*, 1984) have been made, leading to the suggestion that 1 haem group was easily lost during purification of isolated reaction centres. If two haems are indeed present, then the existence of $\alpha\alpha$ and $\beta\beta$ cytochrome dimers has to be considered. However, ion exchange chromatography results (Herrmann *et al.*, 1984) favour the $\alpha\beta$ heterodimer model. Herrmann *et al.*, (1984) showed that DEAE ion exchange chromatography was unable to resolve two distinct fractions corresponding to α_2 and β_2 , even though these two homodimers differ by 10 charge units. Furthermore, the bi-cistronic nature of the mRNA of *psbE* and *psbF* also supports the heterodimer theory (reviewed in Erickson & Rochaix, 1992).

Cyt b559 α subunit (*psbE* product)

	↓
Tobacco	MSGST-GERSFADIITSIRYVWVHSITIPSLFI-AGWLFVSTGLAYDVFGSPRPNEYFTESRQGIPLITG-RPDPL-E-QLDEFSRSF
Spinach	S.....
Pea	I.....T.....S.....
<i>Oenothera bertiana</i>	G.....S.....
<i>Oenothera bookeri</i>	G.....S.....
Rice	D.....S.....
Barley	D.....S.....
Wheat	D.....S.....
Rye	D.....S.....
Maize	D.....S.....
Liverwort	...N...P...S.....N...EV...NS...I...TK...
<i>Euglena gracilis</i>	...A...P...S.....V...V-G...I...IV...I...T...S...T...QA...SD...NA...M...Q...TK
<i>Cyanophora paradoxa</i>	...G...T...P...S...V...TV...F...V...T...D...E...EV...I...NQ...TN
<i>Synechocystis</i> sp. PCC 6803	...T...P...S...V...M...A...T...D...QT...EL...LQE...Y...INQ...I...NQ

Cyt b559 β subunit (*psbF* product)

	↓
Tobacco	NTI--DR---T---YPIFTVRWLAIHGLAV--PTVFPLGSISAMQFIQR
Spinach	S.....
Pea	V.....S.....
<i>Oenothera bertiana</i>	V.....S.....
<i>Oenothera bookeri</i>	V.....S.....
Rice	V.....
Barley	
Wheat	
Rye	
Maize	V.....
Liverwort	V.....A.....
<i>Euglena gracilis</i>	...TNK...R...F...V...A...I.....
<i>Cyanophora paradoxa</i>	...NN--PN---Q-PVS...IGI...A...I...T.....
<i>Synechocystis</i> sp. PCC 6803	A...Q--NPNQPV...V...T...S...V...A...A.....

Figure 1.9 Aligned amino acid sequences of the α and β subunits of cytb559. Figure taken from Satoh, (1993).

Tae *et al.*, (1988) used proteases and a C-terminal specific antibody to the α -subunit, to produce the model of cytochrome shown in Figure 1.10. The authors showed that the N-terminus of the α subunit is exposed to the stroma side of the membrane. The N-terminal methionine residues of both the α and β subunits are absent in the mature polypeptide in spinach (Widger *et al.*, 1984; Ikeuchi

et al., 1989a), pea (Webber *et al.*, 1989b) and wheat (Ikeuchi *et al.*, 1989a). As the single histidine of the α -subunit is near the stroma surface of the membrane (Herrmann *et al.*, 1984) and the haem is ligated by two histidine residues, (Babcock *et al.*, 1985), it is concluded that the NH_2 terminus of the β -subunit is also stromally exposed.

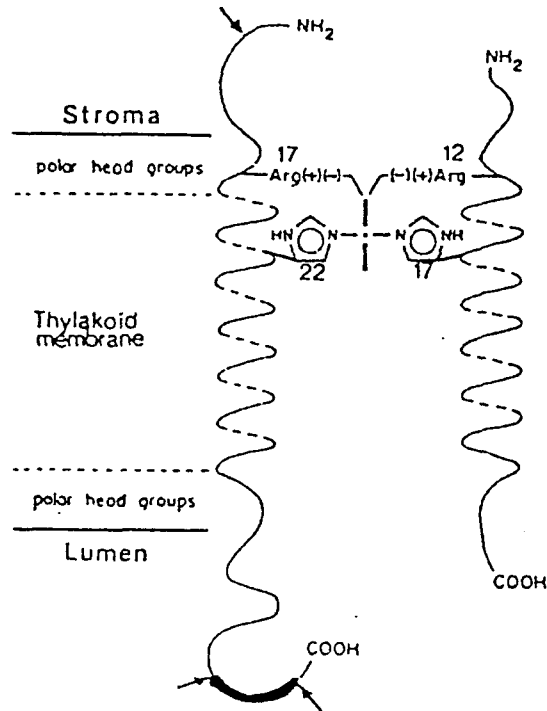


Figure 1.10 Model of cytb559 structure. Figure from Tae *et al.*, (1988).

Although the function of cytb559 is not fully understood, it has frequently been argued that it is involved in the protection of PSII against photoinhibition (Arnon & Tang, 1988; Thompson & Brudvig, 1988; Nedbal *et al.*, 1992; Barber & De Las Rivas, 1993).

Photoinhibition can be divided into donor side and acceptor side mechanisms, both of which are explained by Barber, (1993) (Figure 1.11). Both acceptor and donor side photoinhibition result in damage to the D1 protein. Acceptor side photoinhibition is thought to arise if Q_A becomes doubly reduced, protonated and leaves the binding site (Vass *et al.*, 1992b). If this occurs, the primary radical pair ($\text{P680}^+ \text{Pheo}^-$) undergoes a recombination reaction (back reaction), resulting in the formation of some P680 triplet ($^3\text{P680}$). $^3\text{P680}$ also decays to the ground state (P680) and in the presence of oxygen, singlet oxygen is formed. The singlet oxygen irreversibly bleaches P680 (Telfer *et al.*, 1991) and may result in a conformational change, leading to D1 cleavage. The formation of the 23kDa N-terminal and 10kDa C-terminal fragments are indicative of acceptor side photoinhibition (Greenberg *et al.*, 1987; De Las Rivas *et al.*, 1992b; De Las Rivas *et al.*, 1993; Barber, 1993; Cánovas & Barber, 1993).

Cytochrome can exist in a high or low potential state. Nedbal *et al.*, (1992) and Barber & De Las Rivas, (1993) suggested that the low potential form of cytb559 can accept electrons from pheophytin, thus preventing $P680^+ \text{Phe}^-$ radical pair recombination. As a result, cytb559 may provide some protection against 3P680 and subsequent singlet oxygen formation.

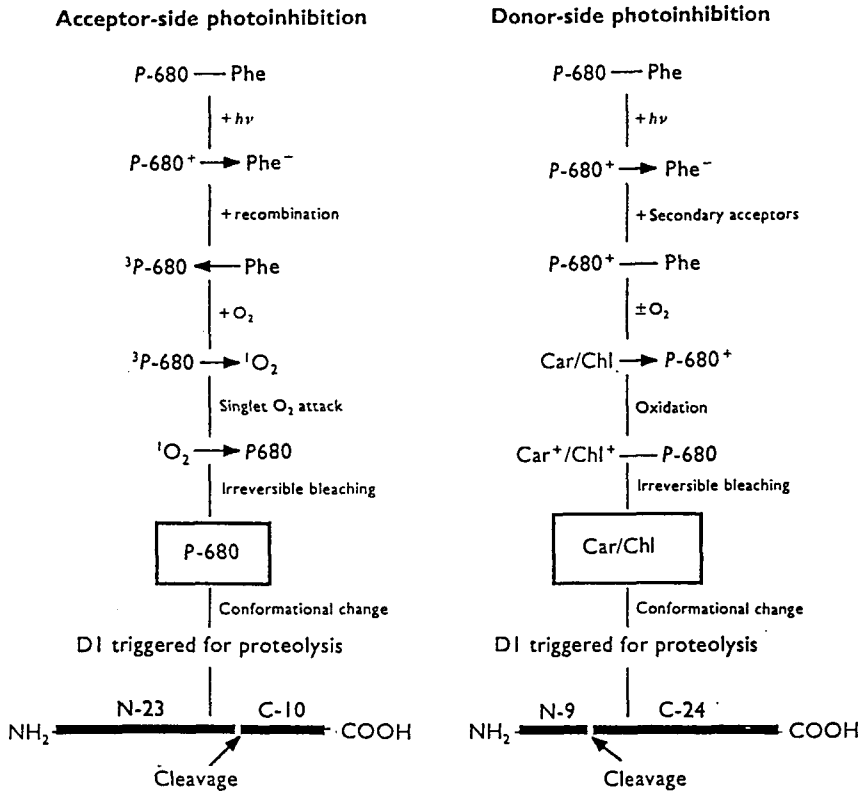


Figure 1.11 Donor and acceptor side photoinhibition diagram. Taken from Barber, (1993).

Donor side photoinhibition also results in D1 damage and is caused by the donor side of PSII being unable to donate electrons to $P680^+$, at a sufficiently fast rate. Under these conditions, $P680^+$ accepts electrons from nearby carotenoid and chlorophyll molecules causing oxidation-induced irreversible bleaching (Telfer *et al.*, 1991). This process appears to trigger D1 cleavage in such a way as to yield a 9kDa N-terminal and a 24kDa C-terminal fragment (Shipton & Barber, 1991; Barbato *et al.*, 1991b; Barber, 1993).

It has been suggested that the high potential form of cytb559 is able to protect PSII against donor side photoinhibition by donating electrons to $P680^+$. This would prevent the irreversible oxidation of carotenoids and chlorophylls closely associated with it (Thompson & Brudvig, 1988; Barber & De Las Rivas, 1993).

Barber & De Las Rivas, (1993) propose that cytb559 is able to drive the cyclic electron transport scheme shown in Figure 1.12.

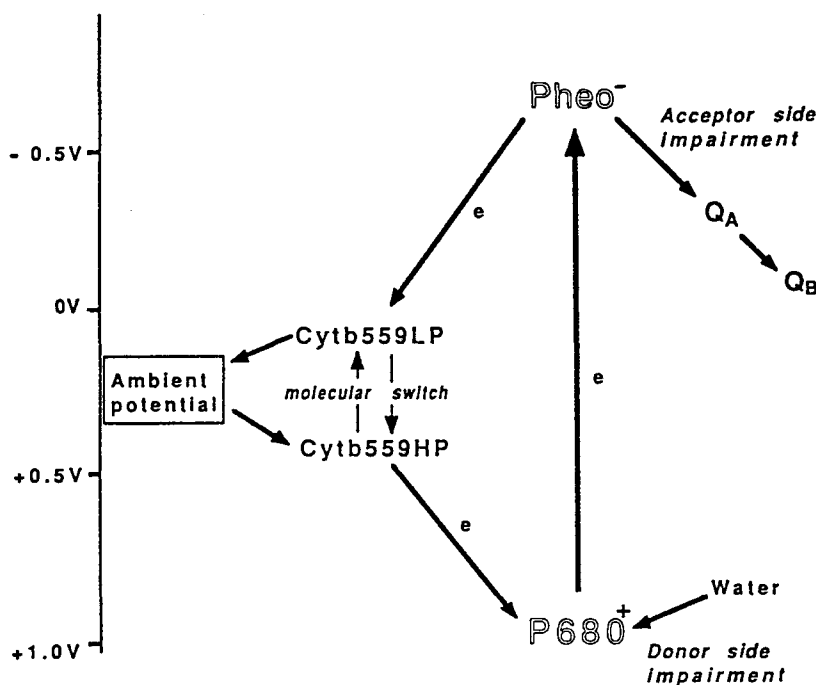


Figure 1.12 The cytb559 driven electron transport scheme proposed by Barber & De Las Rivas, (1993). cytb559LP = low potential form of cytb559, cytb559HP = high potential form of cytb559.

This scheme incorporates the acceptor (Nedbal *et al.*, 1992) and donor (Thompson & Brudvig, 1988) side protection mechanisms, and suggests that cytb559 is able to switch from a high (cytb559HP, Figure 1.12) to a low (cytb559LP, Figure 1.12) potential form to protect the reaction centre of PSII against the two types of photodamage discussed. From this model, it is apparent why cytb559 is so closely associated with the D1 and D2 subunits, which bind the electron transport components of PSII.

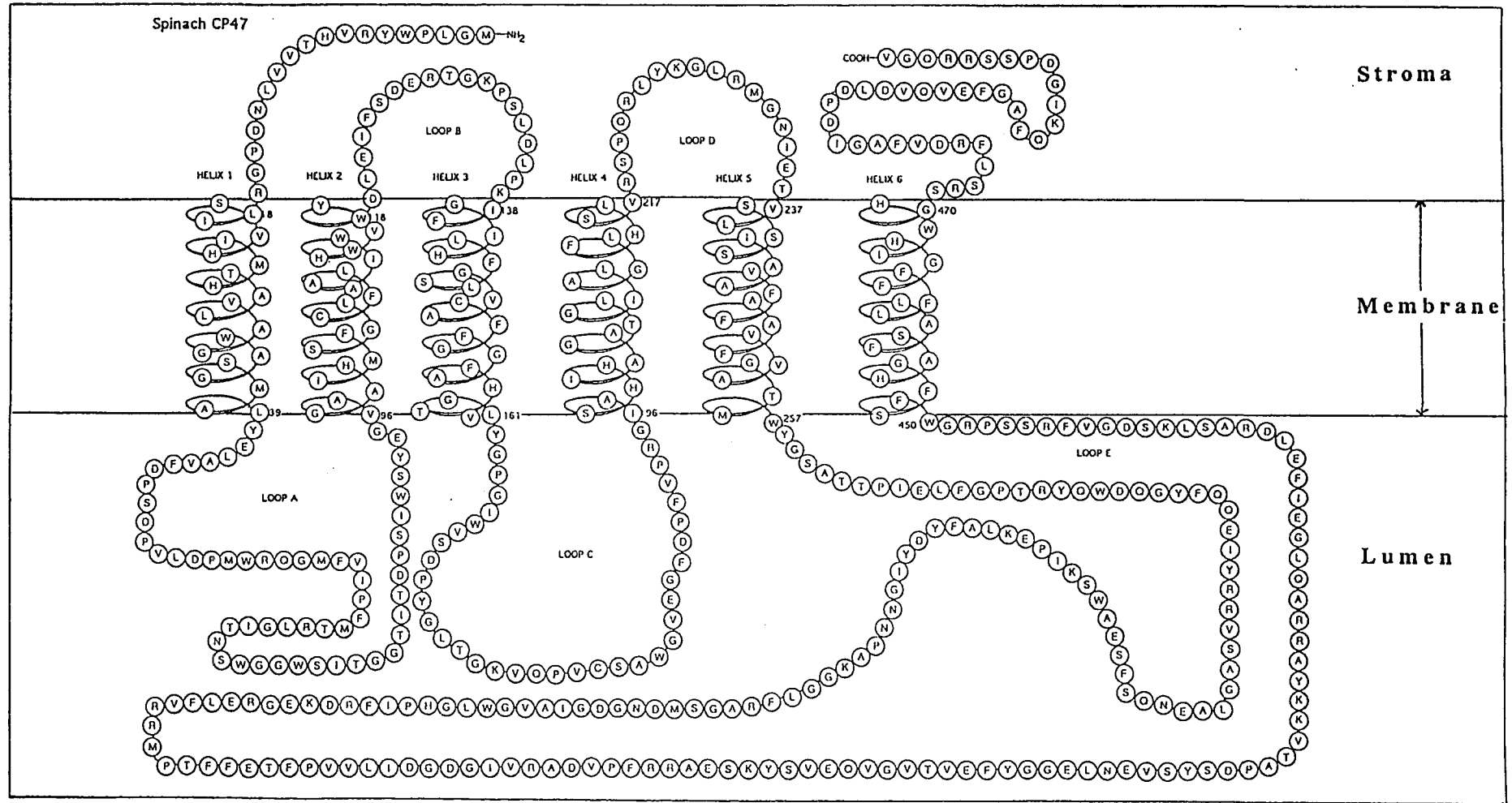
1.4.4 The structure of CP47

CP47 is a pigment binding protein, which is closely associated with the reaction centre of PSII (Dekker *et al.*, 1989). Cross-linking studies showed CP47 to be in direct contact with D2, but not with D1 or cytb559 (Moskalenko *et al.*, 1992). Other studies have shown CP47 to be in contact with CP29 (Rigoni *et al.*, 1992; Dainese *et al.*, 1992a) and CP26 (Dainese *et al.*, 1992a), as well as with the lumenally located extrinsic 33kDa subunit (Bricker & Frankel, 1987; Bricker *et al.*, 1988; Odom & Bricker, 1992; Frankel & Bricker, 1992; Putnam-Evans & Bricker, 1992; Haag *et al.*, 1993). Its close association with all of these subunits explains why its absence inhibits assembly and stability of the oxygen-evolving core of PSII (Vermaas *et al.*, 1986; Vermaas *et al.*, 1988a). Whereas CP47, CP43, D2, D1, cytb559 and the 33kDa subunit are required to support high rates of oxygen evolution *in vitro*, CP29 and CP26 are not (Ikeuchi *et al.*, 1985; Ikeuchi & Inoue 1986). CP29, CP26 and CP24 are

thought to be responsible for the physical and excitonic linkage of LHCII to PSII (see Section 1.6).

CP47 is encoded by the *psbB* gene which is located on the chloroplast genome of higher plants and green algae and the genomic DNA of cyanobacteria (reviewed in Erickson and Rochaix, (1992). *Synechocystis* 6803 (Vermaas *et al.*, 1987), *Marchantia polymorpha* (Ohyama *et al.*, 1986), tobacco (Shinozaki *et al.*, 1986) and spinach (Morris & Herrmann, 1984) amino acid sequences show 72% homology between them. This value rises to 85%, if conserved amino acid changes are included (Bricker, 1990). 40% of the non-conservative amino acid replacements are found in four small regions encompassing only 12% of the protein. These variable regions are residues 73-89, 182-200, 277-296, 407-416 (Bricker, 1990). The positions of the transmembrane helices were predicted using hydropathy plots. This analysis led to the suggestion that either seven (Morris & Herrmann, 1984) or six (Vermaas *et al.*, 1987) helices existed. The latter theory is now favoured as the additional helix was thought to be both too short and too hydrophilic to allow it to span the membrane.

Von Heijne and Gavel (1988) developed a method to predict whether small (< 100 amino acids in length) hydrophilic loops were located on the inner side of the membranes by their relative lysine and arginine content. Loops A and C, which are depleted in arginine and lysine have been predicted to be on the luminal side of the membrane (Von Heijne & Gavel, 1988). Hence loops B,D and the two terminal ends have been positioned on the stroma side. As a result, the N-terminus of CP47 is shown to be exposed to the stroma side of the membrane in Figure 1.13. This N-terminal sequence does not appear to be phosphorylated (Michel *et al.*, 1988).



CP47 binds both chlorophyll *a* and β -carotene, but does not contain pheophytin or chlorophyll *b* (Alfonso *et al.*, 1994). CP47 was estimated to bind 10-12 chlorophyll *a* molecules by Tang & Satoh, (1984) and Barbato *et al.*, (1991a); yet de Vitry *et al.*, (1984) and Alfonso *et al.*, (1994) obtained a value of 20-25 chlorophyll *a* molecules, per CP47 subunit. Alfonso *et al.*, (1994) calculated that each CP47 subunit bound 3 β -carotene molecules. The transmembrane helices responsible for binding the pigments associated with CP47, are highly conserved between species. Whereas, histidine acts as the bacteriochlorophyll ligand in purple bacterial reaction centres (Deisenhofer *et al.*, 1985; Zuber, 1986), Kühlbrandt *et al.*, (1994) identified glutamate, glutamine and glycine, as well as histidine, as chlorophyll ligands in LHCII. Thus, any of the conserved amino acids of this type, located in the transmembrane regions of CP47 may be potential chlorophyll ligands. CP47 contains 11 conserved histidine residues near the membrane surface of the five transmembrane helices. Despite the fact that there is very little homology between the amino acid sequences of CP47 and CP43, both subunits contain 11 histidine residues and are located in very similar positions in the two subunits (Figures 1.13 and 1.14). Furthermore, these histidine residues are arranged in two layers located near the stromal and luminal surfaces of the membrane. As all of the chlorophyll *a* molecules, associated with LHCII, are bound by amino acids in similar positions, these histidine residues appear to be prime candidates for chlorophyll ligands. Shen *et al.*, (1993) mutated all of the conserved histidine residues located within the 6 transmembrane helices of CP47. In all cases, a reduction in the light harvesting capability of PSII was observed, with multiple mutations resulting in cumulative effects. Thus, the authors concluded that all the histidine residues provided ligands for the Mg^{2+} of the bound chlorophyll molecules. Resonance Raman data suggests that chlorophyll can be bound in two orientations, one in which the $C_9=O$ group is relatively free and one in which the $C_9=O$ group interacts with proteins or other chromophores (de Paula *et al.*, 1990). Replacing H469 in helix VI with glutamine, resulted in a drastic reduction in PSII levels, suggesting that this ligand is structurally important (Eaton-Rye & Vermaas, 1992). CP47 exhibits a specific 695nm fluorescence emission band at 77K (Nakatani *et al.*, 1984; Krause & Weis, 1984; Pakrasi *et al.*, 1985; Satoh & Tang, 1984; van Dorssen *et al.*, 1987) which was greatly reduced in the His469 mutant (Eaton-Rye & Vermaas, 1992). The existence of a CP47 specific 695nm fluorescence emission band is indicative of an excitonic trap within this subunit, which would be expected to be shallow enough to allow subsequent excitation energy transfer to the reaction centre of PSII. The studies of Tapie *et al.*, (1984) and van Dorssen *et al.*, (1987) suggest that the 695nm fluorescence emission band probably originates from a monomeric chlorophyll whose fluorescence dipoles are orientated perpendicular to the plane of the membrane. CD spectra showed that some of the chlorophylls in CP47 are excitonically linked to one another (Alfonso *et al.*, 1994).

The conserved nature of the extrinsic loops of CP47 suggests that they may have a critical role to play in ensuring the integrity of the oxygen-evolving PSII core. Loop E is by far the largest of the hydrophilic loops and has been subjected to extensive mutagenic analysis in *Synechocystis sp.* PCC 6803. In doing so, the following regions were shown to be required for the stable assembly of functional PSII; Ile265-Phe268, Thr271-Ile277, Thr304-Ile309, Phe311-Asn317 and Asp440-447 (Haag

et al., 1993). Deletion of domains Lys277-283 and Arg422-428 significantly reduced the level of assembled PSII, as well as resulting in impaired photoautotrophic growth and oxygen evolution. Deleting Ala373-380 enhanced susceptibility to photoinhibition. In contrast, deletion of Gly333-Ile336, Lys347-352, Val392-Gln394, Asp416-420 and a mutation of His343 only slightly reduced the stability of PSII (Haag *et al.*, 1993). Putnam-Evans & Bricker, (1992) showed that mutating 384 and 385Arg to 384 and 385Gly led to a defective association of the oxygen-evolving complex of PSII.

In summary, mutagenic studies have shown various properties of CP47 as being involved in the light harvesting system of PSII and indicate that it plays a critical role in the assembly of this complex. Cross-linking studies further suggest that CP47 may be involved in binding the 33kDa extrinsic polypeptide at the luminal surface of PSII. Assuming this to be correct, it can therefore be concluded that CP47 is a key component of an oxygen-evolving core particle.

1.4.5 The structure of CP43

Like CP47, CP43 is also an intrinsic part of the smallest PSII complex (CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic polypeptide), supporting high rates of oxygen evolution *in vitro* (Ikeuchi *et al.*, 1985; Haag *et al.*, 1990). CP43 and CP47 are both chlorophyll binding proteins, which together form the proximal antenna of PSII, with CP43 being less tightly bound to the reaction centre of PSII than CP47 (Dekker *et al.*, 1989; Garstka & Kaniuga, 1991). In *Euglena*, the 30kDa protein, whose higher plant equivalent is the 33kDa extrinsic polypeptide, has been shown to bind CP47 and CP43 after their incorporation into the PSII complex (Hiramatsu *et al.*, 1991). The involvement of CP43 in PSII core assembly was confirmed using CP43-less mutants, which accumulate less than 10% of wild type PSII core levels (Rögner *et al.*, 1991; Carpenter *et al.*, 1993; de Vitry *et al.*, 1989). Rögner *et al.*, (1991) observed that the cores, which did assemble were able to drive electron transport between Tyr161 and Q_A and thereby confirmed that CP43 was not involved in this process. Carpenter *et al.*, (1993) showed that by inserting a gene, coding for a chimeric *Synechocystis*/spinach CP43 protein, into *Synechocystis* resulted in the loss of the ability of the mutant to grow photoautotrophically. Furthermore, as changes in S-state cycling were observed in the mutant, it appears that CP43 may be directly involved in providing an optimum Mn cluster environment. As cross-linking studies have shown CP43 to be in close contact with CP26 and CP29 (Dainese *et al.*, 1992a), it is possible that the former is also involved in transferring excitation energy from LHCII via the CP proteins to P680.

CP43 (CPa-2) is encoded by the *psbC* gene which is located on the chloroplast genome of higher plants and green algae and the genomic DNA of cyanobacteria (reviewed in Erickson & Rochaix, 1992). This gene has been sequenced in *Synechocystis* 6803 (Chisholm & Williams, 1988), *Synechococcus* 7942 (Golden & Stearns, 1988), *Chlamydomonas reinhardtii* (Rochaix *et al.*, 1989), *Marchantia polymorpha* (Ohayama *et al.*, 1986), spinach (Alt *et al.*, 1984), tobacco (Shinozaki *et al.*, 1986) and pea (Bookjahn *et al.*, 1986). These amino acid sequences were compared by Bricker, (1990) and showed a homology of 67%. This value rises to 80%, if conserved amino acid changes are included. Five regions were identified as being particularly variable (residues 1-37, 101-124, 133-150, 201-216 and 462-473). The positions of the transmembrane helices were predicted using hydropathy plots. This analysis has led to the suggestion that either seven (Alt *et al.* 1984) or six (Chisholm & Williams, 1988) helices exist. The latter theory is now favoured (see Figure 1.14), as the additional helix is thought to be both, too short and too hydrophilic to span the membrane. Loops A and C, which are depleted in arginine and lysine, have been predicted to be on the luminal side of the membrane, using the method of von Heijne & Gavel, (1988). Hence loops B, D and the two terminal ends have been positioned on the stroma side of the membrane in Figure 1.14. This model fits the finding of Michel *et al.*, (1988), who showed that the N-terminus was stromally exposed, as well as being acetylated and phosphorylated at Thr15.

The C-terminal end of CP43 was shown not to be processed (Takahashi *et al.*, 1988). CP43 binds chlorophyll a and β carotene, but not pheophytin or chlorophyll b (Alfonso *et al.*, 1994). Barbato *et al.*, (1991a) and Akabori *et al.*, (1988) reported CP43 to bind 9-11 chlorophyll a molecules

per subunit. In contrast de Vitry *et al.*, (1984) and Alfonso *et al.*, (1994) reported CP43 to bind 20-25 chlorophyll *a* molecules. Furthermore, Alfonso *et al.*, (1994) reported CP43 to be associated with 5 β -carotene molecules. Little is known about the location of the chlorophyll *a* and β -carotene ligands in CP43, or about the function of the extrinsic loops of CP43. The striking similarity in the position of the histidine residues in the transmembrane helices to those in CP47, suggest that they may act as chlorophyll ligands (Bricker, 1990). Loop E is by far the largest of the hydrophilic loops and is thought to be positioned on the luminal side of the membrane. It has been subjected to detailed mutagenic analysis. Deletion of the following sequences resulted in complete loss of oxygen evolution capability, electron transfer between Tyr161 and Q_B and the ability to grow photoautotrophically (Kuhn & Vermaas, 1993):-

Δ Gln298-Val306
 Δ Arg307-Ala313
 Δ Asn314-Thr322
 Δ Gly334-Met343
 Δ Phe345-Glu354
 Δ Trp352-Asn360
 Δ Leu364-Asp370
 Δ Gln375-Ala386.

The dramatic effects observed on mutating only loop E, illustrate the crucial role that CP43 plays in supporting the water photolysis reaction.

It was stated earlier that CP43 has been shown to be in contact with CP26 and CP29 (Dainese *et al.*, 1992a). Bricker (1990) tried to identify possible docking sites for these polypeptides. In cyanobacteria CP29, CP26, CP24 and LHCII are replaced with phycobiliproteins and their associated linker proteins. This difference between higher plants and cyanobacteria was exploited by Bricker, (1990), to identify sequences that were conserved between higher plants or cyanobacteria species, but not in both. In higher plants such sites could be potential CP29 or CP26 binding sites whereas the equivalent cyanobacterial sequences may be involved in phycobilisome linkage. This analysis identified 14 potential "binding residues", eight of which were in the hydrophilic stroma loops. Four of these residues,

cyanobacterium	Arg135, Tyr143, Asp150 and Gln156
higher plants	Ile135, Ser143, Val150 and Lys156

are clustered in loop B which is only 22 residues in length and contains 12 conserved amino acids (Bricker, 1990). Thus, it is possible that loop B may be involved in binding the CP proteins, but this has to be proved experimentally.

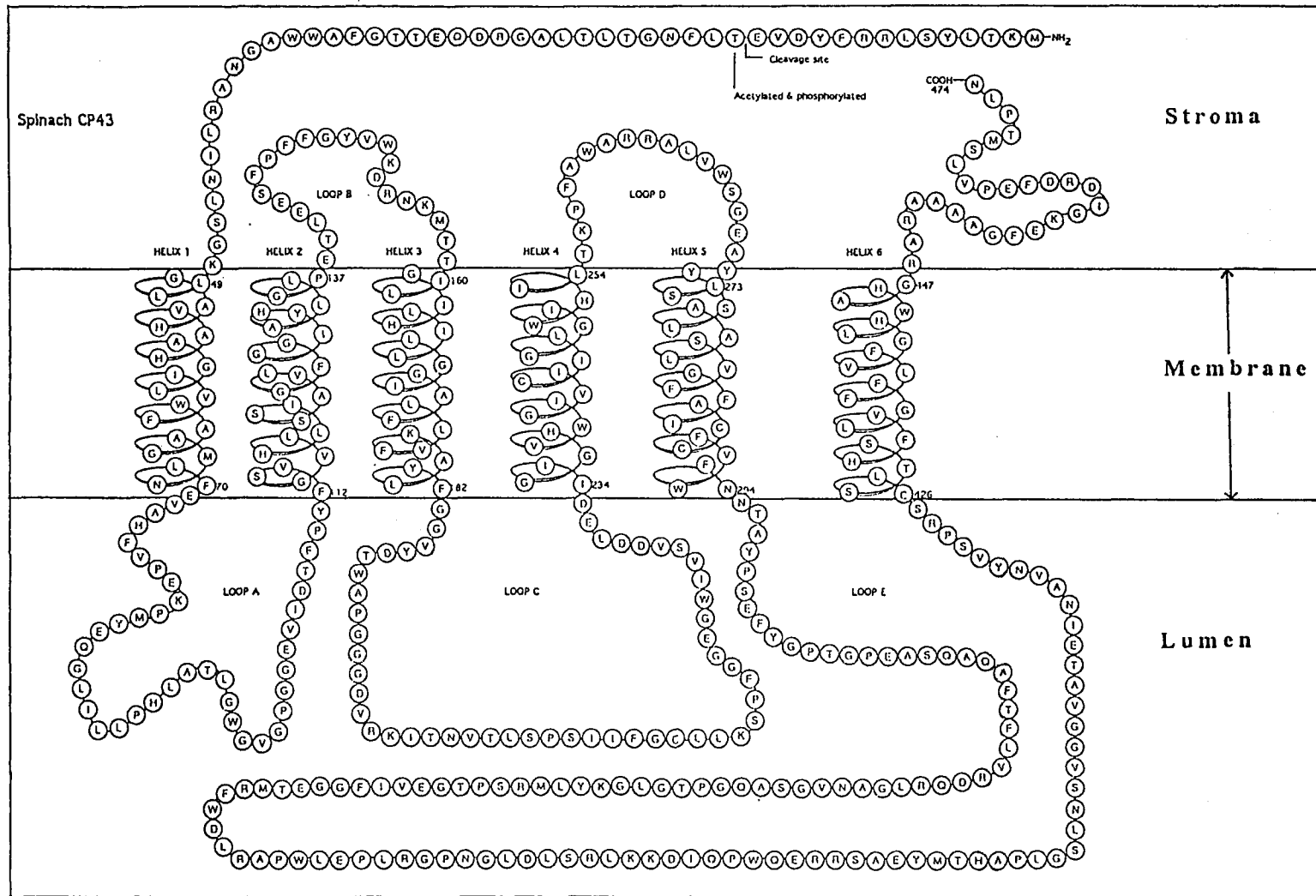


Figure 1.14 Folding model of spinach CP43. Figure was adapted from the review of Bricker, (1990).

1.4.6 The structure of the 33kDa subunit

The 33kDa extrinsic polypeptide of spinach was first purified by Kuwabara & Murata, (1979) who found it to have a pI value of 5.2. By using inside-out vesicles, the 33kDa subunit was shown to bind to the luminal surface of the thylakoid membrane (reviewed in Ghanotakis & Yocum, 1985; Murata & Miyao, 1985; Andersson & Åkerlund, 1987). The 33kDa extrinsic protein has been shown to be in close contact with the extrinsic loop E of CP47 (Bricker & Frankel, 1987; Odom & Bricker, 1992; Frankel & Bricker, 1992; Haag *et al.*, 1993), CP43 (Isogai *et al.*, 1985) and possibly cytb559 (Takahashi & Asada, 1991) and may provide a binding site for the 23 and 18kDa polypeptide in higher plants (Miyao & Murata, 1989). In *Chlamydomonas reinhardtii*, the 33kDa subunit does not appear to be required for efficient binding of the 23kDa subunit (de Vitry, 1989). Although cyanobacterial mutants, lacking the 33kDa extrinsic polypeptide, are able to grow photoautotrophically (Mayes *et al.*, 1991; Burnap & Sherman, 1991; Philbrick *et al.*, 1991), their PSII complexes show apparent structural changes (Vass *et al.*, 1987) typified by impairment of both core assembly and S-state cycling (Boichenko *et al.*, 1992).

It is possible to remove the 33kDa extrinsic polypeptide from the PSII complex without releasing the Mn cluster, using 1M CaCl₂ or 2.6M Urea (Miyao & Murata, 1984a). EPR spectroscopy studies showed that 33kDa-less cores, associated with an intact Mn cluster, are capable of S-State turnover (Styring *et al.*, 1987) and furthermore such cores support low rates of oxygen evolution, in the presence of high concentrations of Cl⁻ and Ca²⁺ (Ono & Inoue, 1984; Miyao & Murata, 1984a & b; Ono & Inoue, 1986; Miyao *et al.*, 1987). The EXAFS studies have shown that the organisation of the Mn cluster is largely unaffected by the removal of the 33, 23 and 17kDa extrinsic polypeptides (Cole *et al.*, 1987), suggesting that these subunits are involved in optimising the function of the Mn cluster rather than in its attachment to the core. Mutants of *Synechocystis* 6803 lacking the 33kDa extrinsic polypeptide show increased sensitivity to photoinhibition (Mayes *et al.*, 1991), while PSII cores lacking this protein have reduced rates of oxygen evolution (Ono & Inoue, 1984) and their higher S-state transition states are more stable (Ono & Inoue, 1986; Miyao *et al.*, 1987). The Q and B band emission signals indicative of S₂Q_A⁻ and S₂Q_B⁻ charge separation states are also reduced (Vass *et al.*, 1992a) by the absence of the 33kDa extrinsic polypeptide in the cyanobacterial mutant.

The involvement of the 33kDa extrinsic polypeptide in maintaining high rates of O₂ evolution, by optimising the S-state cycle, merits a more detailed study of its structure and the way in which it binds to the luminal surface of the core complex. The finding that the 33kDa polypeptide is able to bind to PSII cores lacking the Mn cluster and the fact that it is released from intact cores by a 2.6M urea wash has led to the suggestion that this polypeptide is bound to the luminal core surface via hydrophobic rather than electrostatic interactions.

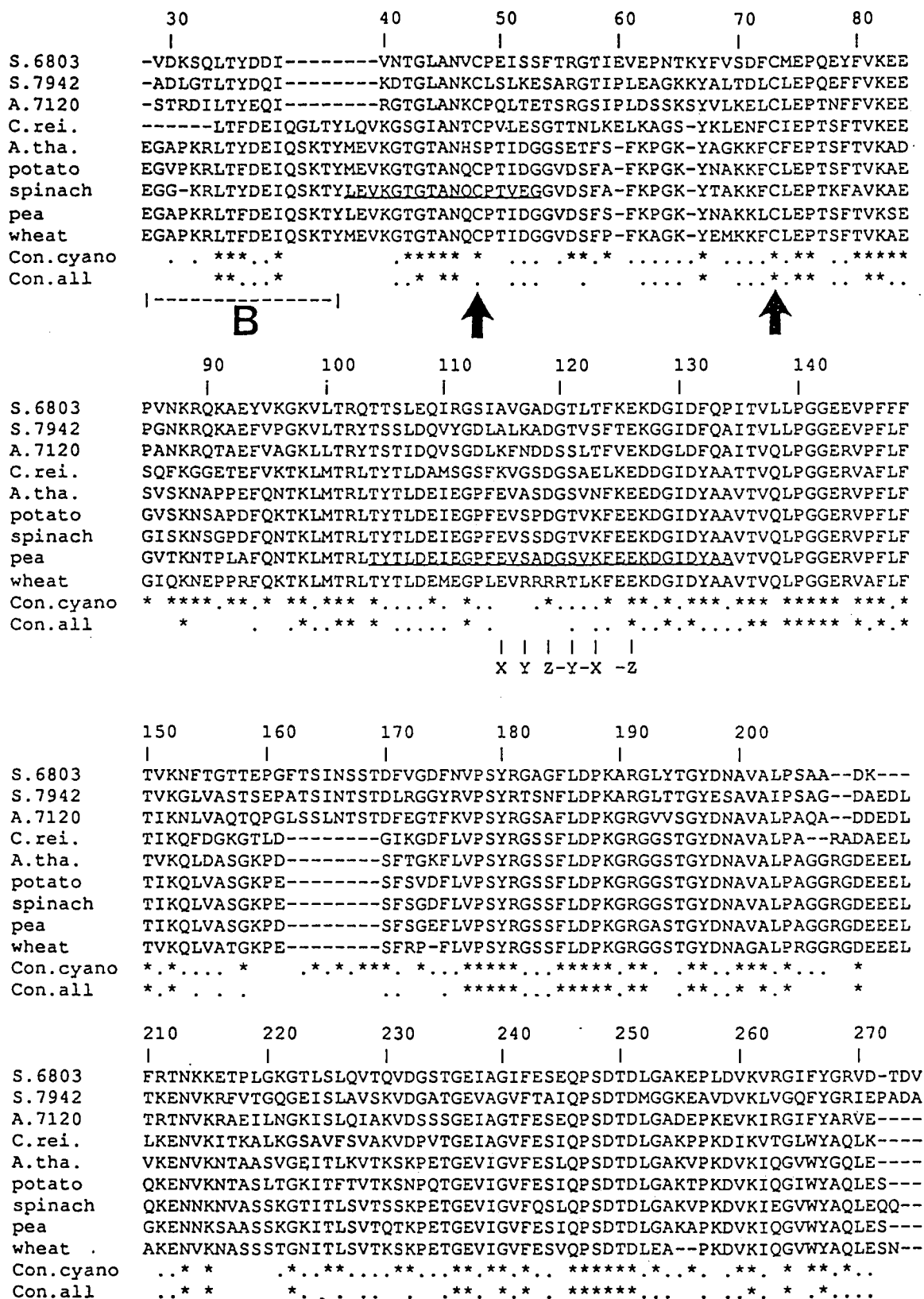


Figure 1.15 Alignment of the mature 33kDa subunit (*psbO*) protein sequences.

This figure is taken from Mayes, (1992). B marks a region involved in the binding of the 33kDa subunit to PSII. Arrows mark cysteine residues which are reported to form a disulphide bridge. X, Y, Z, -X, -Y, -Z are predicted vertices of a putative calcium binding site.

The primary structure of the mature spinach 33kDa protein was first established by direct sequencing (Oh-Oka *et al.*, 1986). Thereafter, a number of 33kDa DNA sequences were reported in both eukaryotes and cyanobacteria. Comparing DNA and mature protein sequences showed that the N-terminus of the pre-protein was absent in the mature 33kDa subunit of wheat. Figure 1.15 shows an amino acid sequence alignment of a number of deduced mature 33kDa extrinsic polypeptide amino acid sequences (Mayes, 1992).

As protease digestion of region B in the spinach 33kDa polypeptide prevented the treated subunit from re-binding to PSII, it appears that this region must act as a binding site to PSII (Eaton-Rye & Murata, 1989). The two cysteine residues (marked with an arrow) are reported to form a cysteine bridge which is essential to the function of the 33kDa extrinsic polypeptide in photosynthetic oxygen evolution (Tanaka & Wada, 1988). The sequence Leu37-Gly53 in spinach was suggested to be a possible Mn binding site because of its sequence similarity to bacterial Mn superoxide dismutases (Oh-Oka *et al.*, 1986). However, later analysis by Wales *et al.*, (1989) suggested it was a Ca^{2+} binding site. Indeed, this latter view does seem more likely in the light of the evidence showing that the 33kDa subunit is not required for the structural integrity of the Mn cluster (see above discussion).

Furthermore, ligand blotting experiments have suggested that the 33kDa subunit may bind Ca^{2+} (Webber & Gray, 1989). The region underlined in the pea sequence seems to form an E-F HAND and bind a Ca^{2+} ion (Wales *et al.*, 1989). Residue marked X, Y, Z, -X, -Y and -Z are predicted vertices of the calcium co-ordinated octahedron.

Ultra-violet circular dichroism (UVCD) measurements showed the ^{secondary} structure of the 33kDa extrinsic subunit to be loose and expansive (Tanaka & Wada, 1988). UVCD and Fourier transform infrared spectroscopy showed it to contain a high proportion of β -sheets and few or no α -helical regions (He, 1991).

From the above results, it is concluded that the 33kDa extrinsic polypeptide plays an important role in maintaining an optimal environment for efficient water photolysis and so should be present in any complexes used for crystallisation trials set up with the ultimate aim of resolving the unique water photolysis reaction of PSII.

1.4.7 The 23kDa extrinsic subunit encoded by the *psbP* gene

The *psbP* gene product is one of the three extrinsic polypeptides associated with the luminal surface of PSII (Andersson & Åkerlund, 1987). Although, the 23kDa extrinsic polypeptide is associated with higher plant PSII, no equivalent polypeptide has been identified in cyanobacteria (reviewed in Erickson & Rochaix, 1992). In spinach the mature polypeptide is formed by the cleavage of 57 amino acids from the N-terminus and consists of 186 residues (Jansen *et al.*, 1987). PSII cores, depleted of the 16 and 23kDa extrinsic polypeptides, show a reduced rate of oxygen evolution, unless supplemented with calcium and chloride ions. Such results have led to the suggestion that the 23kDa subunits regulate the binding affinity of these ions (Andersson *et al.*, 1984; Ghanotakis *et al.*, 1984; Boussac *et al.*, 1985), but calcium and chloride binding sites have not yet been identified.

1.4.8 The 16kDa extrinsic subunit encoded by *psbQ*

The *psbQ* gene product is the smallest of the three extrinsic polypeptides (Andersson & Åkerlund, 1987) associated with the oxygen-evolving complex (i.e. 33, 23 and 16kDa extrinsic polypeptides). Because of discrepancies in the apparent molecular weight of this subunit, the *psbQ* gene product is often referred to as the 17kDa extrinsic subunit. Comparing the N-terminal transit polypeptide motifs of the spinach *psbO* and *psbP* gene products, with that of the spinach *psbQ* gene product, suggests that the latter is cleaved in such a way as to yield a mature protein, 149 amino acids in length. (Jansen *et al.*, 1987). The predicted sequences of spinach (Jansen *et al.*, 1987) and *Chlamydomonas reinhardtii* (Mayfield *et al.*, 1989) are only 28% homologous. The conserved sequences are rich in arginine, lysine and proline residues.

The 16kDa subunit has been implicated in regulating the binding of Ca^{2+} and Cl^- to the donor side of PSII (Andersson *et al.*, 1984; Ghanotakis *et al.*, 1984; Boussac *et al.*, 1985), but no Ca^{2+} and Cl^- binding sites have been identified.

1.4.9 The 22kDa intrinsic polypeptide encoded by *psbS*

The intrinsic 22kDa membrane protein associated with PSII was originally identified by Ljungberg *et al.*, (1984) and Ljungberg *et al.*, (1986a). The nuclear *psbS* gene, which encodes the 22kDa intrinsic polypeptide, has since been identified, sequenced and shown to code for a polypeptide 274 amino acids in length (Wedel *et al.*, 1992; Kim *et al.*, 1992). On finding that core preparations lacking the 22 and 10kDa subunits appeared to be unable to rebind the 23 and 17kDa extrinsic polypeptides, Ljungberg *et al.*, (1986a) suggested that the 22kDa intrinsic polypeptide was involved in binding these extrinsic polypeptides to the PSII core complex.

Preparations lacking the 22 and 10kDa subunits also showed altered acceptor side properties. Such cores support high rates of oxygen evolution in the presence of ferricyanide, but not with PBQ (para-benzoquinone). Ferricyanide is thought to accept electrons from Q_A as its ability to accept electrons from PSII is not impaired by the addition of DCMU, which blocks the Q_B site. PBQ supported oxygen evolution is DCMU sensitive, indicating that it accepts electrons from the Q_B site. As removal of the 22 and 10kDa intrinsic polypeptides interferes with Q_B function, it has been suggested that both subunits may be structurally associated with this plastoquinone binding site (Ikeuchi & Inoue, 1986).

Recent experiments have shown that the 22kDa polypeptide to be a chlorophyll a/b binding protein (Andersson *et al.*, 1994). As the absorption spectrum shows it to have a peak maximum at 674nm and a 77K fluorescence maximum at 675nm, the authors suggest that this protein should be categorised as a chlorophyll a/b (CAB) binding protein. As the 22kDa polypeptide shows considerable sequence homology to CP24, CP26 and CP29, such a classification might indeed be correct.

Interestingly, the 22kDa subunit has been detected both in higher plants and cyanobacteria. It is known that cyanobacteria do not contain chlorophyll b and have replaced the light harvesting

antenna consisting of CP24, CP26, CP29 and LHCII with phycobiliproteins. Thus, it is suggested that the 22kDa polypeptide should be categorised as a CAB ancestor.

1.4.10 The *psbH* gene product

The *psbH* gene product was originally isolated from spinach preparations and its N-terminal sequence determined (Farchaus & Dilley, 1986). The second threonine residue of the spinach (Michael & Bennet, 1987) and *Chlamydomonas reinhardtii* (Dedner *et al.*, 1988) *psbH* gene product was shown to be phosphorylated. However, this threonine residue and the consensus sequence flanking it, is missing in the cyanobacteria *Synechocystis* 6803 and *Synechococcus vulcanus* (Mayes & Barber, 1991; Abdel-Mawgood & Dilley, 1990; Koike *et al.*, 1989). The gene for this polypeptide was subsequently identified in the chloroplast genomes of wheat (Hird *et al.*, 1986) and spinach (Westhoff *et al.*, 1986), by comparison to the amino acid sequence data. The *psbH* gene product is thought to be composed of 72 amino acids and contains one membrane spanning sequence close to its C-terminal end. N-terminal sequencing of the higher plant H protein has shown it to lack the N-terminal methionine residue (Farchaus & Dilley, 1986).

In 1988, Packham suggest that the phosphorylation of the H protein, under conditions in which the plastoquinone pool is reduced, stabilises Q_A^- . This was thought to alter electron transfer between Q_A and Q_B , thereby protecting PSII against photoinhibition. Furthermore, Packham, (1988) suggests that in its dephosphorylated state, the *psbH* gene product fulfils a role similar to that of the H subunit of the bacterial reaction centre, by facilitating photochemical charge separation. However, as the nucleotide sequence (Abdel-Mawgood & Dilley, 1990; Mayes & Barber, 1991) and amino acid (Koike *et al.*, 1989) of this protein from cyanobacteria, showed that the N-terminal threonine was absent in the cyanobacterial *psbH* gene product, the role of phosphorylation as part of a mechanism to control electron transfer between Q_A and Q_B was brought into question. Recently, a mutant of *Synechocystis* 6803 has been constructed, which has had the *psbH* gene deleted (Mayes *et al.*, 1993). The mutant had a fully assembled and active PSII complex and grew photoautotrophically. However, in agreement with Packham's conclusions this deletion mutant had modified Q_A to Q_B transfer. Moreover, it was highly sensitive to photoinhibition. These results suggested that the H subunit protects against photoinhibition and this conclusion is supported by the results of Kuhn *et al.*, (1988).

1.4.11 The *psbI* gene product

The nucleotide sequence of the *psbI* gene has been reported in tobacco (Shinozaki *et al.*, 1986), maize (Katoh *et al.*, 1987), wheat (Howe *et al.*, 1988), liverwort (Umesono *et al.*, 1988), rice (Hiratsuka *et al.*, 1989) and barley (Sexton *et al.*, 1990). This gene is part of the *psbK-I* operon which in the monocotyledons, maize, rice and wheat is located upstream of the *psbD-C* operon (Katoh *et al.*, 1987; Howe *et al.*, 1988; Hiratsuka *et al.*, 1989). In the dicotyledons, tobacco and liverwort, this does

not appear to be the case (Shinozaki *et al.*, 1986; Umesono *et al.*, 1988). The *psbI* gene of the cyanobacterium *Anacystis nidulans* 6301 has also been identified, sequenced and found not to be linked to the *psbK* gene (Chen *et al.*, 1990). The *psbI* gene codes for a polypeptide of 36 amino acids in higher plants, but 39 in the cyanobacterium *Anacystis nidulans* 6301 (Chen *et al.*, 1990). Analysis of isolated reaction centres showed them to be associated with the 4.8kDa I protein, in nearly stoichiometric proportions to the other polypeptides in the complex (Ikeuchi & Inoue, 1988b; Webber *et al.*, 1989b). This polypeptide was isolated from spinach and subjected to N-terminal sequencing (Ikeuchi & Inoue, 1988a) and appeared not to undergo N-terminal processing, as the first amino acid of the mature polypeptide is methionine. Hydropathy analysis of the tobacco *psbI* gene product predicts a structure with a single membrane spanning region of 21 amino acid residues (Ikeuchi & Inoue, 1988a). Despite its close association with the PSII reaction centre, the function of the *psbI* gene product is not known. The isolation of a reaction centre consisting of the D1 and D2 proteins, but lacking cytb559 and the product of the *psbI* gene indicates that this polypeptide does not function in the photochemical reaction (Tang *et al.*, 1990).

1.4.12 The *psbJ* gene product

A review of the literature has shown that a conserved reading frame (ORF) encoding a polypeptide 40 amino acids in length is cotranscribed with *psbE*, *psbF* and *psbL* (Erickson & Rochaix, 1992). Based on its location in this polycistronic transcript, this open reading frame (ORF40) was called *psbJ* (for review see Erickson & Rochaix, 1992). It codes for 40 amino acid residues, the last 30 of which are highly hydrophobic, suggesting that the *psbJ* gene product is anchored in the membrane.

Mutants of *Synechocystis* 6803, lacking a functional *psbJ* gene product, were shown to have reduced growth rates as compared to the wild type cells. Oxygen evolution rates, driven by mutant PSII cores, were 60% lower than those of the wild type. The mutant PSII core complexes were also shown to be DCMU sensitive, suggesting that their Q_B site was still intact (Nyhus & Pakrasi, 1989).

1.4.13 The *psbK* gene product

The N-terminal sequence of the *psbK* gene product has been determined in spinach (Murata *et al.*, 1988; Schröder *et al.*, 1988; Ikeuchi *et al.*, 1989a), wheat (Ikeuchi *et al.*, 1989a) and in *Synechococcus* (Koike *et al.*, 1989). Using this sequence data, Murata *et al.*, (1988) located the open reading frame coding for the *psbK* gene product.

The *psbK* gene has been found to be located just upstream of *psbI* gene in the chloroplast genomes of tobacco (Deno & Sugiura, 1983), liverwort (Umesono *et al.*, 1988), rice (Hiratsuka *et al.*, 1989), mustard (Neuhaus & Link, 1990) and barley (Sexton *et al.*, 1990). This gene has also been identified and sequenced from *Anacystis nidulans* (Fukuda *et al.*, 1989), *Chlamydomonas reinhardtii* (Silk *et al.*, 1990) and *Synechocystis* 6803 (Zhang *et al.*, 1990). By comparing the gene sequence and N-terminal sequencing data, the mature *psbK* gene product was calculated to be 37 amino acids in length and to have an N-terminal lysine residue, indicating its post-translational modification (Murata *et*

al., 1988). Murata *et al.*, (1988) identified a single putative membrane spanning segment. Mature tobacco, mustard and barley sequences show a homology of 95-97%, though this is far lower in the N-terminal leader sequence which is post-translationally cleaved (reviewed in Erickson & Rochaix, 1992).

The *psbK* gene product remains associated with PSII when LHCII is removed, but is absent in more highly purified oxygen-evolving core preparations (Murata *et al.*, 1988). Recently, *psbK* deletion mutants were constructed in *Synechocystis* 6803 (Ikeuchi *et al.*, 1991; Zhang *et al.*, 1993) and showed only slight differences in their photosynthetic activity, when compared with that of the wild type. It was therefore suggested that the K polypeptide plays a structural rather than functional role in the water oxidation complex of PSII.

1.4.14 The *psbL* gene product

Two small open reading frames, encoding 38 (ORF38) and 40 (ORF40) amino acids have been located just downstream of the *psbE* and *psbF* genes in tobacco (Shinozaki *et al.*, 1986), *Euglena gracilis* (Cushman *et al.*, 1988), *Cyanophora paradoxa* (Cantrell & Bryant, 1987), liverwort (Fukuzawa *et al.*, 1988) and rice (Hiratsuka *et al.*, 1989). Although these two small ORFs were originally called *psbI* and *psbJ* (Cushman *et al.*, 1988) respectively, this notation has been abandoned since the same name has been given to another ORF38 (Kato *et al.*, 1987). To avoid confusion, ORF38 has been named *psbL* (Webber *et al.*, 1989a). Although the *psbL* gene product was originally identified in an oxygen-evolving core complex isolated from spinach and wheat (Ikeuchi *et al.*, 1989a) and shown to have an apparent molecular weight of 5kDa, its predicted molecular weight is 4.3kDa (Ikeuchi *et al.*, 1989a; Webber *et al.*, 1989a). The mature polypeptide is 37 amino acids in length after the N-terminal methionine residue is cleaved off to expose Thr2 (Ikeuchi *et al.*, 1989a). 21 of the 22 C-terminal amino acids form a hydrophobic region which may span the membrane. Recent studies have suggested that the *psbL* gene product may be required for normal functioning of the Q_A site, since Q_A activity decreases dramatically, when PSII core complexes are depleted of this polypeptide (Nagatsuka *et al.*, 1991). A 4.1kDa polypeptide may also be involved in this process (Nagatsuka *et al.*, 1991).

1.4.15 The *psbM* gene product

Ikeuchi *et al.*, (1989b) identified a 4.7kDa polypeptide associated with oxygen-evolving cores, isolated from a thermophilic cyanobacterium, *Synechococcus vulcanus*. The N-terminal sequence of this polypeptide matched the predicted product of ORF34 in the tobacco, rice and liverwort chloroplast genomes, allowing it to be identified as the *psbM* gene (Ikeuchi *et al.*, 1989b). The predicted proteins have an absolute homology of 85% (reviewed in Erickson & Rochaix, 1992) but, many of the substitutions are conservative. A single membrane spanning region (residues 3-28) has been predicted in the deduced polypeptide sequences of tobacco and spinach (Ikeuchi *et al.*, 1989b). The predicted molecular weights of the tobacco and liverwort M subunits are 3.8 and 3.7kDa respectively (Erickson & Rochaix, 1992). The function of this polypeptide is not known.

1.4.16 The *psbN* gene product

The *Synechococcus vulcanus psbM* gene product co-migrates with another polypeptide in polyacrylamide gels, containing urea (Ikeuchi *et al.*, 1989b). The authors sequenced 18 of the 19 N-terminal amino acid residues of the *psbN* gene product and deduced that it was encoded for by ORF 43. ORF 43 is located in the chloroplast of tobacco, rice and liverwort, between the *psbB* and *psbH* gene products, but on the complementary DNA strand (reviewed in Erickson & Rochaix, 1992). Mayes & Barber, (1991) showed that the *psbN* gene is closely located to the *psbH* gene in *Synechocystis* 6803. ORF43 was thus designated as *psbN*. Although, the predicted tobacco and liverwort polypeptides are 86% homologous, their similarity to the cyanobacterial polypeptides is lower. Indeed, the deduced higher plant and cyanobacterial *psbN* sequences vary in 10 out of the 19 amino acids. Even so, the substitutions are conservative and structural predictions identify a potential membrane spanning region near the N-terminus in all cases (reviewed in Erickson & Rochaix, 1992).

PsbN transcription is light or developmentally-induced and results in monocistronic mRNA being synthesised in liverwort and pea (Kohchi *et al.*, 1988). As the *psbN* gene lies close to the *psbB* gene operon, which codes for the *psbB*, *psbH*, *petB* and *petD* products, but on the complementary DNA strand, the *psbN* gene may be involved in regulating the expression of this operon. The *psbN* gene product has not yet been detected in higher plants, but its deletion in *Synechocystis* 6803 did not prevent the assembly of PSII or photoautotrophic growth (Mayes *et al.*, 1993).

1.4.17 The 4.1kDa polypeptide

A 4.1kDa polypeptide was detected in the oxygen-evolving PSII core preparations from wheat and spinach (Ikeuchi *et al.*, 1989a) and the cyanobacterium *Synechococcus vulcanus* (Ikeuchi *et al.*, 1989b). As the gene encoding this polypeptide has not been located in the chloroplast genome it has been suggested that the 4.1kDa protein is nuclear encoded. Although, these three polypeptides show low degrees of homology, a lysine residue and predicted membrane spanning sequence appear to be conserved.

The 4.1kDa protein has been shown to be closely associated with cyanobacterial reaction centres (Ikeuchi *et al.*, 1989b). Recent studies on the function of the low molecular weight subunits of PSII have suggested that this polypeptide may be a component required to maintain normal Q_A function along with the *psbL* gene product (Nagatsuka *et al.*, 1991).

1.4.18 The 5kDa extrinsic polypeptide

A 5kDa hydrophilic protein associated with oxygen-evolving core complexes has been purified, characterised and shown to contain no metals or obvious prosthetic groups (Ljungberg *et al.*, 1986b). Despite its apparent hydrophilicity, it was not released from inside-out PSII-enriched membrane vesicles by salt treatments which usually release extrinsic polypeptides. Ikeuchi *et al.*, (1989a), detected this subunit in oxygen core complexes of wheat and determined its N-terminal sequence. However, as yet its function is unknown.

1.4.19 The 6.1kDa polypeptide

Two polypeptides of 7 and 6kDa have been detected in oxygen-evolving PSII core complexes of spinach (Ljungberg *et al.*, 1986b) and their N-terminal sequences determined (Schröder *et al.*, 1988). These sequences appear to be the same and identical to that of a 6.1kDa polypeptide identified in oxygen-evolving core complexes of wheat (Ikeuchi *et al.*, 1989a). As the N-terminal sequence does not correspond to any part of the tobacco and liverwort chloroplast genome, it may be encoded by a nuclear gene.

1.5 The oligomeric nature of PSII

The aggregation state (i.e. monomeric or dimeric) of PSII *in vivo* is still a matter of debate. This subject is discussed here before reviewing some of the models of subunit organisation in Section 1.6.

In 1978, Miller and Cushman, presented electron micrographs of thylakoid membranes isolated from wild type and PSII-deficient mutants of tobacco. Electron micrographs of freeze-fractured thylakoids, showed a tetramer like complex on the luminal surface of wild type membranes. As such particles were absent from the PSII deficient mutants, it was concluded that they were PSII complexes. Siebert *et al.*, (1987) also detected tetramer-like structures protruding from the luminal surfaces of spinach thylakoids. These complexes were estimated to have a height of 8.2nm, perpendicular to the membrane. Sequential removal of the 17kDa, 23kDa and 33kDa extrinsic polypeptides reduced this height to 7.8nm, 7.4nm and 6.1nm, respectively. The removal of all three extrinsic particles revealed the membrane embedded portion of the complex, which appeared to have a dimeric structure. This led the authors to conclude that the extrinsic polypeptides gave the luminal surface of PSII its tetrameric appearance, but that the membrane embedded portion was actually a dimeric PSII-LHCII complex. However, this had to be confirmed by structural studies yielding images of higher resolution.

The granal stacks of the thylakoid membranes analysed by Siebert *et al.*, (1987) contained regions in which ordered PSII arrays could be seen. Although, these arrays were too small to yield meaningful electron^{microscopy} data, Bassi *et al.*, (1989), Holzenburg *et al.*, (1993) and Lyon *et al.*, (1993) were able to increase their size, by subjecting isolated granal stacks to a limited delipidation step. This treatment forced the PSII complexes within the membrane into larger ordered arrays. The two-dimensional arrays reported by Bassi *et al.*, (1989) consisted of complexes having antiparallel two-fold symmetry and showing apparent two-fold symmetry at a 5nm resolution. The apparent symmetry of these complexes led the authors to conclude that they were dimeric complexes of PSII. Each of these complexes contained four prominent regions of protein density and so may explain the tetramer-like appearance of the luminal surface of PSII reported by Miller & Cushman, (1978) and Siebert *et al.*, (1987). These four regions of protein density surrounded a central hole or cavity. Ordered arrays of Tris-washed particles, which lacked the 33, 23 and 17kDa extrinsic polypeptides

were also reported by Bassi *et al.*, (1989). These particles showed two (one per monomer), rather than four, major stain-excluding regions and, once again, this agreed with the results obtained by Siebert *et al.*, (1987).

Lyon *et al.*, (1993) also obtained ordered two-dimensional arrays of PSII-LHCII complexes, lacking the extrinsic polypeptides, by delipidating grana stacks. Using electron diffraction studies, these complexes were once again shown to have two-fold antiparallel, rotational symmetry, at a resolution of 1.7nm. The unit cell of the complex was reported to be 11.5 x 16.1nm in the plane of the membrane. From the images, it was concluded that the two constituent monomers, of which the dimer was thought to be composed, surrounded a central hole. Each monomer contained one major stain excluding region, and were similar in this respect to the images of the Tris-washed particles reported by both Siebert *et al.*, (1987) and Bassi *et al.*, (1989).

Although the structures reported by Bassi *et al.*, (1989) and Lyon *et al.*, (1993), appear to be dimeric, pseudo-symmetry can result in incorrect conclusions being drawn about the aggregation state of a complex. For example, D1 and D2 show a considerable amount of homology having 5 trans-membrane helices each and molecular weights of 38 and 39.4kDa respectively. CP47 and CP43 also have similar molecular weights (55.6 and 50.1kDa, respectively) and both are thought to have six trans-membrane helices and one large lumenally exposed loop. As cytb559 is a relatively small component of PSII, it is possible that a monomeric complex consisting of CP47, CP43, D2, D1 and cytb559 could show pseudo-symmetry and so result in its incorrect identification as a dimer, at low resolution. Indeed, Holzenburg *et al.*, (1993) suggested that their ordered arrays of PSII, prepared by delipidating BBYs, did not show true symmetry. As these particles showed, in some projections, four stain excluding regions surrounding a central hole and were similar in appearance in this respect to the particles reported by Bassi *et al.*, (1989) and Lyons *et al.*, (1993) more structural detail will be required to prove which model is correct. With this dilemma in mind, it is important to consider studies on isolated monomeric and dimeric cores.

Monomeric and dimeric PSII complexes consisting of CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic polypeptide, were first isolated from *Synechococcus elongatus* (Rögner *et al.*, 1987) and were shown to have molecular weights of 300 and 500kDa in the presence of DM, respectively.

Of all the higher plant core preparations listed in Table 3.1, only that of Haag *et al.*, (1990) has been subjected to any form of molecular weight analysis. FPLC size exclusion analysis showed these core particles (CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic polypeptide) to have a molecular weight of 270kDa. This value correlates closely to the theoretical molecular weight (250kDa, see Table 3.3), of a monomeric complex of this subunit composition.

Peter & Thornber, (1991a) resolved two PSII complexes, by non-denaturing gel electrophoresis. They had apparent molecular weights of 140 and 230kDa, respectively. Both particles contained CP47, CP43, D2, D1 and cytb559. The larger core particle was also associated with the 33kDa subunit and evolved oxygen. The theoretical molecular weight of a dimeric higher plant (CP47, CP43, D2, D1, cytb559 and 33kDa extrinsic polypeptide) PSII complex is about 500kDa (see Table

3.3) and so varies quite markedly from the 230kDa value calculated from this gel electrophoresis measurement. Although Peter & Thornber, (1991a) suggested that the two particles were monomeric and dimeric complexes of PSII, this can not be confirmed from their molecular weight estimations.

Sucrose gradient centrifugation has also been used to isolate two types of PSII core particle from higher plants, but consisting of the same subunit composition, namely of CP47, CP43, D2, D1 and cytb559, with markedly different densities (Barbato *et al.*, 1989). Although, these particle types were suggested to be monomeric and dimeric forms of PSII, this was not confirmed by direct analysis of their respective molecular weights.

The above studies show that although monomeric and dimeric PSII core particles have been isolated from cyanobacteria, the existence of higher plant PSII dimers still awaits confirmation. Nor do the results provide a conclusive answer as to whether PSII is in fact monomeric or dimeric *in vivo*. Supporters of the monomer theory can claim that dimers are an artifact of purification while the dimer theory proponents suggest that monomers are the product of dimer dissociation. This dilemma can ultimately be resolved by the direct visualisation of monomeric or dimeric complexes in electron micrographs of the thylakoid membrane.

1.6 Organisation of the major PSII and chlorophyll a/b binding proteins composing the PSII-LHCII supercomplex

PSII is closely associated with a number of chlorophyll a/b binding proteins to form what is referred to here as a PSII-LHCII supercomplex. These polypeptides make up a light harvesting antenna system, whose function it is to collect sunlight and transfer the derived excitation energy to PSII, in a controlled manner. The antenna system is able to adapt to heat stress (Larsson *et al.*, 1987), cold stress (Hayden *et al.*, 1986) and light induced state-1/state-2 transitions (Allen *et al.*, 1981; Trissl & Wilhelm, 1993). The state-1/state-2 transition is a process whereby the plant balances the rate of electron transfer through photosystems I and II, to optimise the rate of NADPH and ATP production.

This section will review some of the models illustrating the subunit organisation within the PSII-LHCII supercomplex, but before doing so the chlorophyll a/b binding proteins and their nomenclature will be very briefly reviewed.

Six chlorophyll a/b binding proteins are thought to be associated with the light harvesting antenna of PSII. These subunits are often referred to as the CAB proteins (CP24, CP26 and CP29) since they are encoded by nuclear *cab* genes and the components of LHCII (25, 27 and 28kDa). All have three transmembrane helices and bind varying amounts of chlorophyll a, b, β -carotene and xanthophylls (Dainese *et al.*, 1992b; Janssen, 1994). The CAB proteins have been shown to be more closely associated with PSII than the 25, 27 and 28kDa components of the LHCII fraction as they remain bound to the core complex under conditions which result in LHCII dissociation (see Table 3.1). Several pieces of evidence point to CP24, CP26 and CP29 being involved in transferring excitation energy from the LHCII components to PSII. The first is that the LHCII components bind

50-65% of the chlorophyll associated with PSII, while the CP proteins together only bind 20% of this pigment type (Peter & Thornber, 1991b; Green *et al.*, 1992). Clearly, the larger number of chlorophylls organised by the LHCII subunits would suggest that it is these polypeptides which act as the predominant light harvesting components of the antenna system. Thus, although it is likely that the CP proteins can harvest light energy as well, their major role is thought to be one of facilitating controlled excitation energy transfer from LHCII to PSII. The finding that the absorption spectra of CP24, CP26 and CP29 are blue shifted with respect to those of the PSII core components is consistent with this hypothesis (Bassi *et al.*, 1987; Bassi & Wollman, 1991; Bassi & Dainese 1992). Uncontrolled excitation energy transfer could, however result in an excessively high rate of P680 turnover and so in either rapid acceptor or donor side photoinhibition and subsequent D1 damage (see Figure 1.10). This would clearly be undesirable and so it is interesting to note that the CP proteins bind 78% of the total violoxanthin associated with a PSII-LHCII supercomplex. Strong illumination converts violoxanthin to xeaxanthin, which in turn is able to dissipate excess excitation energy, which if transferred to P680 could result in photoinhibition (Demmig-Adams, 1990). This result is important in that it lends support to the theory that the CP proteins are not only involved in transferring excitation energy but in controlling its rate of transfer to PSII.

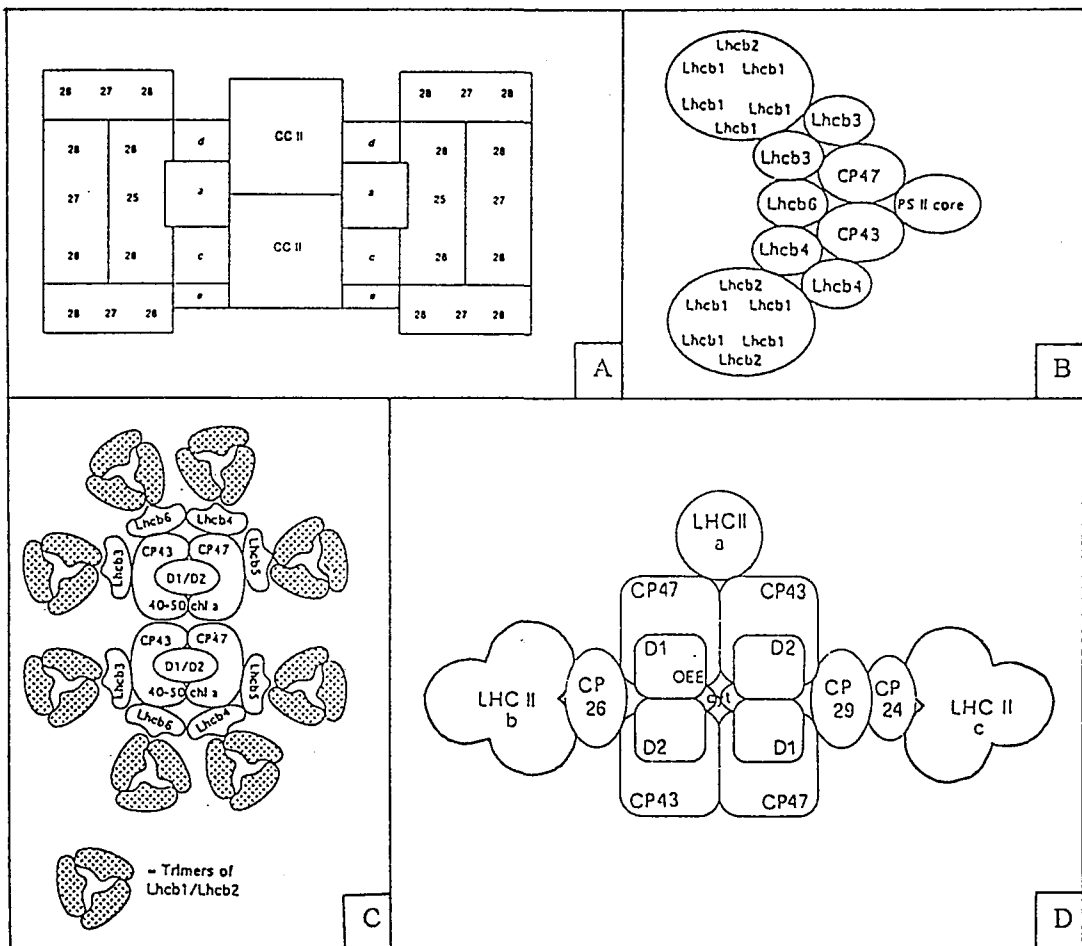
Studies of the CAB proteins have been complicated by the fact that they exhibit striking sequence homology. This in turn results in antibodies raised against a specific chlorophyll a/b binding protein, cross-reacting with other polypeptides belonging to the CAB and LHCII class. The similarity of their sequences has also hampered their separation by SDS-PAGE. As a result, there has until recently been a considerable amount of confusion in the literature, regarding the nomenclature of these polypeptides. Before presenting models of the possible organization of the subunits composing the PSII-LHCII supercomplex, this nomenclature must be reviewed. Table 1.2 is taken from the review of Janssen, (1994) in which the author has clarified the different nomenclature.

Table 1.2 A conversion table relating the nomenclature of Janssen, (1994), Green *et al.*, (1991), Peter & Thornber, (1991b), Bassi *et al.*, (1990) and Harrison & Melis, (1992).

Gene product	Green <i>et al.</i> , 1991	Peter & Thornber, (1991b)	Bassi <i>et al.</i> , (1990)	Harrison & Melis, (1992)
Lhcb1	Type I LHCII	LHCII b 28kDa	LHCII	b
Lhcb2	Type II LHCII	LHCII b 27kDa	LHCII	c
Lhcb3	Type III LHCII	LHCII b 25kDa	LHCIIa	d?
Lhcb4	Type II CP29	LHCII a	CP29	a
Lhcb5	Type I CP29	LHCII c	CP26	?
Lhcb6	CP24	LHCII d	CP24	e

Figures 1.15A-D illustrate 4 of the more recent models of subunit organisation within a PSII-LHCII supercomplex. Table 1.2 allows the inter conversion of the nomenclature used in the diagrams.

Having discussed the current debate on whether PSII is actually a monomeric or dimeric complex *in vivo* in the previous section, this topic will not be entered into again here. It is interesting to note that a similar debate is currently in progress concerning the existence of monomeric and trimeric forms of LHCII (Janssen, 1994). Kühlbrandt *et al.*, (1994) have recently published a 3.4Å resolution structure of an LHCII homotrimer consisting of three 28kDa LHCII subunits. However, whether or not this homotrimer exists *in vivo* remains to be seen. Peter & Thornber, (1991b) suggested the existence of a two LHCII heterotrimer types (28:27:28 and 28:25:28kDa) and that they are associated with PSII according to the model shown in Figure 1.16A. Although the models shown in Figure 1.16A-D all suggest the existence of the 28:27:28 trimer type, Model 1.15A is the only one to suggest that the 25kDa LHCII component is ever present as part of a trimeric LHCII complex.



Published subunit cross-linking data are still not comprehensive enough to allow the positions of the core subunits composing the PSII complex to be unequivocally located. These cross-linking studies are detailed in Section 6.3.4, and so will not be elaborated on here. However, in summary there are at present two favoured models: In the first, the reaction centre components are placed symmetrically between CP43 and CP47. In the second, a linear CP43-CP47-reaction centre model is proposed. Either way, the CP43 and CP47 subunits are likely to be involved in transferring excitation energy from the CAB proteins to P680. Bricker, (1990) has tentatively proposed CAB protein docking sites on the stroma side of CP43. Indeed, cross-linking studies showed that CP26 and CP29 are both in close contact with CP43, as well as CP47 (Dainese *et al.*, 1992a). As the model of Peter & Thornber, (1991b) shown in Figure 1.16A, does not depict the subunits composing the central PSII core dimer, the results of this cross-linking study cannot be used to validate it. While the model shown in Figure 1.16D does take these interactions into account, Figures 1.15B and C do not. Based on this result alone, the model depicted in Figure 1.16D seems to fit the available experimental information best. It has another interesting feature; during the state 1/state 2 transition, a 'mobile' LHCII fraction dissociates from PSII, following the phosphorylation of the LHCII components (Barber, 1982a). However, some LHCII ('bound fraction') remains associated with the core complex. Interestingly, Bassi & Dainese, (1992), isolated a CP29-CP24-(28:27:28 LHCII trimer) complex which dissociated on phosphorylation. Thus, it is possible that the LHCII trimer connected to PSII via CP29 and CP24 in Figure 1.16D, represents the mobile LHCII fraction. No information is currently available on the function of the other two putative LHCII trimers depicted in Figure 1.16D. However, based on the experimental evidence available, it is not possible to discount the possibility that two sets of CP24, CP26 and CP29 flank a central dimeric complex.

1.7 Summary

This chapter has outlined the reactions catalysed by PSII as part of the photosynthetic electron transport chain (Section 1.3). Our current knowledge on the subunits associated with PSII, their structure, function and location was reviewed in Section 1.4. A literature review on the aggregation state of PSII *in vivo* is provided in Section 1.5 while Section 1.6 presents some of the current models of subunit organisation within a PSII-LHCII supercomplex. From the literature reviewed in this chapter, it is apparent that considerable amount of biochemical and biophysical information relating to the structure of PSII has been gained over the passed few years. However, the models of PSII shown in Figure 1.16 serve to illustrate how little we do in fact know about the three-dimensional structure of this complex which is able to drive the unique water photolysis reaction. Such structural detail can currently only be obtained from highly ordered crystals. A prerequisite to such crystals being obtained is the availability of pure, intact and active oxygen-evolving cores. The introduction to Chapter 3 describes the lack of information on isolated PSII core complexes, regarding these factors. To rectify this situation two distinct core preparations were subjected to detailed characterisation (Chapters 3 and 4). The information gained was used to develop novel core preparation procedures which allowed the

isolation of pure monomeric and dimeric cores differing in subunit composition (Chapter 5). Biochemical characterisation of these distinct core types and their subsequent study by electron microscopy and single particle analysis (Chapter 6), provides not only a useful insight into the suitability of these complexes for two- and three-dimensional crystallisation trials, but yielded structural information at a resolution of 25Å. This information has enabled me to propose possible models of subunit organisation not solely on the basis of cross-linking studies but by direct visualisation. Chapters 7 and 8 present data obtained from preliminary two- and three-dimensional crystallisation trials. Though preliminary, these trials provide useful information for future crystallisation studies. Suggestions for future work are presented in Chapter 9.

Chapter 2 Methods

2.1 PSII particle isolation

2.1.1 Isolation of sub-chloroplast PSII enriched membranes (BBYs)

Pisum sativum var. 'Feltham First' seeds were sown densely into 8 (20 x 30cm) trays filled with vermiculite and grown under greenhouse conditions, for 14 days. The seedlings were then incubated in the dark for 16h before harvesting their leaves. Subsequent operations were performed in dim light. The leaves were suspended in 1l of ice cold grind medium (50mM KH_2PO_4 pH7.5, 0.35M KCl, 0.5mM EDTA) before being homogenised three times (10s) at high speed in a Waring blender. The homogenate was filtered through a layer of cotton wool sandwiched between 8 layers of muslin, the filtrate collected and centrifuged for 10min at 8000g (4°C). The pellets obtained by this centrifugation step were gently re-suspended in 500ml of SNT buffer (0.2M sucrose, 0.1M NaCl, 50mM tricine pH8.0, 5mM MgCl_2) before being centrifuged for a further 15min at 8000g (4°C). This pellet was enriched in thylakoid membranes. To isolate the PSII enriched granal stacks from these thylakoids, the membrane pellets were first re-suspended in HSB buffer (5mM MgCl_2 , 15mM NaCl, 2mM Mes pH6.5) so as to give a final chlorophyll concentration of $1\text{mg}\cdot\text{ml}^{-1}$. This thylakoid membrane suspension was incubated in the dark and on ice for 60-90min. A volume of 10% (v/v) Triton X-100 dissolved in HSB buffer, was then added to the thylakoid suspension, so as to give a final detergent concentration of 3.3%. The mixture was then gently stirred on ice and in the dark for precisely 30 min. The granal stacks were then pelleted by subjecting this mixture to a 20,000g, 35min centrifugation step at 4°C. The pelleted granal stacks were then re-suspended in MAGIC buffer (20mM Mes pH6.3, 5mM MgCl_2 , 15mM NaCl, 10% w/v glycerol, before being frozen in liquid nitrogen and stored at -80°C.

2.1.2 Isolation of oxygen-evolving PSII cores from peas

Oxygen-evolving cores isolated from pea (*Pisum sativum*), were purified using a method based on that of De Las Rivas *et al.*, (1992a). Essentially, the method used was as follows. BBYs (15mg chlorophyll) were washed in 40mM Mes-NaOH (pH 6.0), 0.5M sucrose and re-suspended in 3.0ml of a buffer containing 72mM Mes-NaOH (pH 6.0), 1.8M sucrose, 72mM MgCl_2 , 18mM NaCl. To this was added 2ml of 10% n-heptyl-thioglucopyranoside (HTGP) to obtain a final detergent concentration of 4% and a chlorophyll concentration of $3\text{mg}\cdot\text{ml}^{-1}$. The mixture was homogenised, incubated on ice in the dark for 1h, subjected to a 2.5 fold dilution with the first buffer and then centrifuged at 30,000g for 15min at 4°C. The supernatants were decanted, rediluted 2 fold with the first buffer without sucrose and centrifuged at 175,000g for 2h at 4°C. After this centrifugation a dark green pellet of LHCII was obtained. The pale green supernatant was again diluted twice in the first buffer without sucrose and centrifuged at 150,000g for 1h at 4°C to obtain a PSII core pellet.

2.1.3 Isolation of PSII reaction centres from peas

The procedure used here for the isolation of PSII reaction centres (D1, D2, cytb559 and the *psbI* gene product) was a modification of that reported by Gounaris *et al.*, (1991); this method originated from the work of Nanba & Satoh, (1987), which basically employs Triton X-100 as a detergent for separation of the PSII reaction centre proteins from other intrinsic membrane proteins. For standard 'full size' preparations, 200mg chlorophyll of BBYs were used. All operations were performed in low light and at 4°C.

To remove any extrinsic proteins from the BBYs, they were diluted to 0.8mg.ml⁻¹ chlorophyll in 50mM Tris-(hydroxymethyl)-aminomethane-HCl (Tris-HCl) (pH 9.5) and homogenised to ensure uniform suspension. Pellets were collected by centrifugation at 40,000g for 20min and re-suspended in 50mM Tris-HCl (pH 7.2). Homogenisation was carried out again, and the sample then solubilised by the addition of 30% w/v Triton X-100 stock (at room temperature) resulting in a detergent to chlorophyll ratio of 50:1 (w/w). The solution was homogenised thoroughly, diluted to 0.8mg.ml⁻¹ chlorophyll and stirred on ice in the dark for 1h. After solubilisation, the insoluble material was removed by centrifugation at 48,000g for 90min. The pellets were discarded, and the supernatant loaded (flow rate 1.2-1.5 ml.min⁻¹) onto a 16 x 300mm column containing Fractogel TSK DEAE-650 (S) (Merck-BDH), which had previously been equilibrated with 50mM Tris-HCl (pH 7.2), 0.2% (w/v) Triton X-100, 1mM DM (Tris-TX). When loading was completed, the flow rate was changed to 0.4 ml.min⁻¹ and the column washed with a large volume of Tris-TX, also containing 40mM NaCl. The loading and washing steps resulted in the removal of more than 95% of the initial chlorophyll. The column was eluted with 150ml of 40-400mM NaCl gradient in Tris-TX (flow rate 0.6 ml.min⁻¹) and the absorption spectrum of each fraction determined; the main peak of interest usually eluted at around 110mM NaCl. Those fractions with acceptable absorption characteristics were combined. A second column (9 x 100mm) was prepared containing Fractogel, as above, and equilibrated with 50mM Tris, pH 7.2, 2mM dodecyl maltoside (DM) (Tris-DM). The combined acceptable fractions from the first column were diluted four-fold with Tris-DM, then loaded onto the column at a flow rate of 0.7ml.min⁻¹. Washing was carried out with 60mM NaCl in Tris-DM, and the column eluted with 60-400mM NaCl gradient in Tris-DM (flow rate 0.5ml.min⁻¹). Each fraction was checked for purity on the basis of the absorption characteristics, and acceptable fractions were combined for the final preparation analysis. The 'red peak' was accurately determined, and the chlorophyll and cytochrome concentrations assessed. The completed preparation was then subdivided, and flash-frozen for storage at -80°C.

2.1.4 Isolation of the CP47-D1-D2-cytb559 complex and CP43 from peas

The methods used to purify the CP47-D1-D2-cytb559 complex and CP43 are described below and were modified from the procedures developed by Ghanotakis *et al.*, (1987a) and Dekker *et al.*, (1989).

BBYs (125mg chlorophyll) isolated from pea (*Pisum sativum*) leaves (see Section 2.1.1), were washed with solution A (50mM Mes, 10mM NaCl, 0.4M sucrose) and precipitated by centrifugation

(48,400g for 15min, at 4°C). The pellet was re-suspended in solution A (50ml) and homogenised with five passes of the plunger. The chlorophyll concentration of the homogenised solution was then measured (Arnon, 1949) and corrected to 2.5mg.ml⁻¹ with solution A. The sample was then mixed with an equal volume of solution B (50mM Mes pH6.0, 0.8M NaCl, 10mM CaCl₂, 1.0M sucrose, 70mM octyl glucoside (OG) (Calbiochem) and homogenised 10 times. Following this the sample was incubated (4°C for 15min) in the dark with continuous stirring. Two volumes of cold solution C (50 mM Mes pH6.0, 0.4M NaCl, 5mM CaCl₂, 1.0M sucrose) were added and the resultant mixture incubated in the dark at 4°C for a further 15min, with continuous stirring. Centrifuging this solution at 48,400g for 90min yielded a pellet of LHCII. The supernatant was poured into a Spectrapor No.6 dialysis bag (50,000kDa cut-off) and dialysed (3 x 30m) against three 1,000ml volumes of solution D (50mM Mes pH6.0, 10mM NaCl, 5mM CaCl₂). The dialysed sample was sufficiently depleted of sucrose, to allow the oxygen-evolving PSII cores to be pelleted by a 1h, 48,400g, 4°C centrifugation step. The pellet was then re-suspended in solution E (50mM Mes pH6.0, 10mM NaCl, 0.4M sucrose, 5mM CaCl₂ and 0.03% DM) and its chlorophyll concentration analysed. To remove the extrinsic polypeptides, the above PSII cores were mixed with 0.8M Tris-HCl (pH8.0) to give a final chlorophyll concentration of 0.1mg.ml⁻¹. The sample was left on ice and in the dark for 15min, before being centrifuged at 140,000g for 30min at 4°C. This Tris-washing step was repeated once more, to ensure efficient removal of the extrinsic proteins, before the remaining core pellet was re-suspended in solution H (20mM Bis-Tris pH6.5, 20mM NaCl, 10mM MgCl₂, 0.75% taurine) supplemented with 0.5% DM. The volume was corrected to give a final chlorophyll concentration of 0.5mg.ml⁻¹ and the sample homogenised 5 times, before being incubated in the dark and on ice for 10min. Following this, the sample was then passed through an S-Sepharose column which had been equilibrated in solution H. The green fraction was eluted with solution H, supplemented with 0.03% DM. A flow rate of 1ml.min⁻¹ was used. This ion exchange step was carried out at room temperature and in the dark, although the eluent was collected on ice. The green fraction was then loaded onto a Q-Sepharose column equilibrated with solution H. The first fraction (CP43) was eluted in solution H, containing 0.03% DM (flow rate 1ml.min⁻¹). A second green fraction (CP29) was eluted during a 0-40mM MgSO₄ gradient step (60ml), run at a flow rate of 0.5ml.min⁻¹. Crude CP47-D1-D2-cytb559 was eluted by increasing the MgCl₂ concentration to 120mM.

The CP43 fraction was dialysed at 4°C overnight against solid sucrose to concentrate it. The dialysis tubing volume was then restricted and the sucrose and MgCl₂ concentration of the sample reduced by dialysing against solution H, supplemented with 0.03% DM, for 15min at 4°C. The CP43 fraction was flash frozen in liquid nitrogen and stored at -80°C.

The CP47-D1-D2-cytb559 fraction was diluted by adding three volumes of solution H (containing 0.03% DM), before being loaded onto a second Q-Sepharose column. Using a 25ml, 30-120mM MgSO₄ gradient, the last fraction to be eluted was the CP47-D1-D2-cytb559 complex. Samples were frozen in liquid nitrogen and stored at -80°C.

2.1.5 Isolation of oxygen-evolving cores from spinach (*Spinacea oleracea*) leaves using HTGP or OG

Market spinach leaves (*Spinacea oleracea*) were bought a day before starting the PSII core purification procedure described in this section. The leaves were washed and stored in water, overnight at 4°C, before being de-stemmed the following morning. The de-stemmed leaves were placed into a Waring blender and mixed with 1l of grind solution (50mM Hepes pH7.5, 400mM NaCl, 10mM MgCl₂, 1mM EDTA, 10mM sodium ascorbate and 2g.l⁻¹ protease free, delipidated bovine serum albumin). Bovine serum albumin was delipidated by using the procedure described by (Siegenthaler *et al.*, 1987). The spinach leaves and grind solution were subjected to three 10s blending steps, before being squeezed through a single layer of muslin. The filtered extract was then quickly passed through a second filter, consisting of four layers of muslin and a layer of cotton wool. This crude chloroplast extract was centrifuged at 12,400g for 10min at 0°C. To reduce protease damage, the resultant crude chloroplast pellets were quickly re-suspended in 500ml of re-suspension buffer 1 (50mM Mes pH6.0, 150mM NaCl, 5mM MgCl₂, 2.5mM sodium ascorbate and 1g.l⁻¹ delipidated bovine serum albumin), before being subjected to a second 10min centrifugation step at 12,400g at 0°C. The thylakoid membrane pellets were re-suspended with 40ml of re-suspension buffer 2 (50mM Mes pH6.0, 5mM MgCl₂, 15mM NaCl, 2.5mM sodium ascorbate). The chlorophyll concentration of this solution was measured using the method described by Arnon, (1949). The thylakoid membranes were solubilised in a solution having a final concentration of 2.5% Triton X-100 and 2mg.ml⁻¹ chlorophyll. This solution was gently stirred in the dark and on ice for 30min before being subjected to a 30min, 43,700g centrifugation step at 0°C. The supernatant was discarded and the pellet re-suspended in 80ml of SMNCB (0.3M sucrose, 25mM Mes pH6.5, 10mM NaCl, 5mM CaCl₂, 10mM NaHCO₃), to dilute the Triton-X100 associated with the crude granal stacks (BBYs). BBYs were precipitated by another 30min, 43,700g centrifugation step at 0°C. The pellets were re-suspended with 40ml SMNCB before the solutions chlorophyll concentration was determined.

To prepare oxygen-evolving PSII cores from these granal stacks, BBYs (15mg chlorophyll) were re-suspended in 20ml of core buffer A (0.5M sucrose, 40mM Mes pH6.0) before being homogenised five times. The solution was then centrifuged at 48,400g for 15min at 0°C. The washed BBY pellet was carefully re-suspended in 3ml of core buffer B (72mM Mes pH6.0, 1.8M sucrose, 72mM MgCl₂, 18mM NaCl) and 2ml of either HTGP (346mM) or OG (346mM). The detergents were of the Ultrol grade and were obtained from Calbiochem. In both cases, samples were homogenised with 15 passes of the plunger before being incubated in the dark, with constant stirring for 75min at 4°C. The solubilised PSII core/LHCII mixture was diluted with 7.5ml of core buffer A and centrifuged at 48,400g for 10min at 4°C, in order to pellet any unsolubilised material. The resultant supernatant of HTGP or OG solubilised cores was then diluted with 12.5 or 15.5 ml of 40mM Mes pH6.0, respectively. The samples were then centrifuged at 150,000g at 4°C for 1h in order to precipitate LHCII. The supernatant, which contained the oxygen-evolving PSII cores characterised in Chapters 3 and 4 respectively, was diluted with an equal volume of 40mM Mes (pH6.0) and centrifuged for 30min at

150,000g at 4°C. These precipitated cores were solubilised with SMNCB, supplemented with 2mM DM, before being flash frozen in liquid nitrogen and stored at -80°C. Cores isolated using HTGP are referred to as HTGP cores while OG cores were isolated from BBYs using OG.

2.1.6 Isolation of monomeric and dimeric oxygen-evolving PSII cores from spinach

Monomeric and dimeric oxygen-evolving cores containing CP47, CP43, D1, D2, cytb559 and the extrinsic 33kDa subunit were isolated according to the method detailed in Chapter 5. Essentially PSII cores (75µg chlorophyll) isolated using OG (see Section 2.1.5 and Chapter 4) were dissolved in MNCB (25mM Mes pH6.5, 10mM NaCl, 5mM CaCl₂ and 10mM NaHCO₃) and supplemented with DM to give a detergent concentration of 30mM in a final volume of 150ml. The solubilised sample was homogenised and loaded onto a freshly prepared sucrose gradient. To prepare sucrose gradients, centrifuge tubes were filled with sucrose gradient mix solution (25mM Mes pH6.5, 0.5M sucrose, 10mM NaCl, 5mM CaCl₂, and 0.03% DM) and frozen at -20°C before being thawed slowly at 4°C. Slow thawing resulted in the formation of a sucrose density gradient. OG cores (75µg chlorophyll at a 0.5mg.ml⁻¹ chlorophyll) were loaded onto the gradients and centrifuged over night at (90,000-150,000g) at 4°C in a Beckmann SW41 swing out rotor. The three green bands were resolved from one another. The middle (PSII monomer) and lower (PSII dimer) bands were removed from the sucrose gradients, frozen in liquid nitrogen and stored at -80°C.

2.1.7 Isolation of dimeric PSII particles lacking the 33kDa extrinsic polypeptide

PSII dimers lacking the 33kDa extrinsic polypeptide were prepared by washing the above dimers (Section 2.1.6) with Tris buffer according to the following procedure. A 980mM Tris-HCl/2mM DM stock solution was prepared and cooled to 4°C. 34µl of a spinach dimer stock, having a chlorophyll a/b ratio of 41:1 and chlorophyll concentration of 2.5mg.ml⁻¹, were mixed with 1ml of the 980mM Tris/2mM DM stock. This solution was dispensed into a 100kDa cut-off, micro-concentrator (Amicon) and centrifuged at 1500g. The remaining 50ml were washed twice more with 1ml of the 980mM Tris/2mM DM stock and concentrated as before. Excess Tris was removed by washing the concentrated sample with 500ml MNCB supplemented with 2mM DM and concentrating it to 50ml as before. The resultant cores were flash frozen in SMNCB supplemented with 2mM DM and stored at -80°C.

2.1.8 The isolation of a dimeric PSII/LHCII complex from spinach

BBY particles were isolated from spinach using the method described in Section 2.1.5. They were then solubilised (150µg of chlorophyll at a final chlorophyll concentration of 0.5mg.ml⁻¹) in MNCB in the presence of 30mM DM. The solution was homogenised five times before 150µl were loaded onto a sucrose gradient and centrifuged overnight at (150,000g) in a Beckmann SW41 swing-out rotor.

Sucrose gradients were prepared as described in Section 2.1.6. The purified complexes were removed from the gradients, flash frozen and stored at -80°C .

2.2 Subunit analysis

2.2.1 Preparing gels for SDS polyacrylamide gel electrophoresis

Discontinuous, dissociating SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels were prepared and run using the LKB 2001 vertical electrophoresis apparatus and a variation of the Laemmli buffer systems (Laemmli, 1970). Gels of 1.5mm thickness were prepared according to the two methods outlined below. Method 1 was superseded by Method 2. Both methods gave similar PSII subunit resolution, but gels prepared by Method 2 polymerised more quickly and showed fewer irregularities in the polyacrylamide gradient. The latter improvement was achieved by the addition of glycerol to the 17% acrylamide mix (Table 2.1). The glycerol-induced density gradient supported the acrylamide gradient during polymerisation.

Table 2.1 The composition of solutions used to prepare two types of 10-17% linear gradient resolving gel.

Stocks	Method 1 resolving gel solutions		Method 2 resolving gel solutions	
	10%	17%	10%	17%
40% BDH Acrylogel (ml)	7.5	12.75	7	11.5
3M Tris (pH 8.9) (ml)	8.0	8.0	7.5	7.5
0.1M EDTA (ml)	0.6	0.6	---	---
10% SDS (ml)	0.3	0.3	---	---
Urea (g)	10.81	10.81	10	10
H ₂ O (ml)	5	---	6.5	6.5
80% Glycerol (ml)	---	---	---	1.4
TEMED (μl)*	1	1	8	8
10% AMPS (μl)**	130	130	65	65

* TEMED (NNNN' Tetramethylethylenediamine) was added just before the gradient was poured.

** 10% AMPS (ammonium persulphate) was added just before the gradient was poured.

Polymerised resolving gels prepared by the two methods outlined in Table 2.1 were overlaid with the corresponding stacking gels (Table 2.2).

Table 2.2 The composition of two types of stacking gel

Stocks	Method 1	Method 2
	stacking gel mix	stacking gel mix
40% Acrylogel (ml)	3.0	3.0
0.313M Tris (pH 6.8) (ml)	8.0	8.0
0.1M EDTA (ml)	0.4	---
10% SDS (ml)	0.2	---
H ₂ O (ml)	7.9	13
TEMED (μ l)*	10	15
10% AMPS (μ l)**	500	250

* TEMED was added just before the gradient was poured.

** 10% AMPS was added just before the gradient was poured.

2.2.2 Sample solubilisation and loading

Samples were solubilised with two different solubilisation buffers. Solubilisation buffers 1 (250mM Tris-HCl pH8.9, 4% SDS, 2% β -mercaptoethanol, 40% sucrose) and 2 (62.5mM Tris-HCl pH6.8, 5% SDS, 0.1M dithiothreitol, 50% glycerol) could be used interchangeably as the polypeptide resolution was unaffected. Both solubilisation buffers were three times the working concentration and so were added in a ratio of 1:2 to the samples being loaded. Samples appeared to be fully solubilised after a 1-2min incubation at room temperature. Gel loadings depended on the lane width and whether gels were Coomassie stained or Western blotted. Table 2.3 lists thylakoid, BBY, PSII core and LHCII loadings used for these two visualisation procedures.

Table 2.3 Table showing sample loading for gels which were Coomassie stained and Western blotted

Sample *	Coomassie stained gels		Western blots and Silver stained gels
	15 lane gel	20 lane gel	20 lane gel
Thylakoids($\mu\text{g Chl}$)	10	6.5	1
BBYs ($\mu\text{g Chl}$)	10	6.5	1
PSII cores ($\mu\text{g Chl}$)	10	6.5	1
LHCII ($\mu\text{g Chl}$)	4	2.6	---

*Sample concentrations were determined on a mg.ml^{-1} chlorophyll basis using the method described by Arnon, (1949).

Unstained molecular mass markers were obtained from Pharmacia: phosphorylase b (94kDa), bovine serum albumin (67kDa), ovalbumin (43kDa), carbonic anhydrase (30kDa, soy bean trypsin inhibitor (20.1kDa), and myoglobin (14.4kDa) and were used only in Coomassie stained gels. Pre-stained markers were purchased from Biorad. The pre-stained markers (phosphorylase b (106kDa), bovine serum albumin (80kDa), ovalbumin (49.5kDa), carbonic anhydrase (32.5kDa), soybean trypsin inhibitor (27.5kDa) and lysozyme (18.5kDa)) were used both for Western blots and Coomassie stained gels.

2.2.3 Gel running conditions

The electrophoresis running buffer (52mM glycine, 24.7mM Tris-base and 0.1% (w/v) SDS) was cooled to 12°C. Gels were run overnight under current limiting conditions at 11mA (per gel).

2.2.4 Staining gels with Coomassie R250

To visualise the protein bands, gels were stained with Coomassie R250. Gels were gently shaken in a 0.1% Coomassie R250, 25% isopropanol, 10% glacial acetic acid solution for 4h. This solution simultaneously fixed and stained the protein bands. Excess dye was removed by destaining the gels in a 25% isopropanol, 10% glacial acetic acid solution. Subjecting them to a further overnight stain and second destaining step, increased the stain intensity of the protein bands.

2.2.5 Silver Staining

The silver stain reagents were obtained from Daiichi Pure Chemicals Co., Ltd., (Tokyo, Japan), in the form of the 2D-Silver Stain II "Daiichi" kit. Samples were resolved using the PAGE (polyacrylamide gel electrophoresis) conditions described in Sections 2.2.1-2.2.3. The 150 x 100 x 1.5mm slab gels were silver-stained by using the procedure outlined in Table 2.4.

Table 2.4 Procedure used to silver stain PSII polypeptides resolved by SDS-PAGE

Solution	Incubation Time (min)	Water (ml)	Methanol (ml)	Acetic acid (ml)	Reagent	Reagent vol. (ml)
Fix Sol. 1	15	80	100	20	---	---
Fix Sol. 2	22.5	110	60	20	Reagent 1	10
Pre-treatment	15	90	100	---	Reagent 2	10
Water	7.5	200	---	---	---	---
Silver stain	20	180	---	---	Reagent 3+4	10+10
Water	3 x 3.5	200	---	---	---	---
Developer	1-10	190	---	---	Reagent 5	10
Stopper	10	---	---	---	Reagent 6	10

2.3 Western blotting

2.3.1 Transfer and detection of SDS-PAGE polypeptides

Protein samples resolved by SDS-PAGE were transferred to nitrocellulose (using a method modified from that reported by Towbin *et al.*, (1979), prior to their analysis with antibodies raised to specific PSII subunit and subunit fragments. Western blotting with specific antibodies allowed the detection of PSII subunits and their breakdown fragments.

After electrophoresis the gel was placed into transfer buffer (3mM Na₂CO₃, 10mM NaHCO₃ in 20% methanol). The blotting cassette was opened and placed, black side down, into a container filled with transfer buffer. Two layers of Scotchbrite were placed onto the black surface of the cassette. These were overlaid with two layers of 3MM Whatman paper, a layer of nitrocellulose (Schleicher and Schuell reinforced nitrocellulose of 0.45µm pore size), the gel, two layers of 3MM Whatman paper and another layer of Scotchbrite. At all stages great care was taken to prevent any air bubbles being trapped as they would interfere with protein transfer. On completion of this procedure the cassette was closed and placed into a temperature-controlled blotting tank which had been pre-cooled to 4°C. The black side of the cassette was placed nearest to the anode. Proteins were transferred from the gel onto the nitrocellulose, by blotting at a constant 40V (1 gel) or 50V (2 gels) for 2h.

After transfer, the cassette was dismantled and the membrane blocked with a 10% solution of supermarket skimmed milk powder, dissolved in TBS/Tween (50mM Tris-HCl pH7.4, 150mM NaCl, 0.1% Tween 20) for 30min. Tween 20 was used to reduce the non-specific binding of immunochemicals. The membrane was then briefly washed in TBS/Tween and primary antibody (diluted 1:400-1:2000 in TBS/Tween) was added. After incubating the membrane for 1.5h at room temperature it was washed with TBS/Tween (6 x 5min) to remove non-specifically bound primary

antibody. Antirabbit IgG (whole molecule) biotin conjugate (diluted to 1:2000 in TBS/Tween) was then incubated with the membrane at room temperature for another hour. To reduce non-specific binding, the blot was once again washed 6 times in TBS/Tween, before being incubated for 30min with Extravidin-alkaline phosphatase conjugate (Sigma) diluted to 1:2000 with TBS/Tween. Extravidin specifically binds biotin. Once bound to the biotinylated secondary antibody, the alkaline phosphatase part of the conjugate can be induced to catalyse a colour development reaction which allows the visualisation of antibody-labelled bands. Non-specifically bound Extravidin was removed with a final series of 6 x 5min washes in TBS/Tween. The blot was then quickly rinsed in AP buffer (100mM Tris pH9.5, 100mM NaCl, 50mM MgCl₂). This buffer was removed and replaced with 10ml of fresh AP buffer, containing 33 μ l BCIP (5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt) stock and 66 μ l NBT (nitroblue tetrazolium chloride) stock. Stock solutions of BCIP (50mg.ml⁻¹ in 100% dimethyl formamide) and NBT (75mg.ml⁻¹ in 70% dimethyl formamide) were stored at 4°C until required.

2.3.2 Primary antisera

Antibodies raised against CP47, CP43, D2, D1 and the 33kDa extrinsic polypeptide were used in this study. CP47, CP43 and D2 specific antibodies were a kind gift from Dr. R. Barbato. The CP47 specific antibody was raised against spinach CP47, which had been electrophoretically purified according to the method of Dekker *et al.*, (1989). The spinach CP43, used as the antigen to raise the CP43 specific antibody, was purified by a similar method (Barbato *et al.*, 1991c), as was the D2 polypeptide. The preparation of the D2 antibody is outlined in (Barbato *et al.*, 1992c).

The C-terminal D1 (Dupont 304) antibody was a kind gift from Dr. P. Nixon. It was raised against a synthetic polypeptide, homologous to the 29 amino acid of the C-terminal pea D1 precursor. During processing of the D1 precursor *in vivo*, the last 9 amino acids are cleaved away from the C-terminal end, suggesting that the D1 antibody should only recognise the last 20 amino acids of mature D1.

The antibody specific for the 33kDa extrinsic polypeptide was the kind gift of Dr. D. Chapman and was prepared according to Chapman *et al.*, (1989).

2.4 Oxygen evolution

Oxygen evolution rates of thylakoids, BBYs and a number of PSII cores were measured using a Clark type oxygen electrode (Hansatech). Samples (1ml) were illuminated with white light (approx. 4000 μ E.m⁻²s⁻¹) in the presence of electron acceptors. DCBQ (2,6-dichloro-p-benzoquinone) was purchased from Kodak. DMBQ (2,5-dimethyl-benzoquinone) was obtained from the Sigma-Aldrich Library of Rare Chemicals. Oxygen evolution measurements were made in SMNCB buffer supplemented with additives, depending on the type of PSII preparation. These additives are listed in Table 2.5.

Table 2.5 Conditions used to measure the oxygen evolution rates of thylakoids, BBYs and PSII cores

Additives	Thylakoids		BBY Particles		PSII Cores	
	DMBQ	DCBQ	DMBQ	DCBQ	DMBQ	DCBQ
DM %	0	0	0	0	0.03%	0.03%
FeCN (mM)	0.5	0.5	0	0	0	0
DMBQ(mM)	1	0	1	0	1	0
DCBQ(mM)	0	1	0	1	0	1
PSII (µg chl)	20	20	10	10	5	5

DCBQ is thought to accept electrons from both Q_A and Q_B , while the methylated benzoquinone (DMBQ) is only able to accept electrons from the Q_B site (Graan & Ort, 1986). During illumination of fully functional PSII particles, electrons are passed from pheophytin to Q_A and on to Q_B . As DMBQ is only able to accept electrons from Q_B , the rate of oxygen evolution on its addition was taken as a measure of fully functional PSII cores. In contrast, DCBQ is able to accept electrons from both the Q_A and the Q_B site and as a result provides a measure of the sum of inactive and actively reducing Q_B particles. Equation 2.1 was used to calculate the percentage of fully functional PSII particles (Y) with respect to the total number of complexes having a functional donor side.

$$Y = [\text{OER}(\text{DMBQ}) / \text{OER}(\text{DCBQ})] \times 100$$

Equation 2.1

Where OER = O_2 evolution rate.

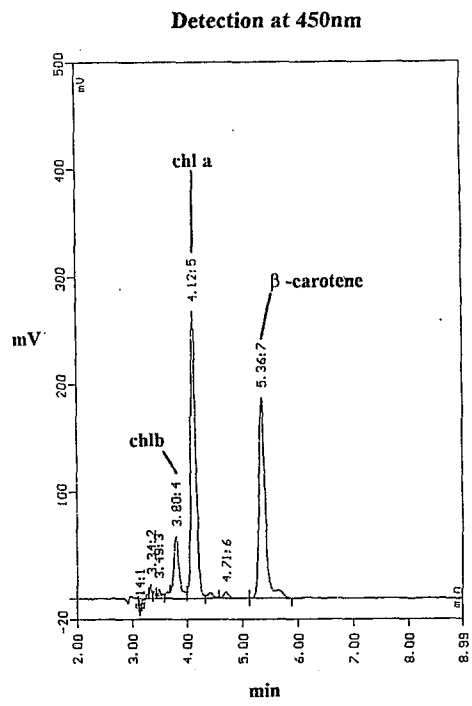
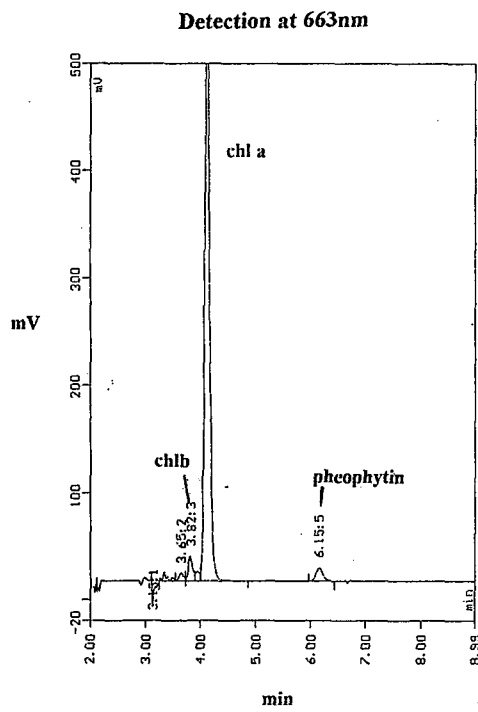
2.5 HPLC size exclusion analysis

HPLC size exclusion analysis was completed under the guidance of Dr. M. Rögner, (Westfälische Wilhelms-Universität, Münster).

HTGP and OG cores isolated from spinach BBYs according to the method described in Section 2.1.5 were stored in SMNCB buffer supplemented with 2mM DM. As detergents have different micelle sizes these core preparations were subjected to a detergent exchange step to remove residual amounts of HTGP or OG, prior to size exclusion analysis. Detergent exchange was achieved by loading 100-200µg of HTGP or OG cores in SMNCB buffer, which had been supplemented with 2mM DM, onto an HA:Econopak 10DG column obtained from Biorad. The purified core complexes were then eluted in TSB-30 Buffer (20mM Mes pH6.5, 10mM MgCl_2 , 30mM CaCl_2 , 0.5M mannitol and 0.03% DM).

PSII monomers and dimers (prepared according to the method outlined in Section 2.1.6) were not detergent-exchanged, as they had undergone extensive washing with DM during sucrose gradient

Resolution of pigments of sucrose gradient purified monomeric PSII core complexes using the reverse phase HPLC method described in section 2.6



purification. In some cases, samples were concentrated in Amicon centricon-100 micro concentrator units, but this did not cause an increase in the PSII aggregation number.

The PSII samples (1-15 μ g chlorophyll) were applied to a TSK4000sw (Amicon) HPLC gel filtration column in a volume of 70 μ l, with TSB-30 running buffer. A flow rate of 0.5ml.min⁻¹ was used to separate the components of HTGP or OG cores purified according to the methods developed in Chapters 3 and 4, but was reduced to 0.4ml.min⁻¹ for the analysis of the two sucrose gradient purified oligomeric forms (Chapter 5). PSI trimer (820kDa), invertase (540kDa), PSI monomer (300kDa), myoglobin (17kDa) and vitamin B₁₂ (1350) were used to calibrate the column. PSI monomers and trimers were only used as standards after extensive calibration against other standards (Rögner, personal communication). This column had previously been calibrated with a large number of proteins and the above standards gave the most linear calibration curve (Rögner, unpublished results).

2.6 Pigment analysis

PSII core particle concentrations were measured on a total chlorophyll basis following the method of Arnon, (1949). Absorption spectra of PSII core preparations were recorded with a DW2000 dual wavelength spectrophotometer purchased from SLM Instruments, using a band width of 2nm. Sample spectra were measured at a final concentration of approximately 5 μ g.ml⁻¹.

The pigment composition of various PSII particles were determined using reverse phase HPLC. For the purposes of such analysis, PSII cores were mixed with 100% acetone so as to give a final concentration of 80% acetone and a chlorophyll concentration of 75-100 μ g.ml⁻¹. After precipitating the proteinaceous fraction by centrifugation (1min in a bench top centrifuge), the supernatant was passed through a 13mm Whatman PVDF filter having a pore size of 0.2 μ m. 20 μ l of the filtered pigment extract were injected onto an ODS-1 column (5mm particle size, 250mm by 4.6mm ID, obtained from Anachem) which had been equilibrated in the solvent mixture used for the analysis (68% methanol: 32% ethylacetate). ODS-1 is a non-end capped 6% carbon, spherical silica material. The ODS-1 guard column was obtained from Phase separation. The pigments were resolved in the above mentioned solvent system at a constant flow rate of 1ml.min⁻¹. The eluting pigment peaks were detected using a Kontron-430 dual-wavelength detector set to absorption at 450 and 663nm. Chlorophyll a, chlorophyll b and pheophytin concentrations were calculated from the peak area measured at 663nm. The β -carotene content was calculated from the peak area measured at 450nm. The extinction coefficients (E mM.cm⁻¹) of the respective pigments, measured at the above mentioned wavelengths, were as follows: chlorophyll a (46.55), chlorophyll b (22.92), pheophytin a (32.93) and β -carotene (165.9). To calculate the number of chlorophyll a, chlorophyll b, and β -carotene molecules associated with a given monomeric core complex, the respective pigment amounts were normalised to two pheophytin molecules. This normalisation is based on the finding that higher plant reaction centres contain two pheophytin molecules (Kobayashi *et al.*, 1990; Gounaris *et al.*, 1990), while CP47 (Alfonso *et al.*, 1994), CP43 (Alfonso *et al.*, 1994) and the CAB proteins CP24, CP26 and CP29 (see Thornber *et al.*, 1993) are not associated with this pigment.

2.7 Stability trials

2.7.1 The thermostability of oxygen-evolving PSII cores in the presence of 0.3 and 2.4M sucrose

One way of measuring the degree of damage of isolated PSII core preparations is to measure changes in its absorption spectrum. Intact PSII particles absorb strongly at 437 and 419nm. The 437nm absorption peak is predominantly due to the presence of chlorophyll a in the preparation, while the 419nm peak is the pheophytin absorption peak maximum. The loss of a single Mg^{2+} ion from chlorophyll a, results in the formation of pheophytin a. Thus the rate of pheophytinisation (the formation of pheophytin) can be determined by measuring the change in the 419:437 absorption ratios.

Two sets of nine Eppendorfs were filled with 1.485ml of MNCD (10mM NaCl, 5mM $CaCl_2$, 25mM Mes pH6.5 and 2mM DM) supplemented with sucrose, to give a final sucrose concentration of either 0.3M or 2.4M. HTGP PSII cores isolated from peas (Section 2.1.5), were then added, giving a final chlorophyll concentration of $10\mu g.ml^{-1}$. All samples were incubated in the dark.

Samples were set up for each of the six treatments listed downwards in Table 2.6. The effect of incubating PSII cores in 0.3 and 2.4M sucrose at three different temperatures (4, 18 and 27°C) was tested using the following experimental matrix (Table 2.6).

Table 2.6 Experimental design to test the effect of temperature and sucrose concentration on PSII core stability

Incubation temperature (°C)	Sucrose concentration (M)	Incubation 0h (Replicate No.)	Incubation 5h (Replicate No.)	Incubation 23h (Replicate No.)	Incubation 261h (Replicate No.)
4	0.3	3	3	3	3
4	2.4	3	3	3	3
18	0.3	3	3	3	3
18	2.4	3	3	2	3
27	0.3	3	3	3	3
27	2.4	3	3	3	3

After the appropriate incubation time, a 300 μ l aliquot was removed from the sample being analysed and diluted to 1ml in MNCD supplemented with either 0.3M or 2.4M sucrose. The spectra of these 1ml samples were analysed using a DW2000 dual wavelength spectrophotometer. The band width was set to 2nm and the scan speed to 5nm.sec⁻¹.

2.7.2 Method of determining the effect of freezing PSII cores, on D1 and D2 degradation.

PSII cores isolated from spinach according to the methods described in Section 2.1.5 and 2.1.6 (also see Chapters 3-5) were dissolved in SMNCB buffer supplemented with 2mM DM. As a control, freshly prepared cores were mixed with solubilisation buffer 2 (see Section 2.2.2) immediately after

their purification. To test whether the freezing of isolated core preparations caused D1 and D2 subunit damage, freshly prepared cores were flash frozen in liquid nitrogen and thawed again before being solubilised with solubilisation buffer 2, for SDS-PAGE (see Sections 2.2.1-2.2.3) and Western blotting (see Sections 2.3.1-2.3.2) with D1 and D2 specific antibodies.

2.8 Image enhancement by single particle analysis

Transmission electron microscopy can be used to gain structural information on detergent solubilised PSII complexes. The resolving power of a transmission electron microscope is 2-3Å. The theoretical resolution attainable using this method of analysis, would be expected to be of a similar order of magnitude. However, for biological material it is usually much lower because of the low contrast level of the sample and their susceptibility to electron beam damage. Contrast levels are improved by negatively staining the protein complex of interest with metal salts, such as uranyl acetate. Image enhancement techniques are designed to increase the signal to noise ratio still further, and the procedures to achieve this aim are described below.

The electron micrographs of a large number of negatively-stained particle images can be scanned by a digitising CCD camera and the information analysed by computerised image processing (Boekema & Böttcher, 1992; Penczek *et al.*, 1992; Van Heel & Keegstra, 1981). The digitised images are first treated with a band pass filter which suppresses image detail outside the 1-30nm range (de Haas *et al.*, 1993). This treatment also limits the effect of small defocus differences, as well as large stain modulations and so retains only the detail relevant at a resolution of 15-25Å. The band pass treated particle images are then circled by a tightly fitting mask, which excludes image detail outside of the particle of interest. Having eliminated a considerable amount of irrelevant image detail by the band pass and masking steps, the filtered particle images are rotationally and translationally aligned. Rotational alignment, allows each of the particles within a specific group to be rotated about a central axis, so that all of them have the same orientation. During the translational alignment step, all of the rotationally aligned particles are aligned along their X and Y axes, with respect to a given reference particle. As the averaged image, obtained in this manner, is a more accurate representation of the true structure of the complex, than that obtained from any individual particle, it is used as a reference during a second alignment step. By subjecting all of the particles to a second alignment step with respect to the average image, the quality of the image is improved still further. This process is repeated until the cross-correlation value (a rough measurement of the degree of alignment) of the respective particles can no longer be improved (de Haas *et al.*, 1993). Next, a proportion of particles having the highest cross-correlation values are selected and subjected to another series of alignment cycles. The final, optimally aligned images are then subjected to multivariate statistical analysis and hierarchical ascendant classification, to sort individual particles into distinct classes according to their degree of similarity. This process usually discriminates between projections differing subtly due to the attachment of particles to the carbon support (Van Heel & Frank, 1981; Van Heel, 1984).

Sucrose interferes with single particle analysis as it binds uranyl acetate and so reduces the signal to noise ratio. To avoid this problem, core samples (5-10 μ g chlorophyll in 50 μ l) were dialysed against a sucrose free wash buffer (10mM Mes pH6.5, 5mM MgCl₂, 5mM CaCl₂, 2mM DM), overnight at 4°C. A small aliquot (3 μ l) of the sucrose depleted sample was dispensed onto a carbon coated 400 mesh copper grid and left at room temperature for 2min to allow the protein to bind to the carbon support. The sample droplet was then brought into contact with a 1ml drop of filtered de-ionised water, in order to remove any unbound protein. The washed grid was then held against the surface of a 100 μ l drop of 2% uranyl acetate for 10s before being pulled away. This procedure left a small stain droplet on the surface of the grid. The grid was left to stain for a further 2min before the rest of the uranyl acetate was blotted off using fine mesh filter paper (Schleicher and Schuell filter paper disks 1575). Stained grids can be damaged if blotted dry with coarser mesh filter paper, as such materials absorb the surface moisture too quickly. Rapid stain removal can also cause uneven staining or physical damage to the grid and is to be avoided.

2.9 Two-dimensional crystallisation

2.9.1 Method of studying dimyristoyl phosphatidyl choline:PSII core bilayer formation

The experiments described in Section 7.2.1 were carried under the guidance of Prof. Kühlbrandt, (1992) at the European Molecular Biology Laboratories in Heidelberg. PSII cores used in these experiments were isolated from pea sub-chloroplast membranes according to De Las Rivas, (1992a)(Section 2.1.2). DMPC (dimeristyl phosphatidyl choline), the bilayer-forming lipid used in these experiments, was dissolved in chloroform and stored as a stock solutions at -20°C . DMPC was a kind gift from Prof. W. Kühlbrandt.

For crystallisation trials aliquots of the DMPC stock were dispensed into Eppendorf tubes and dried down under nitrogen. Care was taken to remove the chloroform completely and to prevent the exposure of the dried lipid to oxygen. PSII cores (100 μg chlorophyll), solubilised in 2mM DM, were added to the dried lipid. The sample volume was then corrected to 100 μl , by the addition of 50mM Mes pH6.1/1.75mM DM, thus ensuring that the final chlorophyll concentration was 1mg.ml $^{-1}$. The lipid-detergent-protein mixture was sonicated for 2min and then incubated at 27°C for 30min to allow the formation of mixed micelles. At this stage, the sample had a transparent green appearance indicating that the PSII cores were well solubilised. The solubilised samples were then dispensed into L-shaped glass capillary tubes sealed at one end with a piece of dialysis membrane (Spectrapore 12,000-14,000 M.W. cut off), before being dialysed against 50mM Mes pH6.1 at 27°C . As the detergent was dialysed away, the solution took on a cloudy appearance. Prior to analysis, samples were subjected to a 37°C heat shock to aid the fusion of lipid-protein-detergent mixed micelles. As the detergent was dialysed from the solution the dialysate took on a turbid appearance, indicating the conversion of the mixed micelles to lipid-protein vesicles and sheets. To monitor the development and packing order of two-dimensional crystals during the trials, samples were taken from the dialysate at various time points and analysed by electron microscopy.

Electron microscope grids were prepared according to Wang & Kühlbrandt, (1991). Carbon films were prepared by evaporating carbon rods (Agar Aids) onto freshly cleaved mica at 5.0×10^{-6} T in an Edwards 306 evaporator. The carbon film was then floated onto a clean surface of water and picked up onto the shiny side of a 400 mesh copper EM grid. A 1-3 μl droplet of the sample to be analysed, was gently placed onto the carbon layer and left for 10s before being blotted dry using Whatman No.4 filter paper. This was done by applying the filter paper to the side of the grid, while holding the grid with the shiny side up. The grid was then overlaid with a droplet of 2% (w/v) uranyl acetate. After 10s the droplet was blotted off as before and the staining process repeated. After negatively staining the samples in this manner, they were viewed on a liquid nitrogen cooled stage in a JOEL 100C electron microscope.

2.9.2 Crude estimation of the amount of thylakoid lipid associated with various PSII core preparations

The experiments presented in Section 7.2.3 were completed at the University of Groningen under the guidance of Dr. Boekema. The aim of these experiments was to determine whether various PSII preparations (characterised in Chapters 3-5), were associated with an excess, the correct amount or too little thylakoid lipid, to allow the complexes to pack tightly into the forming bilayers. HTGP and OG core preparations (Section 2.1.5) were stored at -80°C in SMNCB supplemented with 2mM DM. Sucrose density gradient purified cores (Section 2.1.6), were concentrated in Centricon-100 microconcentrators (Amicon) before being flash frozen and stored at -80°C . The sucrose gradient mixture is the same as that of SMNCB buffer except that the sucrose concentration increases down the gradient. As sucrose interferes with uranyl acetate staining of protein complexes core samples (5-10 μg in 50 μl) were dialysed overnight at 4°C against a sucrose free wash buffer (10mM Mes pH6.5, 5mM MgCl_2 , 5mM CaCl) supplemented with 2mM DM overnight at 4°C .

To get an approximate idea of how much thylakoid lipid was still associated with PSII cores, the sucrose free samples were incubated with hydrophobic beads (Bio-beads SM7, 20-50 mesh) for a further 16h at 4°C . Bio-beads selectively bind detergent, forcing the thylakoid lipid and membrane protein complexes to aggregate. The formation of bilayers, in which the protein complexes are not forced into close association, indicates the presence of a high lipid:PSII core ratio. In contrast, the formation of precipitate after detergent removal suggests that the amount of thylakoid lipid still associated with isolated cores is too low to support the formation of two-dimensional crystals.

To analyse the effect of detergent removal using Bio-beads, samples were negatively stained using the procedure described in Section 2.8. Electron micrographs were taken using a JEOL 1200-EX microscope.

2.10 Three-dimensional crystallisation trials

Purified CP43, a CP47-D1-D2-cytb559 complex and various oxygen-evolving PSII cores (Section 2.1) were subjected to initial three-dimensional crystallisation screens covering 120 conditions, in which precipitant, pH and additive conditions were varied as described by Jankarik & Kim, (1991).

2.10.1 Preparation of concentrated protein stock solutions

All complexes and isolated subunits used in the crystallisation trials described in Chapter 8, were stored at -80°C in the presence of sucrose prior to use. The chlorophyll and protein concentrations of the samples used were determined using the method of Arnon, (1949) and the BCA protein assay kit (Pierce), respectively. The required amount of sample was dispensed into a micro concentrator. Millipore Ultrafree-MC 10,000, 100,000 NMWL (Millipore) and Centricon-100 micro concentrators units (Amicon) were used. The Millipore ultrafiltration units were fitted with a low protein binding PLGC membrane, as opposed to the polysulfone type (PTHK) to prevent the core complexes from sticking to the filter surface. The samples were diluted with a wash buffer (2mM DM, 50mM Mes pH6.1) before being subjected to a centrifugal concentration step. The Millipore concentrators were microfuged, whereas the Amicon ultrafiltration units were centrifuged at 1500g. Centrifugation times were dependent on sample viscosity and volume. The samples were washed twice more with the wash buffer before being concentrated to the volume yielding the desired protein or chlorophyll stock concentration. A typical oxygen-evolving core stock concentration used was $30\text{mg}\cdot\text{ml}^{-1}$ protein.

2.10.2 Setting up crystallisation trials

Crystallisation screens were set up with the aid of the automated batch dispensing system designed at Imperial College (Chayen *et al.*, 1990). Figure 2.1 depicts this system, which allows the simultaneous mixing of the protein stock with up to three filtered (using 0.2 μ m filter) solutions. It is a computer-controlled syringe-driven dispenser, capable of accurately mixing four stock solutions into a single droplet, as small as 1.2 μ l. The four bore tip (Figure 2.2), from which the stock solutions are dispensed, is positioned at the bottom of a paraffin oil-filled Terazaki plate well (Figure 2.3) before the solutions are ejected.

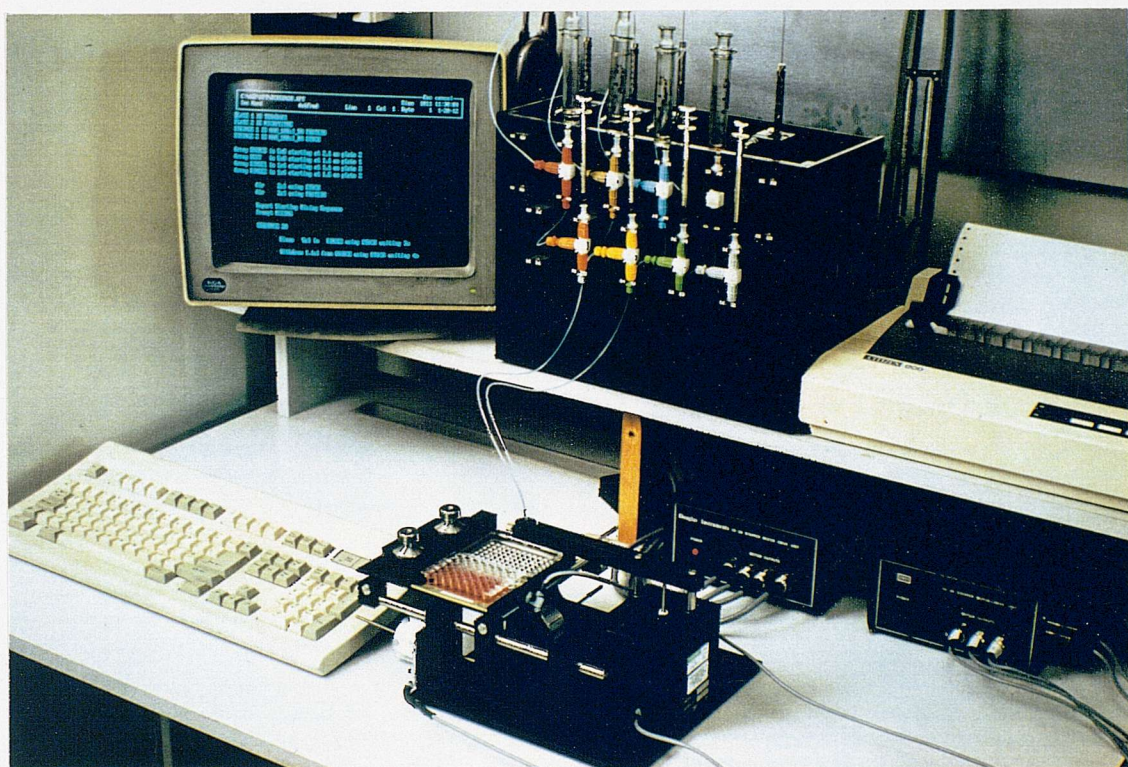


Figure 2.1 The automated Micro-Batch Protein Crystallisation System (Chayen *et al.*, 1990).

As the tip is withdrawn, it is wiped clean by the oil thus preparing it for the next sample well. More importantly though, the oil reduces the evaporation rate to allow slow concentration of the mother liquor.



Figure 2.2 The four bore tip from which the stock solutions are dispensed. The diameter of the four bore tube is approximately 1mm.

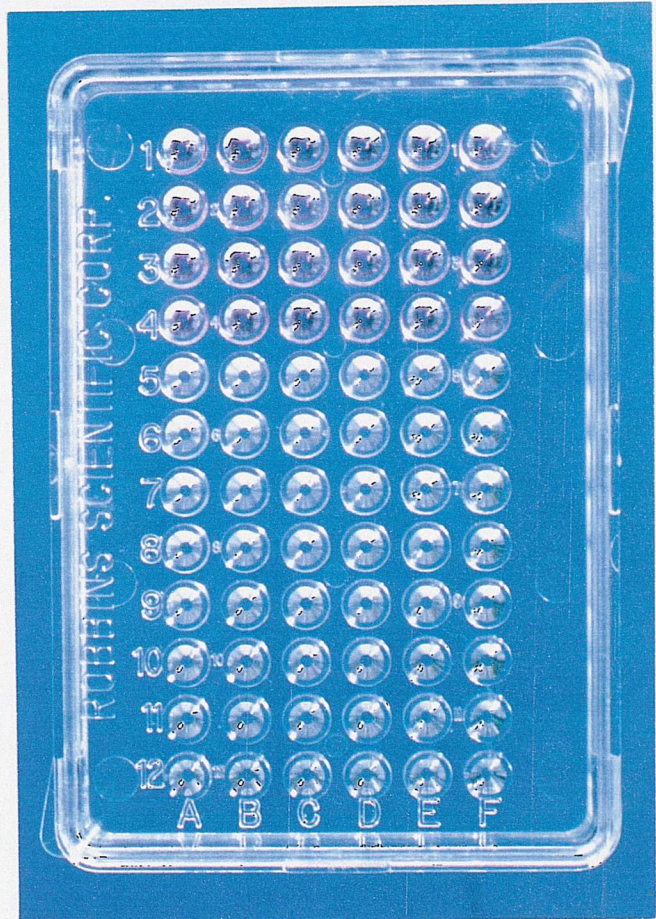


Figure 2.3 A Terazaki plate in which wells in lanes 1-4 have been filled with paraffin oil.

The four bore tip is positioned at the bottom of a paraffin oil-filled well before the solutions are dispensed.

Initial screening of crystallisation conditions was carried out by adding 1 μ l of protein stock (Section 2.10.1) to 1 μ l of the stock solutions listed in Table 2.7.

Table 2.7 Table of crystallisation stocks used to screen PSII core complexes for possible crystallisation conditions

Stock number	Precipitant	Buffer
1	1.1M (27.5%) (NH ₄) ₂ SO ₄	0.1M Acetate pH 4.6
2	1.4M (35%) (NH ₄) ₂ SO ₄	0.1M Acetate pH 4.6
3	1.7M (42.5%) (NH ₄) ₂ SO ₄	0.1M Acetate pH 4.6
4	2.0M (50%) (NH ₄) ₂ SO ₄	0.1M Acetate pH 4.6
5	2.3M (57.5%) (NH ₄) ₂ SO ₄	0.1M Acetate pH 4.6
6	1.1M (27.5%) (NH ₄) ₂ SO ₄	0.1M Mes pH 6.1
7	1.4M (35%) (NH ₄) ₂ SO ₄	0.1M Mes pH 6.1
8	1.7M (42.5%) (NH ₄) ₂ SO ₄	0.1M Mes pH 6.1
9	2.0M (50%) (NH ₄) ₂ SO ₄	0.1M Mes pH 6.1
10	2.3M (57.5%) (NH ₄) ₂ SO ₄	0.1M Mes pH 6.1
11	1.1M (27.5%) (NH ₄) ₂ SO ₄	0.1M PIPES pH 6.8
12	1.4M (35%) (NH ₄) ₂ SO ₄	0.1M PIPES pH 6.8
13	1.7M (42.5%) (NH ₄) ₂ SO ₄	0.1M PIPES pH 6.8
14	2.0M (50%) (NH ₄) ₂ SO ₄	0.1M PIPES pH 6.8
15	2.3M (57.5%) (NH ₄) ₂ SO ₄	0.1M PIPES pH 6.8
16	1.1M (27.5%) (NH ₄) ₂ SO ₄	0.1M Tris pH 8.3
17	1.4M (35%) (NH ₄) ₂ SO ₄	0.1M Tris pH 8.3
18	1.7M (42.5%) (NH ₄) ₂ SO ₄	0.1M Tris pH 8.3
19	2.0M (50%) (NH ₄) ₂ SO ₄	0.1M Tris pH 8.3
20	2.3M (57.5%) (NH ₄) ₂ SO ₄	0.1M Tris pH 8.3
21	5% PEG4000	0.1M Acetate pH 4.6
22	10% PEG4000	0.1M Acetate pH 4.6
23	15% PEG4000	0.1M Acetate pH 4.6
24	25% PEG4000	0.1M Acetate pH 4.6
25	5% PEG4000	0.1M Mes pH 6.1
26	10% PEG4000	0.1M Mes pH 6.1
27	15% PEG4000	0.1M Mes pH 6.1
28	25% PEG4000	0.1M Mes pH 6.1
29	5% PEG4000	0.1M PIPES pH 6.8
30	10% PEG4000	0.1M PIPES pH 6.8
31	15% PEG4000	0.1M PIPES pH 6.8
32	25% PEG4000	0.1M PIPES pH 6.8
33	5% PEG4000	0.1M Tris pH 8.3

34	10% PEG4000	0.1M Tris pH 8.3
35	15% PEG4000	0.1M Tris pH 8.3
36	25% PEG4000	0.1M Tris pH 8.3
37	10% MPD	0.1M PIPES pH 6.8
38	20% MPD	0.1M PIPES pH 6.8
39	30% MPD	0.1M PIPES pH 6.8
40	40% MPD	0.1M PIPES pH 6.8
41	50% MPD	0.1M PIPES pH 6.8
42	5% Isopropanol	0.1M PIPES pH 6.8
43	10% Isopropanol	0.1M PIPES pH 6.8
44	15% Isopropanol	0.1M PIPES pH 6.8
45	20% Isopropanol	0.1M PIPES pH 6.8
46	25% Isopropanol	0.1M PIPES pH 6.8
47	1.1M Na/K phosphate	pH 7.0
48	1.4M Na/K phosphate	pH 7.0
49	1.7M Na/K phosphate	pH 7.0
50	2.0M Na/K phosphate	pH 7.0
51	2.3M Na/K phosphate	pH 7.0
52	1.2M Na citrate	pH 6.4
53	1.6M Na citrate	pH 6.4
54	2.0M Na citrate	pH 6.4
55	5% PEG4000 + 1M LiCl	0.1M PIPES pH 6.8
56	10% PEG4000 + 1M LiCl	0.1M PIPES pH 6.8
57	20% PEG4000 + 1M LiCl	0.1M PIPES pH 6.8
58	5% PEG4000 + 2M NaCl	0.1M PIPES pH 6.8
59	10% PEG4000 + 2M NaCl	0.1M PIPES pH 6.8
60	20% PEG4000 + 2M NaCl	0.1M PIPES pH 6.8

NOTE:

PEG4000 = polyethylene glycol (Mr 4000)

MPD = 2-methyl 2,4-pentanediol

Na/K phosphate was prepared by adding 4M K_2HPO_4 to 4M NaH_2PO_4 until the pH of the solution was 7.0. This solution was then diluted to 1.1M, 1.4M, 1.7M, 2M and 2.3M so as to prepare stock solutions 47-51 (Table 2.7).

After dispensing the samples, the trays were stored in the dark at 4°C or 18°C. To prevent photodamage, crystallisation trials were analysed in dim green light.

Chapter 3 The characterisation of oxygen-evolving PSII cores isolated from BBYs using HTGP

3.1 Introduction

Over the past decade or so, considerable research have gone into the isolation of discrete PSII complexes capable of driving water photolysis. Although chloroplast isolation procedures were well established by 1967 (Cockburn *et al.*, 1967), it was only from 1981 onwards that granal stacks (BBYs), highly enriched in PSII were isolated (Berthold *et al.*, 1981; Ford & Evans, 1983; Dennenberg *et al.*, 1986). Such preparations catalysed water photolysis, but they were nevertheless membrane fragments containing a large number of proteins, including the light harvesting complex (LHCII). Discrete, detergent solubilised, PSII core preparations were only reported from 1985 onwards (Ikeuchi *et al.*, 1985). Since then, a number of preparations have been reported (Table 3.1). Despite extensive characterisation of their polypeptide composition and ability to evolve oxygen, none of these preparations have been simultaneously analysed for lipid depletion, homogeneity and subunit damage. All of these factors can affect crystal quality and so should be characterised before embarking on an extensive PSII crystallisation programme. The aim of the work presented in this chapter was to characterise a spinach PSII core preparation, isolated using the HTGP core preparation method outlined in Section 2.1.5. The theory underlying this isolation procedure is introduced in Sections 3.1.1-3.1.3. The properties of published core preparations, and the methods used to analyse them, are presented in Section 3.1.4. This chapter describes the first step of the development of a core purification method, yielding pure, active and undamaged PSII cores suitable for crystallisation trials.

3.1.1 Chloroplast and thylakoid isolation

The possible exposure of PSII to protease and free radical attack during purification is a major consideration in the design of any PSII purification strategy. Purification of intact chloroplasts prior to thylakoid isolation is perhaps the most elegant way of minimising the damage to the PSII complex, due to proteases and free radicals present in the crude leaf extract. Using this approach, the chloroplast envelope provides a physical barrier against damaging agents which can be removed by a series of washing steps, before the chloroplast is ruptured to release the thylakoid membranes. Intact chloroplasts have been isolated by centrifuging the filtered leaf extract in an isotonic buffer (0.2mM MgCl₂, 0.33M sorbitol solution), having a very low ionic strength (Nakatani & Barber, 1977). The low salt concentrations used caused thylakoid membranes, released from damaged chloroplasts, to unstack. The integrity of the intact chloroplasts was maintained by the isotonic conditions. As unstacked thylakoids are less dense than either their stacked counterparts or intact chloroplasts, the latter could be readily precipitated in a pure form using a 20s centrifugation step (2,200g).

Highly oxygen-evolving BBY-type membranes have been purified from spinach chloroplasts, isolated by this procedure (Ford & Evans, 1983). The use of this initial purification step has also yielded discrete PSII cores, which showed the highest rates of oxygen evolution reported in any of the preparations reviewed in Table 3.1 (Ikeuchi & Inoue, 1986). Unfortunately, for the purposes of

obtaining the large quantities of cores required for crystallisation screens, this approach is not suitable as thylakoid yields are low. The low yields are due to the large proportion of chloroplasts split, during the maceration step and the gentle centrifugation step employed to precipitate them. The most commonly used purification procedures achieve increased thylakoid yields by making two basic changes to the above procedure. Firstly, the ionic strength of the hypertonic chloroplast wash medium was raised, in particular by increasing the MgCl_2 concentration from 0.2 to 5mM. This is sufficient to maintain complete stacking of the large proportion of thylakoids released from ruptured chloroplasts (Barber & Chow, 1979; Barber, 1982a). The second change involves the use of a stronger centrifugation step, which enables the simultaneous precipitation of intact chloroplasts and the stacked thylakoid membranes. These changes form the basis of the thylakoid isolation steps used by Berthold *et al.*, (1981) and underlies the method used to prepare the thylakoids characterised in this chapter.

The improved thylakoid yields are of course achieved at a cost of their increased exposure to free radicals and cytoplasmic proteases. To prevent thylakoid damage a number of precautions were taken. Firstly, all purification stages were performed as quickly as possible, at 0°C to reduce protease-induced damage. Oxygen free radicals, which are able to oxidise proteins and lipids, were removed from solution by the addition of sodium ascorbate. Lysolipids, the products of lipid oxidation, can inhibit PSII mediated electron transport. Delipidated bovine serum albumin (BSA) was therefore added to the media used, as it binds lysolipids and reduces the electron transport inhibition they induce (Siegenthaler *et al.*, 1987). The BSA simultaneously acts as a competitive protease inhibitor. To maintain the integrity and protection afforded to PSII by the chloroplast envelope, intact chloroplasts were only burst by osmotic shock, to release the encapsulated thylakoid membranes, after extensive washing.

3.1.2 The purification of BBYs from thylakoids

BBYs, which are enriched in PSII and the LHCII complex, were first separated using a limited Triton X-100 solubilisation at pH 7.5 (Berthold *et al.*, 1981). Exposing the donor side to alkaline solutions can result in the release of the extrinsic polypeptides, which make up the oxygen-evolving complex (Kuwabara & Murata, 1982). To prevent this, the pH was reduced to 6.5 during the Triton X-100 solubilisation and in subsequent purification steps (Ford & Evans, 1983; Dennenberg *et al.*, 1986). Limited Triton X-100 solubilisation cleaves the stromal lamellae and non-appressed regions of the grana from the appressed regions. The latter are free of PSI (Andersson & Anderson, 1980). The ionic strength of the solubilisation medium and subsequent solutions is kept high to maintain granal stacking. The dense granal stacks are purified from the stromal lamellae fraction by a simple centrifugation step.

3.1.3 Isolation of detergent solubilised PSII core complexes from BBYs

The PSII core complexes listed in Table 3.1 were isolated from spinach, pea, wheat and rice BBYs using procedures similar to those outlined in Sections 3.1.1 and 3.1.2. BBYs contain two major protein complexes: PSII cores and LHCII. OG, HTGP and DM are the detergents most commonly

used to solubilise these two core complexes from the membranes in which they are embedded (see Table 3.1). The solubilised complexes can be separated by density gradient centrifugation (Ikeuchi *et al.*, 1985; Ikeuchi & Inoue, 1986; Haag *et al.*, 1990). An alternative approach involves the addition of $MgCl_2$ to the solubilised mixture. This salt normally induces granal stacking (see Section 3.1.2) but in solubilised membranes causes the LHCII to aggregate. This aggregate can be precipitated, leaving the solubilised PSII core in the supernatant (Ghanotakis & Yocum, 1986; Ghanotakis *et al.*, 1987a & b; De Las Rivas *et al.*, 1992a). Indeed it was the method of De Las Rivas *et al.*, (1992a) which formed the basis of the procedure used to isolate the cores characterised in this chapter (see Section 2.1.5). The methods used to characterise previously published cores, as well as the ones presented in this chapter, are described in Section 3.1.4.

3.1.4 Characterisation of the purity, activity and stability of isolated PSII core preparations

This section outlines the procedures used to characterise isolated PSII core complexes. Particular emphasis is placed on the parameters measured in Section 3.2, to determine the suitability of HTGP cores for crystallisation purposes.

A large number of oxygen-evolving PSII core preparations had been reported prior to the work described in this chapter (Table 3.1). The major subunits CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic subunit were identified in all cases. However, varying amounts of CP29, CP26, CP24, the 22kDa and 10kDa intrinsic, as well as the 23 and 17kDa extrinsic polypeptides were also present. Partial removal of these subunits during purification would lead to undesirable sample heterogeneity. To date, the smallest core complex capable of driving high rates of oxygen evolution *in vitro*, contains CP47, CP43, D2, D1, cytb559 and the 33kDa polypeptide, but lacks the chlorophyll a/b binding proteins CP24, CP26, CP29 and LHCII, as well as the intrinsic 22 and 10kDa and extrinsic 23 and 17kDa subunits (Ikeuchi & Inoue, 1986; Ghanotakis *et al.*, 1987a).

To understand fully the unique water splitting capability of PSII, a core complex containing all of the components required to drive this process will have to be induced to form highly ordered crystals. As the resolution to which a crystal diffracts is dependent on the number of repeating units (in this case a unit is a single core complex) and because large crystals are often physically unstable, it seems sensible to crystallise the smallest oxygen-evolving complex available. This suggests that PSII cores, similar in subunit composition to those reported by Ikeuchi & Inoue, (1986) and Ghanotakis *et al.*, (1987a), may be useful candidates for the above mentioned crystallisation trials. The aim of the work presented in this chapter was to characterise PSII cores purified from spinach using a method (Section 2.1.5) adapted from that of De Las Rivas, (1992a). Particular emphasis was placed on determining whether the cores isolated by this procedure (HTGP cores) were homogeneous, active, free of subunit breakdown fragments and thus suitable for future crystallisation trials.

To ensure the purity of a complex, its subunit and pigment composition, as well as its HPLC size exclusion profile, should be known. SDS-PAGE analysis procedures used to characterise the core preparations of Ikeuchi & Inoue, (1986), Ghanotakis *et al.*, (1987a), as well as most of the other PSII

cores listed in Table 3.1, do not resolve the LHCII polypeptides well from the D1 subunit band. Although CP26 and CP29 can be resolved from D1, they are poorly resolved from one another in such gel systems. The use of SDS-PAGE analysis has shown that the preparations of Ghanotakis & Yocum, (1986), De Las Rivas *et al.*, (1992a), Haag *et al.*, (1990) and the "PSII RC complex" preparation of Ghanotakis *et al.*, (1987a), all appear to contain some CP26 or CP29. To ensure that a core preparation is free of chlorophyll a/b binding protein contamination, it is useful to complement SDS-PAGE analysis with detailed pigment analysis. Such analysis has suggested that PSII reaction centres (D1, D2, cytb559 and the *psbI* gene product) bind 5-7 chlorophyll a molecules (Barber *et al.*, 1987; Nanba & Satoh, 1987; Dekker *et al.*, 1989; Gounaris *et al.*, 1990).

Table 3.1 Characteristics of published photosystem II core preparations

Reference	Ikeuchi <i>et al.</i> , (1985)	Ikeuchi M. & Inoue Y., (1986)	Ghanotakis & Yocum, (1986) and Ghanotakis <i>et al.</i> , (1987b)	Ghanotakis <i>et al.</i> , (1987a) PSII RC complex	Ghanotakis <i>et al.</i> , (1987a) PSII RC core	De Las Rivas <i>et al.</i> , (1992a)	Haag <i>et. al.</i> , (1990)/Gleiter <i>et al.</i> , (1993)	Enami <i>et. al.</i> , (1989)	Shen <i>et al.</i> , (1988)
Leaves used	Spinach	Wheat	Spinach	Spinach	Spinach	Pea	Spinach	Spinach	Rice mutant
Detergents used	Triton /OG	Triton /OG	Triton /OG	Triton /OG	Triton /OG	Triton /HTGP	Triton /DM	Triton /HTGP	Triton /OG
Subunits identified	CP47, CP43, 33, D2, D1, cytb559	CP47, CP43, 33, D2, D1, cytb559	CP47, CP43, 33, D2, D1, cytb559	CP47, CP43, 33, D2, D1, cytb559	CP47, CP43, 33, D2, D1, cytb559	CP47, CP43, 33, D2, D1, cytb559	CP47, CP43, 33, D2, D1, cytb559	CP47, CP43, 33, D2, D1, cytb559	CP47, CP43, 33, D2, D1, cytb559
Other subunits	approx. 10kDa	-	10,20, & 29kDa	10,20 & 28kDa	-	29kDa	approx. 65kDa CP29 ?	10 & 20kDa prob. intrinsic, CP29,23 and 17kDa extrinsic	21(23ext?), 15(17ext?) (22kDa & 10kDa int?) CP24
O ₂ evolution assay medium	40mM Mes (pH6.5) 0.4M Sucrose 5mM CaCl ₂ - - 25°C	40mM Mes (pH6.5) 0.4M Sucrose 10mM CaCl ₂ - 0.1% digitonin 25°C	40mM Mes (pH6.5) - 10mM CaCl ₂ - - -	50mM Mes (pH6.4) - 10mM CaCl ₂ 10mM NaCl, 0.01% DM -	50mM Mes (pH6.4) - 10mM CaCl ₂ , 10mM NaCl, 0.01% DM -	50mM Mes (pH6.5), 0.4M Sucrose - 10mM NaCl, 2mM DM -	20 mM Mes (pH6.5) - 50mM CaCl ₂ 10mM NaCl - 20°C	40mM Mes (pH6.0) 0.4M sucrose 5mM CaCl ₂ 10mM NaCl 5mM MgCl ₂ -	40mM Mes (pH6.5) 0.4M sucrose - 10mM NaCl 5mM MgCl ₂ 27°C
O ₂ evol. DCBQ	461 (0.3mM)	765 (0.6mM)	940 (0.5mM)	1050 (0.5mM)	640 (0.5mM)	-	1300 (1mM)	799 (0.4mM)	1070 (0.4mM)
O ₂ evol. DMBQ	2 (1mM)	84 (2mM)	-	-	-	-	-	-	470 (1mM)

O ₂ evol. FeCN	846 (1mM)	1481 (1mM)	530 (2mM)	620 (2mM)	1100 (2mM)	-	1300 (1mM)	371 (0.4mM)	270 (1mM)
O ₂ evol. PBQ	2 (0.3mM)	258	-	-	-	133 (0.5mM)	-	1114 (0.4mM)	1100 (0.4mM)
O ₂ evol. DCIP	55	-	-	-	-	56	-	-	200
DCMU sensitive	No (see Ghanotakis & Yocum.,(1986))	No	80% sensitive	75% sensitive	80% sensitive	-	30% sensitive	Yes	Yes
Pigment analysis	No	39.2 Chl 4Mn	-	-	-	Chl a 38.4 Pheophytin a 2 β-carotene 3.8 Xanthophylls 2 PQ A-9 1.2 Cytb559 1.15	-	80 Chl a:Q _A 76 Chl a:4Mn	53 Chl : Q _A 56 Chl: Ca ²⁺ 52 Chl: 4Mn
Spectrum	No	No	No	No	No	Yes	Yes	Yes	Yes
HPLC / FPLC size exclusion	No	No	No	No	No	No	250-300kDa	No	No

Considerably greater variation is seen between the estimates of the number of chlorophyll *a* molecules associated with CP47 and CP43 (Sections 1.4.4 and 1.4.5). Using the values of Barbato *et al.*, (1991a), who concluded that CP47 and CP43 each bind 9-12 chlorophyll *a* molecules, suggests that cores consisting of CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic polypeptide would bind 23-31 molecules of this pigment. The reports of de Vitry *et al.*, (1984) and Alfonso *et al.*, (1994) suggest that cores of this subunit composition are more likely to bind a larger number of chlorophyll *a* molecules. The pigment compositions of CP24, CP26, CP29 and the LHCII polypeptides are reviewed in Thornber *et al.*, (1993). This information is provided in Table 3.2. Interestingly, the pigment composition data, obtained by De Las Rivas *et al.*, (1992a) and summarised in Table 3.1, correlates well with the findings of Barbato *et al.*, (1991a) and Thornber *et al.*, (1993). If CP29 binds 9 chlorophyll *a* molecules, a core complex containing CP47, CP43, D2, D1, cytb559, the 33kDa extrinsic polypeptide, as well as a single CP29 subunit, would according to Barbato *et al.*, (1991a) and Thornber *et al.*, (1993) contain up to 40 chlorophyll *a* molecules per reaction centre. De Las Rivas *et al.*, (1992a) showed that a core composed of these subunits was associated with about 38 chlorophyll *a* molecules. This value is close to that theoretically expected from the results of Barbato *et al.*, (1991a) and Thornber *et al.*, (1993), yet is actually lower than that obtained by Ikeuchi & Inoue, (1986), who reported their core complexes to contain about 39 chlorophyll *a* molecules. In the absence of any analysis of the chlorophyll *b* content of these particles, the possibility that the preparation of Ikeuchi & Inoue, (1986) contains chlorophyll *a/b* binding protein contaminants must be considered. As Ghanotakis *et al.*, (1987a) provide no data on the pigment composition of their "PSII RC cores", it is possible that these preparations may also be associated with contaminating chlorophyll *a/b* binding proteins. In order to determine whether the HTGP cores, analysed in this chapter, contained any chlorophyll *a/b* binding proteins and to facilitate the quantification of any contaminants of this type, these preparations were subjected to SDS-PAGE and reverse phase HPLC analysis (Section 3.2).

The chlorophyll *a/b* binding proteins are not the only subunits whose removal has to be ensured if CP47, CP43, D2, D1, cytb559 and 33kDa complexes are to be isolated. PSII is associated with a large number of other subunits whose properties were reviewed in Chapter 1. Two of these, the 23kDa *psbP* and 17kDa *psbQ* gene products, are associated with the cores isolated by Enami *et al.*, (1989) and Shen *et al.*, (1988). Their removal does not cause a significant drop in oxygen evolution capability as long as the medium is supplemented with CaCl_2 (Table 3.1)(Ikeuchi *et al.*, 1985; Ikeuchi & Inoue, 1986; Ghanotakis *et al.*, 1987a; Haag *et al.*, 1990). These two subunits can easily be identified by SDS-PAGE analysis, as they are readily dissociated from the core complex using a NaCl wash (Åkerlund *et al.*, 1982; Miyao & Murata, 1989).

Table 3.2 The pigment composition of the chlorophyll a/b binding proteins associated with PSII

Chlorophyll a/b binding proteins associated with PSII	Molecular mass (kDa)	Chlorophyll		Xeaxanthin		
		a	b	Neoxanthin	Violoxanthin	Lutein
LHCIIa (CP29)	31	9	4	1	2	2
LHCIIb (CP2)	28/27/25	8	6	1	0.5	2
LHCIIc (CP26)	29/26.5	9	5	1	0.7	2
LHCIIId (CP24)	21	6	7	1	1	2
LHCIIe	13	2	1	1	3	2

* taken from Thornber *et al.*, (1993)

The 10kDa *psbH* and 22kDa *psbS* gene products are also commonly associated with isolated core preparations (Ikeuchi *et al.*, 1985; Ghanotakis & Yocum, 1986; Enami *et al.*, 1989; Shen *et al.*, 1988) and the "PSII RC complex" preparation of Ghanotakis *et al.*, (1987a). Barbato *et al.*, (1989) show where the 10 and 22kDa subunits run in an SDS-PAGE system, which is virtually identical to the one used to resolve the HTGP core preparation characterised in this chapter. Using this information, it is possible to identify whether HTGP cores contain these subunits, despite the lack of specific antibodies.

A large number of low molecular weight subunits have also been shown to be associated with PSII and were reviewed in Chapter 1. The *psbI* gene product is closely associated with the reaction centre of PSII (Webber *et al.*, 1989b; Ikeuchi *et al.*, 1989a) while the *psbL* gene product is thought to be involved in Q_A binding (Nagatsuka *et al.*, 1991). These two low molecular weight components at least, are likely to be associated with the core preparations listed in Table 3.1. None of the preparations listed in Table 3.1 have been subjected to analysis of their low molecular weight components. Due to a lack of specific antibodies to the low molecular weight *psbI*, *psbJ*, *psbK*, *psbL*, *psbM* and *psbN* gene products their presence in the HTGP cores will not be focused on in this chapter.

Table 3.3 Estimating the theoretical molecular weight of a PSII core (CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic polypeptide) from its polypeptide, chlorophyll a and pheophytin composition.

	CP47 subunit	CP43 subunit	D1 subunit	D2 subunit	α and β subunits of cytb559	33kDa extrinsic subunit	Core Complex *
Polypeptide molecular weight (kDa) **	55.6	50.1	38	39.4	13.7	26.5	223.3
No. of chlorophyll a molecules per subunit***	9-12	9-12	5-7 (per RC)	see D1	0	0	23-31
Total mass of chlorophyll a (kDa) (Mr=893.5)	8-10.7	8-10.7	4.5-6.2 (per RC)	see D1	0	0	20.5-27.6
No. of pheophytin molecules per subunit	0	0	2 (per RC)	see D1	0	0	2
Total mass of pheophytin (kDa) (Mr=871)	0	0	1.7	see D1	0	0	1.7
Estimated mass of individual subunits and complex (kDa)	64.2-67	58-60.7	95.8-97.5 (per RC)	see D1	13.7	26.5	245-252

* Core complex consisting of CP47, CP43, D2, D1, cytb559 and 33kDa extrinsic polypeptide complex. Low molecular weight polypeptides and minor pigments are not accounted for in this calculation.

** For review of polypeptide molecular weights see Nixon *et al.*, (1992).

*** Chlorophyll content according to Tang & Satoh, (1984), Barber *et al.*, (1987), Nanba & Satoh, (1987), Dekker *et al.*, (1989), Gounaris *et al.*, (1990) and Barbato *et al.*, (1991a).

Another parameter other than subunit and pigment composition must be analysed, if the purity of an isolated PSII complex is to be ensured. Subunit and pigment analyses provide no information on the aggregation state of the isolated core particle. Core preparations containing randomly aggregated PSII complexes are not suitable for crystallisation purposes, as the aggregates can associate with a growing crystal and so disrupt the formation of an ordered lattice. The preparation of

Haag *et al.*, (1990) is the only higher plant core preparation listed in Table 3.1 which has been subjected to any form of size exclusion analysis. It was shown to consist of complexes having a molecular weight of 270kDa in the presence of DM. As a monomeric complex consisting of CP47, CP43, D2, D1, cytb559 and the 33kDa polypeptide is calculated to have a molecular weight of about 250kDa (see Table 3.3) it is concluded that this preparation is indeed composed of monomeric complexes. The HTGP cores, characterised in this chapter, were subjected to HPLC size exclusion analysis to determine whether they consisted of a single population of particles and whether they could be identified as being monomeric or dimeric complexes of PSII. Such analysis is important in the light of the current debate on whether the active form of PSII is a monomeric or dimeric complex *in vivo* (Barbato *et al.*, 1992a; Santini *et al.*, 1994).

Being a membrane protein, it is quite likely that isolated PSII cores are still associated with a quantity of lipid molecules. The presence of excess lipid can inhibit the formation of ordered three-dimensional crystals. None of the preparations listed in Table 3.1 have been analysed for lipid content. Although detailed lipid analysis is time consuming, a simple method of determining the presence of large quantities of lipid is introduced here. If a solution containing detergent solubilised PSII cores is dialysed to remove the detergent, the remaining protein complexes are forced to aggregate. Thus PSII cores, associated with fairly large amounts of lipid, form lipid-protein sheets (see Section 7.2.3). In the absence of lipid, electron dense protein precipitate is observed. It is therefore possible to use this dialysis procedure to estimate the lipid content of a PSII core preparation. This analysis was carried out on HTGP cores and the results obtained are presented in Chapter 7.

So far, this section has outlined how SDS-PAGE, HPLC size exclusion, dialysis and pigment analysis procedures can be used to determine the purity and homogeneity of isolated PSII core complexes. However, the most highly purified PSII complexes are virtually useless for the purposes of crystallisation, if they have been damaged during the purification procedure. Oxygen evolution assays are the most direct method of measuring the activity of the PSII core complex. Oxygen evolution rates listed in Table 3.1 ranged from 2 to 1,481 $\mu\text{moles O}_2 \text{ mg chl}^{-1} \text{ h}^{-1}$ for various preparations. As all preparations contain CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic subunit, some of this variation may be explained by differences in the assay conditions used. They varied in pH (6.0-6.5), NaCl (0-10mM), CaCl_2 (0-50mM) and sucrose concentration (0-0.4M) as well as in the detergent used (digitonin (0.1%) or DM (2mM)) and temperature (20-27°C). As cores, lacking the 23 and 17kDa extrinsic polypeptides, require NaCl (Ghanotakis & Yocum, 1986) and CaCl_2 (Ghanotakis & Yocum, 1986; Haag *et al.*, 1990) to maintain high rates of oxygen evolution, the rate differences are perhaps not so surprising.

The oxygen evolution rates observed are also dependent on the electron transport acceptor used (see Table 3.1). The larger complexes (Enami *et al.*, 1989; Shen *et al.*, 1988) showed highest oxygen evolution rates with PBQ (Table 3.1). These cores are associated with the 22 and 10kDa intrinsic subunits, as well as the complete oxygen-evolving complex. Smaller cores are commonly assayed in the presence of DCBQ or FeCN (Table 3.1). FeCN is able to accept electrons from the non

haem iron between Q_A and Q_B (Diner & Petrouleas, 1988). This explains its ability to support high rates of oxygen evolution in cores having a damaged Q_B site (Haag *et al.*, 1990; Gleiter *et al.*, 1993). DCBQ is thought to accept electrons from Q_A and Q_B (Graan & Ort, 1986) and supported equally high rates of oxygen evolution in these core preparations. DMBQ is thought to accept electrons only from the Q_B site (Graan & Ort, 1986). Measuring oxygen evolution in the presence of either DCBQ or DMBQ therefore allows changes at the Q_B site to be estimated at various stages of purification (Section 2.4).

The HTGP cores characterised in this chapter were subjected to oxygen evolution rate measurements in the presence of either DCBQ or DMBQ. Such measurements provide a limited amount of information about the Q_B site activity of an isolated PSII complex. Further information can be obtained by Western blot analysis, which identifies whether specific subunits are damaged during the isolation procedure. This chapter describes CP47, CP43, D2, D1 and 33kDa subunit specific Western blot analyses of SDS-PAGE resolved HTGP core preparations. Mapping the breakdown fragments in this way is the first step toward identifying the sites of PSII most susceptible to damage. Once identified, steps can be taken to prevent their formation. None of the preparations listed in Table 3.1 have been subjected to such analysis.

Section 3.2 describes the results of the characterisation of the purity, stability and activity of HTGP cores isolated according to the methods outlined in Section 2.1.5.

3.2 Results and discussion

3.2.1 SDS/gel electrophoresis

3.2.1.1 Identification of the subunits associated with HTGP cores

The experiments presented in this section were designed to identify the major PSII subunits associated with HTGP cores. In the absence of antibodies, raised against the low molecular weight components of PSII, no attempt was made to identify polypeptides having apparent molecular weights of less than 9kDa.

Isolated thylakoids, BBYs, PSII cores and their Tris-washed products were solubilised with buffer 1 (Section 2.2.2) before being subjected to SDS-PAGE (Sections 2.2.1-2.2.3). An HTGP core sample was run in parallel with a PSII core preparation, known to bind the 23 and 17kDa extrinsic subunits, to ensure the identification of all three extrinsic subunit bands. On completion the gel was stained with Coomassie R250 (Section 2.2.4). The visualised subunit bands seen in Figure 3.1, were labelled according to the nomenclature used by Barbato *et al.*, (1989).

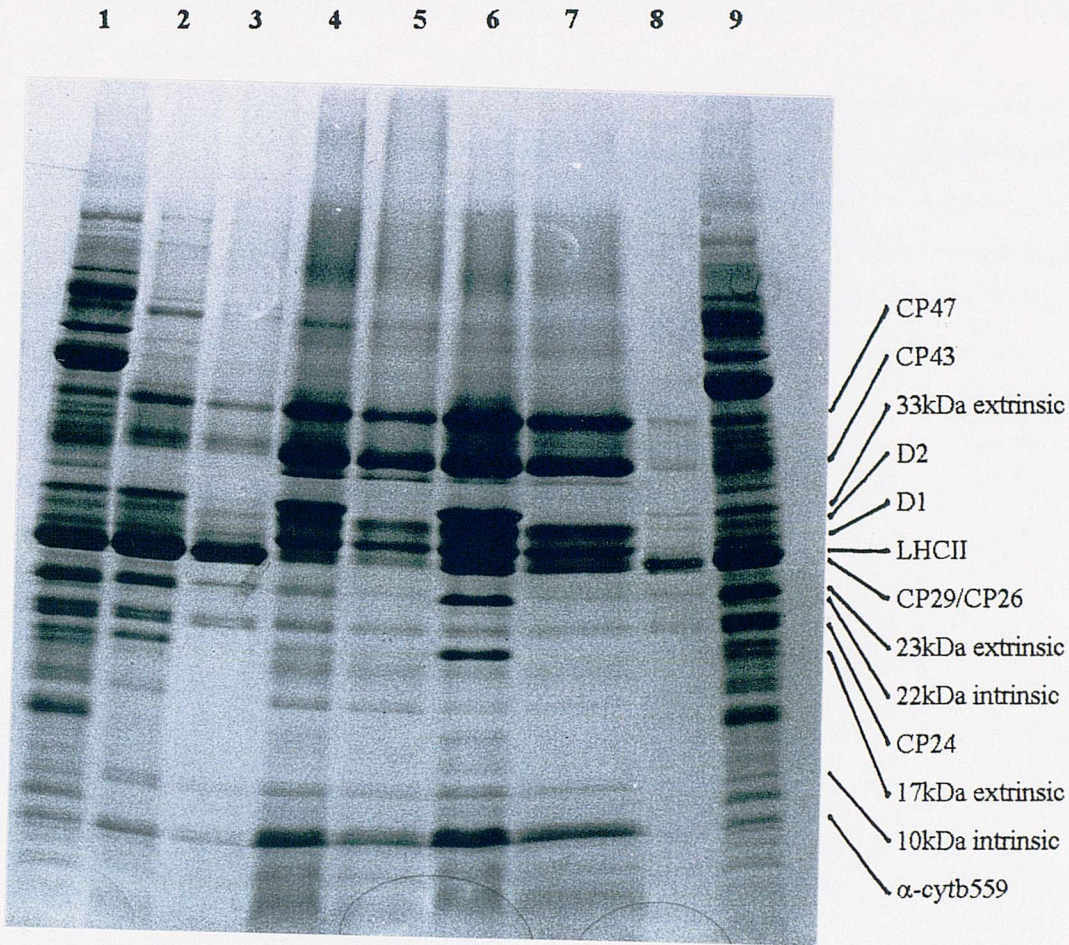


Figure 3.1 Identification of subunits of SDS-PAGE resolved thylakoid, BBY, PSII core and LHCII samples. Lane 1:Thylakoids, Lane 2:BBYs, Lane 3:Tris-washed BBY, Lane 4: HTGP cores, Lane 5:Tris-washed HTGP cores, Lane 6: PSII cores (OG cores see Chapter 4), Lane 7:Tris-washed PSII cores (OG cores see Chapter 4), Lane 8:LHCII pellet, Lane 9:Thylakoids. Subunits are labelled according to the nomenclature used by Barbato *et al.*, (1989).

3.2.1.2 Determining the variation of subunit composition between triplicate thylakoid, BBY and HTGP core preparations

The following experiment was conducted to determine whether the HTGP core preparation procedure, described in Section 2.1.5, allowed the isolation of PSII cores of reproducible quality. To test whether or not this was the case, three HTGP core preparations (preps. 1-3), as well as the thylakoid (preps. 1-3, respectively) and BBY particles (preps. 1-3, respectively) from which they were isolated, were subjected to simultaneous SDS-PAGE analysis. The results are shown in Figure 3.2.

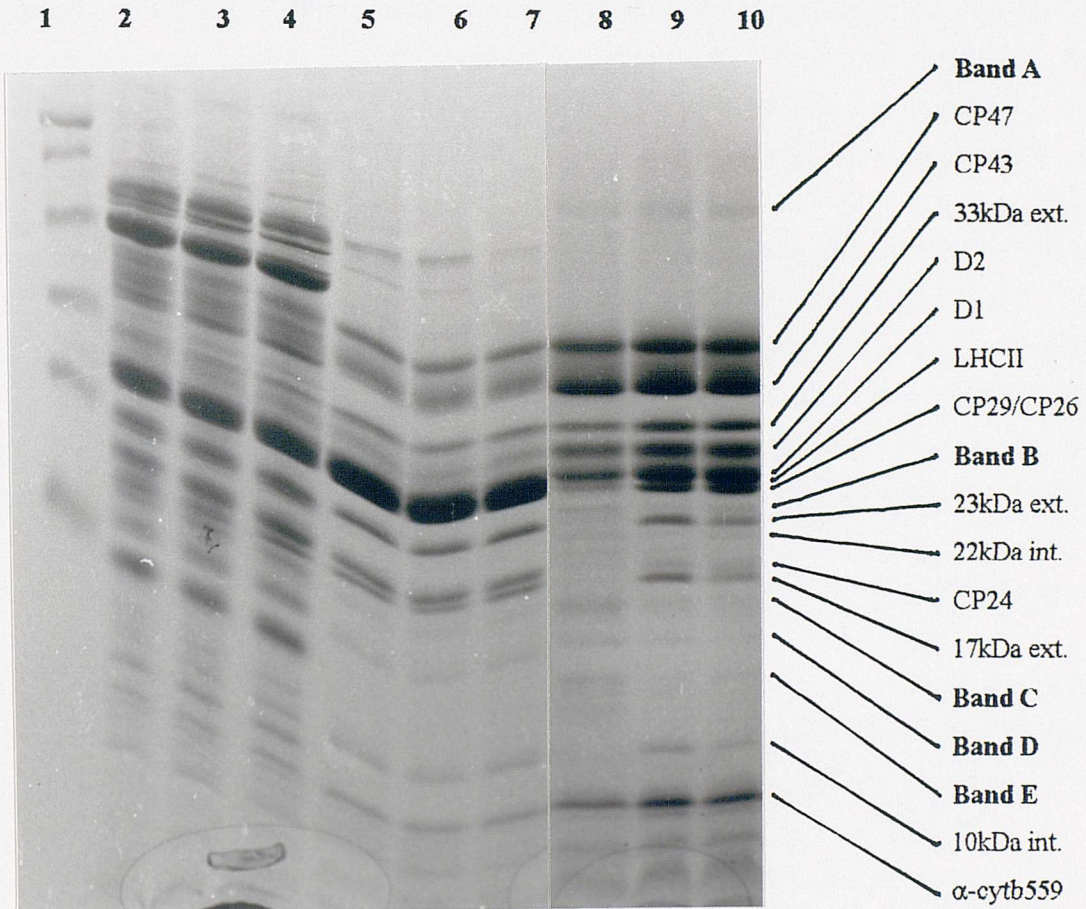


Figure 3.2 Variation in subunit composition between triplicate PSII preparations at the thylakoid, BBY and isolated core stage. Lane 1: Biorad Markers, Lane 2: Thylakoids (prep. 1), Lane 3: Thylakoids (prep. 2), Lane 4: Thylakoids (prep. 3), Lane 5: BBYs (prep. 1), Lane 6: BBYs (prep. 2), Lane 7: BBYs (prep. 3), Lane 8: PSII cores (prep. 1), Lane 9: PSII cores (prep. 2), Lane 10: PSII cores (prep. 3). In addition to the labelled PSII subunit bands, five unidentified bands (A-E) are labelled. The Biorad marker polypeptides had molecular weights of 106kDa, 80kDa, 49.5kDa, 32.5kDa, 27.5kDa and 18.5kDa. Extrinsic and intrinsic proteins are labelled ext. and int., respectively.

Lanes 2-4 (Figure 3.2) show that there is no noticeable difference in the subunit composition of the three thylakoid preparations analysed. The triplicate BBY preparations, resolved in Lanes 5-7 (Figure 3.2) also differed little from one another, in their subunit composition. This confirmed that the isolation procedure described in Section 2.1.5, yielded thylakoid and BBY type membranes of reproducible quality. However, variation was observed between the three HTGP core preparations. The subunit composition (Figure 3.2, Lane 8) of HTGP core (prep. 1) was very similar to that reported by De Las Rivas *et al.*, (1992a). However, preparations 2 and 3 show that HTGP cores were more typically associated with the 23 and 17kDa extrinsic polypeptides, as well as CP29, CP26 and CP24 (Figure 3.2, Lanes 9 & 10). The 22kDa and 10kDa polypeptides also appear to be present in these preparations. The above results suggest that the differences between the subunit compositions of the three HTGP core samples are the result of slight variations in the way in which they were isolated from their respective BBY preparations.

Five unidentified bands (Bands A-E) can be seen to be associated with the HTGP core preparations resolved by SDS-PAGE in Lanes 8-10 (Figure 3.2). Bands B, C, D and E appeared to be enriched in preparation 1. As this sample was depleted of CP29/CP26, CP24, as well as the 23 and 17kDa extrinsic polypeptides, it seemed likely that bands B, C, D and E represent PSII subunit breakdown fragments, rather than residual contaminants. To determine whether this was the case, a HTGP core preparation was subjected to the Western blot analysis presented in Section 3.2.2.

3.2.2 Western blot analysis of thylakoids, BBYs and isolated core particles with antibodies raised against CP47, CP43, D2, D1 and the 33kDa extrinsic subunit.

The aim of the work presented in this section was to determine whether Bands A, B, C, D, and E (Figure 3.2) could be identified as aggregation or breakdown products of CP47, CP43, the 33kDa extrinsic polypeptide, D2 or D1. To test whether this was the case, an SDS-PAGE resolved HTGP core preparation was subjected to Western blot analysis using antibodies specific to the above subunits. Samples of the thylakoid and BBY preparations, from which the HTGP cores analysed in this section were isolated, were subjected to similar Western blot analysis. In this way, I aimed to test whether any breakdown fragments detected in the HTGP core preparation, could also be detected in the thylakoid and BBY samples from which they were isolated. Equal amounts (on the basis of chlorophyll concentration) of thylakoids (Figure 3.4), BBYs (Figure 3.5) and HTGP core particles (Figure 3.6) were Western blotted according to Sections 2.2.1-2.3.2. As the antigenic subunits are enriched during the purification process, relative band intensities at the three purification stages are not indicative of variations in the amount of breakdown products detected. The results are therefore only used to identify the presence and position of any antigenic bands, but not to compare their relative intensities. It should be noted that D1 specific Western blots were done using C-terminal specific antibodies.

Western blot analysis allowed the identification of a number of bands in thylakoids (Figure 3.4), BBYs (Figure 3.5) and isolated cores (Figure 3.6). The apparent molecular weights of these antigenic components were calculated using the standard curve shown in Figure 3.3. The intact

subunits of CP47, CP43, the 33kDa extrinsic polypeptide, D2 and D1 are labelled in bold type in Figures 3.4-3.6. Thylakoids, BBY and HTGP core preparations all contained polypeptides which were labelled by specific antibodies, but had higher apparent molecular weights than the intact PSII subunits. It was therefore concluded that such polypeptides were PSII subunit aggregates rather than contaminants. The largest HTGP core component aggregate (Figure 3.6) had an apparent molecular weight of 91kDa and was only labelled by the CP47 specific antibody. As intact CP47 had an apparent molecular weight of 55kDa (Figure 3.6), it is possible that this band represented a dimeric CP47 aggregate. Comparison of Figure 3.6 and Figure 3.2 shows that the 91kDa band labelled by the CP47 specific antibody (Figure 3.6) corresponds to Band A (Figure 3.2). It seems likely that Band A is either formed as a result of incomplete sample solubilisation or SDS induced subunit aggregation, rather than any purification-induced artifact. Indeed, this view is supported by the finding that similar bands were detected in both BBYs (100kDa) and thylakoids (101kDa). Although, the molecular weights of these bands were higher than those observed in the HTGP core preparation (91kDa), so were those of the intact CP47 which ran at 58kDa in BBYs and thylakoids (Figures 3.5 and 3.4, respectively).

Barbato *et al.*, (1992d) have shown that photoinhibitory treatments, which lead to the damage of isolated PSII reaction centres, can result in the cross-linking of the α -subunit of cytb559 and the D1 polypeptide. If photoinhibitory treatments can cause subunit cross-linking, it is also possible that purification procedures might have a similar effect. Thus, at this stage, the possibility that the "CP47 dimer" aggregate is the product of purification induced subunit cross-linking, cannot be excluded.

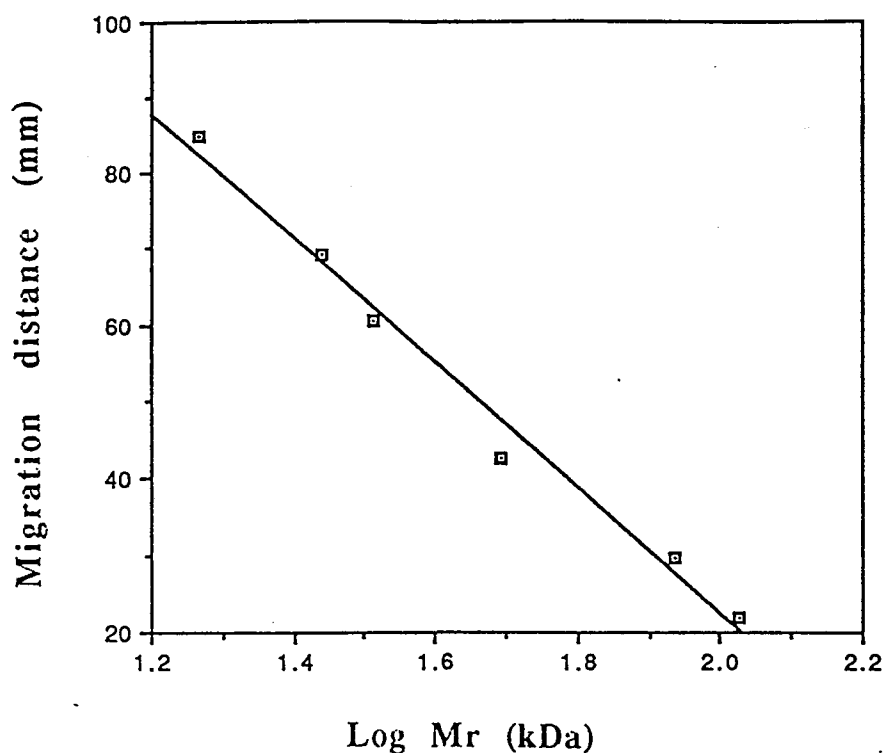


Figure 3.3 Standard curve of molecular weight markers used to calculate the molecular weights of the bands shown in Figures 3.4-3.6. The markers used were phosphorylase b (106kDa), bovine serum albumin (80kDa), ovalbumin (49.5kDa), carbonic anhydrase (32.5kDa), soybean trypsin inhibitor (27.5kDa) and lysozyme (18.5kDa).

It should be noted that the detection of high molecular weight bands, associated with PSII cores, is not unique to this preparation. Haag *et al.*, (1990) showed that their PSII core preparation contained a polypeptide having a higher apparent molecular weight than that of the intact CP47 subunit. The other publications describing the characterisation of isolated PSII core preparations, reviewed in Table 3.1, also provide photographs of Coomassie stained SDS-PAGE gels. However, in most cases the SDS-PAGE resolved core samples, presented by the respective authors (Table 3.1), were obtained using lower sample loadings, and so would fail to detect any of the aggregation bands reported in this chapter.

A 79kDa D1/D2 heterodimer band was the second largest aggregation band detected in cores and corresponded well to the 77kDa D1/D2 heterodimers, detected in both BBYs (Figure 3.5) and thylakoids (Figure 3.4). As the apparent molecular weights of intact D2 and D1 subunits in the core particles (Figure 3.6) were calculated to be 38 and 35kDa respectively it is quite possible that the 77-79kDa bands correspond to a heterodimer consisting of intact subunits. Whether or not this 79kDa band is the product of purification-induced subunit damage, incomplete sample solubilisation or SDS induced subunit aggregation, remains to be determined. Whatever the cause, the low stain intensity of this polypeptide band suggests that it is present in thylakoids, BBYs and HTGP cores in very low amounts.

The 58kDa band in Figure 3.6 was also detected by both the D1 and the D2 antibody. The band was labelled as being detected by CP47, as well. Although this cannot be discounted, it is possible that the colour development in this region of the CP47 specific blot is due to over-development of the intact CP47 subunit band. A similar band was observed in HTGP cores (Figure 3.6)

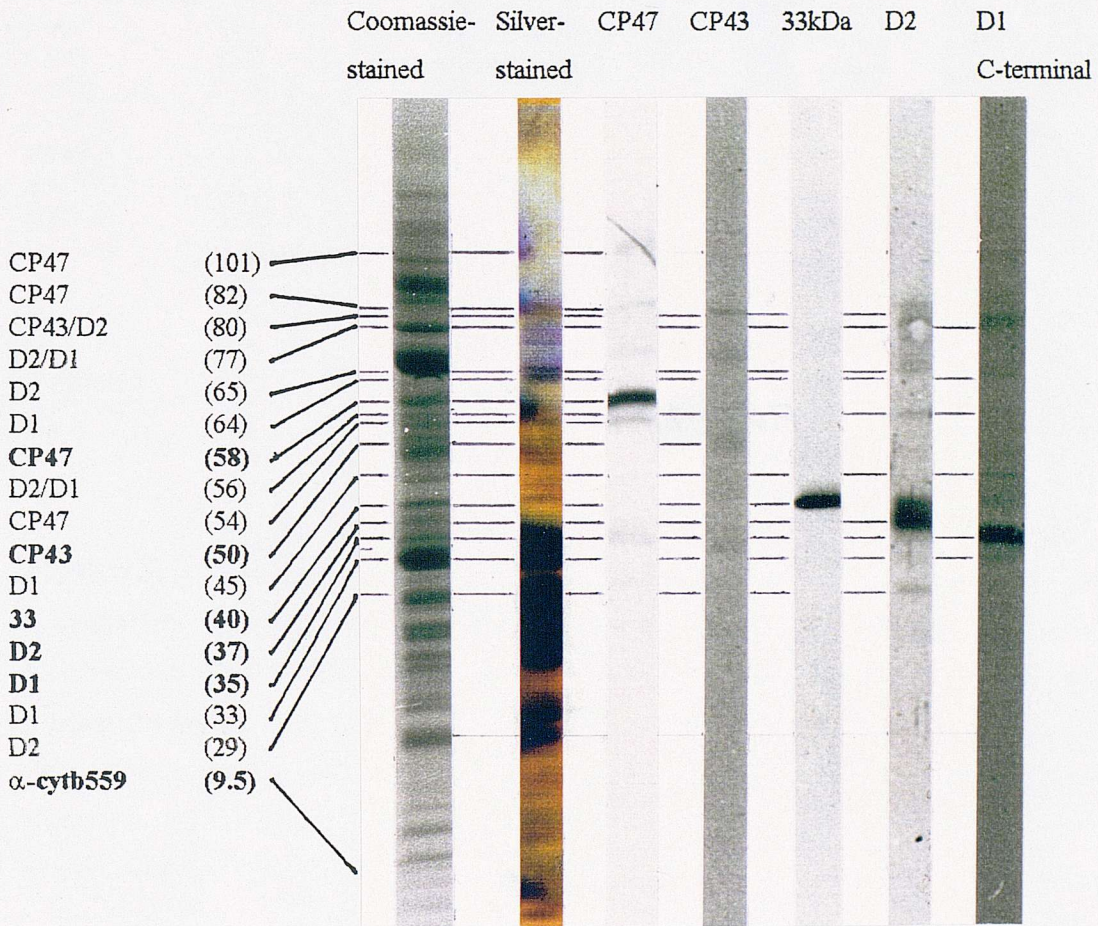


Figure 3.4 The detection of CP47, CP43, 33kDa extrinsic, D2 and D1 subunit aggregation and breakdown products in thylakoids using Western blot analysis. The combined use of Coomassie-stained and silver-stained gels allows sensitive detection of the polypeptides present in the preparation. Western blotting allows the determination of their origin. Bold labels indicate the intact subunits. Plain text indicates aggregation or breakdown products detected by specific antibodies. The numbers in brackets are the molecular weights, calculated using the standard curve shown in Figure 3.3.

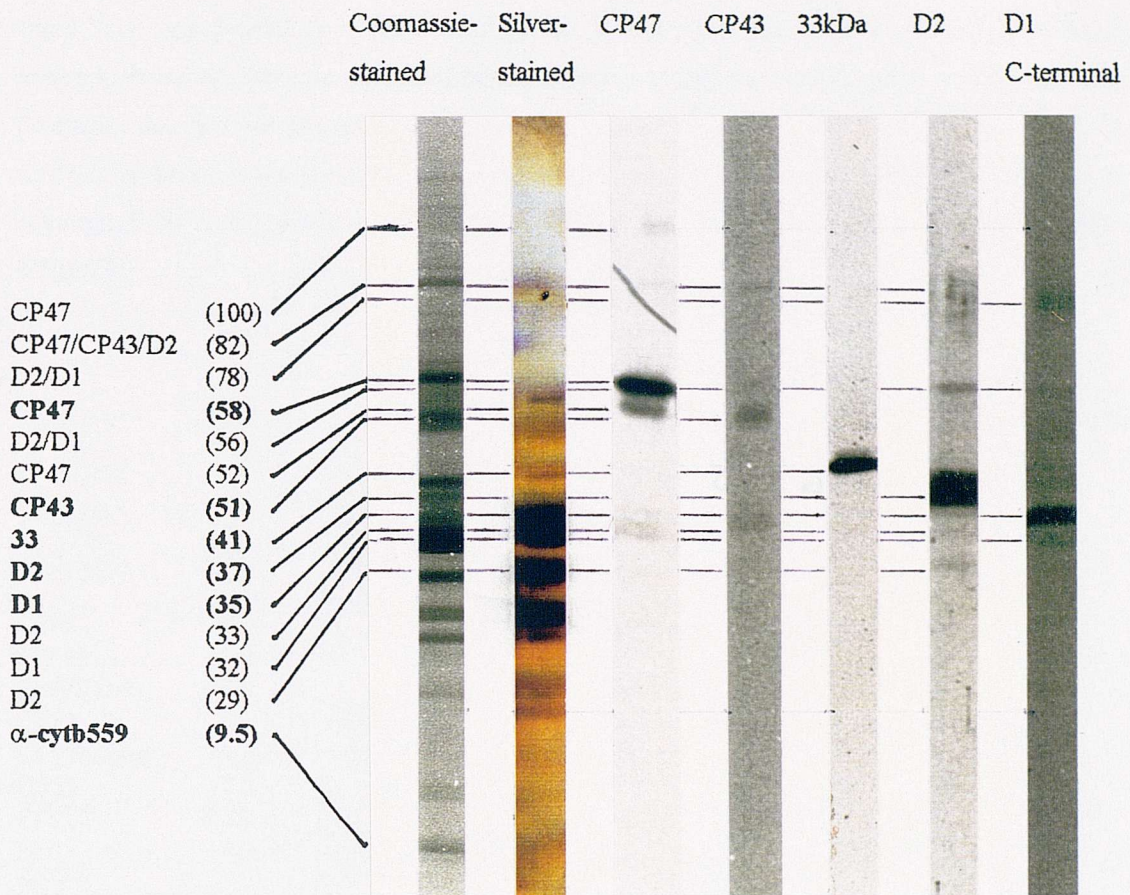


Figure 3.5 The detection of CP47, CP43, 33kDa extrinsic, D2 and D1 subunit aggregation and breakdown products in BBYs using Western blot analysis. The combined use of Coomassie-stained and silver-stained gels allows sensitive detection of the polypeptides present in the preparation. Western blotting allows the determination of the origin of some of the detected bands. Bold labels indicate the intact subunits. Plain text indicates aggregation or breakdown products detected by specific antibodies. The numbers in brackets are the molecular weights, calculated using the standard curve shown in Figure 3.3.

The intact CP47 (55kDa), CP43 (47kDa), 33kDa extrinsic (42kDa), D2 (38kDa) and D1 (35kDa) subunits were clearly detected by their specific antibodies (Figure 3.6), thus confirming the labelling of the Coomassie-stained gels depicted in Figure 3.2. Their apparent molecular weights fitted the true molecular weights quite well, except for the 33kDa extrinsic polypeptide (see Table 3.3). This protein has a molecular weight of 26.5kDa, yet in this system the apparent molecular weight was calculated to be 42kDa.

Several breakdown products running below the intact D1 band, were detected. A 33kDa D1 fragment was observed just below the intact D1 subunit. Although a C-terminal specific antibody was used, this fragment could be formed as a result of D1 cleavage at two possible positions. If a small proportion of the total number of D1 polypeptides was cut near the N-terminal end, a faint 33kDa band would be expected to be detected by this C-terminal specific antibody. Conversely, if a polypeptide of

about 2kDa was cleaved from the C-terminal end it is possible that there would still be sufficient antigenic structure attached to the large fragment to allow detection using this antibody. The 33kDa D1 fragment does not correspond to any of the Bands labelled B-E in Figure 3.2. However, as the CP29/CP26 band runs at the same position as this putative 33kDa D1 fragment, it is likely that the chlorophyll a/b binding proteins prevented its detection in the Coomassie-stained gels shown in Figure 3.2 and 3.6.

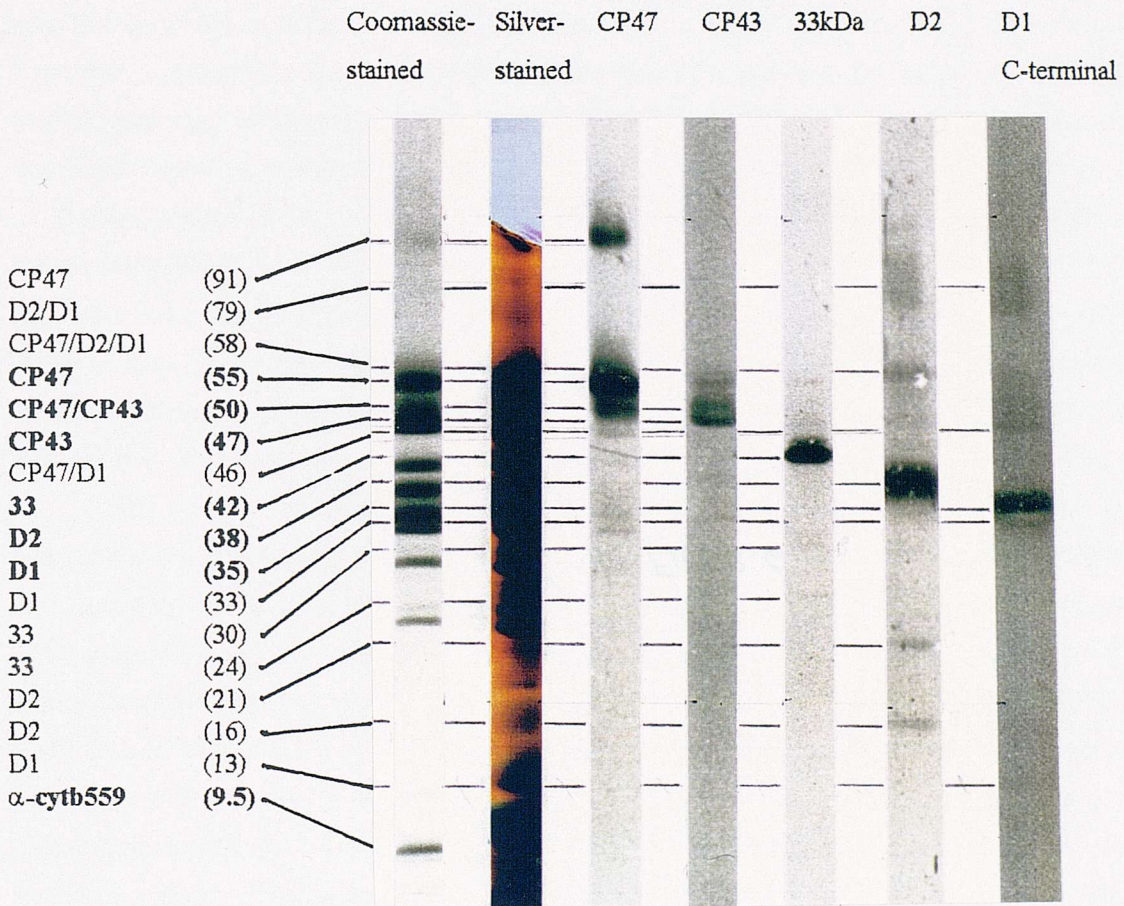


Figure 3.6 The detection of CP47, CP43, 33kDa extrinsic, D2 and D1 subunit aggregation and breakdown products in isolated HTGP cores, using Western blot analysis. The combined use of Coomassie-stained and silver-stained gels allows sensitive detection of the polypeptides present in the preparation. Western blotting allows the determination of the origin of some of the detected bands. Bold labels indicate the intact subunits. Plain text indicates aggregation or breakdown products detected by specific antibodies. The numbers in brackets are the molecular weights, calculated using the standard curve shown in Figure 3.3.

A very faint band, having a molecular weight of 30kDa, was detected in the 33kDa extrinsic polypeptide specific Western blot shown in Figure 3.6. This band ran in the same position as Band B (Figure 3.2). It is therefore possible that Band B is a breakdown fragment of the 33kDa subunit. The

fact that the 30kDa band, detected by the 33kDa extrinsic specific antibody (Figure 3.6), is so weakly stained in this Western blot and Coomassie stained gel, suggests that this fragment is present in very low quantities in this particular HTGP core preparation.

HTGP cores were also shown to be associated with two D2 breakdown products, having molecular weights of 21 and 16kDa. These molecular weights add up to 37kDa, which is remarkably close to the apparent molecular weight of the intact D1 subunit (38kDa) observed in this gel system. Similar sized D2 bands were not detected at either the thylakoid or BBY stages of the preparation. This may be because higher thylakoid and BBY loadings would be required to allow the detection of such fragments. Alternatively, it is of course possible that the D2 subunit was cleaved during the HTGP solubilisation step. Comparing Figures 3.2 and 3.6 shows that the 21 and 16kDa D2 breakdown fragments (Figure 3.6) correspond to Bands C and E (Figure 3.2), respectively. Interestingly, Friso *et al.*, (1994) reported a 22kDa D2 fragment containing the N-terminus. The authors suggested that it was the product of cleavage in the hydrophilic loop, linking the putative transmembrane helices IV and V of D2. This region contains ligands involved in the binding of Q_A (see Section 1.4.2). Furthermore Ponticos *et al.*, (1994) have recently detected a 20kDa D2 fragment in acceptor side photoinhibited oxygen-evolving core preparations. It is therefore possible that the 21kDa and 16kDa D2 fragments, detected in the HTGP core preparation (Figure 3.6), are the products of D2 cleavage near the Q_A site.

The smallest breakdown product detected in HTGP cores was a 13kDa C-terminal D1 polypeptide (Figure 3.6). It was only weakly stained in the D1 specific Western Blots and no equivalent band was detected in the Coomassie-stained sample. It has been suggested that the cleavage of D1 in the hydrophilic loop connecting transmembrane helices IV and V, results in the formation of a 10kDa C-terminal fragment (Greenberg *et al.*, 1987; De Las Rivas *et al.*, 1992b; Cánovas & Barber, 1993; De Las Rivas *et al.*, 1993). Cleavage in the hydrophilic loop connecting transmembrane helices III and IV of D1 is thought to result in the formation of a 16kDa fragment (Ponticos *et al.*, 1993). The 13kDa C-terminal D1 fragment detected in the HTGP core preparation, (Figure 3.6), may therefore be formed as a result of D1 cleavage near transmembrane helix IV. Whether the cleavage site is on the stromal or luminal side of the PSII complex remains to be determined.

Although the identity of Band D (Figure 3.2) was not accounted for by the Western blot analysis shown in Figure 3.6, it is quite possible that it is an N-terminal D1 fragment not detected with the C-terminal specific antibody used in these experiments. However as the remainder of the Bands A, B, C and E were identified as a CP47 aggregate (A), and breakdown products of the 33kDa (B), D2 (C) and D2 (E) subunits respectively, it seems reasonable to conclude that HTGP cores are free of any non-PSII related contamination. Having established this, HTGP core preparations were subjected to pigment analysis in order to estimate their chlorophyll a and b composition and hence the number of chlorophyll a/b binding subunits with which each reaction centre is associated.

3.2.3 Pigment analysis

3.2.3.1 Spectra of isolated thylakoids, BBYs, HTGP cores and the LHCII fraction

Figure 3.7 compares the absorption spectra of thylakoids, BBYs and a crude LHCII fraction with that of isolated HTGP cores, using the method described in Section 2.6. The four spectra shown in Figure 3.7, were normalised at the 437nm peak. Chlorophyll a is known to absorb predominantly at 437nm and 667nm, whereas chlorophyll b has peaks in the 470-480nm and 650nm region of the spectrum, when associated with PSII. HTGP cores absorb much less strongly in the 470-480nm region than the thylakoid, BBY and LHCII samples (Figure 3.7) leading to the conclusion that these cores are depleted, but are not free of chlorophyll b. This conclusion is in complete agreement with the SDS-PAGE analysis (Figure 3.2, Lanes 8-10), which ^{suggested}, all three HTGP core preparations to be associated with the chlorophyll a/b binding proteins CP24, CP26 and CP29, but virtually free of LHCII. As chlorophyll a, chlorophyll b and cytb559 absorption spectra overlap, it is not possible to calculate the pigment compositions of HTGP cores directly from their spectra.

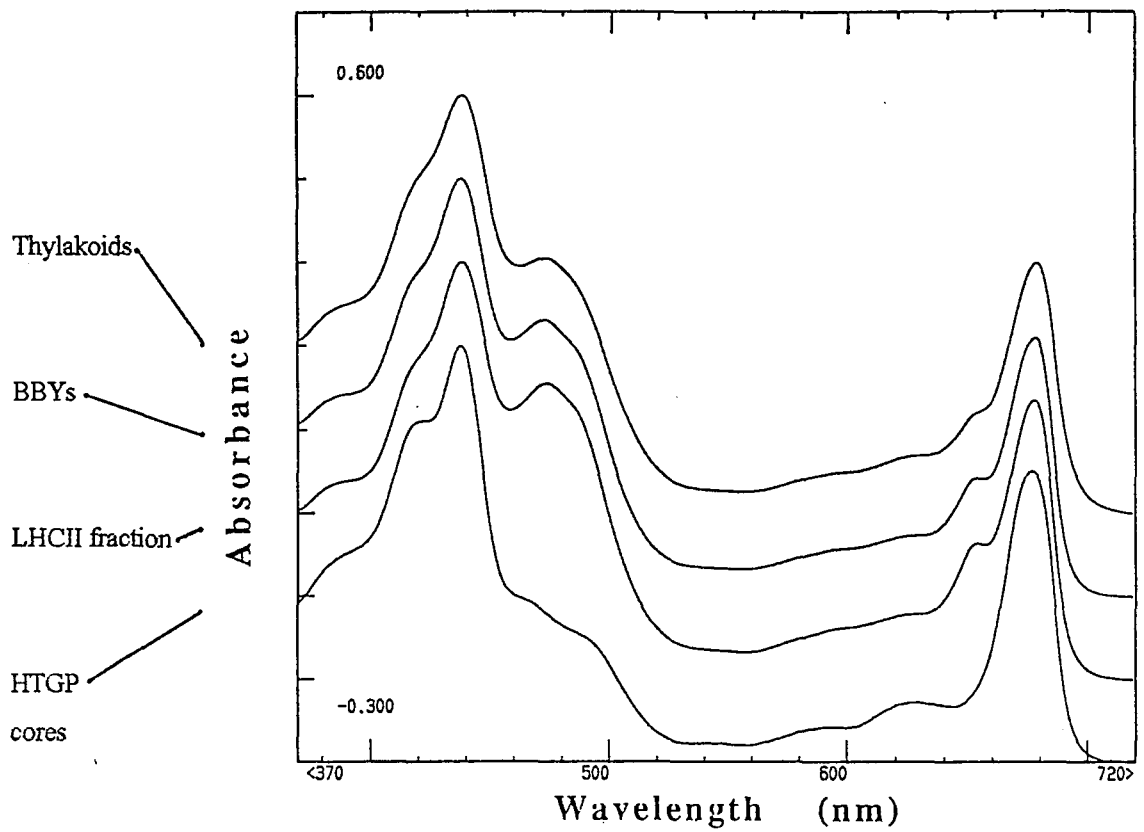


Figure 3.7 Spectra of isolated thylakoids, BBYs, HTGP cores and the LHCII fraction spectra. The absorption spectra of thylakoids BBYs, HTGP cores and the LHCII fraction were measured in SMNCB buffer supplemented with 2mM DM, using the method described in Section 2.6.

3.2.3.2 Triplicate spectra

The absorption spectra of the isolated core preparations (1, 2 and 3), which were analysed by SDS-PAGE in Figure 3.2 (lanes 8-10), are compared in Figure 3.8. The spectral similarities suggest that the chlorophyll a: chlorophyll b ratios are fairly constant between preparations, making them suitable for more detailed pigment analysis. Determining the number of chlorophyll a and b molecules associated with a given core particle gives an estimation of the number of chlorophyll a/b binding proteins associated with each monomeric core complex.

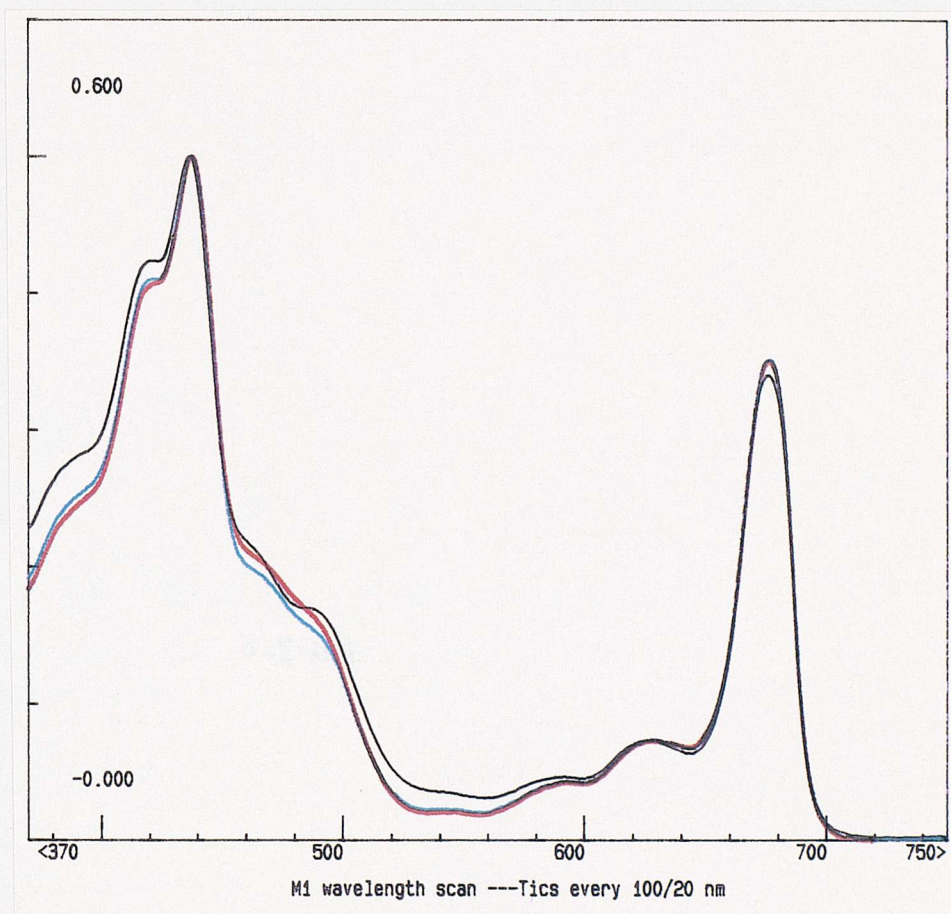


Figure 3.8 Spectra of three HTGP PSII core preparations. The isolated core preparations 1 (black), 2 (green) and 3 (red), whose subunits were resolved by SDS-PAGE in Figure 3.2 (lanes 8-10), were solubilised with SMNCB supplemented with 2mM DM. The spectra were normalised at 437nm.

3.2.3.3 Determining the number of chlorophyll a, chlorophyll b and β -carotene molecules associated with each of the triplicate HTGP core complexes.

In order to determine the pigment composition of the three HTGP core preparations characterised by SDS-PAGE in Figure 3.2, these preparations were subjected to reverse phase HPLC analysis using the method described in Section 2.6. Using this procedure it was possible to quantify their chlorophyll a, chlorophyll b, β -carotene and pheophytin composition.

Higher plant PSII reaction centres (D1, D2, cytb559 and the *psbI* gene product) are thought to contain two pheophytin molecules (Kobayashi *et al.*, 1990). In contrast, CP47 (Alfonso *et al.*, 1994), CP43 (Alfonso *et al.*, 1994), CP24, CP26, CP29 and the components of LHCII (see Thornber *et al.*, 1993) do not appear to contain this pigment. Thus, the pigment content of the HTGP core particles could be calculated by normalising the amount of chlorophyll a, chlorophyll b and β -carotene to two pheophytin molecules. The results of such analysis are shown in Table 3.4.

Table 3.4 The pigment composition of three HTGP core preparations

Sample	Chlorophyll a	Chlorophyll b	β -carotene	Pheophytin
HTGP core prep. 1	40.8	5.0	8.6	2
HTGP core prep. 2	41.8	6.1	7.3	2
HTGP core prep. 3	40.3	6.9	6.8	2
HTGP core prep. Mean (SD)	41.0 (0.8)	6.0 (1.0)	7.6 (0.7)	2

The average number of chlorophyll b molecules associated with each HTGP core complex was calculated to be 6.0 (see Table 3.4). Figure 3.2 shows that the CP29/CP26 band in the three core preparations analysed, is far more intense than that of CP24. Most of the chlorophyll b associated with these core preparations is therefore likely to be associated with CP26 and/or CP29. As CP26 and CP29 have been reported to bind 5 and 4 molecules of chlorophyll b respectively (see Thornber *et al.*, 1993), the chlorophyll b data suggests that up to 1.5 molecules of CP29 or 1.2 molecules of CP26 are associated with each monomeric PSII core complex.

If CP43 and CP47 each bind 12 molecules of chlorophyll a, as suggested by Tang & Satoh, (1984) and Barbato *et al.*, (1991a), while the reaction centre binds 6 chlorophyll a molecules, a PSII core complex consisting of CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic polypeptide, would be expected to be associated with 30 molecules of chlorophyll a in total. The chlorophyll b data shown in Table 3.4 suggests that HTGP cores are associated with approximately 1 molecule of CP26 or CP29 (see Thornber *et al.*, 1993). On this basis HTGP cores would theoretically be expected to bind 39 molecules of chlorophyll a. As reverse phase HPLC analysis yielded a value of 40.3 chlorophyll a molecules per HTGP core, it does indeed seem reasonable to suggest that these PSII core preparations are associated with approximately 1-1.5 molecules of either CP26, CP29 or a mixture of these two polypeptides or with a residual amount of LHCII.

HTGP core preparation 1 was associated with the lowest amount of chlorophyll b. This result is in agreement with SDS-PAGE analysis (Figure 3.2, Lane 8) which suggests that this HTGP core

preparation is associated with less CP29/CP26 and CP24 than preparations 2 and 3 (Figure 3.2, Lanes 9 and 10).

3.2.4 Oxygen evolution rates driven by triplicate core preparations

Having established the subunit and pigment compositions of the three HTGP core preparations, resolved by SDS-PAGE analysis in Figure 3.2, these same PSII core preparations were subjected to oxygen evolution analysis according to Section 2.4. Table 3.5 shows the results of such analysis.

Table 3.5 Oxygen evolution rates of thylakoids, BBYs and HTGP cores using DCBQ and DMBQ as electron acceptors

Sample	O ₂ evolution ($\mu\text{moles O}_2 \text{ mg chl}^{-1} \text{ h}^{-1}$) DCBQ	O ₂ evolution ($\mu\text{moles O}_2 \text{ mg chl}^{-1} \text{ h}^{-1}$) DMBQ	DMBQ:DCBQ O ₂ evolution rate ratio x 100 (Y)
Thylakoids	124	83	66
BBYs	493	181	37
Prep. 1 (SD)	326 (71)	69.6 (1)	21
Prep. 2 (SD)	730 (15)	200 (10)	27.3
Prep. 3 (SD)	807 (322)	165 (35)	20.4

Preparation 1, which lacked the 23 and 17kDa extrinsic subunits, showed considerably lower rates of oxygen evolution than core preparations 2 and 3. The values obtained, using DCBQ and DMBQ as electron acceptors, were at the lower end of the range reported for other preparations in the presence of DCBQ and DMBQ (see Table 3.1). DCBQ is thought to accept electrons from the Q_A and Q_B sites, whereas DMBQ probably only accepts electrons from Q_B (Graan & Ort, 1986). The DMBQ:DCBQ oxygen evolution rate ratio, expressed as a percentage has been denoted as Y in Section 2.4 (see Table 3.5). Using this model, Y represents the percentage of oxygen-evolving cores having functional Q_A to Q_B transfer. The results show that the Y values obtained from the HTGP core preparations are lower than those obtained from BBYs, which in turn are lower than those of thylakoids. It is possible that the purification procedure used affects electron transfer between Q_A and Q_B. Such changes might be due to a number of reasons. The first is that the solubilisation of thylakoids with Triton X-100, or BBYs with HTGP, may result in the release of polypeptides which maintain the integrity of the Q_A and Q_B pockets. Mayes *et al.*, (1993) have shown that *Synechocystis* sp. PCC6803 mutants lacking the *psbH* gene product exhibit impaired electron transport between Q_A to Q_B. Furthermore, Nagatsuka *et al.*, (1991) suggested that the *psbL* gene product and possibly other low molecular weight subunits may be required to allow the Q_A site to function normally. As I did not complete Western blot analyses, to determine whether these subunits were associated with the HTGP core complexes, I cannot say if the low Y values obtained for these preparations are the result of purification induced subunit dissociation.

A second reason for the observed purification induced Y value change might be a reduction in the hydrophobicity of the acceptor side environment. Successive detergent solubilisation steps are likely to deplete the PSII complexes of lipid. As oxygen evolution measurements were made in the presence of DM, it is expected that at least some detergent molecules are associated with the HTGP cores. So, as DMBQ is a less polar molecule than DCBQ, the presence of detergent may reduce the ability of DMBQ to bind to the Q_B pocket more than the ability of DCBQ to bind. This in turn could result in the purification induced Y value reduction shown in Table 3.5.

A third explanation for purification induced Y value changes observed is that the acceptor side of PSII is damaged during the purification process. The Western Blot analysis shown in Section 3.2.1.2, identified D2 fragments having apparent molecular weights of 16 and 21kDa as well as a 13kDa D1 fragment. Sections 1.4.1 and 1.4.2 reviewed the literature on the structure of the D1 and D2 subunits. As the D1 subunit provides ligands for Q_B while D2 binds Q_A , these two polypeptides are known to be integral parts of these two quinone binding sites. Their damage is likely to result in altered acceptor side properties. Indeed, the results of Friso *et al.*, (1994), suggest that the 16 and 21kDa D2 fragments observed in HTGP cores (Figure 3.6) could have been formed as the result of damage near the Q_A site. Damage to this site would be expected to result in a reduction in the oxygen evolution rates observed in the presence of DCBQ or DMBQ. It would not, however be expected to result in a change in the DCBQ:DMBQ rate ratio. This suggests that there might be some changes to the Q_B site. A detailed characterisation of the PSII cores of Haag *et al.*, (1990) indicated that this preparation showed signs of Q_B site damage. In these oxygen-evolving cores Yz to P680⁺ (Eckert *et al.*, 1992) and Pheo⁻ to Q_A electron transport (Bernarding *et al.*, 1991) are the same as in PSII membrane fragments. These results confirm that the electron transport chain is intact between Yz and Q_A in the core preparations of Haag *et al.*, (1990) which contained CP47, CP43 D2, D1, cytb559 and the 33kDa extrinsic subunit.

Gleiter *et al.*, (1993) also reported that the Q_B sites of cores prepared by Haag *et al.*, (1990) were damaged. These results suggest that the Q_B site is most sensitive to purification induced damage. Removal of CP29 was not the cause of the observed impairment of Q_B , as its removal did not alter the flash-induced variable fluorescence from that observed in PSII membrane fragments (Gleiter *et al.*, 1993). Interestingly, mild trypsin treatment selectively modified the Q_B site and cytb559 of membrane embedded PSII complexes (Renger *et al.*, 1985). Such trypsin treated samples were able to donate electrons to FeCN, but lacked DCMU sensitivity. As the cores purified by Haag *et al.*, (1990) had lost their DCMU sensitivity, they resembled mildly trypsinised PSII thylakoids and membranes, and so it was suggested they showed signs of damage to the loop connecting the transmembrane helices IV and V of D1 (Gleiter *et al.*, 1993).

The detection of a 13kDa D1 fragment in HTGP cores (Figure 3.6) suggests that the D1 subunit is cleaved near transmembrane helix IV (Greenberg *et al.*, 1987; De Las Rivas *et al.*, 1992b; Cánovas & Barber, 1993; De Las Rivas *et al.*, 1993; Ponticos *et al.*, 1993) and so might be expected to reduce the integrity of the Q_B site. If this is indeed the case, it might at least in part explain the

purification related reduction in the Y value, as the 13kDa D1 band was not detected in the Western blots of thylakoids (Figure 3.4) or BBYs (Figure 3.5).

3.2.5 HPLC size exclusion analysis of HTGP core preparations

HTGP core preparations 1 and 2 were subjected to size exclusion analysis (Section 2.5) to determine their aggregation state and homogeneity. Figure 3.9 shows the calibration curve obtained using myoglobin (17kDa), monomeric PSI(300kDa) and invertase (540kDa).

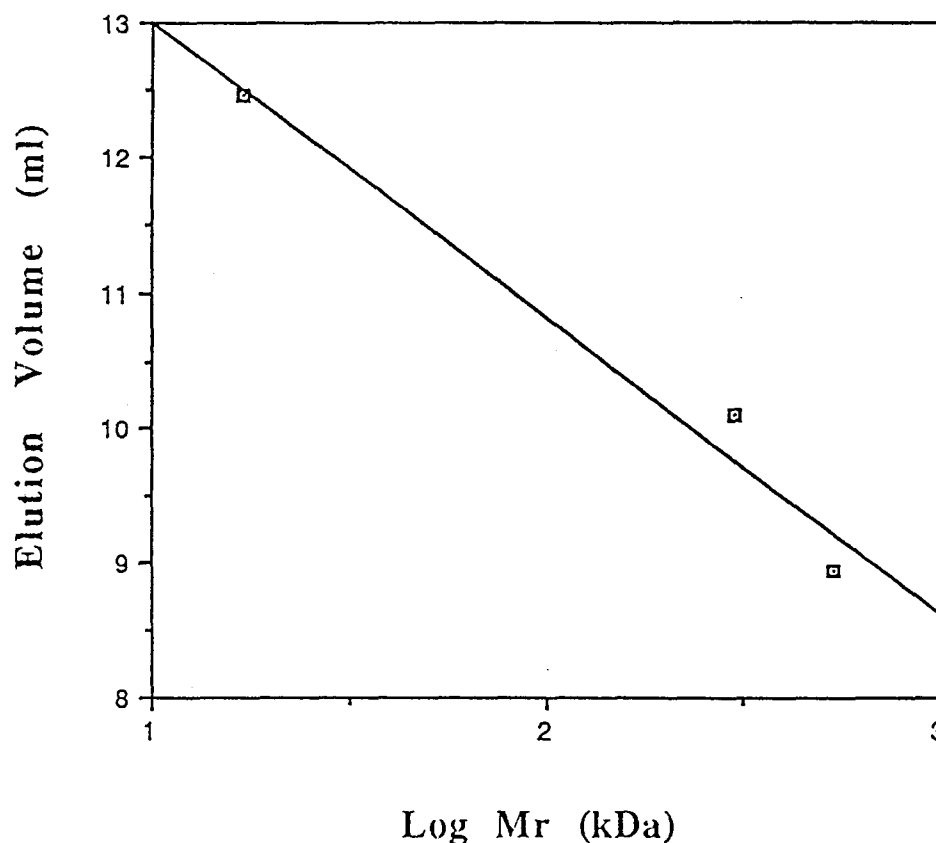


Figure 3.9 Standard curve for HPLC size exclusion analysis The standards used were myoglobin (17kDa), PSI monomer (300kDa) and invertase (540kDa). The value of 300kDa for the DM solubilised PSI monomer was obtained by calibrating the molecular weight of this membrane protein complex to a large number of other standards (Rögner , personal communication).

The calibration curve (Figure 3.9) was used to calculate the molecular weights of the complexes eluting in the peaks shown in Figure 3.10. The elution volumes of the four fractions were 9.2, 9.9, 11.2 and 14.2ml. These correspond to the molecular weights of 550kDa, 263kDa, 70kDa and 2.8kDa, respectively. The 14.2ml peak may contain free pigment. As PSII is a membrane protein, its hydrophobic regions are thought to be associated with the DM present in the running buffer. The preparation of Haag *et al.*, (1990) which contained CP47, CP43, D2, D1, cytb559 and the 33kDa subunit, was shown to have an apparent molecular weight of 270kDa in the presence of DM. As a

monomeric PSII complex has a theoretical molecular weight of about 250kDa (Table 3.3), the authors concluded that the complex was monomeric. The difference between the theoretical (Table 3.3) and apparent molecular weights reported by (Haag *et al.*, 1990) can be explained by an experimental error of 8%, as well as the mass contributed to the complex by associated low molecular weight polypeptides and DM. Unfortunately, insufficient quantities of the four HPLC resolved HTGP core fractions (Figure 3.10) were isolated to allow direct characterisation of their respective subunit compositions. However, in the light of the results of Haag *et al.*, (1990) it is suggested that the 263kDa peak (Figure 3.10) corresponds to a monomeric PSII core of similar subunit composition.

Rögner *et al.*, (1987) isolated dimeric (CP47, D2, D1, cytb559 and the 33kDa extrinsic polypeptide) PSII core complexes from *Synechococcus sp.* and showed them to have a molecular weight of about 500kDa. The HPLC size exclusion analysis procedure used to analyse these *Synechococcus* dimers (Rögner *et al.*, 1987) and the HTGP cores characterised in this chapter have an experimental error of about 20%. Thus, the 550kDa peak (Figure 3.10) may well correspond to a dimeric PSII complex of this subunit composition. The 70kDa peak may contain DM solubilised chlorophyll a/b binding proteins such as CP24, CP26 and CP29.

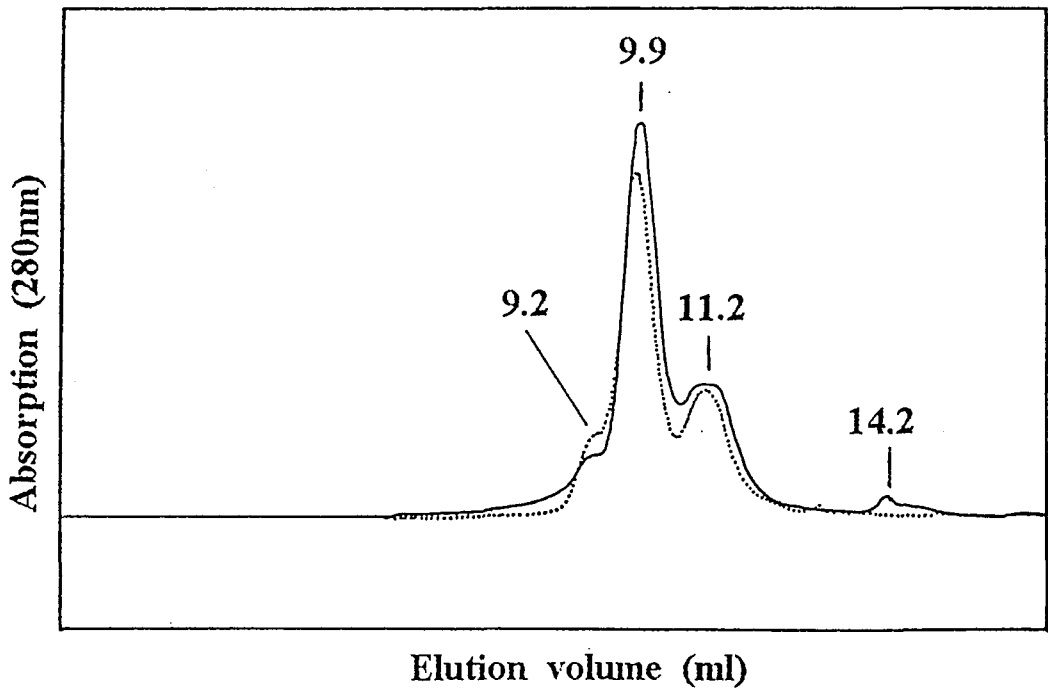


Figure 3.10 Elution profile of two HTGP core preparations. HTGP core preparations 1 and 2 were subjected to HPLC size exclusion separation.

Clearly this hypothesis has to be confirmed by direct characterisation of the subunit composition of each HPLC size exclusion resolved fraction. However, Figure 3.10 does suggest that

HTGP cores consist of mainly monomeric PSII core complexes and so may be useful for initial crystallisation screens, though they are insufficiently pure for more advanced trials.

3.2.6 Lipid analysis

The lipid analysis of this preparation is presented in Section 7.2.3

3.3 Conclusion

HTGP cores, isolated according to the method described in Section 2.1.5, were shown to contain CP47, CP43, D2, D1, cytb559, the 33kDa extrinsic as well as the 22 and 10kDa intrinsic and variable amounts of the 23 and 17kDa extrinsic polypeptides. SDS-PAGE analysis also showed these cores to be associated with CP29 and/or CP26 as well as CP24. Using reverse phase HPLC analysis, HTGP cores were calculated to be associated with 41 chlorophyll a, 6.0 chlorophyll b and 7.6 β -carotene molecules per 2 molecules of pheophytin. Taken together, the subunit and pigment analyses suggest that HTGP cores are associated with approximately 1-1.5 molecules of CP29 or CP26, or a mixture of these two polypeptides, per reaction centre. Apart from being contaminated with chlorophyll a/b binding proteins, the HTGP core preparation was also shown to have undergone considerable D1 and D2 subunit damage. A 13kDa C-terminal D1 as well as two D2 fragments having molecular weights of 16 and 21kDa were detected in HTGP cores but not in BBYs or thylakoids. Comparison of these fragment sizes with previously published studies suggest that all three fragments may be formed as a result of purification induced damage to the acceptor side of PSII. The 13kDa C-terminal fragment may originate from cleavage in the hydrophilic loop connecting the putative transmembrane helices IV and V of D1. The 16 and 21kDa fragments may originate from cleavage in the corresponding loop of D2. The hypothesis that HTGP cores show acceptor side damage is in agreement with direct oxygen evolution measurements. HPLC size exclusion analysis suggests that the HTGP cores contain predominantly monomeric PSII core complexes as well as a small proportion of dimeric complexes. Although, the HTGP cores are apparently enriched in monomeric PSII core complexes and so may be suitable for initial crystallisation screens, they are unsuitable for more advanced crystallisation trials. It is desirable to obtain PSII complexes free of the contaminating chlorophyll a/b binding proteins, but there is little point in subjecting HTGP core preparations to a further purification step, as these PSII complexes are already damaged. As the major D1 and D2 breakdown fragments detected in HTGP cores were not detected in BBYs or thylakoids, it is suggested that the purification procedure used to isolate cores from BBYs should be improved. As PSII cores isolated with DM (Haag *et al.*, 1990) and HTGP (this chapter) are seen to be partially damaged, it seems logical to replace HTGP with OG during the BBY solubilisation step.

Chapter 4.0 The characterisation of an oxygen-evolving PSII core preparation isolated using octyl glucoside

4.1 Introduction

One of my aims was to isolate pure, intact and active PSII complexes consisting of CP47, CP43, D2, D1, cytb559, the 33kDa extrinsic polypeptide and any closely associated low molecular weight subunits, for crystallisation purposes. HTGP cores (Chapter 3) contained a number of subunits in addition to the ones listed above. However, as they were associated with damaged D1 and D2 subunits, there was little point in subjecting such HTGP core complexes to additional purification steps, to remove CP24, CP26, CP29, as well as the 23 and 17kDa extrinsic and 22 and 10kDa intrinsic subunits. This chapter describes the characterisation of PSII cores (OG cores) isolated from BBYs using OG rather than HTGP. Section 2.1.5 shows that the OG and HTGP core preparation procedures are very similar, with the obvious exception that they employ different detergents at the BBY solubilisation stage. Comparing the properties of the two PSII core types should identify whether the use of OG rather than HTGP, during the BBY solubilisation step, improves the purity and activity and reduces the level of subunit damage observed in HTGP core preparations. To facilitate a direct comparison between the properties of HTGP and OG cores, OG cores are characterised using the procedures employed to analyse HTGP cores (Chapter 3).

4.2 Results and discussion

4.2.1 Subunit composition

4.2.1.1 Determining subunit composition of triplicate OG core preparations.

The experiments presented in this section were designed to identify the major PSII subunits associated with OG cores and to determine whether the procedure used to isolate them (Section 2.1.5) yielded PSII cores of reproducible quality. To achieve this, three OG core preparations (OG core preps. 1-3) were isolated, and subjected to SDS-PAGE analysis (Figure 4.1, Lanes 2-4), using the methods described in Sections 2.2.1-2.2.3. For the purposes of comparison HTGP core preparation 2 (Figure 3.2 Lane 9) was run on the same gel (Figure 4.1, Lane 1).

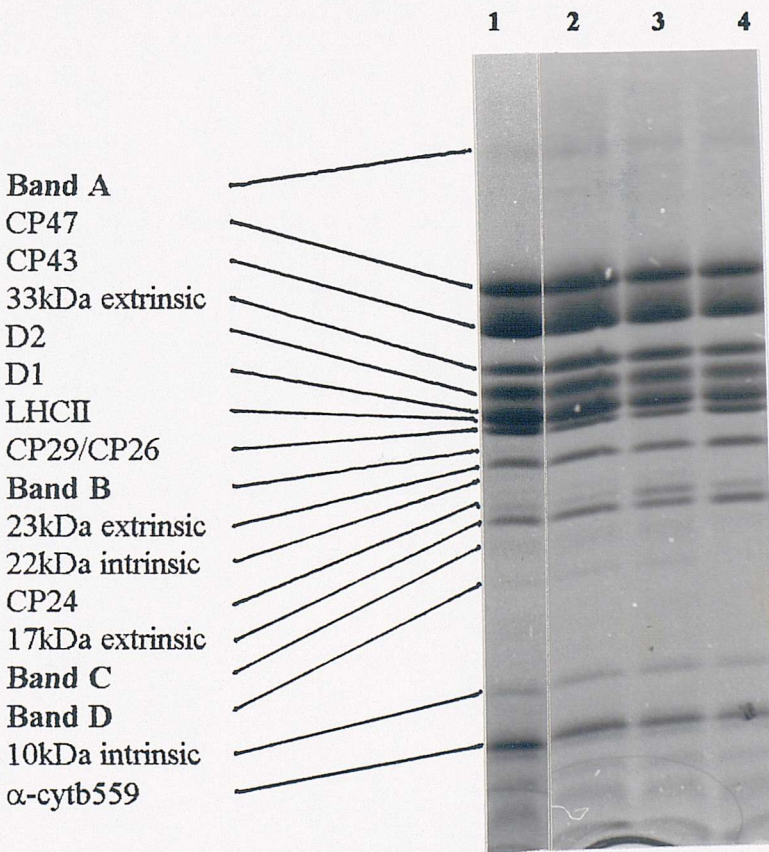


Figure 4.1 A comparison of the subunit composition of a HTGP core sample with three OG core preparations. Lane 1: HTGP core preparation 2, Lane 2: OG core preparation 1, Lane 3: OG core preparation 2, Lane 4: OG core preparation 3. OG cores contain CP47, CP43, D2, D1, cytb559, CP29, CP26 and CP24, the 33, 23 and 17kDa extrinsic polypeptides as well as the 22 and 10kDa intrinsic subunits. Four additional bands (Bands A-D) are also labelled.

Figure 4.1 shows that OG and HTGP cores are similar in that they consist of CP47, CP43, D2, D1, cytb559, the 33, 23 and 17kDa extrinsic polypeptides, as well as the 22 and 10kDa intrinsic subunits. CP24 and CP29/CP26 were also present in both preparation. In contrast to HTGP cores (Figure 3.2, Lanes 8-10), OG cores consistently bound the 23 and 17kDa extrinsic polypeptides (Figure 4.1, Lanes 2-4). This result shows that the OG core preparation procedure yields PSII cores of reproducible quality. Four bands labelled A-D are also marked in Figure 4.1. These bands seem to correspond to Bands A-D, characterised in Chapter 3 (Figure 3.2 and Section 3.2.2). To determine whether this is the case, an OG core preparation was subjected to the Western blot analysis as described in Section 4.2.2.

4.2.2 Western blot analysis of OG cores

4.2.2.1 Western blot analysis of isolated OG cores using antibodies raised against CP47, CP43, D2 D1 and the 33kDa extrinsic subunit

The aim of the work presented in this section was to determine whether the origin of Bands A, B, C and D (Figure 4.1) could be identified using antibodies raised against CP47, CP43, D2, the C-terminus of D1 and the 33kDa extrinsic polypeptide. To test this, an SDS-PAGE resolved OG core preparation was subjected to Western blot analysis using antibodies specific to the above subunits (Sections 2.2.1-2.3.2).

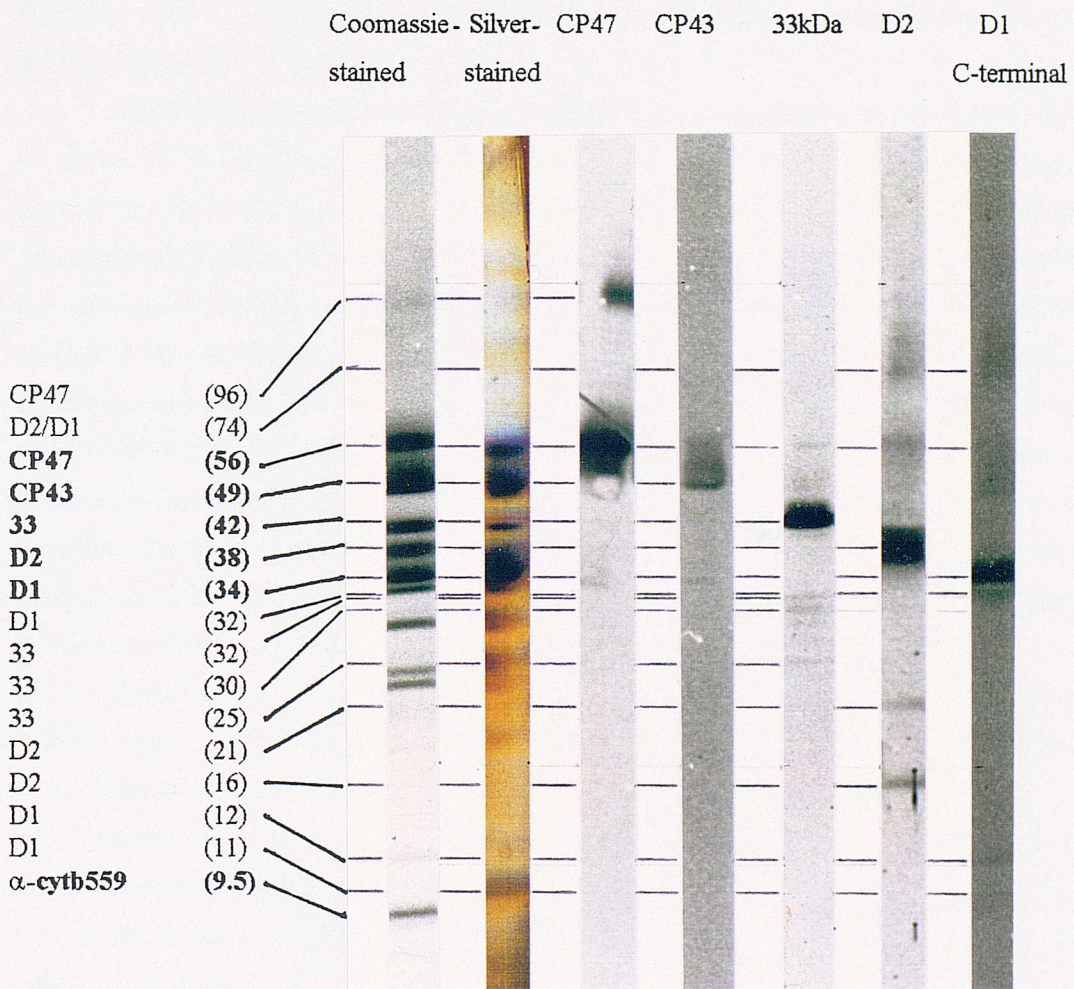


Figure 4.2 The detection of CP47, CP43, 33kDa extrinsic, D2 and D1 aggregation and breakdown products in isolated OG cores using Western blot analysis. The combined use of Coomassie stained and silver stained gels allows sensitive detection of the polypeptides present in the preparation. Western blotting allows the origin of some of the detected bands to be determined. Bold labels indicate the intact subunits. Plain text indicates aggregation or breakdown products detected by specific antibodies. The numbers in brackets are the molecular weights, calculated using the standard curve shown in Figure 3.3.

Figure 4.2 shows the intact forms of CP47(56kDa), CP43 (49kDa), the 33kDa extrinsic polypeptide (42kDa), D2 (38kDa) and D1 (34kDa) to have the apparent molecular weight values given in the respective brackets.

Two polypeptides having higher apparent molecular weights than any of the intact subunits, were labelled in the Western blots (Figure 4.2). The CP47 specific antibody labelled a band having an apparent molecular weight of 96kDa. As intact CP47 has an apparent molecular weight of 56kDa, it is possible that the 96kDa band corresponds to a dimeric aggregate of CP47. Comparison of Figures 4.1 and 4.2 shows this 96kDa band (Figure 4.2) to correspond to Band A (Figure 4.1). As similar bands were detected in thylakoids (101kDa band, Figure 3.4), BBYs (100kDa Band, Figure 3.5) and HTGP cores (91kDa, Figure 3.6) it seems likely that this CP47 aggregate is either formed as a result of incomplete sample solubilisation or SDS induced subunit aggregation, rather than any purification induced artifact.

The second aggregation band seen in Figure 4.2, has an apparent molecular weight of 74kDa. As the sum of the molecular weights of the intact D1 and D2 subunits (Figure 4.2) is 72kDa, it is possible that the 74kDa aggregate is a D1/D2 heterodimer consisting of the intact forms of the two subunits. A similar band of 79kDa was also detected in HTGP cores (see Figure 3.6). It is possible that this aggregation is also observed as a result of incomplete solubilisation using SDS prior to SDS-PAGE analysis. However, Barbato *et al.*, (1992d) have shown that photoinhibitory treatments, which lead to the damage of isolated PSII reaction centres, can also result in the cross-linking of the α -subunit of cytb559 and the D1 polypeptide. If photoinhibitory treatments can cause subunit cross-linking, it is possible that harsh purification procedures might have a similar effect. Thus, at this stage, the possibility that the 96kDa and 74kDa bands are the product of purification induced subunit cross-linking, cannot be excluded. It should be noted that aggregation products are present in detectable levels in other PSII core preparations (Haag *et al.*, 1990; De Las Rivas *et al.*, 1992b)

A number of breakdown products were also detected in this OG core preparation. The sample used for this experiment had been stored at -80°C for several months. Using such a preparation allowed the identification of the subunits most susceptible to subunit damage. The antibody raised against the 33kDa extrinsic polypeptide labelled three bands having molecular weights of a 32, 30 and 25kDa respectively (Figure 4.2). The 30 and 25kDa fragment were also detected in the HTGP cores (Figure 3.6, 30kDa and 24kDa fragments), suggesting that the damage is not detergent specific. Oxygen free radicals and/or proteases could be two causative agents of the observed damage. As no studies on the breakdown of this subunit are available, little can be said about the sites of cleavage. However, the 30kDa fragment appears to correspond to Band B (Figure 4.1).

Three D1 breakdown fragments were also visualised. They were calculated to have molecular weights of 32, 12 and 11kDa. Although a C-terminal specific antibody was used, the faint band detected at the 32kDa position, could be due to D1 cleavage at one of two possible positions. If a small proportion of the D1 polypeptides were cut close to the N-terminus, a faint 32kDa band would be detected by this C-terminal specific antibody. Conversely, if a small fragment was cleaved from the C-

terminus of D1, it is possible that there would still be sufficient antigenic structure attached to the large fragment, to allow detection of an N-terminal (32kDa) fragment, using this antibody. The molecular weight of this D1 fragment corresponds closely with a 33kDa D1 specific band, seen in HTGP cores (Figure 3.6), suggesting that its formation is not detergent specific. The other two D1 fragments had molecular weights of 12 and 11kDa respectively. As was discussed in Section 3.2.2, C-terminal fragments of this size are thought to arise from cleavage of the D1 subunit in the hydrophilic loop connecting the putative transmembrane helices IV and V (Greenberg *et al.*, 1987; De Las Rivas *et al.*, 1992b; Cánovas & Barber, 1993; De Las Rivas *et al.*, 1993). This hydrophilic loop is thought to be an integral part of the Q_B site (Section 1.4.1).

Two D2 fragments having molecular weights of 21 and 16kDa were also detected. The sum of their molecular weights is 37kDa and therefore similar to that of the intact subunit (38kDa). This suggests that these two fragments may be formed as a result of a single cleavage. As was discussed in Section 3.2.2, these two fragments may be formed as a result of a single cleavage in the hydrophilic loop of D2 connecting transmembrane helices IV and V (Andersson *et al.*, 1994; Friso *et al.*, 1994; Ponticos *et al.*, 1994). Fragments of the same size were detected in HTGP cores (Figure 3.6). Comparison of Figures 4.1 and 4.2 show that the 21kDa D2 band (Figure 4.2) correspond to Band C (Figure 4.1). Interestingly, a model has recently been proposed in which it is suggested that the hydrophilic loops, connecting transmembrane helices IV and V of the D1 and D2 subunits, are more sensitive to cleavage, if the Q_A and Q_B sites are unoccupied (Trebst *et al.*, 1994). As the above Western blot analyses suggest, the D1 and D2 subunits of OG cores may both be susceptible to proteolytic cleavage near the Q_B and Q_A sites respectively, it is possible that the quinones associated with these binding sites were extracted during the purification procedure. Ponticos *et al.*, (1994) have shown that DCMU can reduce the rate of D1 degradation of PSII cores exposed to photoinhibitory conditions. However, the decrease in D1 degradation rate was accompanied by an increase in the rate of D2 degradation. It is therefore possible that both the Q_A and Q_B site must be occupied if acceptor side damage is to be prevented. Clearly it would be beneficial to analyse the quinone content of OG cores in the future.

To summarise, the OG cores, used in the Western blot analysis described in this section had been stored at -80°C for a prolonged period of time, and clearly contained detectable levels of D1, D2 and 33kDa extrinsic subunit breakdown products. Their presence allowed the identification of Bands A (91kDa CP47 aggregate), B (30kDa fragment of the 33kDa extrinsic polypeptide) and C (21kDa fragment of D2) shown in Figure 4.1. In addition a 16kDa D2, as well as a 12kDa C-terminal D1 fragment, were identified. Clearly, the formation of these subunit breakdown products is undesirable. For OG cores to be useful as a starting material for additional purification steps, it was therefore necessary to try to reduce the level of subunit damage. The similarity in subunit fragment sizes detected in HTGP and OG cores, suggested that their formation was not the result of detergent specific damage. Experiments were therefore set up (Section 4.2.2.2) to determine whether freshly prepared

OG cores were associated with equally high levels of D1 and D2 subunit damage, or whether these polypeptides might have been damaged during storage.

4.2.2.2 D1 and D2 specific Western blot analysis of freshly prepared OG cores

PSII subunit breakdown fragments were detected in OG cores, which had been exposed to prolonged storage at -80°C . To determine whether freshly prepared OG cores were less damaged, such preparations were immediately solubilised and resolved by SDS-PAGE (Sections 2.2.1-2.2.3) and subsequently Western blotted using D2 and C-terminal D1 specific antibodies (Sections 2.3.1-2.3.2). The D2 and C-terminal D1 specific Western blots of freshly prepared OG cores are shown in Figure 4.3.

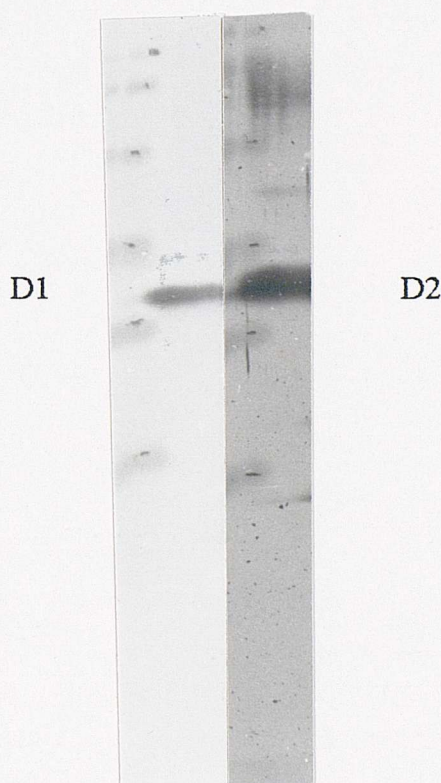


Figure 4.3 D1 and D2 specific Western blots of a freshly prepared OG core preparation. A freshly prepared OG core sample was resolved by SDS-PAGE, before being Western blotted with D2 and C-terminal D1 specific antibodies. In contrast to the core preparation used in Figure 4.2, this freshly prepared sample was free of any D1 and D2 breakdown fragments.

Figure 4.3 shows that freshly prepared OG cores appeared to be free of any proteolytically cleaved D1 or D2 subunits. The intact nature of freshly prepared OG cores suggests that subunit breakdown detected in Figure 4.2 may have been storage induced. More importantly, freshly prepared OG cores appear to be suitable as a starting material for additional purification steps, to remove chlorophyll a/b binding proteins, the 23 and 17kDa extrinsic and 22 and 10kDa intrinsic polypeptides.

4.2.3 Oxygen evolution rates driven by triplicate OG core preparations

The work presented in this section describes the oxygen evolution capability of OG cores. OG core preparations 1-3, whose subunit composition was shown in Figure 4.1 (Lanes 2-4), were subjected to oxygen evolution measurements according to the method described in Section 2.4. These measurements were carried out at the same time as those of HTGP core preparations (Table 3.5), allowing the values presented in Tables 3.5 and 4.1 to be compared directly.

Table 4.1 Oxygen evolution rates of three OG core preparations using DCBQ and DMBQ as electron acceptors

Sample	O ₂ evolution ($\mu\text{moles O}_2 \text{ mg chl}^{-1} \text{ h}^{-1}$)	O ₂ evolution ($\mu\text{moles O}_2 \text{ mg chl}^{-1} \text{ h}^{-1}$)	DMBQ:DCBQ O ₂ evolution rate ratio x 100 (Y)
	DCBQ	DMBQ	
OG core Prep. 1 (SD)	1165 (108)	208 (96)	18
OG core Prep. 2 (SD)	1372 (499)	305 (114)	22
OG core Prep. 3 (SD)	1202 (110)	417 (5)	35
OG core mean (SD)	1246 (110)	310 (104)	25 (8.9)

The average rate of oxygen evolution attained by the three OG core preparations was 1246 $\mu\text{moles O}_2 \text{ mg chl}^{-1} \text{ h}^{-1}$. HTGP core preparations 2 and 3, which still retained their 23 and 17kDa extrinsic polypeptides evolved oxygen at a mean rate of 768.5 $\mu\text{moles O}_2 \text{ mg chl}^{-1} \text{ h}^{-1}$. Comparing these two values, shows that the OG core preparations evolve oxygen at a rate 62% higher than that of HTGP cores. Indeed, the oxygen evolution rates driven by OG cores are amongst the best of any of the published PSII core preparations listed in Table 3.1. The mean rate of oxygen evolution of OG cores in the presence of DMBQ (310 $\pm$ 104 $\mu\text{moles O}_2 \text{ mg chl}^{-1} \text{ h}^{-1}$) increased by 59% from that attained by HTGP cores. As all three OG core preparations used in the oxygen evolution rate measurements, showed signs of subunit damage (Figure 4.1, Lanes 2-4), it is possible that freshly prepared core preparations may support even higher rates of oxygen evolution.

The DMBQ:DCBQ oxygen evolution rate ratio (Y), calculated for OG cores (Table 4.1) was similar to that reported for the HTGP cores (Table 3.5). This ratio was taken as a measure of the ability of a PSII core preparation to facilitate electron transport between Q_A and Q_B (Sections 2.4 and 3.2.4). Western blot analysis (Figures 4.2 and 4.3) suggests that these OG core preparations may have suffered damage on the acceptor side during storage. In the absence of detailed analysis of the low molecular weight subunits associated with OG cores, one cannot exclude the possibility that the low Y value, reported in Table 4.1, is the result of purification induced dissociation of such subunits. These low Y values could for example be caused by acceptor side changes such as the removal of the *psbL* gene product, which has been proposed to protect the Q_B binding site (Nagatsuka *et al.*, 1991). Another

reason why OG cores show low Y values, might be that purification induced alterations in the hydrophobicity of the acceptor side environment, have taken place. Successive detergent solubilisation steps are likely to deplete the PSII complexes of lipid. As oxygen evolution measurements were made in the presence of DM, it is expected that at least some detergent molecules are associated with the OG cores. So, as DMBQ is a less polar molecule than DCBQ, the presence of detergent may reduce the ability of DMBQ to bind to the Q_B pocket more than the ability of DCBQ to bind. This in turn could result in the purification induced Y value reduction shown in Table 4.1. If this is indeed the case, the Y value may actually not be the best way of measuring acceptor side alterations. For purposes of comparison, it should be noted that Y values calculated for the preparations of Ikeuchi *et al.*, (1985), Ikeuchi & Inoue, (1986) and Shen *et al.*, (1988) are 0.4, 11 and 44 respectively.

The above results have shown OG cores to be able to support high rates of oxygen evolution in the presence of DCBQ and reasonable rates in the presence of DMBQ. Western Blot analyses suggest that freshly prepared OG cores are associated with only very low levels of PSII subunit breakdown fragments. As OG core preparations are active and appear to have undergone little subunit damage, they fulfil two of the criteria set to measure the suitability of a PSII core preparation for crystallisation purposes. However OG cores are associated with CP24, CP26, CP29, the 23kDa and 17kDa extrinsic and 22kDa and 10kDa intrinsic polypeptides. These subunits will have to be removed if PSII core complexes, consisting of CP47, CP43, D2, D1, cytb559, the 33kDa extrinsic polypeptide and any associated low molecular weight subunits are to be isolated for crystallisation purposes. In the following section OG core preparations are subjected to pigment analysis, to determine the levels of chlorophyll a and b with which they are associated. Such analysis allows an estimation of the number of chlorophyll a/b binding proteins associated with each PSII core complex.

4.2.4 Pigment analysis of OG cores

4.2.4.1 Triplicate OG core spectra

SDS-PAGE analysis of three OG core samples (preps 1-3, Figure 4.1) showed all three to be associated with CP24 as well as CP26/CP29. The absorption spectra of these three OG core preparations were obtained using the method described in Section 2.6, and are shown in Figure 4.4.

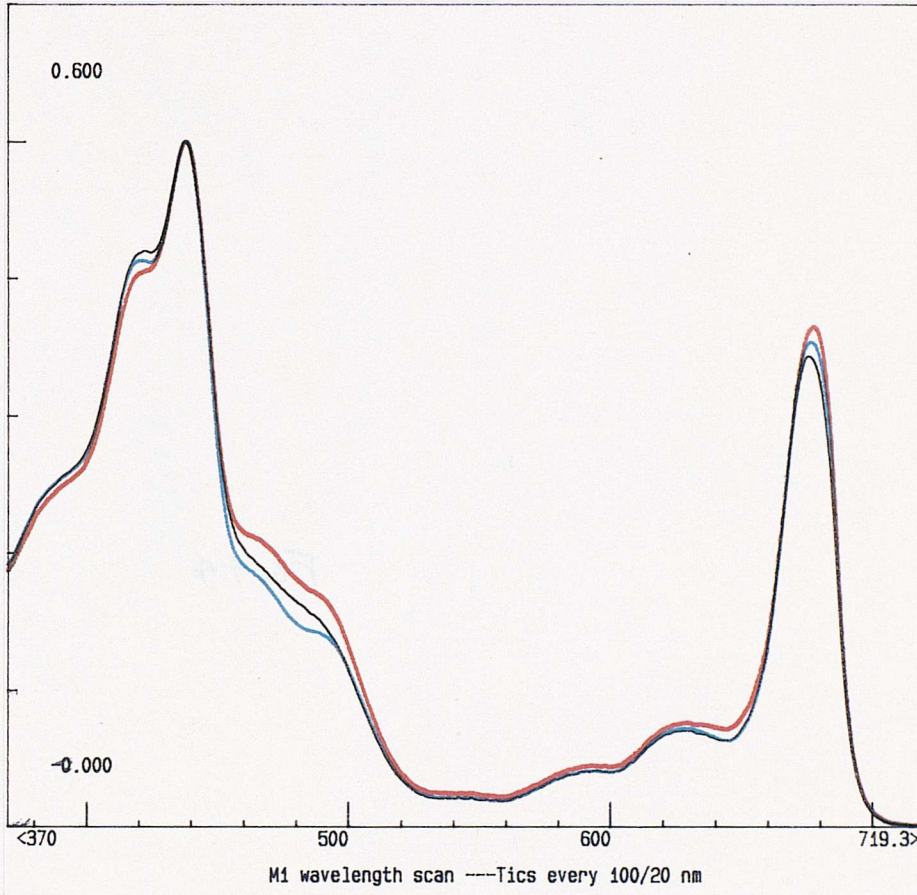


Figure 4.4 Spectral analysis of triplicate OG cores The isolated core preparations 1 (black), 2 (green) and 3 (red) whose subunits were resolved by SDS-PAGE in Figure 4.1 (lanes 2-4), were solubilised with SMNCB supplemented with 2mM DM. The three spectra were normalised at the 437nm peak. Chlorophyll a absorbs predominantly at 437nm and 667nm, whereas chlorophyll b has peak maxima in the 470-480nm and 650nm region of the spectrum, when associated with PSII. Chlorophyll a and b, as well as cytb559, mask the pheophytin peak (417nm), as they absorb in this region. β -carotene absorbs predominantly in the 483nm region.

The three absorption spectra shown in Figure 4.4. were normalised at the 437nm peak, yet show a variation in the 460-510nm region of the spectrum. This indicates some differences in the chlorophyll *b* and carotenoid content of the three OG cores preparations. The absorption peaks of the three OG core preparations in the 666-668nm region of the spectrum vary slightly. As all three cores were shown to be highly active (Table 4.1), this shift does not appear to be damage related. I have noticed that the red peak (666-668nm peak) of OG cores shifts to the red in the absence of detergents such as DM, presumably due to aggregation. However, as the three spectra shown in Figure 4.4 were measured in the presence of DM, the observed difference in the absorption characteristics should not be due to a lack of detergent.

4.2.4.2 Comparing the average HTGP and OG core spectra

To compare the spectra of HTGP (preps 1-3, Figure 3.8) and OG cores (preps 1-3, Figure 4.4), the triplicate traces presented in Figure 3.4 and 4.4 were averaged respectively, and the resultant traces overlaid (Figure 4.5).

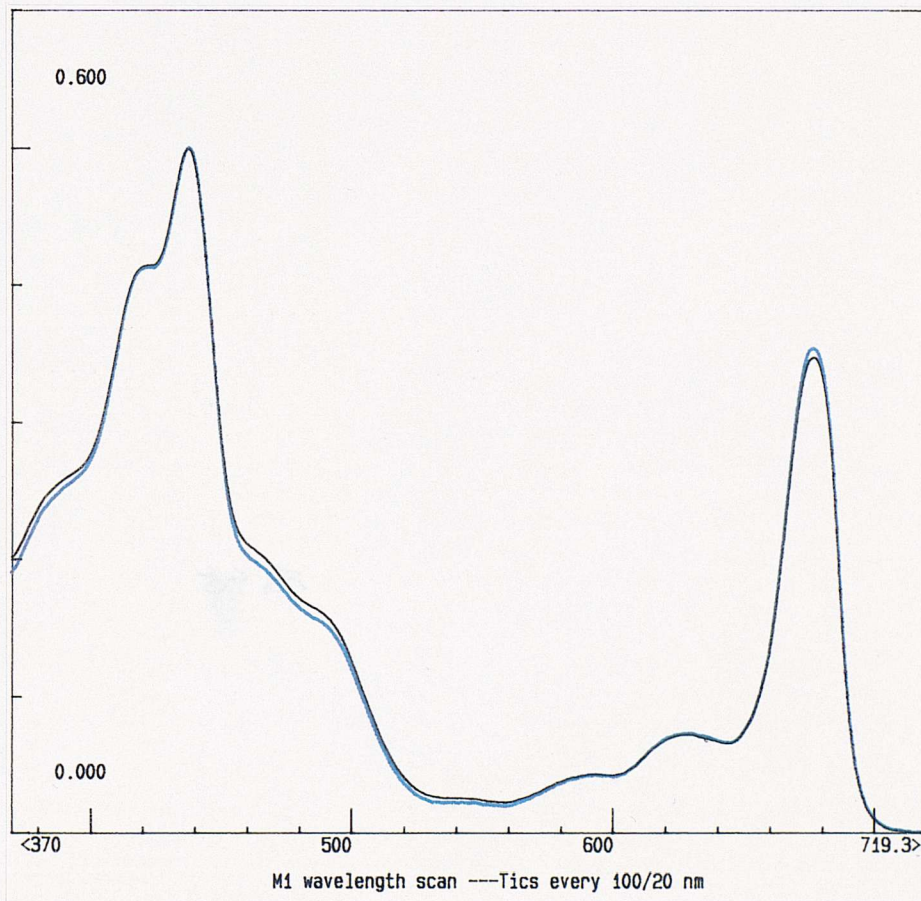


Figure 4.5 Comparison of average OG and HTGP core spectra. The averaged HTGP spectrum (Figure 3.8) is traced in black and the blue trace depicts the average of the OG core spectra shown in Figure 4.4.

The two averaged spectra shown in Figure 4.5 are almost identical, suggesting that the stoichiometries of the pigments they each bind, are very similar. To determine whether this is the case, OG cores were subjected to reverse phase HPLC analysis.

4.2.4.3 Determining the number of chlorophyll a, chlorophyll b and β -carotene molecules associated with each of the OG core preparations 1,2 and 3.

In order to determine the pigment composition of the three OG core preparations characterised by SDS-PAGE in Figure 4.1(Lanes 2-4), these preparations were subjected to reverse phase HPLC analysis using the method described in Section 2.6. The pigment content of the OG core preparations was calculated by normalising the amount of chlorophyll a, chlorophyll b and β -carotene, associated with each sample, to two pheophytin molecules. The results of such analysis are shown in Table 4.2.

Table 4.2 The pigment composition of three OG core preparations

Sample	Chlorophyll a	Chlorophyll b	β -carotene	Pheophytin
Core prep. 1	40.5	3.5	9.1	2
Core prep. 2	40.5	3.9	9.7	2
Core prep. 3	45.0	7.0	9.6	2
OG core mean (SD)	42 (2.6)	4.8 (1.9)	9.5 (0.3)	2

The results in Table 4.2 show the average number of chlorophyll b molecules, associated with each core particle, to be 4.8. This value is not significantly different from the corresponding HTGP core value of 4.2 (Table 3.4). CP29, CP26 and CP24 bind 4, 5 and 7 molecules of chlorophyll b per subunit, respectively. Thus, approximately one of any of these subunits may be associated per reaction centre. Indeed, it is suggested that each monomeric PSII complex in an OG core preparation can bind approximately 1.2 CP29, 1.0 CP26 or 0.7 molecules of CP24. Figure 4.1 shows less CP24 than CP26/CP29 to be present in these preparations. This suggests that most of the chlorophyll b detected in these preparations is associated with CP26 or CP29. The results of Kobayashi *et al.*, (1990), Tang & Satoh, (1984) and Barbato *et al.*, (1991a) suggest that PSII cores containing the chlorophyll a binding proteins CP47, CP43, D2, D1 can bind up to 31 chlorophyll a molecules (Barbato *et al.*, 1991a). CP29 and CP26 have been reported to bind 9 chlorophyll a molecules each, while CP24 is only thought to be associated with 6 molecules of this pigment type (see Thornber *et al.*, 1993). A core complex containing CP47, CP43, D2, D1, cytb559, the 33kDa extrinsic polypeptide, as well as a single molecule of CP29 would be expected to bind 40 chlorophyll a and 4 chlorophyll b molecules for every two molecules of pheophytin. A core complex in which CP29 is replaced with a single subunit of CP26, would be expected to bind 40 chlorophyll a and 5 chlorophyll b molecules for every two molecules of

pheophytin, whereas a core complex plus a single molecule of CP24 would be expected to bind 37 chlorophyll a and 7 chlorophyll b molecules per two molecules of pheophytin. It therefore seems likely that the OG cores are associated with more CP29 or CP26, than CP24.

The subunit and pigment analyses described so far do not provide any information on whether the subunits making up OG cores are organised in such a way as to form monomeric or dimeric PSII core complexes. To establish this, and to estimate the homogeneity of the OG cores, such preparations were subjected to HPLC size exclusion analysis as described in Section 4.2.5.

4.2.5 HPLC size exclusion analysis of the OG core preparation

OG core preparations 1 and 2 were subjected to size exclusion analysis (Section 2.5) to determine their aggregation state and homogeneity. The calibration curve (Figure 3.9) was used to calculate the molecular weights of the OG core components resolved by HPLC size exclusion analysis, as shown in Figure 4.6.

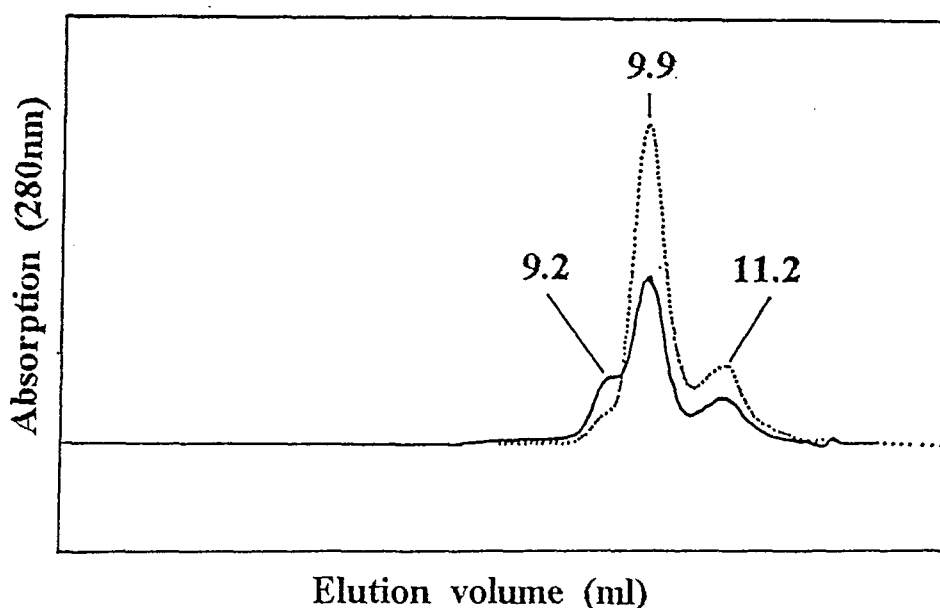


Figure 4.6 Elution profile of two OG core preparations subjected to HPLC size exclusion analysis. OG core preparations 1 and 2 were subjected to HPLC size exclusion separation. Using the standard curve shown in Figure 3.9, the molecular weights of the peak components eluting at 9.2, 9.9 and 11.2ml, were calculated to be 550kDa, 263kDa and 70kDa, respectively.

The elution volumes of the major fractions absorbing at 280nm, were 9.2, 9.9 and 11.2 ml and their components were calculated to have molecular weights of 550, 263 and 70kDa in the presence of DM. The elution profiles of the two OG core preparations, shown in Figure 4.6 and the apparent molecular weights of the resolved particle classes are very similar to those reported for HTGP cores (Figure 3.10). As a result, similar conclusions are drawn (Section 3.2.5). It is therefore suggested that the OG core preparation consists predominantly of a monomeric PSII core fraction, having an apparent molecular weight of 263kDa in the presence of DM. The 550kDa fraction may correspond to a dimeric PSII core complex (Rögner *et al.*, 1987), while the 70kDa fraction may contain DM solubilised chlorophyll *a/b* binding proteins. However, these suggestions could not be confirmed by SDS-PAGE analysis, as insufficient quantities of the three HPLC resolved fractions were available for such analysis. However, Figure 4.6 does show that OG cores are not homogeneous and therefore insufficiently pure for advanced crystallisation trials.

Santini *et al.*, (1994) have recently reported that the granal regions of the thylakoid membrane contain predominantly dimeric PSII cores, whereas the stromal regions contain predominantly monomeric PSII complexes. HTGP cores, OG cores and the PSII core preparations of Haag *et al.*, (1990) were isolated from the granal regions of the thylakoid (BBYs), yet consisted predominantly of monomeric core complexes. It is therefore possible that the purification procedures used to isolate these core complexes may have resulted in the dissociation of PSII dimers into their monomeric constituents. To determine whether the 550kDa and 263kDa complexes, reported in this section, are indeed monomeric or dimeric PSII cores, these two particle types will have to be isolated in large enough quantities to allow their characterisation. Comparing the properties of isolated PSII monomers and dimers should allow the suitability of such complexes to be gauged for crystallisation purposes.

4.2.6 Lipid analysis

The lipid analysis of this preparation is presented in Section 7.2.3.

4.3 Conclusions

OG cores, isolated according to the method described in Section 2.1.5, were shown to contain CP47, CP43, D2, D1, cytb559, the 33kDa extrinsic as well as the 22 and 10kDa intrinsic and variable amounts of the 23 and 17kDa extrinsic polypeptides. SDS-PAGE analysis also showed these cores to be associated with CP29/CP26 and CP24. OG cores were calculated to be associated with 42 chlorophyll a, 4.8 chlorophyll b and 9.5 β -carotene molecules per 2 molecules of pheophytin. Taken together, the subunit and pigment analyses suggest that OG cores are associated with 1-1.5 molecules of CP29 or CP26, or a mixture of these two polypeptides, per reaction centre.

Despite being associated with a large number of subunits, OG cores were amongst the most active, in terms of oxygen evolution capacity of any of the published PSII core preparations listed in Table 3.1. Furthermore, they were able to drive far higher rates of oxygen evolution than the HTGP cores characterised in Chapter 3. No D1 or D2 breakdown fragments were detected in freshly isolated OG core, suggesting that such preparations might be suitable for use in further PSII purification steps, designed to remove the 23 and 17kDa extrinsic, 22 and 10kDa intrinsic and CP24, CP26 and CP29 subunits. As HPLC size exclusion analysis suggests that OG cores may consist of monomeric and dimeric PSII cores, and possibly a chlorophyll a/b binding protein rich fraction, it will be necessary to resolve these PSII preparations into their three constituent components. Furthermore, these components will have to be characterised. If the 550kDa and 263kDa fractions do indeed correspond to monomeric and dimeric PSII core complexes, their characterisation should allow a decision to be made on which form is more suitable for advanced crystallisation trials.

Chapter 5 The use of sucrose gradient centrifugation to purify discrete monomeric and dimeric PSII complexes.

5.1 Introduction

Chapter 4 established that freshly prepared OG cores were able to support higher rates of oxygen evolution and free of detectable levels of D1 and D2 subunit breakdown fragments. This chapter describes the use of sucrose density gradient centrifugation to separate CP24, CP26/CP29, the 23 and 17kDa extrinsic as well as the 22 and 10kDa intrinsic polypeptides from monomeric and dimeric PSII core complexes consisting of CP47, CP43, D2, D1, cytb559, the 33kDa subunit and associated low molecular weight polypeptides. Section 5.2.1 compares how pure and active such monomeric and dimeric core complexes are and determines the level of breakdown fragments, with which each preparation is associated. The properties of these preparations are discussed with respect to their suitability for single particle analysis, as well as two- and three- dimensional crystallisation trials. Section 5.2.2 describes the purification of a dimeric PSII core complex, lacking the 33kDa extrinsic polypeptide, for single particle analysis. Section 5.2.3 describes the isolation and characterisation of a dense sucrose gradient band obtained by the mild solubilisation of BBYs, with the view to isolating a discrete PSII-LHCII super complex for single particle analysis.

5.2 Results and discussion

5.2.1 Experiment to optimise the detergent concentration used to resolve oligomeric core complexes from OG core preparations

HPLC size exclusion analysis of OG cores showed them to consist of particles, having apparent molecular weights of 550kDa, 263kDa and 70kDa in the presence of DM (Figure 4.6). From their molecular weights, it was suggested that the three particle types might correspond to dimeric and monomeric PSII core complexes and a chlorophyll a/b binding rich fraction, respectively. Barbato *et al.*, (1989) described the use of sucrose density gradient centrifugation to resolve PSII cores (similar to OG cores), into three chlorophyll rich fractions. The least dense of these was shown to contain LHCII, CP29 and CP26. The middle (putative PSII monomer) and lower (putative PSII dimer) bands contained PSII complexes, having identical subunit compositions (CP47, CP43, D2, D1 and cytb559). This section describes the development of a sucrose density gradient centrifugation procedure, which was able to resolve OG cores into three distinct chlorophyll binding fractions, similar to those reported by Barbato *et al.*, (1989). It should be noted that the putative PSII monomers and dimers reported by Barbato *et al.*, (1989) lacked the 33kDa extrinsic polypeptide. To obtain oxygen-evolving cores for crystallisation purposes, it was necessary to alter the conditions used by Barbato *et al.*, (1989) to prevent the dissociation of the 33kDa extrinsic polypeptide. To achieve this, the pH of the sucrose gradients was reduced from 7.2 to 6.5, according to Section 2.1.6.

To test the effect that detergent concentration has on the resolution of the bands in the sucrose gradient, the experiment shown in Figure 5.1 was set up. OG cores solubilised in 5mM DM only yielded a single chlorophyll rich band ("5mM Tube" Figure 5.1). Electron microscopy showed this fraction to contain a number of different protein complexes (electron micrograph, Figure 5.1). This suggests that the conditions used to obtain the banding pattern shown in the "5mM tube" (Figure 5.1), did not favour the separation of these particle types. Raising the DM concentration to 10mM allowed the separation of a less dense chlorophyll rich band (Upper fraction, Figure 5.1). Solubilising OG cores with 20-60mM DM yielded a third green band (Lower fraction). The colour intensities of the upper and lower fractions increased with detergent concentration, reaching a stable ratio at a concentration of about 30mM DM, taking into consideration the slight overloading in the "60mM tube". These results suggested that the mixture of protein complexes present in the single band of the "5mM tube" (Figure 5.1) were well resolved from one another using the conditions which gave the banding pattern seen in the "30mM tube" (Figure 5.1). This banding pattern was very similar to that reported by Barbato *et al.*, (1989). To determine whether these three green fractions corresponded to those reported by Barbato *et al.*, (1989), they were subjected to SDS-PAGE analysis (Section 5.2.2).

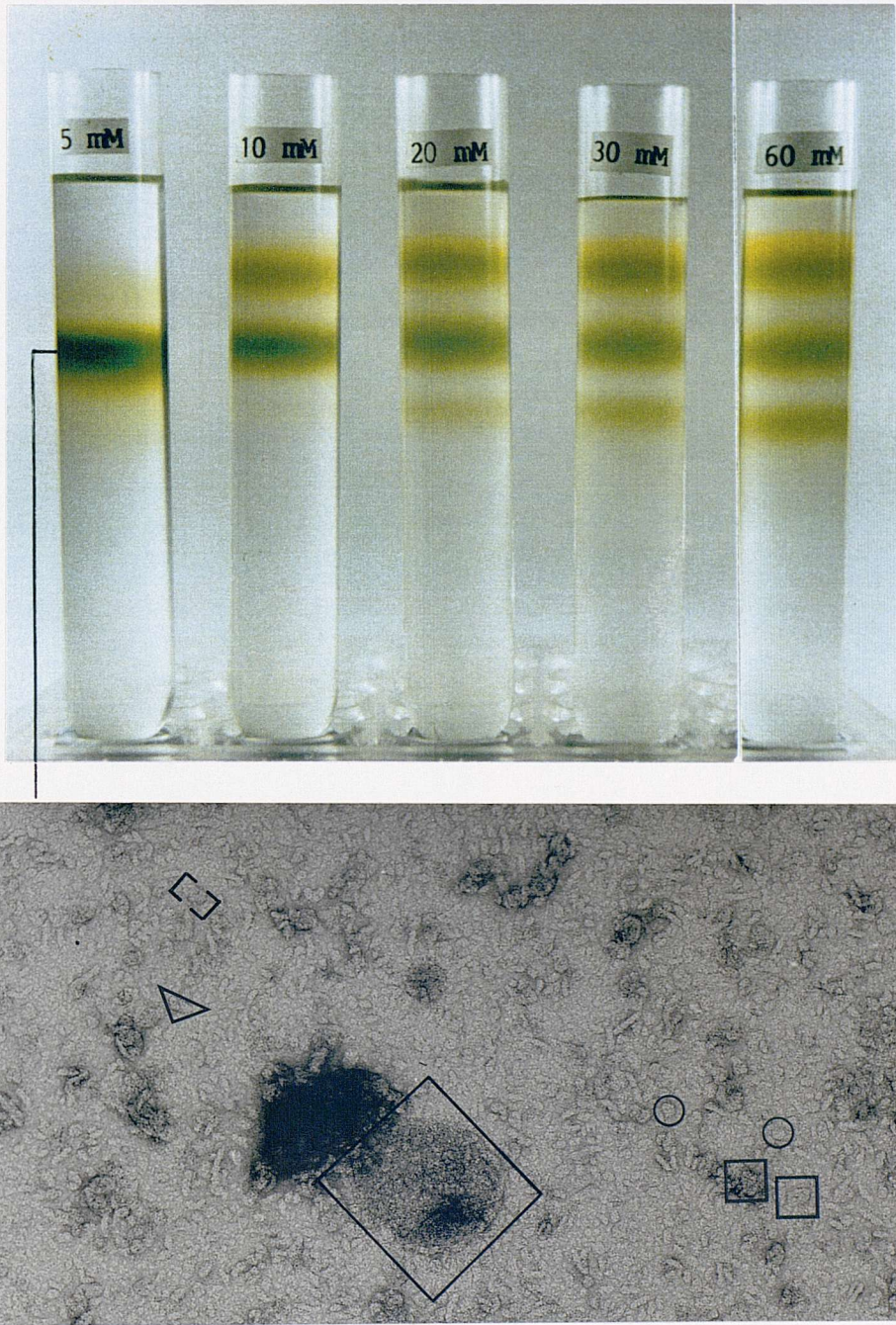


Figure 5.1 The effect of detergent concentration on the separation of three chlorophyll binding complexes from OG cores using sucrose density centrifugation. 75 μ g OG cores were suspended in MNCB buffer to give a final chlorophyll concentration of 0.5mg.ml⁻¹ chlorophyll on solubilisation with 5, 10, 20, 30 and 60mM DM. The DM concentrations used are marked on the individual tubes. The electron micrograph inserted, shows a negatively stained sample of the single fraction seen in the "5mM tube". Protein aggregates which may be lipid-protein bilayers (large square) and discrete particles of different shapes are marked with circles, small squares, triangles and brackets. The scale bar measures 200nm.

5.2.2 The subunit composition of the chlorophyll binding fractions resolved from OG cores by sucrose density gradient centrifugation

To determine the subunit composition of the three chlorophyll binding complexes resolved by sucrose density gradient centrifugation ("30mM Tube", Figure 5.1), the three green bands were extracted and subjected to SDS-PAGE analysis, using the methods described in Sections 2.2.1-2.2.4. The results of such analysis are shown in Figure 5.2.

BBYs (Figure 5.2, Lane 1) and OG cores (Figure 5.2, Lane 2) were run as controls. The LHCII and CP29/CP26 components of the BBY sample (Lane 1, Figure 5.2), can be seen to be resolved from one another. OG cores are associated with CP29 and/or CP26, but are depleted of LHCII (Lane 2, Figure 5.2). The subunit compositions of the middle and lower sucrose gradient fractions ("30mM tube", Figure 5.1) are shown in Lanes 3 and 4 (Figure 5.2). These sucrose gradient fractions have almost identical subunit compositions, consisting of CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic polypeptide. The low molecular weight subunits associated with these two complexes were not characterised. A comparison of Lanes 2, 3 and 4 (Figure 5.2), shows that the two sucrose gradient purified PSII core complexes (Lanes 3 and 4, Figure 5.2) are depleted of CP29 and/or CP26 as well as LHCII and suggests that these chlorophyll a/b proteins compose the upper fraction ("30mM tube", Figure 5.1). Although, the upper sucrose gradient fraction contains a faint band running at the expected CP29/CP26 position (Lane 5, Figure 5.2), a more intense band is seen to run below it. The reason why these subunits run at a lower position than observed in BBYs or OG cores is not clear. To confirm the chlorophyll b rich nature of the upper sucrose gradient fraction, its spectrum is presented in Figure 5.9.

The results suggest that sucrose density gradient centrifugation can be used to isolate PSII cores consisting of CP47, CP43, D2, D1, cytb559, the 33kDa subunit and associated low molecular weight polypeptides. The isolation of complexes of this subunit composition, for the purposes of crystallisation, was one of the aims of my thesis. Having isolated two complexes of this subunit composition, which differ in density, I decided to determine their respective aggregation states and whether one core type was more pure, undamaged and active than the other. Figure 5.2 (Lanes 3 and 4) shows both sucrose gradient core types to be associated with a number of unidentified bands (Bands A-F). The middle fraction ("30mM tube", Figure 5.1) resolved by SDS-PAGE in Lane 3 (Figure 5.2) contains higher levels of these unidentified bands than the lower fraction ("30mM tube", Figure 5.1) resolved in Lane 4 (Figure 5.2). Similar bands were detected in HTGP cores (Figure 3.2) and OG cores (Figure 4.1) and are thought to be aggregation and breakdown products of CP47, CP43, D2, D1, cytb559 and the 33 extrinsic polypeptide (Figures 3.6 and 4.2). To identify any aggregation and breakdown fragments associated with these two core preparations, both were subjected to Western blot analysis in Section 5.2.7. Before conducting such experiments, it was important to establish whether the middle and lower fractions ("30mM tube", Figure 5.1) corresponded to monomeric and dimeric PSII core complexes, as suggested by Barbato *et al.*, (1989).

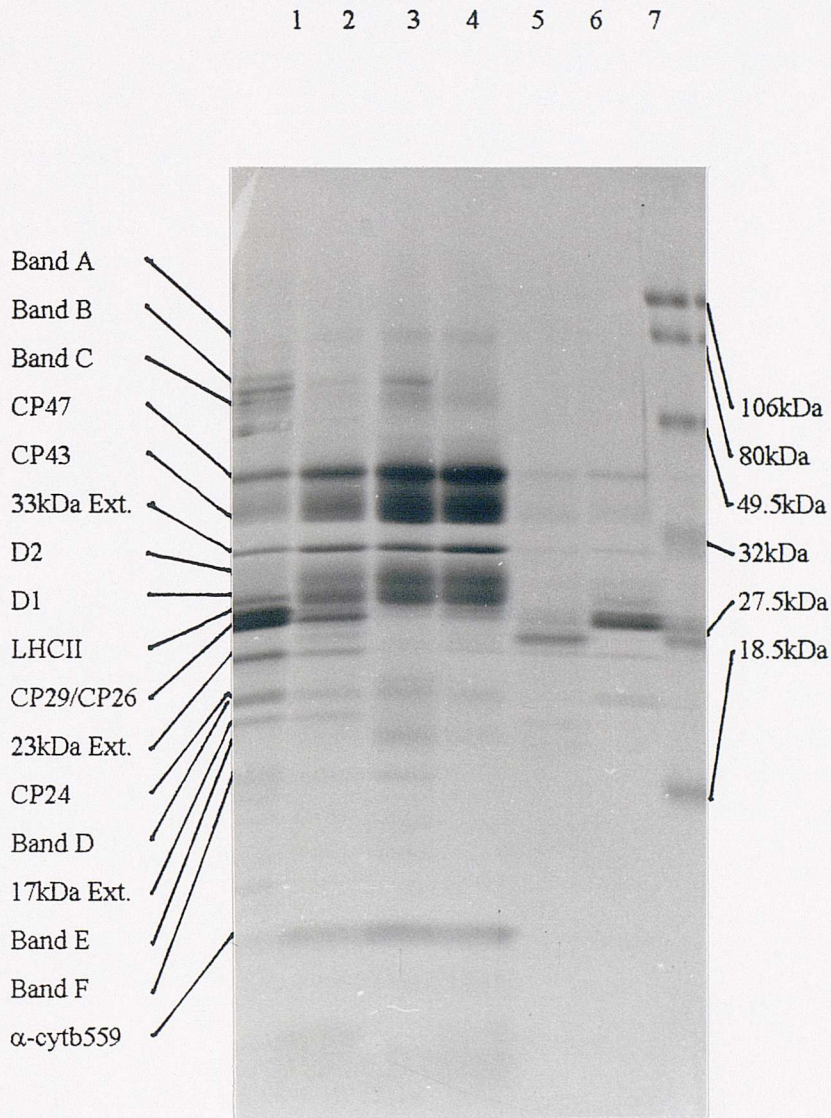


Figure 5.2 The subunit composition of the three chlorophyll binding fractions resolved by sucrose density gradient centrifugation. For purposes of comparison, the three sucrose gradient fractions, extracted from the "30mM" tube shown in Figure 5.1, were resolved by SDS-PAGE, in parallel with BBYs, OG cores and crude LHCII. Lane 1 - BBYs (6.5 μ g chlorophyll); Lane 2 - OG cores (4 μ g chlorophyll); Lane 3 - middle sucrose gradient fraction (4 μ g chlorophyll); Lane 4 - lower sucrose gradient fraction (4 μ g chlorophyll); Lane 5 - upper sucrose gradient band (1 μ g chlorophyll); Lane 6 - crude LHCII pellet (1 μ g chlorophyll); Lane 7 - Biorad Markers. The gel was stained with Coomassie.

5.2.3 Molecular weight determination of the three sucrose gradient purified chlorophyll binding fractions.

This section describes the characterisation of the three sucrose gradient purified fractions ("30mM tube", Figure 5.1) using HPLC size exclusion analysis. To calculate their molecular weights, standards were first subjected to HPLC size exclusion analysis, according to the method described in Section 2.5. The standard curve, which was obtained, is provided in Figure 5.3.

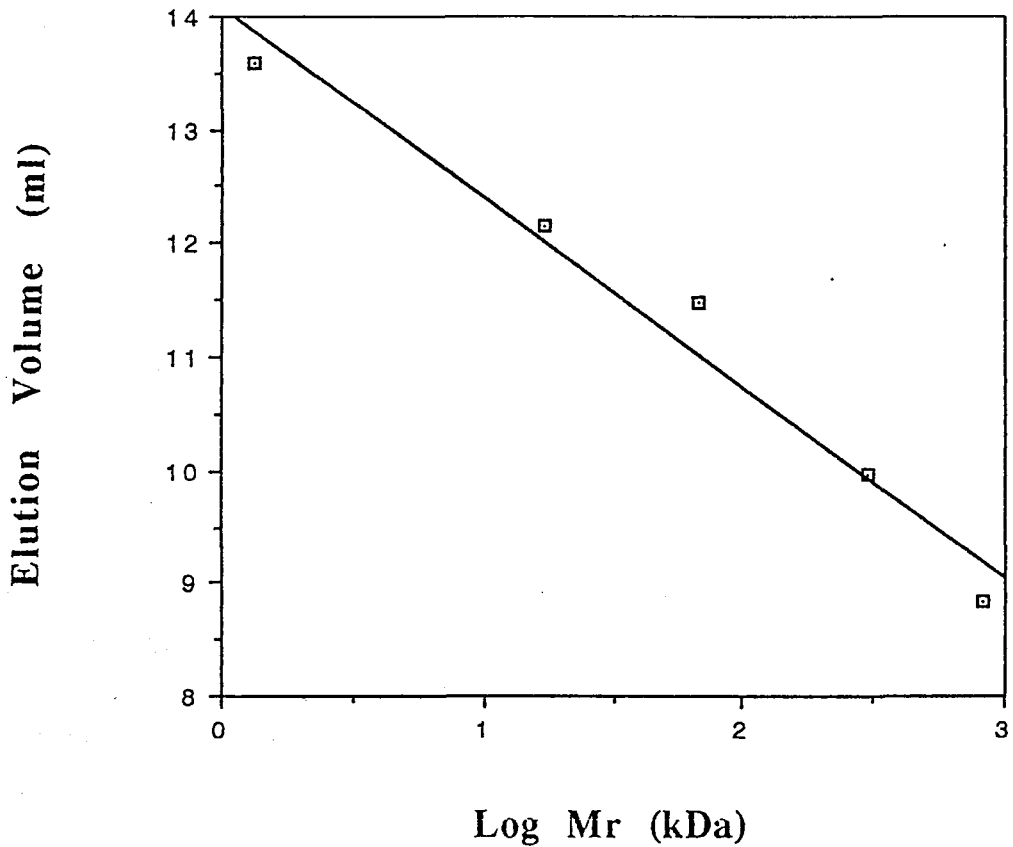


Figure 5.3 Molecular weight calibration curve. PSI trimer (820kDa), invertase (540kDa), PSI monomer (300kDa), bovine serum albumin (67kDa), myoglobin (17kDa) and vitamin B12 (1.35kDa) were used to produce this calibration curve. PSI monomers and trimers were used as standards in this experiment after calibration with a large number of other standards (Rögner, personal communication).

The HPLC size exclusion elution profiles of the two PSII core fractions (i.e. the middle and lower fractions of the "30mM tube" in Figure 5.1) are given in Figure 5.4. The elution profile of the

middle sucrose gradient fraction is marked by the dotted line (Figure 5.4). Its major peak had an elution volume of 10.1ml. From Figure 5.3, the complexes composing this peak were calculated to have an apparent molecular weight of $240\text{kDa} \pm 48\text{kDa}$ in the presence of DM. The PSII complexes composing the middle sucrose gradient fraction (Figure 5.1) are known to consist of CP47, CP43, D2, D1, cytb559, the 33kDa extrinsic polypeptide and associated low molecular weight subunits (Figure 5.2, Lane 3). The molecular weight of this peak (240kDa) corresponds closely to the theoretical value (250kDa) of a monomeric PSII complex having the same subunit composition (Table 3.3). This strongly suggests that the middle fraction ("30mM tube", Figure 5.1) consists of monomeric (CP47, CP43, D2, D1, cytb559 and the 33 extrinsic subunit) PSII core complexes and suggests that the corresponding 9.9ml elution peaks shown in Figures 3.10 (HTGP cores) and Figure 4.6 (OG cores) may be composed of such complexes.

The HPLC size exclusion elution profile of the lower sucrose gradient band ("30mM tube", Figure 5.1) is marked by a solid line in Figure 5.4. From this elution profile, it can be seen that the lower fraction consists predominantly of complexes having an elution volume of 9.5 ml. From Figure 5.3, the molecular weight of the complexes, composing this elution peak is calculated to be $540\text{kDa} \pm 108\text{kDa}$, in the presence of DM. This corresponds closely to the theoretical molecular weight (500kDa) of a PSII dimer (CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic polypeptide). This strongly suggests that the peaks eluting at 9.2ml in Figure 3.10 and Figure 4.6 are dimeric PSII complexes.

Both the PSII monomer and dimer fractions showed low levels of cross-contamination (Figure 5.4). It is likely that this contamination occurred during the extraction of the two bands from the sucrose gradient. However, they also contained low levels of a contaminant having an elution volume of 11.5ml. The molecular weight of the particles composing this peak was calculated to be $35\text{kDa} \pm 7\text{kDa}$. Due to the small yields, the individual peaks were not harvested for direct SDS-PAGE analysis. To test whether the peak, eluting after 11.5ml, was composed of contaminating chlorophyll a/b binding proteins, the upper sucrose gradient fraction was subjected to HPLC size exclusion analysis (Figure 5.5). Its major peak eluted after 11.2ml and corresponded to a molecular weight of $52\text{kDa} \pm 10\text{kDa}$. Thus, the precise nature of the contaminating particles seen to elute after 11.5ml in Figure 5.4, remains to be determined.

The molecular weight of the particles eluting after 14.3ml in both Figure 5.4 and 5.5 was calculated to be approximately 0.75kDa, suggesting that they may be free pigments. The purified dimers did not contain this "free pigment" fraction (Figure 5.4).

The results presented in this section suggest that the middle and lower bands ("30mM tube", Figure 5.1) consist of purified monomeric and dimeric PSII complexes. These core complexes were subjected to spectral (Section 5.2.4) and reverse phase HPLC analysis (Section 5.2.5) to gain a more complete picture of their respective pigment compositions.

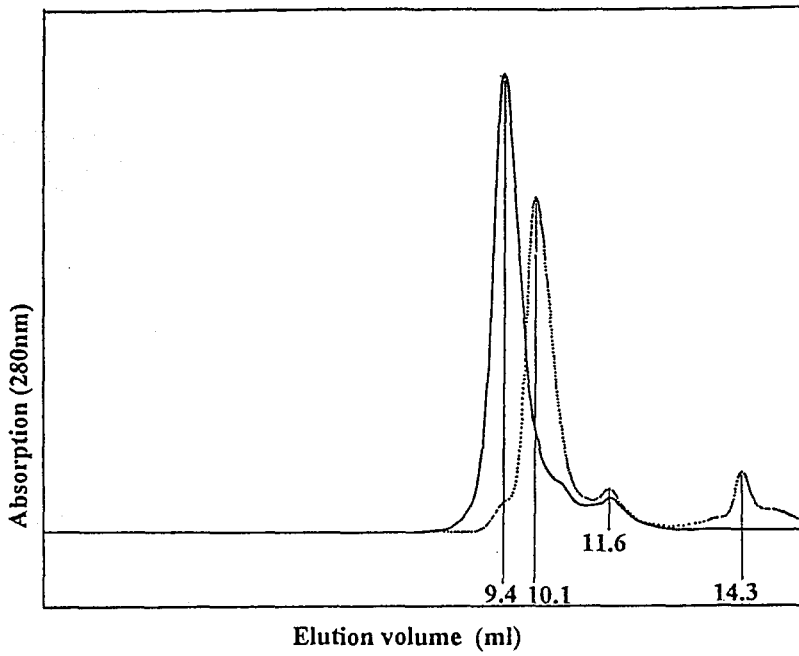


Figure 5.4 HPLC elution profile of the two sucrose gradient purified core fractions. The elution profile of the middle fraction of the sucrose gradient ("30mM tube", Figure 5.1) is represented by the dotted line. The elution profile of the lower fraction is represented by the solid line.

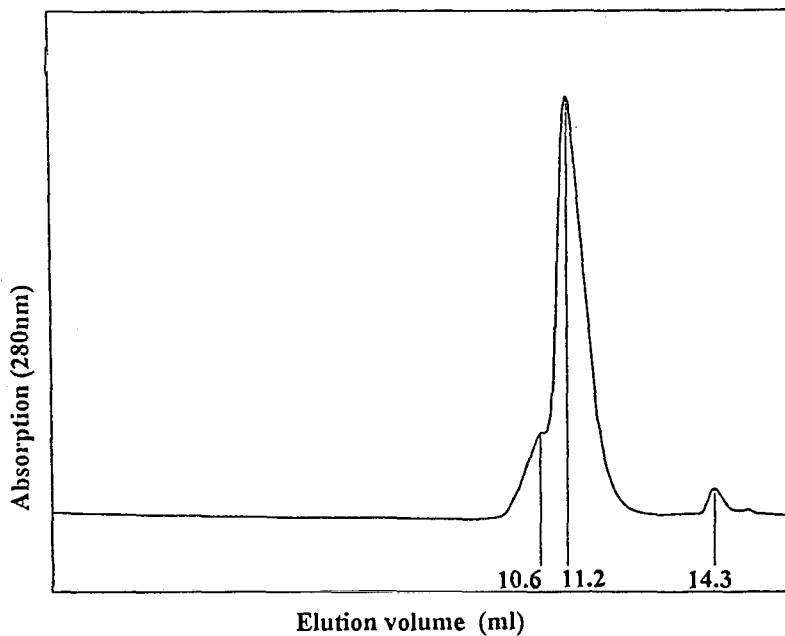


Figure 5.5 HPLC size exclusion analysis of the upper sucrose gradient fraction. The elution profile identifies peaks eluting at 10.6ml, 11.2ml and 14.3ml. These elution volumes correspond to molecular weights of 119kDa, 52kDa and 0.75kDa.

5.2.4 Comparison of the spectra of OG cores, sucrose gradient purified PSII core monomers and dimers

The spectra of two replicate sucrose gradient purified monomer fractions (middle band, Figure 5.1) were recorded using the method described in Section 2.6 and are overlaid in Figure 5.6. The two monomer spectra (Figure 5.6) are seen to be virtually identical.

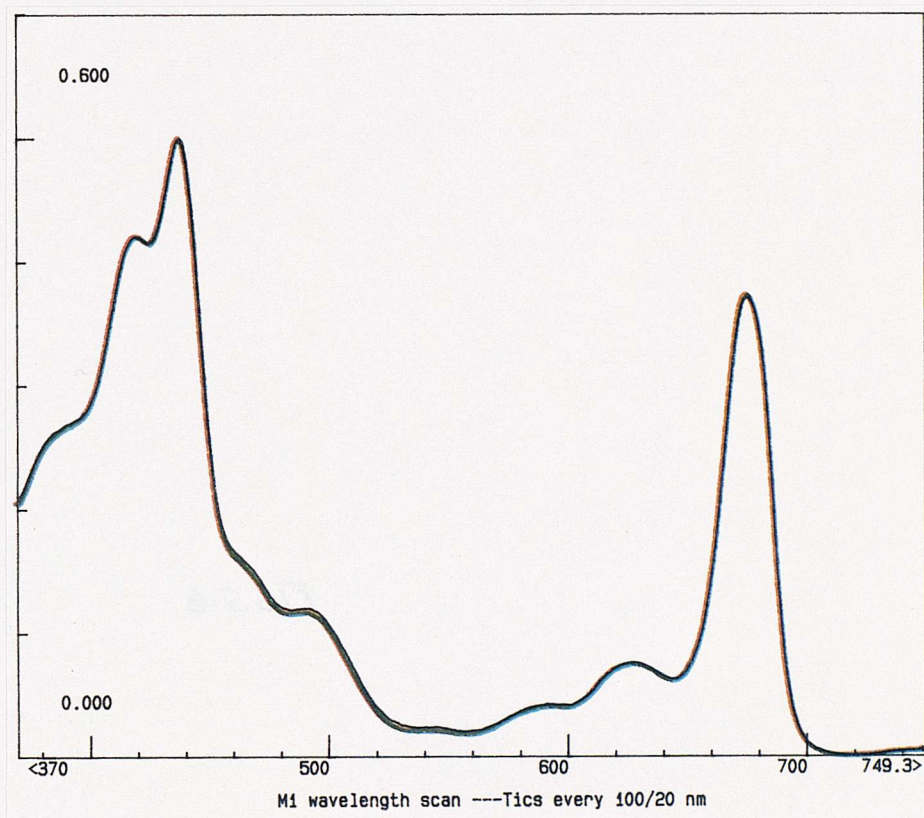


Figure 5.6 Spectral comparison of two sucrose gradient purified PSII monomer preparations. PSII monomers in SMNCB supplemented with 2mM DM. The 437nm peaks of the recorded spectra were normalised. The red peak λ_{max} values of these two monomeric PSII core preparations were found to be 675.5nm.

The spectra of three replicate sucrose gradient purified dimer fractions are overlaid in Figure 5.7. The dimeric core particles showed greater variation in the 460-500nm range of the spectrum, than the monomeric complexes. This suggests that the dimer preparations analysed (Figure 5.7) may be associated with slightly different amounts chlorophyll b or carotenoids, or that the baseline is somewhat skewed as a result incomplete sample solubilisation.

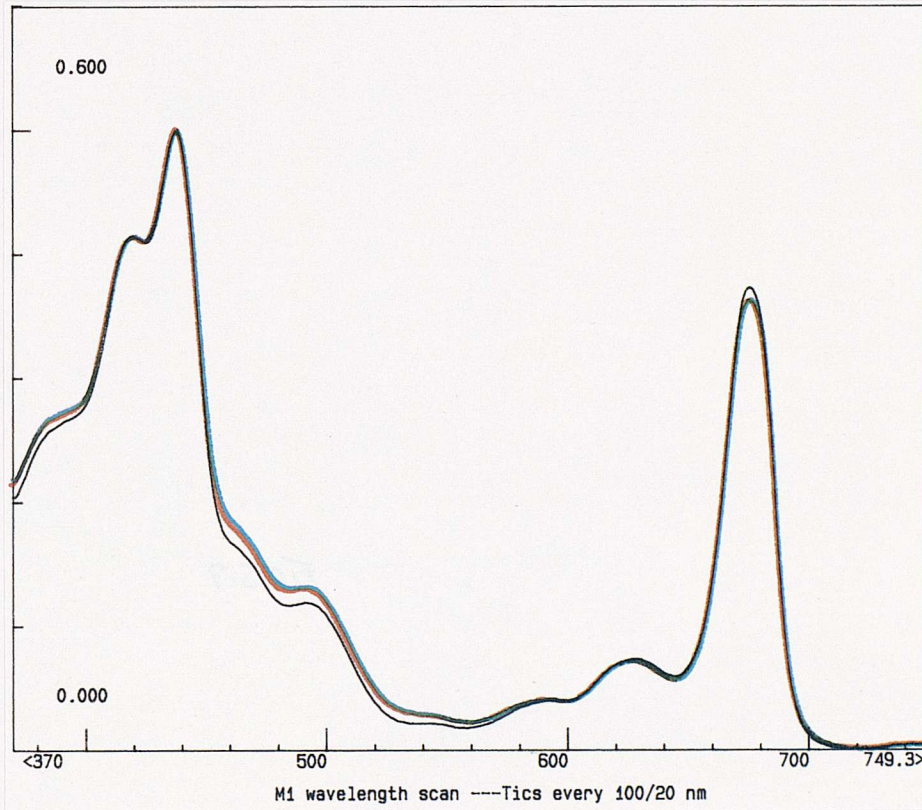


Figure 5.7 Spectral comparison of three sucrose gradient purified PSII dimer preparations. PSII dimers in SMNCB supplemented with 2mM DM. The 437nm peaks of the recorded spectra were normalised to 0.500 absorbance units before being overlaid. The red peak λ_{max} values of the three dimeric PSII core preparations was 676nm.

The average of the two monomer (Figure 5.6) and three dimer (Figure 5.7) spectra are compared in Figure 5.8. As a reference, the average of three OG core spectra taken from Figure 4.5 (green trace, in Figure 4.5), is also drawn in Figure 5.8 (black trace). The results shown in Figure 5.8 suggest that the dimer (blue trace) either has a slightly higher carotenoid content than the monomer (orange trace) as it absorbs more strongly in the 460-500nm range of the spectrum, but not at 645nm, or that the higher absorbance in this region is related to the aggregation state of the complex. The red peak of the monomeric complexes is blue shifted with respect to that of the dimeric complex, which in turn is blue shifted to that of OG cores. It is possible that this blue shift is related to level of detergent with which each complex is associated. The sucrose gradient purified monomers and dimers absorb less strongly in both the 460-500nm and 650nm region of the spectrum than OG cores (black trace). This is expected as Figure 5.2 shows that sucrose gradient purified monomers and dimers, are both depleted of the chlorophyll a/b binding proteins (CP24, CP26, CP29 and LHCII).

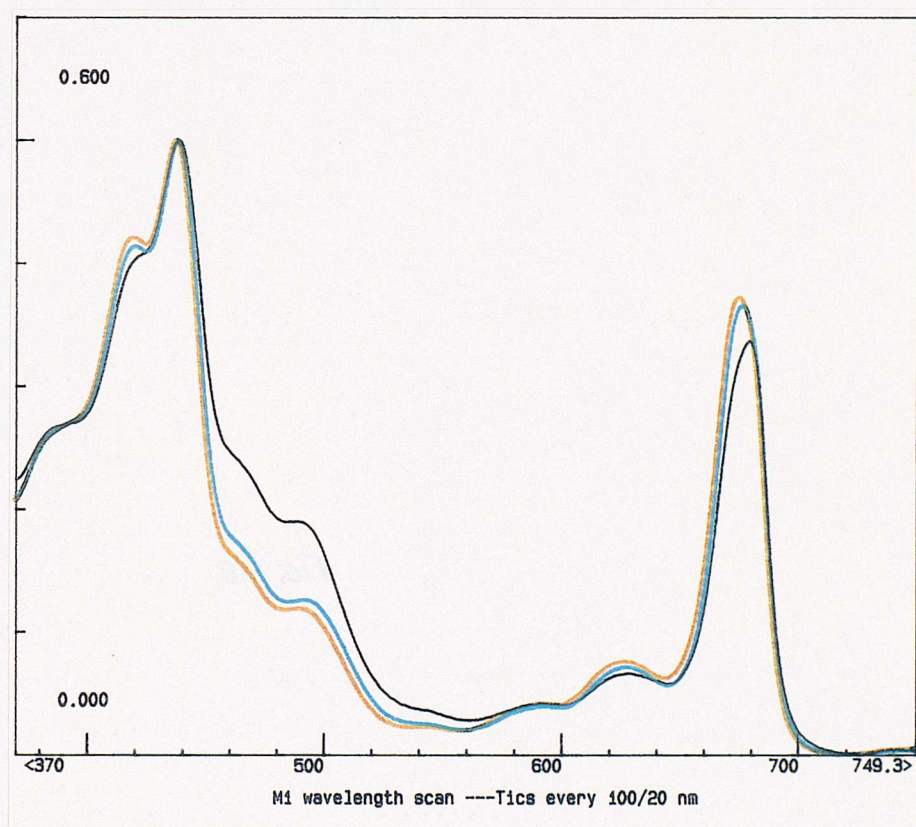


Figure 5.8 Comparison of average OG core, monomer and dimer spectra. The average of three OG cores (Figure 4.5), two monomer (Figure 5.6) and three dimer (Figure 5.7) spectra are overlaid in this figure. OG core, monomer and dimer spectra are traced in black, orange and blue respectively. All samples were analysed in SMNCB supplemented with 2mM DM. The 437nm peaks of the recorded spectra were normalised to 0.500 absorbance units before being overlaid.

The orange trace (Figure 5.9) shows that the upper sucrose gradient fraction ("30mM tube", Figure 5.1) is chlorophyll b rich, as it absorbs more strongly than OG cores (black trace, Figure 5.9) in the 460-500nm and 645nm region of the spectrum. This confirms that the loss of chlorophyll b from the sucrose gradient purified monomers and dimers can be accounted for by its presence in the upper fraction ("30mM tube", Figure 5.1).

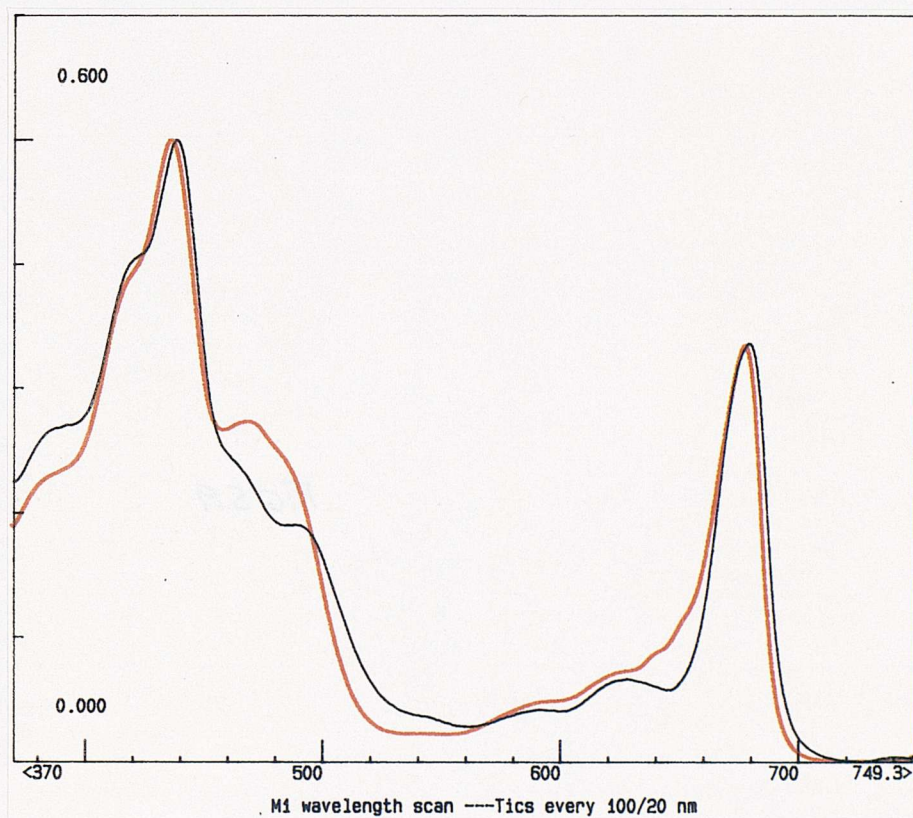


Figure 5.9 Comparison of OG core and upper sucrose gradient fraction spectra. The average of three OG core spectra (black trace), is compared with a typical spectrum of the upper sucrose gradient fraction (orange trace). All samples were analysed in SMNCB supplemented with 2mM DM. The 437nm peaks of the recorded spectra were normalised to 0.500 absorbance units before being overlaid.

5.2.5 Determining the number of chlorophyll a, chlorophyll b and β -carotene molecules associated with each of the triplicate PSII core complexes

This section describes the HPLC reverse phase pigment analyses of the monomeric and dimeric PSII core fractions isolated by sucrose density gradient centrifugation (middle and lower bands of "30mM tube", Figure 5.1). The chlorophyll a: chlorophyll b: β -carotene: pheophytin ratios of these core types were obtained using the method described in Section 2.6. Pigment ratios were normalised to 2 pheophytin molecules, for the reasons previously discussed in Sections 3.1.4 and 3.2.3.3. Since it is thought that each reaction centre binds 2 pheophytin molecules (Sections 3.1.4 and 3.2.3.3), the number of pigments associated with each monomeric core particle can be calculated directly. The pigment ratios of the dimeric cores shown in Table 5.1 were also normalised to two pheophytin molecules. This allows the number of pigment molecules, associated with each monomeric segment of the dimer, to be compared with that of the pure monomeric form of PSII. As dimeric core particles contain two reaction centres and presumably 4 pheophytin molecules, multiplying their pigment ratios by a factor of two, allows the total number of pigments associated with each dimer to be calculated.

Table 5.1 The pigment stoichiometry of sucrose gradient purified PSII monomer and dimer preparations containing CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic polypeptide.

Sample	Chlorophyll a	Chlorophyll b	β -carotene	Pheophytin
Monomer mean (SD)	34.3 (0.9)	2.2 (0.05)	7.7 (0.3)	2
Dimer mean (SD)	31.4 (0.8)	1.4 (0.06)	7.6 (0.2)	2

On the basis of the results of Kobayashi *et al.*, (1990), Nanba & Satoh, (1987), Gounaris *et al.*, (1990) and Barbato *et al.*, (1991a) a complex consisting of CP47, CP43, D2, D1, cytb559 and the 33kDa subunit would be expected to bind up to 31 molecules of chlorophyll a per 2 pheophytin molecules. Thus, the number of chlorophyll a molecules calculated to be associated with the sucrose gradient purified monomers and dimers characterised in Table 5.1, appear to be reasonable. However, the sucrose gradient purified monomers and dimers both contain some chlorophyll b contamination. As CP29, CP26 and CP24 are reported to bind 4, 5 and 7 chlorophyll b molecules respectively (Thorner *et al.*, 1993), the PSII monomers analysed by reverse phase HPLC analysis (Table 5.1) are estimated to be associated with (0.6) CP29, (0.4) CP26 or (0.3) CP24 subunits per PSII reaction centre, or a combination thereof. On the same basis the sucrose gradient purified dimers are estimated to be associated with (0.4) CP29, (0.3) CP26 or (0.2) CP24 subunits per PSII reaction centre. In view of the fact that the PSII monomer band runs just below the chlorophyll a/b protein rich band ("30mM tube", Figure 5.1), it is possible that the slightly higher chlorophyll a and chlorophyll b values presented for the PSII monomers (Table 5.1) may be the result of contamination by the chlorophyll b rich upper

band components, during extraction. However it cannot be excluded that the presence of chlorophyll b in the two core preparations reflects that they are still directly linked, rather than contaminated by, residual amounts of the chlorophyll a/b binding proteins. No significant difference was observed between the levels of β -carotene associated with the monomeric and dimeric PSII core preparations analysed. The difference in absorption between monomeric and dimeric PSII core complexes in the 460-500nm region of the spectrum (Figure 5.8) may therefore be due to them being associated with varying amounts of carotenoids, other than β -carotene. Alternatively, the spectral differences between the monomeric and dimeric PSII core complexes, may be directly related to their respective aggregation states.

The SDS-PAGE (Section 5.2.2), HPLC size exclusion (Section 5.2.3) and reverse phase HPLC analysis (Section 5.2.5) have established that the sucrose gradient purified monomers and dimers have almost identical subunit and pigment compositions. To determine whether the two complexes differ in oxygen evolution capability, the experiments described in Section 5.2.6 were set up.

5.2.6 Oxygen evolution rates driven by sucrose gradient purified PSII monomers and dimers

The oxygen-evolving ability of two PSII monomer and two PSII dimer preparation were determined using the method described in Section 2.4. The oxygen evolution rates are calculated on the basis of chlorophyll concentration and the results are shown in Table 5.2. The DMBQ:DCBQ oxygen evolution rate ratio was used as a measure of the ability of a given core preparation to support electron transport between Q_A and Q_B (see Section 2.4).

Table 5.2 Oxygen evolution rates of isolated PSII monomers and dimers, in the presence of DCBQ and DMBQ as electron acceptors

Sample	O ₂ evolution ($\mu\text{moles O}_2 \text{ mg chl}^{-1} \text{ h}^{-1}$) DCBQ	O ₂ evolution ($\mu\text{moles O}_2 \text{ mg chl}^{-1} \text{ h}^{-1}$) DMBQ	DMBQ:DCBQ O ₂ evolution rate ratio x 100 (Y)(%)
Monomer prep. 1 (SD)	337 (11)	15 (10)	4.4
Monomer prep. 2 (SD)	306 (3)	16 (7)	5.3
Dimer prep. 1 (SD)	495 (33)	56 (8)	11.3
Dimer prep. 2 (SD)	595 (7)	108 (17)	18.2

Comparing the oxygen evolution rates driven by the two monomer and two dimer samples in the presence of DCBQ (Table 5.2), shows that the dimeric complexes analysed were considerably more active than their monomeric counterparts. This difference in the rates of oxygen evolution

driven by PSII monomers and dimers, in the presence of DCBQ, may be an indication that the Q_A site or the donor side of the dimeric PSII core complexes are less damaged than those of their monomeric counterparts. The fact that both the sucrose gradient purified PSII monomer and dimer preparations evolve oxygen at lower rates, than OG cores, may be related to the loss of the 23 and 17kDa subunit. It should however, be noted that the oxygen evolution assay medium was supplemented with 15mM $CaCl_2$; a concentration which has been reported to be high enough to compensate for the removal of the 23kDa extrinsic polypeptide (Miyao & Murata, 1984b). Thus, it is possible that the monomeric and dimeric PSII core preparations evolve oxygen at a lower rate than OG cores for reasons other than the loss of the 17 and 23kDa extrinsic subunits.

The oxygen evolution rates driven by the dimeric PSII core complexes, in the presence of DMBQ, were also considerably higher than those of the monomeric complexes. This is reflected in the Y value (Table 5.2), which was taken as a measure of Q_A to Q_B electron transfer (see Section 2.4 and 3.2.4). As the Y values of the dimers were found to be higher than those of their monomeric counterparts, it is possible that the latter exhibit a greater degree of acceptor side damage.

A 10kDa polypeptide, which might correspond to either the *psbR* or *psbH* gene product, is present in OG cores (Figure 4.1), but absent from the monomeric and dimeric PSII core complexes (Figure 5.2). If this polypeptide is the *psbH* gene product it is possible that its dissociation from the core complex during sucrose density gradient centrifugation can partially account for the fact that the monomeric and dimeric PSII cores have lower Y values than were reported for OG cores (Table 4.1)(Packham, 1987; Mayes *et al.*, 1993). It does not, however explain why the Y values of the PSII monomer and dimer preparations differ from one another so much. The low-molecular-weight subunit composition of these two core complexes were not characterised. I cannot therefore suggest whether the removal of any low molecular weight subunits, such as the *psbL* gene product, resulted in the differences in the Y values of these two core preparations. As Nagatsuka *et al.*, (1991) have suggested that the *psbL* gene product and possibly some of the other low molecular weight subunits are involved in the normal functioning of the Q_A site, it would be interesting to determine whether the monomers and dimers are associated with differing amounts of these polypeptides. The reduced oxygen evolution capacity of the monomers, with respect to the dimers, may however be due to PSII subunit damage, rather than the removal of any low molecular weight subunits.

To test whether subunit breakdown products could be detected in these preparations, monomeric and dimeric PSII core preparations were subjected to Western blot analysis (Section 5.2.7).

5.2.7 Study of the PSII subunits breakdown fragments associated with monomeric and dimeric sucrose gradient purified PSII core complexes

5.2.7.1 Western blot analysis of sucrose gradient purified PSII monomers and dimers using antibodies raised against CP47, CP43, D2, D1 and the 33kDa extrinsic subunit

Figure 5.2 showed that the monomeric (Lane 3, Figure 5.2) and dimeric (Lane 4, Figure 5.2) sucrose gradient purified PSII core complexes were associated with a number of unidentified bands (Bands A-F). This section describes Western blot analysis of such monomeric and dimeric PSII core complexes, using antibodies raised against CP47, CP43, D2, the C-terminal end of D1 and the 33kDa extrinsic polypeptide. The aim of this experiment was to identify PSII subunit aggregation products and breakdown fragments associated with these preparations. To achieve this, samples were resolved by SDS-PAGE, using the methods described in Sections 2.2.1-2.2.3. Coomassie and silver staining procedures, as well as the Western blot methodology, are provided in Sections 2.2.4, 2.2.5 and 2.3.1-2.3.2, respectively. Figures 5.10 (PSII monomers) and 5.11 (PSII dimers) summarise the information gained by comparing Coomassie and silver stained SDS-PAGE resolved samples with CP47, CP43, 33kDa extrinsic polypeptide, D2 and C-terminal D1 specific Western blots.

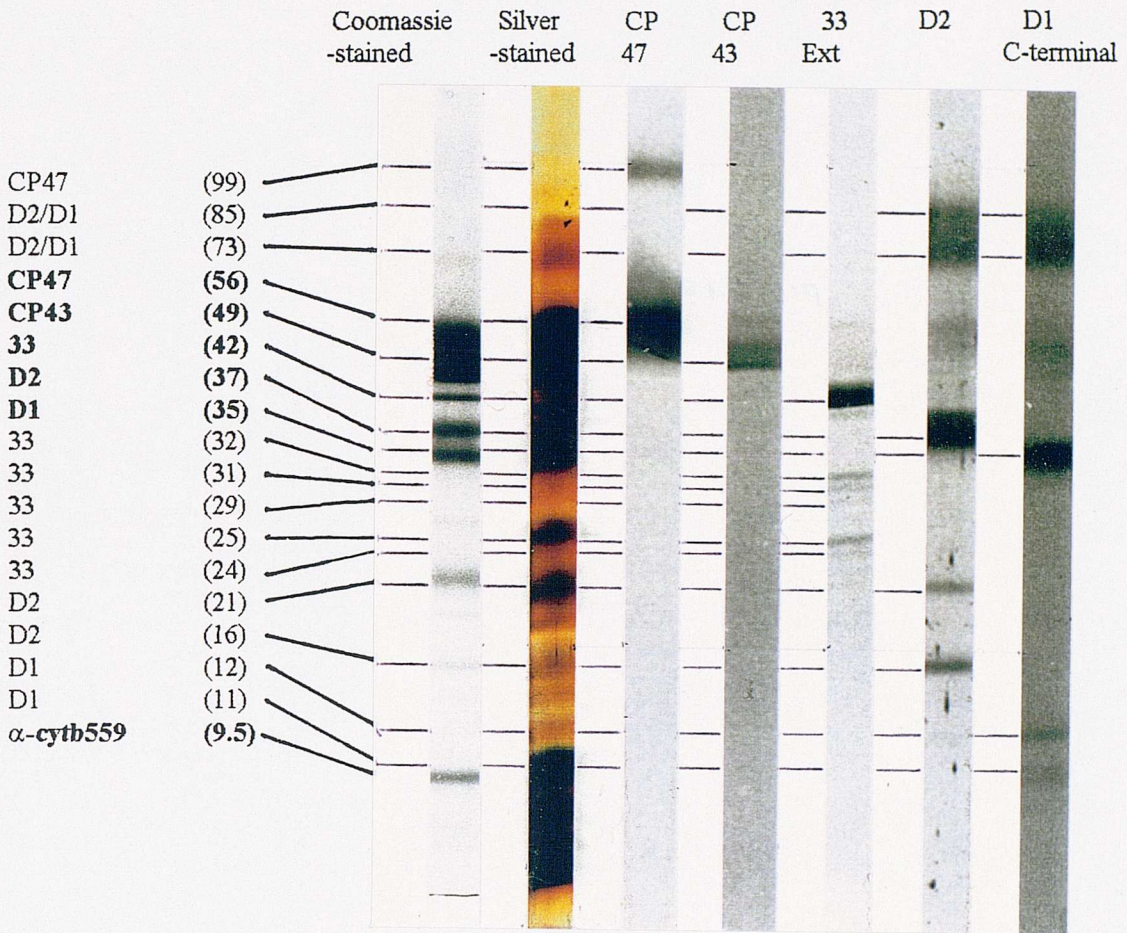


Figure 5.10 The detection of CP47, CP43, D2 and D1 and the 33kDa extrinsic subunit aggregation and breakdown products, in isolated PSII monomer preparations. The amount of PSII monomer resolved in each of the 7 lanes (shown above) is given in μg of chlorophyll. Lane 1 - Coomassie stained ($10\mu\text{g}$ PSII monomer); Lane 2 - silver stained ($1\mu\text{g}$ PSII monomer); Lane 3 - CP47 specific Western blot ($1\mu\text{g}$ PSII monomer); Lane 4 - CP43 specific Western blot ($1\mu\text{g}$ PSII monomer); Lane 5 - 33kDa extrinsic polypeptide specific Western blot ($1\mu\text{g}$ PSII monomer); Lane 6 - D2 specific Western blot ($1\mu\text{g}$ PSII monomer); Lane 7 - D1 specific Western blot ($1\mu\text{g}$ PSII monomer). Bold labels indicate intact subunits. Plain text indicates aggregation and breakdown products.

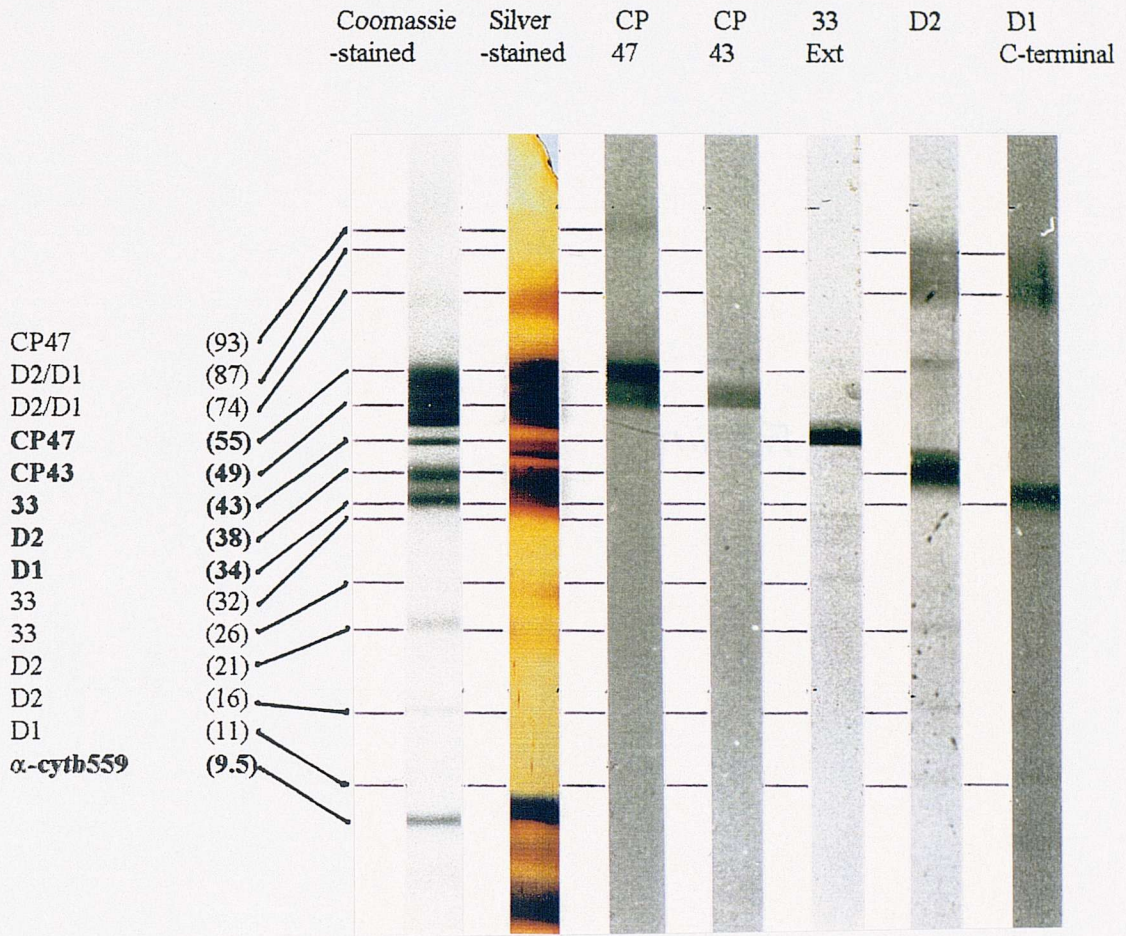


Figure 5.11 The detection of CP47, CP43, D2 and D1 and the 33kDa extrinsic subunit aggregation and breakdown products, in isolated PSII dimer preparations. The amount of PSII dimer resolved in each of the 7 lanes shown above is given in μ g of chlorophyll. Lane 1 - Coomassie stained (10 μ g PSII dimer); Lane 2 - Silver stained (0.8 μ g PSII dimer); Lane 3 - CP47 specific Western blot (0.8 μ g PSII dimer); Lane 4 - CP43 specific Western blot (0.8 μ g PSII dimer); Lane 5 - 33kDa extrinsic polypeptide specific Western blot (0.8 μ g PSII dimer); Lane 6 - D2 specific Western blot (0.8 μ g PSII dimer); Lane 7 - D1 specific Western blot (0.8 μ g PSII dimer). Bold labels indicate intact subunits. Plain text indicates aggregation and breakdown products.

The estimated molecular weights (Figures 5.10 and 5.11) of the intact CP47, CP43, D2 and D1 subunits are very similar to their true values (Table 3.3). In contrast, the intact 33kDa extrinsic subunit, whose actual molecular weight is 27kDa (Table 3.3), had an apparent molecular weight of 42-43kDa in the gel systems used (Figures 5.10 and 5.11).

Figure 5.10 shows three polypeptides, having apparent molecular weights higher than those of any of the intact subunits. The largest of these was detected by the CP47 specific antibody, in both the PSII monomer (99kDa band, Figure 5.10) and PSII dimer (93kDa band, Figure 5.11) preparations. As the intact CP47 subunit was estimated to have a molecular weight of 55-56kDa (Figures 5.10 and 5.11) and high molecular weight estimations are less accurate, the 93-99kDa band detected by the CP47 specific antibody may be a dimeric form of this polypeptide. Aggregation products detected by the CP47 specific antibody and having similar apparent molecular weights were also detected in OG cores (96kDa band, Figure 4.2), HTGP cores (91kDa band, Figure 3.6), BBYs (100kDa band, Figure 3.5) and thylakoids (101kDa band, Figure 3.4) (see Figure 5.11), respectively. Their presence in all of these preparations suggests that the samples were either not completely solubilised or that these aggregation products are formed as a result of SDS treatment, prior to SDS-PAGE analysis. The possibility of damage related subunit cross-linking cannot be excluded. Comparing Figures 5.10 and 5.11 with Figure 5.2, suggests that this CP47 aggregate corresponds to Band A (Figure 5.2).

Two other aggregation products were detected in monomers (Figure 5.10). Both were labelled by the D1 and D2 antibodies, suggesting that they may be D1/D2 heterodimer complexes. Their molecular weights were calculated to be 85 and 73kDa (Figure 5.10). The sum of the molecular weights of the intact D2 (38kDa) and D1 (34kDa) subunits is 72kDa (Table 3.3). This suggests that the smaller of the two complexes (73kDa band, Figure 5.11), which corresponds to Band C (Figure 5.2), may consist of the two intact subunits. A similar band was also detected in OG cores (74kDa band, Figure 4.2), as well as in sucrose gradient purified dimers (74kDa band, Figure 5.11). The nature of the 85kDa D1/D2 aggregate (Figure 5.7) remains unclear, though its molecular weight is consistent with the hypothesis that this polypeptide complex may also be associated with the α -subunit of cytb559. Although, such a band was not detected in OG cores, an 87kDa band was seen in the dimeric core (Figure 5.8). A comparison of Figures 5.10 and 5.11 with Figure 5.2, suggests that the 85-87kDa D1/D2 aggregate (Figures 5.10 and 5.11) corresponds to Band B (Figure 5.2). Whether or not the 85-87kDa band (Figure 5.10-5.11) and the 73kDa band (Figure 5.10) are formed as a result of incomplete sample solubilisation, SDS induced subunit aggregation, or whether they are the product of damage related subunit cross-linking (Section 3.2.2) remains to be determined.

A number of PSII subunit breakdown fragments were detected in both the PSII monomer and dimer preparations. The antibody raised against the 33kDa subunits, labelled five fragments in the PSII monomer preparation (Figure 5.10). Their apparent molecular weights were calculated to be 32, 31, 29, 25 and 24kDa. Only the 32 and 25kDa bands were detected in the dimer preparation (Figure 5.11). Three fragments, having apparent molecular weights of 32, 30 and 25kDa, were detected in the OG core (Figure 4.2). As the true (27kDa, Table 3.3) and apparent molecular weight (42-43kDa,

Figures 5.10 and 5.11) of the 33kDa extrinsic subunit differed markedly, the cleavage positions, resulting in the formation of the five 33kDa extrinsic polypeptide fragments detected in monomer and dimer preparations, could not be determined. However, as there is considerable similarity between the molecular weights of the detected 33kDa extrinsic polypeptide fragments in the four core preparations (HTGP cores, OG cores, monomers and dimers), it is suggested that the process is not random.

No CP47 or CP43 specific breakdown was detected in either the isolated monomer (Figure 5.10) or dimer (Figure 5.11) preparations.

The C-terminal D1 specific antibody detected two breakdown fragments having apparent molecular weights of 11 and 12kDa in the monomeric PSII preparation (Figure 5.10). Dimers also contained the 11kDa C-terminal D1 fragment, but no 12kDa C-terminal fragment was detected in this preparation. As was discussed in Section 3.2.2 and 4.2.2.1, the 11 and 12kDa C-terminal D1 products may be formed as a result of proteolytic cleavage in the hydrophilic loop connecting the putative transmembrane helices IV and V (Greenberg *et al.*, 1987; De Las Rivas *et al.*, 1992b; Cánovas & Barber, 1993; De Las Rivas *et al.*, 1993).

Two D2 fragments having molecular weights of about 21 and 16kDa were detected in both monomeric (Figure 5.10) and dimeric (Figure 5.11) core preparations. The 21kDa D2 fragment (Figures 5.10 and 5.11) appears to correspond to Band E (Figure 5.2). The sum of the molecular weights of these two fragments is 37kDa, which is similar to that of the intact subunit (37-38kDa). This suggests that they may be derived by a single cleavage. As was discussed in Sections 3.2.2 and 4.2.2.1, the 21 and 16kDa D2 fragments may have been formed as a result of cleavage in the hydrophilic loop of D2, connecting the putative transmembrane helices IV and V, though this remains to be confirmed (Friso *et al.*, 1994; Ponticos *et al.*, 1994).

To summarise, comparison of Figure 5.2 with Figures 5.10 and 5.11, shows that Bands A, B, C and E (Figure 5.2) appear to correspond to a 93-99kDa CP47 aggregate, 85-87kDa D1/D2 heterodimer, a 73-74kDa heterodimer and a 21kDa D2 fragment respectively. The origins of Bands D and F (Figure 5.2) were not determined. Although, the silver stained gels of PSII monomers (Figure 5.10) and PSII dimers suggest that the former are associated with higher levels of subunit damage, this could not be confirmed for certain from the results presented, as slightly higher loadings of PSII monomer (1µg chlorophyll) than PSII dimer (0.8µg chlorophyll) were used. A more quantitative comparison of the D1 and D2 subunit fragment levels is presented in Section 5.2.7.2.

5.2.7.2 Comparison of the levels of D1 and D2 subunit breakdown fragments associated with freshly prepared and frozen OG core, PSII monomer and PSII dimer preparations

D1 and D2 subunits of sucrose gradient purified monomers and dimers were shown to be damaged in Figure 5.10 and 5.11. The aim of the experiment presented in this section was to determine whether isolated PSII cores were damaged before, during, or after sucrose gradient centrifugation. To determine whether the isolated cores were damaged before sucrose gradient centrifugation, OG cores, as well as the monomers and dimers isolated from them, were analysed simultaneously. The effect of freezing cores was tested to determine whether cores were damaged after isolation. Figure 5.12 shows the result of blotting freshly isolated OG core, as well as PSII monomer and dimer, preparations with C-terminal D1 antibody, before and after freezing in liquid nitrogen. Similar D2 specific blots are shown in Figure 5.13. The methods used for this experiment are described in Sections 2.3 and 2.7.2.

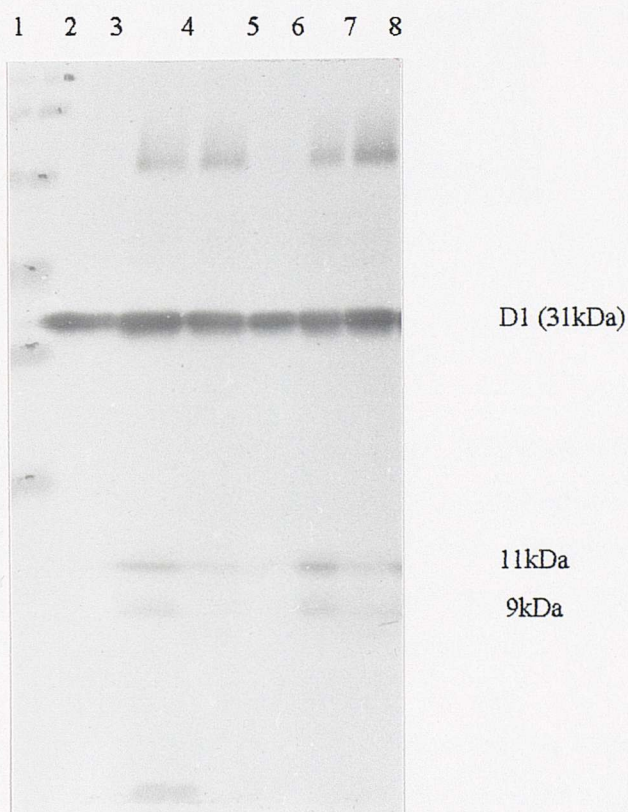


Figure 5.12 The effect of freezing on D1 damage of OG cores and sucrose gradient purified monomers and dimers. 1 μ g chlorophyll of each core type was loaded in Lanes 3-8. Lane 1 and 2 - Biorad markers; Lane 3 - OG cores before freezing; Lane 4 - monomers before freezing; Lane 5 - dimers before freezing; Lane 6 - OG cores after freezing; Lane 7 - monomers after freezing; Lane 8 - dimers after freezing.

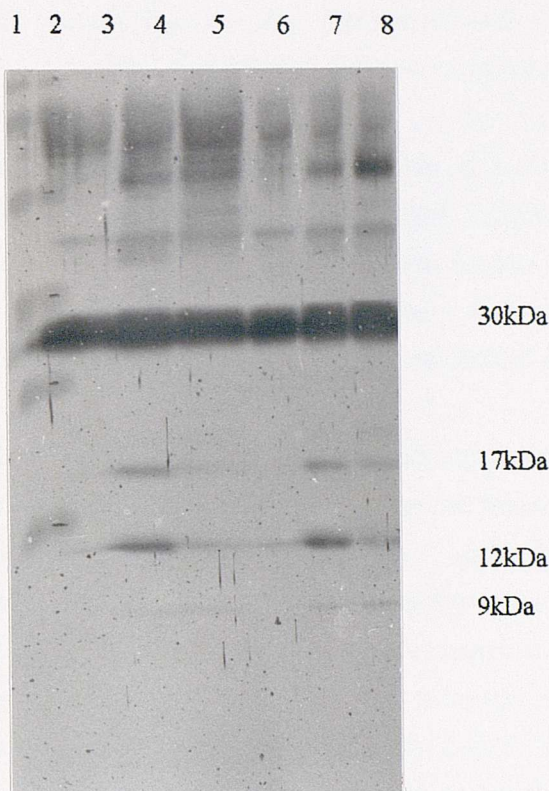


Figure 5.13 The effect of freezing on D2 damage of OG cores and sucrose gradient purified monomers and dimers. 1 μ g chlorophyll of each core type was loaded in Lanes 3-8. Lanes 1 and 2 - Biorad markers; Lane 3 - OG cores before freezing; Lane 4 - monomers before freezing; Lane 5 - dimers before freezing; Lane 6 - OG cores after freezing; Lane 7 - monomers after freezing; Lane 8 - dimers after freezing.

D1 (Figure 5.12) and D2 (Figure 5.13) specific blots show freshly isolated OG core preparations (Lane 3) to be virtually free of breakdown fragments of these subunits, confirming their intact nature. Freshly prepared monomers contained detectable levels of D1 breakdown fragments, having apparent molecular weights of 11 and 9kDa (Lane 4, Figure 5.12). However, as the intact D1 subunit, shown in Figure 5.12, had an apparent molecular weight of 31kDa, rather than 35kDa (Figure 5.10), and because of the similarity in banding, it is thought that the 9 and 11kDa fragments in Figure 5.12 correspond to the 11 and 12kDa bands seen in Figures 5.10 and 5.11.

Comparing the C-terminal specific D1 Western blots of OG cores (Lane 3, Figure 5.12) with those of freshly prepared PSII monomers isolated from them (Lane 4, Figure 5.12), shows that the detected D1 fragment levels are much higher in the latter. Freshly prepared dimers (Lane 5, Figure

5.12) contained far lower levels of C-terminal D1 fragments than were found to be associated with the corresponding monomers (Lane 4, Figure 5.12). These results indicate that, although both PSII monomers and dimers were damaged during their isolation from OG cores, the dimers were far less susceptible to such damage.

Comparing the C-terminal D1 specific Western blots of freshly prepared OG cores, PSII monomer and PSII dimer preparations before (Lanes 3-5, Figure 5.12) and after freezing (Lanes 6-8, Figure 5.12), shows that the freezing treatment does not greatly increase the C-terminal D1 fragment levels in any of these preparations. It is therefore concluded that the C-terminal D1 fragments detected in the PSII monomer and dimer preparations were formed during the sucrose gradient purification step.

D2 specific Western blots (Figure 5.13) yielded similar results to those obtained from the C-terminal D1 specific Western blots shown in Figure 5.12. Whereas, freshly prepared OG cores did not show any sign of D2 subunit damage, the monomer (Lane 2, Figure 5.13) and dimer preparations (Lane 3, Figure 5.13) were associated with fragments having apparent molecular weights of 17 and 12kDa. As the intact D2 subunit, seen in Figure 5.13 had an apparent molecular weight of 30kDa rather than 37kDa (Figure 5.10) and because of the similarity in banding pattern, it is thought that the 17 and 12kDa D2 fragments in Figure 5.13 correspond to the 21 and 16kDa D2 fragments seen in Figure 5.10. A very faint 9kDa D2 fragment was also detected in all of the PSII monomer and dimer preparations (Figure 5.13). Freezing OG cores, PSII monomers and PSII dimers (Lanes 6-8, Figure 5.13) did not result in a detectable increase in these three D2 fragments.

The experiments presented in this section show that the D1 and D2 subunits of the PSII monomers and dimers are damaged during the sucrose gradient purification step and that freezing had little effect on increasing this damage. More importantly however, Western blots presented in this section clearly show the PSII dimer to be far less susceptible to this damage.

5.3 The isolation of a dimeric PSII core complex lacking the 33kDa extrinsic polypeptide

Section 1.5 described the current debate on whether the ordered arrays of Bassi *et al.*, (1989), Lyon *et al.*, (1993), Holzenburg *et al.*, (1993) and Santini *et al.*, (1994) consist of monomeric or dimeric PSII core complexes. Subjecting the monomeric and dimeric PSII core complexes, characterised in Section 5.2, to single particle analysis (Chapter 6) may yield useful structural information at a resolution of about 25Å (Boekema *et al.*, 1994). It is hoped that such analysis may help to resolve the above mentioned controversy. In addition, though it should be possible to use single particle analysis to start mapping the location of individual subunits, associated with PSII. One way of achieving this is to isolate discrete complexes, differing in subunit composition. Sections 5.3.1 and 5.3.2 describe the isolation and characterisation of a PSII core complex lacking the 33kDa extrinsic subunit.

5.3.1 Subunit composition of Tris-washed dimers of PSII

This section describes the characterisation of PSII dimers (CP47, CP43, D2, D1, cytb559, the 33kDa extrinsic polypeptide and associated low molecular weight subunits) and their Tris-washed counterparts, using SDS-PAGE (Sections 2.2.1-2.2.4) and Western blot analysis (Sections 2.3.1-2.3.2). These analyses made it possible to determine whether the 33kDa subunit was completely removed by the Tris-washing treatment. The results are shown in Figure 5.14.

The Coomassie stained gel (Lanes 1 and 2, Figure 5.14) shows that Tris-washing facilitates the discrete and effective dissociation of the 33kDa extrinsic polypeptide from the sucrose gradient purified PSII dimer core complexes. Western blots of SDS-PAGE resolved dimers (Lane 3, Figure 5.14) and Tris-washed dimers (Lane 4, Figure 5.14), showed the 33kDa polypeptide to be present in both, but greatly reduced in the latter. An additional, but important point to be noted is that the 33kDa band seen in Lane 4, (Figure 5.14) is considerably more intense than the 33kDa breakdown fragments seen in Lane 3. This suggests that the 33kDa specific antibody used is very sensitive and that the breakdown fragment levels detected in the dimers (Lane 4, Figure 5.14) are therefore, negligibly small.

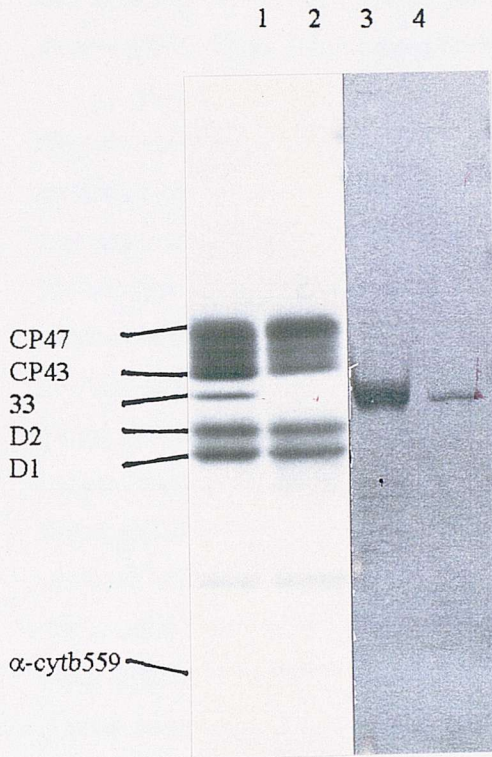


Figure 5.14 SDS-PAGE and 33kDa specific Western blot analysis of PSII dimers before and after washing with 980mM Tris pH8.0, 2mM DM. Lane 1 - Coomassie stained PSII dimers (10 μ g chlorophyll), before Tris-wash; Lane 2 - Coomassie stained PSII dimers (10 μ g chlorophyll), after Tris-wash; Lane 3 - 33kDa extrinsic subunit specific Western blot of PSII dimers (1 μ g chlorophyll) before Tris-wash; Lane 4 - 33kDa extrinsic subunit specific Western blot of dimers (1 μ g chlorophyll) after Tris-wash.

5.3.2 HPLC size exclusion analysis to compare the molecular weights of isolated PSII monomers and dimers (CP47, CP43, D2, D1, cytb559 and 33kDa extrinsic polypeptide) with Tris-washed dimers (CP47, CP43, D2, D1 and cytb559)

Monomers and dimers (CP47, CP43, D2, D1, cytb559 and 33kDa extrinsic polypeptide and associated low molecular weight subunits) and Tris-washed dimers (CP47, CP43, D2, D1, cytb559 and associated low molecular weight subunits) were analysed by HPLC size exclusion analysis to compare their apparent molecular weights. This approach (see Section 2.5) was taken to determine whether Tris-washing dissociated dimeric PSII core complexes into their monomeric constituents. The standard curve (Figure 5.15), used to calculate the molecular weights of complexes, shows the highest R^2 value of all those reported in this thesis. It was obtained at 15°C using monomeric (300kDa) and trimeric (820kDa) PSI and monomeric (67kDa) and dimeric (134kDa) BSA as standards. Using this standard curve, PSII monomers and dimers were calculated to have molecular weights of 230kDa $\pm$ 46kDa and 445kDa $\pm$ 89kDa in the presence of DM (Figure 5.16). Tris-washed dimer samples consisted of three particle types having apparent molecular weights of 426kDa $\pm$ 85kDa, 210kDa $\pm$ 42kDa and 90kDa $\pm$ 18kDa. It is thought that the 426kDa peak corresponds to PSII dimers, lacking the 33kDa extrinsic polypeptide. The 210kDa peak corresponds to PSII monomers lacking the 33kDa extrinsic polypeptide. The 90kDa peak was previously shown to consist of DM solubilised pigments (Rögner, personal communication).

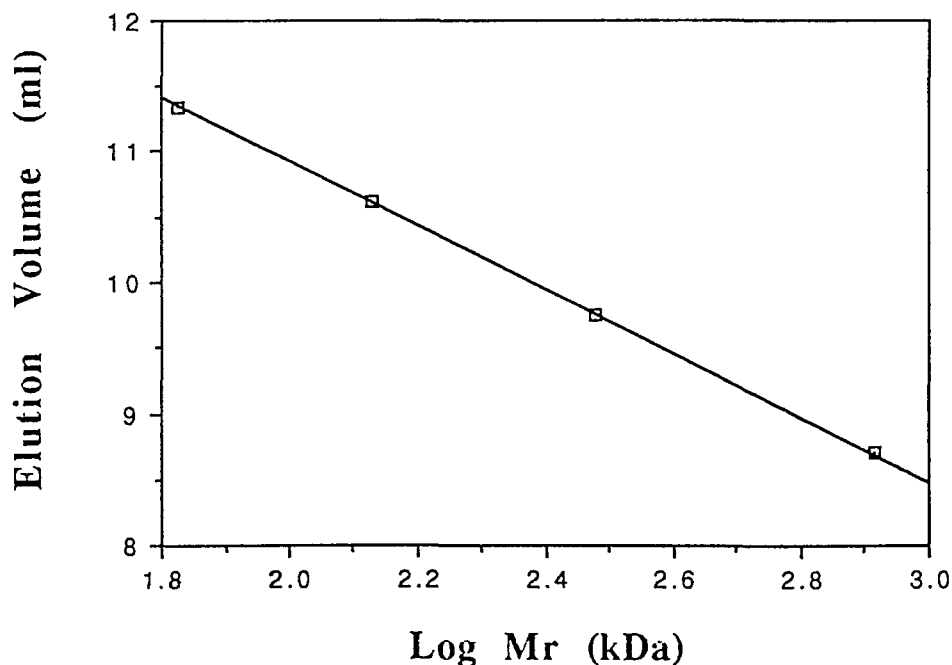
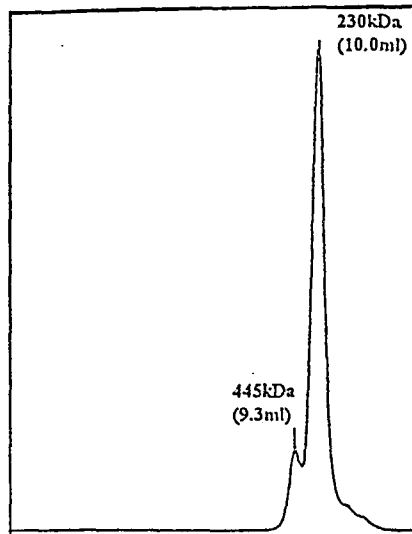


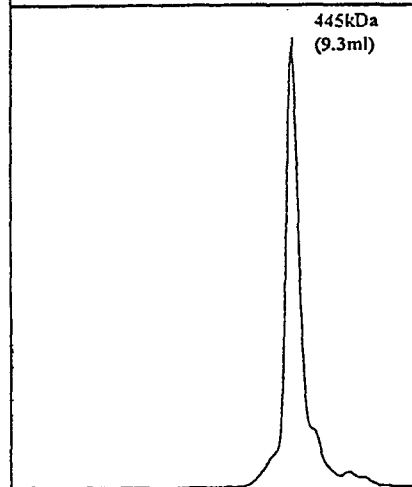
Figure 5.15 Standard curve for HPLC analysis of Tris-washed and control samples. This standard curve was obtained at 15°C using monomeric (300kDa) and trimeric (820kDa) PSI, as well as monomeric (67kDa) and dimeric (134kDa) BSA. The value of 300kDa for the DM solubilised monomeric and dimeric PSI was obtained by calibrating the molecular weight of this membrane protein complex to a large number of other standards (Rögner, personal communication).

MONOMERS

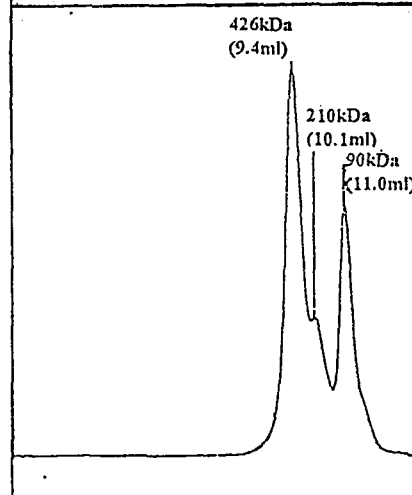
Absorbance
280nm

**DIMERS**

Absorbance
280nm

**TRIS-WASHED DIMERS**

Absorbance
280nm



Elution volume (ml)

Figure 5.16 HPLC size exclusion elution profiles of PSII monomers, dimers and Tris-washed PSII complexes. The elution profiles of PSII monomers, dimers and Tris-washed dimers are presented. The elution volumes and corresponding molecular weights of the particles composing each peak, were calculated using the standard curve shown in Figure 5.15, and are indicated above.

As the Tris-washed dimer sample was shown to be almost free of the 33kDa extrinsic polypeptide, but contained a large proportion of complexes having an apparent molecular weight of 426kDa, it is concluded that it consisted predominantly of dimeric PSII core complexes. Although, the Tris-washed PSII dimer preparation consists of a mixture of monomeric and dimeric core complexes lacking the 33kDa subunits, it should be possible to determine the aggregation state of individual particles, within this preparation using single particle analysis (Chapter 6). Comparing the images of these particles with those of the monomeric and dimeric cores, associated with the 33kDa subunit, may allow the location of this subunit to be identified.

5.4 Isolation and characterisation of dimeric PSII-LHCII dimers from BBYs

5.4.1 Isolation of dimeric PSII-LHCII dimers from BBYs

This section describes the use of sucrose density gradient centrifugation to resolve DM solubilised PSII core complexes associated with CP24, CP26, CP29 and LHCII, from BBYs. Such PSII core complexes were purified using the method described in Section 2.1.8 and the results of sucrose gradient centrifugation are shown in Figure 5.17.

BBYs (150 μ g chlorophyll) solubilised with 20mM DM at a final concentration of 1mg.ml⁻¹ chlorophyll were resolved into four chlorophyll-binding fractions by sucrose density gradient centrifugation (Figure 5.17).

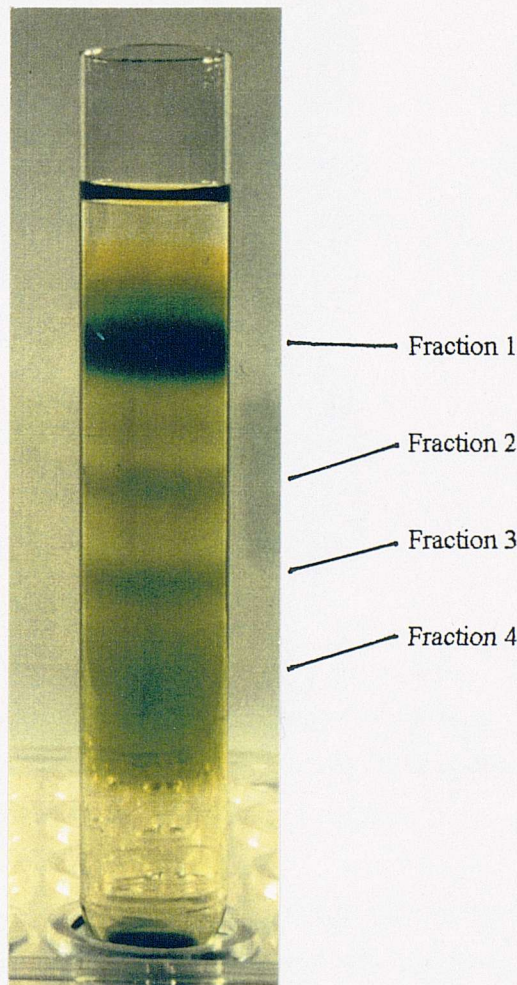


Figure 5.17 Sucrose density gradient centrifugation of DM solubilised BBYs.

BBYs (150 μ g chlorophyll) were suspended in MNCB in the presence of 20mM DM to give a final chlorophyll concentration of 1mg.ml⁻¹, before being subjected to sucrose density centrifugation, using the method described in Section 2.1.8.

5.4.2 Subunit composition of chlorophyll binding complexes resolved by sucrose density gradient centrifugation after solubilising BBYs with 30 mM DM

The chlorophyll rich fractions (1-4) resolved from DM solubilised BBYs, using sucrose density gradient centrifugation (Figure 5.17), were analysed by SDS-PAGE (Figure 5.18). The SDS-PAGE method used in this section is described in Section 2.2.

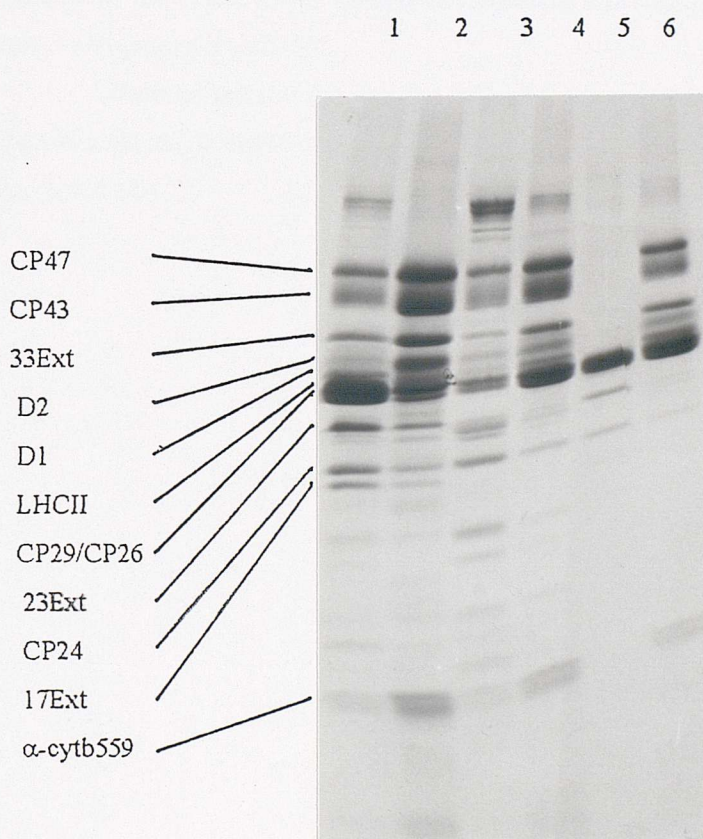


Figure 5.18 Subunits composition of the chlorophyll binding fractions resolved from BBYs by sucrose density gradient centrifugation (Figure 5.17).

10µg chlorophyll of each sample were loaded per lane. Lane 1, BBYs; Lane 2, OG cores; Lane 3, Fraction 2; Lane 4, Fraction 3; Lane 5, Fraction 1; Lane 6, Fraction 4.

For the purposes of comparison, the subunit composition of the four chlorophyll rich fractions resolved according to Figure 5.18, were compared with those of BBYs (Lane 1) and OG cores (Lane 2) in Figure 5.19. Fraction 1 (Lane 5), contained LHCII, CP29 and/or CP26 as well as CP24, but none of the major PSII subunits. Fractions 2 and 3 contained CP47, CP43, D2, D1, cytb559, the 33kDa extrinsic polypeptide, LHCII, CP29 and/or CP26 as well as CP24. These fractions also associated with a number of high molecular weight components (possibly non-PSII

polypeptides). Fraction 4 contained CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic subunit, as well as LHCII, CP29/CP26, CP24 and associated low molecular weight subunits. This fraction did, however, appear to contain very low levels of any other subunits. Fraction 4, the most dense of the three PSII containing fractions, was associated with the most chlorophyll (Figure 5.17), suggesting that it is the most abundant form of PSII isolated from BBYs, under these conditions. Furthermore, its simple subunit composition suggests that it may be suitable for single particle analyses. Comparing the enhanced images of PSII core monomers, dimers and the PSII-LHCII complexes composing Fraction 4, may yield useful information regarding the way in which the chlorophyll a/b binding complexes associate with PSII.

However, before subjecting the PSII-LHCII complex (Fraction 4, Figure 5.17) to such single particle analysis, its apparent molecular weight was determined using HPLC size exclusion analysis (Section 5.4.3).

5.4.3 HPLC size exclusion analysis of Fraction 4

The PSII-LHCII complexes resolved in Fraction 4 (Figure 5.17) were subjected to HPLC size exclusion analysis, using the method described in Section 2.5. The Fraction 4 sample was analysed at the same time as the Tris-washed dimers (Figure 5.16). As a result, the standard curve shown in Figure 5.15 is used to calculate the molecular weights of the Fraction 4 complexes resolved by HPLC size exclusion analysis (Figure 5.19).

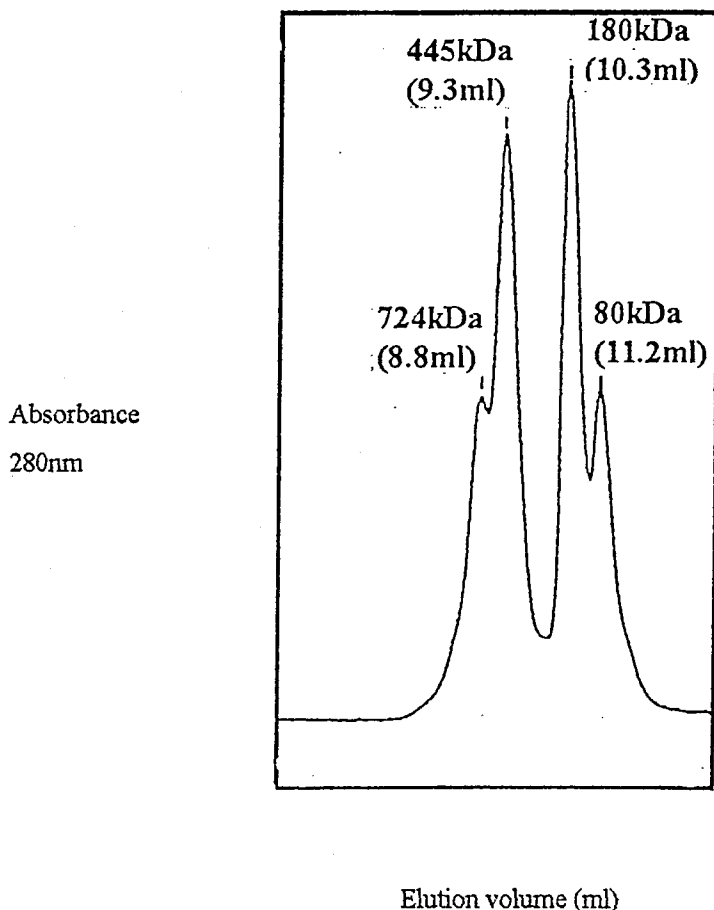


Figure 5.19 HPLC size exclusion profile of Fraction 4. This figure shows the HPLC size exclusion profile of Fraction 4. The elution volumes of each peak and corresponding molecular weights are indicated.

Figure 5.19 shows that Fraction 4 (Figure 5.17) was resolved into four peaks whose component complexes, had apparent molecular weights of $724\text{kDa} \pm 145\text{kDa}$, $445\text{kDa} \pm 90\text{kDa}$, $180\text{kDa} \pm 36\text{kDa}$ and $80\text{kDa} \pm 16\text{kDa}$ in the presence of DM. Although, the results are not shown here, our collaborators Dirk Bald, Jochen Krup and Matthias Rögner, subjected the components of these four peaks to SDS-PAGE analysis. The 724kDa peak consisted of components having the same subunit composition as Fraction 4 (Lane 6, Figure 5.18) and so corresponded to a PSII-LHCII complex. The 445kDa peak contained PSII core complexes consisting of CP47, CP43, D2, D1,

cytb559, the 33kDa extrinsic subunit and associated low molecular weight subunits. The 445kDa peak was shown to correspond to dimeric PSII core complexes (Figure 5.16). The 180kDa peak (Figure 5.19) which contained LHCII is suggested to consist of LHCII trimers. The 80kDa peak contained CP29, CP26 and CP24, probably in their monomeric forms.

From the above analysis it was not possible to determine whether Fraction 4 consisted of a mixture of complexes, or whether the 724kDa PSII-LHCII component had dissociated during the HPLC size exclusion analysis. To address this question a purified 724kDa PSII-LHCII component fraction was subjected to a second HPLC size exclusion analysis run and yielded a similar elution profile to that shown in Figure 5.19. This result is in agreement with the hypothesis that Fraction 4 consists of pure PSII-LHCII complexes, which dissociate during the HPLC size exclusion analysis procedure. It is interesting to note that the 724kDa PSII-LHCII complex dissociates most readily into a 445kDa PSII dimer and a putative 180kDa LHCII trimer component, but that no 230kDa monomeric PSII core complexes (see Figure 5.16) were observed. The relative areas of the four HPLC resolved peaks (Figure 5.19) suggests that the PSII dimers and LHCII trimers are relatively stable, but that they are easily dissociated from one another.

It should be noted that, the higher the molecular weights of the complexes being subjected to HPLC size exclusion analysis are, the less accurate the molecular weight predictions will be. I would therefore, not like to draw too many conclusions about the number of LHCII, CP29, CP26 and CP24 subunits associated with each dimeric PSII core complex, based on these results alone. To obtain a more detailed picture of the subunit stoichiometry of the PSII-LHCII complexes, they were subjected to the pigment analyses reported in Section 5.4.4.

5.4.3 Pigment analysis of Fraction 4

5.4.3.1 Spectral analysis of Fraction 4

In Figure 5.20 the spectra of PSII (CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic polypeptide) monomers and dimers, isolated according to the procedure depicted in Figure 5.1), are compared with the spectrum of Fraction 4 isolated according to Figure 5.19. Fraction 4 (blue trace is shown to be chlorophyll b rich, by its strong absorption in both the 460-500 and 650nm regions of the spectrum. The red peaks of isolated monomers (black trace) and dimers (orange trace) are blue shifted with respect to that of the PSII-LHCII complexes (blue trace) of which Fraction 4 is composed.

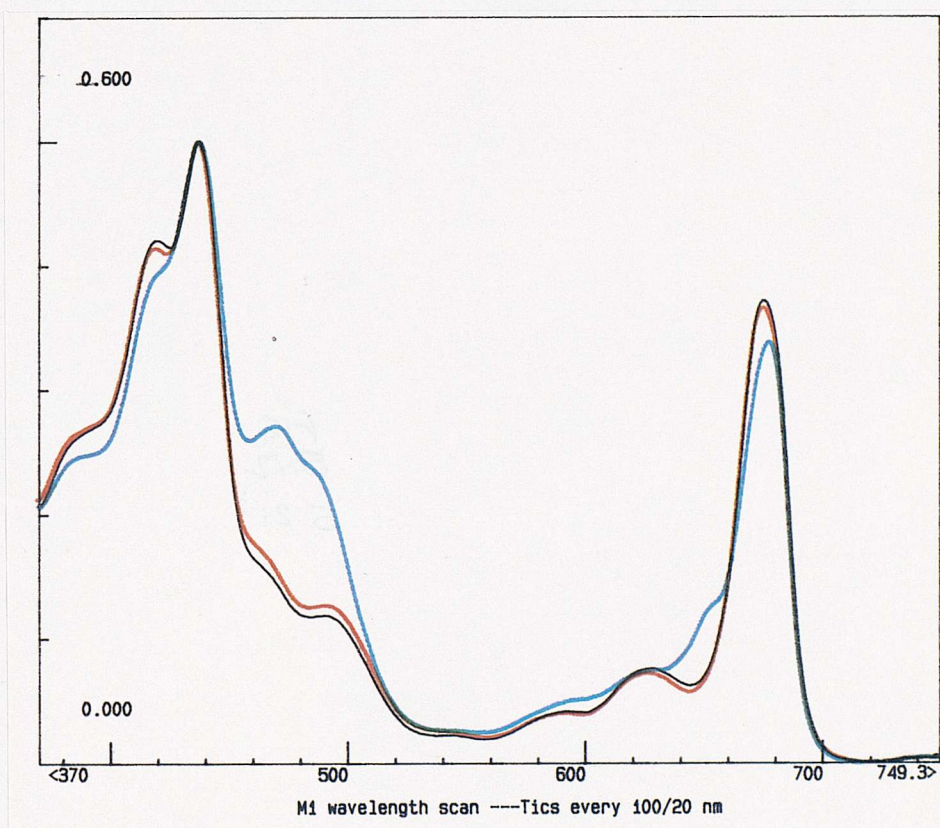


Figure 5.20 Spectral comparison of isolated (CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic polypeptide) monomers and dimers with that of Fraction 4. PSII monomers and dimers (CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic polypeptide) were isolated using the procedure described in Section 5.2.1. Fraction 4 was isolated using the procedure described in Section 5.4.1. The black trace is the average of two monomer spectra. The orange trace is the average of three dimer spectra. The blue trace is the average of three Fraction 4 spectra. All samples were analysed in SMNCB supplemented with 2mM DM. The 437nm peaks of the recorded spectra were normalised to 0.500 absorbance units before being overlaid. The red peak λ max value of the PSII-LHCII complexes composing Fraction 4 was 677nm.

5.4.3.2 Analysis of the pigment composition of Fraction 4 using reverse phase HPLC analysis

In order to determine the pigment composition of Fraction 4 (Figure 5.17), characterised by SDS-PAGE (Figure 5.18, Lane 6), this preparation was subjected to reverse phase HPLC analysis using the method described in Section 2.6. The chlorophyll a, chlorophyll b and β -carotene associated with this preparation was normalised to two pheophytin molecules. The results of such analysis are shown in Table 5.3.

Table 5.3 The pigment composition of the PSII-LHCII complex (Fraction 4, Figure 5.18)

Sample	Chlorophyll a	Chlorophyll b	β -carotene	Pheophytin
Fraction 4	75 (5.6)	32 (2.4)	13 (0.9)	2

The results presented in Table 5.3 show the number of chlorophyll a, chlorophyll b and β -carotene molecules associated per monomeric core complex. The pigment data presented here will be discussed in Section 6.3.2, in conjunction with the single particle analysis of the PSII-LHCII components composing Fraction 4.

5.4 Conclusion

Monomeric and dimeric PSII core complexes consisting of CP47, CP43, D2, D1, cytb559, the 33kDa extrinsic subunit and associated low molecular weight polypeptides, were isolated and shown to have molecular weights of 445-540kDa and 240-230kDa in DM, respectively. The dimeric PSII cores were associated with lower levels of both D1 and D2 subunit breakdown fragments than the monomeric complexes. Furthermore, they were able to support higher rates of oxygen evolution than their monomeric counterparts, in the presence of both DCBQ and DMBQ. Reverse phase HPLC analysis showed the monomeric PSII core complexes to have a pigment stoichiometry of 34.3 chlorophyll a: 2.2 chlorophyll b: 7.7 β -carotene: 2 pheophytin, while the dimers were associated with 31.4 chlorophyll a, 1.4 chlorophyll b and 7.6 β -carotene per 2 pheophytin molecules. The slightly higher levels of chlorophyll b associated with the monomeric core complexes may be due to contamination from the chlorophyll a/b-binding protein rich fraction (Upper Fraction, Figure 5.1). However it cannot be excluded that the presence of chlorophyll b in the two core preparations reflects that they are still directly linked rather than contaminated by, residual amounts of the chlorophyll a/b binding proteins. Western blot analysis and oxygen evolution measurements suggest that the acceptor side of the dimeric core complexes may be less damaged than that of the monomeric cores. HPLC size exclusion analysis suggests that both preparations are sufficiently pure to be useful for single particle analysis (Chapter 6).

Dimeric PSII cores were Tris-washed in order to prepare complexes lacking the 33kDa subunit, for single particle analysis. HPLC size exclusion analysis showed them to consist of a mixture of monomeric and dimeric core complexes. However, as a pure monomeric and dimeric cores associated with the 33kDa were also isolated for single particle analysis, it should be possible to determine the aggregation state of individual particles in the Tris-washed sample, using this technique.

Mild solubilisation of BBYs and subsequent sucrose density gradient centrifugation yielded a chlorophyll-a/b-binding protein rich band and three PSII containing fractions. The most dense of the three PSII containing fractions (Fraction 4) was associated with the most chlorophyll, suggesting that it is the most abundant form of PSII isolated under these conditions. SDS-PAGE analyses showed that this fraction consisted of complexes composed of CP47, CP43, D2, D1, cytb559, the 33kDa subunit, LHCII, CP24, CP26 and/or CP29, as well as associated low molecular weight subunits. HPLC size exclusion analysis showed these complexes to have molecular weight of $724\text{kDa} \pm 144\text{kDa}$ and induced the dissociation of these complexes into dimeric PSII core complexes and LHCII trimers. As the 724kDa fraction was not dissociated into monomeric PSII core complexes, it is possible that the 724kDa PSII-LHCII complexes contain dimeric PSII cores. In the absence of lipid reconstitution experiments, I cannot categorically state that the PSII dimers and PSII-LHCII complexes isolated in Section 5.4 are not the product of fortuitous aggregation. It does however, seem unlikely considering the mild solubilisation conditions used to obtain them and so may indicate that these 724kDa PSII-LHCII supercomplexes may exist *in vivo*. Pigment analysis showed the PSII-LHCII complexes

composing Fraction 4, to have a pigment stoichiometry of 75 chlorophyll a: 32 chlorophyll b: 13 β -carotene: 2 pheophytin.

The monomeric and dimeric PSII core complexes, Tris-washed dimers and the 724kDa PSII-LHCII preparation may yield useful structural information at a resolution of about 25Å, if subjected to single particle analysis.

Chapter 6 Single particle analysis of dimeric PSII complexes differing in subunit composition

6.1 Introduction

Several research groups have, to date, published electron diffraction images (17-50Å) obtained from ordered arrays of PSII-LHCII complexes (Bassi *et al.*, 1989; Holzenburg *et al.*, 1993; Lyon *et al.*, 1993; Santini *et al.*, 1994). Because of the inherent symmetry of the images obtained by Bassi *et al.*, (1989), Lyon *et al.*, (1993) and Santini *et al.*, (1994), all three sets of authors concluded that their ordered arrays were composed of dimeric PSII complexes. Holzenburg *et al.*, (1993) also presented images of PSII complexes obtained from two-dimensional arrays but concluded that their stromal surfaces lacked symmetry and therefore could not be dimeric. Furthermore, Holzenburg *et al.*, (1993) suggested that the luminal side of the complex showed pseudo- rather than true two-fold symmetry. The images of Bassi *et al.*, (1989), Holzenburg *et al.*, (1993), Lyon *et al.*, (1993) and Santini *et al.*, (1994) had similar top view dimensions and structural features such as a central hole or cavity. Thus the hypothesis that the PSII complexes reported by Bassi *et al.*, (1989), Lyon *et al.*, (1993) and Santini *et al.*, (1994) were dimeric was brought into question. However, the results of Holzenburg *et al.* (1993) are not quite as conclusive as they might at first appear. Analysis of the subunits composing PSII suggests that CP47, CP43, D2, D1 and the α and β subunits of cytb559 and associated low molecular weight subunits, only have small stromally exposed loops (see Section 1.4). As the contours of such small loops probably cannot be accurately mapped at a resolution of around 20Å, a final conclusion about the symmetry of the stromal surface awaits higher resolution detail. The current debate about the oligomeric state of PSII in the above mentioned preparations might be answered if the dimensions and contours of discrete detergent solubilised PSII core monomers and dimers of known subunit composition, could be determined.

I have isolated monomeric and dimeric PSII core complexes (CP47, CP43, D2, D1, cytb559, the 33kDa extrinsic polypeptide and associated low molecular weight subunits) as well as Tris-washed core dimers and PSII-LCHII supercomplexes, from spinach. The apparent molecular weights, subunit and pigment compositions of these complexes were reported in Chapter 5. With advances in specimen preparation and image processing technology, it is now possible to obtain 15-25Å resolution electron density maps of detergent solubilised membrane proteins, by averaging the electron micrograph images of a large number of discrete and similar projections (Boekema *et al.*, (1994). By analysing top- and side-view projections, a considerable amount of structural information can be gained. This chapter presents such images of the complexes isolated in chapter 5. It is hoped that these images may help to resolve the current debate on the aggregation state of PSII in the ordered arrays reported by Bassi *et al.*, (1993), Lyon *et al.*, (1993), Holzenburg *et al.*, (1993) and Santini *et al.*, (1994) and aid identification of specific subunit positions within PSII.

6.2 Results

Several different PSII and PSII-LHCII complexes described in Chapter 5 were analysed by single particle averaging. Image enhanced side and top-views of these spinach particles are compared with each other and with those obtained from *Synechococcus* complexes. All of the cyanobacterial core preparations were prepared by our collaborators: Dirk Bald, Jochen Kruip and Matthias Rögner, Universität Münster. All of the image enhancement analysis was carried out by Egbert Boekema and Arjen Boonstra at the University of Groningen, according to the procedure described in Section 2.8.

6.2.1 Electron micrographs of the spinach and *Synechococcus* particles analysed in this chapter

Figure 6.1 shows electron micrographs of all of the negatively-stained spinach core complexes analysed in this chapter. The images shown are a representative sample of the crude data used to generate the enhanced images shown in later figures. Figure 6.1A shows a typical electron micrograph of the PSII-LHCII complex preparation (Fraction 4) characterised in Section 5.4. Several particle types were observed in their top-view position. The first type is marked by a single circle (Figure 6.1A). Thicker stain embedding indicates a top-view projection, resulting from two stacked complexes and is marked by a double circle. In their side-view position, these large particles could often be seen to aggregate into pairs. Each of the paired complexes is marked with an arrow. Sometimes a specific part of the PSII-LHCII complex (single circle, Figure 6.1A) was missing from these complexes (marked by a triangle, Figure 6.1A). Figure 6.1B shows an electron micrograph of negatively-stained dimeric *Synechococcus* (CP47, CP43, D2, D1, cytb559, the 33kDa extrinsic polypeptide and associated low molecular weight subunits) complexes in top- (circled) and side-view (large arrows) projection. The small arrows mark two protrusions which are often seen in the side-view position. Figure 6.1C is an electron micrograph of negatively-stained dimeric spinach PSII (CP47, CP43, D2, D1, cytb559, the 33kDa extrinsic polypeptide and associated low molecular weight subunits) cores (prepared as described in Section 5.2) in top- (circled) and side-view (large arrows) position. Figure 6.1D shows negatively-stained Tris-washed *Synechococcus* dimers consisting of CP47, CP43, D2, D1 and cytb559, in top- (circled) and side-view (large arrows) positions. Figure 6.1E shows negatively-stained Tris-washed spinach dimers of similar subunit composition, in top- (circled) and side-view (large arrows) positions. All of the complexes shown in Figure 6.1 are often seen to aggregate into pairs when in the side-view position. Two protrusions are visible in the *Synechococcus* dimer images (Figure 6.1B). Such protrusions have previously been reported to be the 33kDa extrinsic polypeptides (Dekker *et al.*, 1988). Thus, it is concluded that the stromal surfaces of the various complexes have a tendency to aggregate.

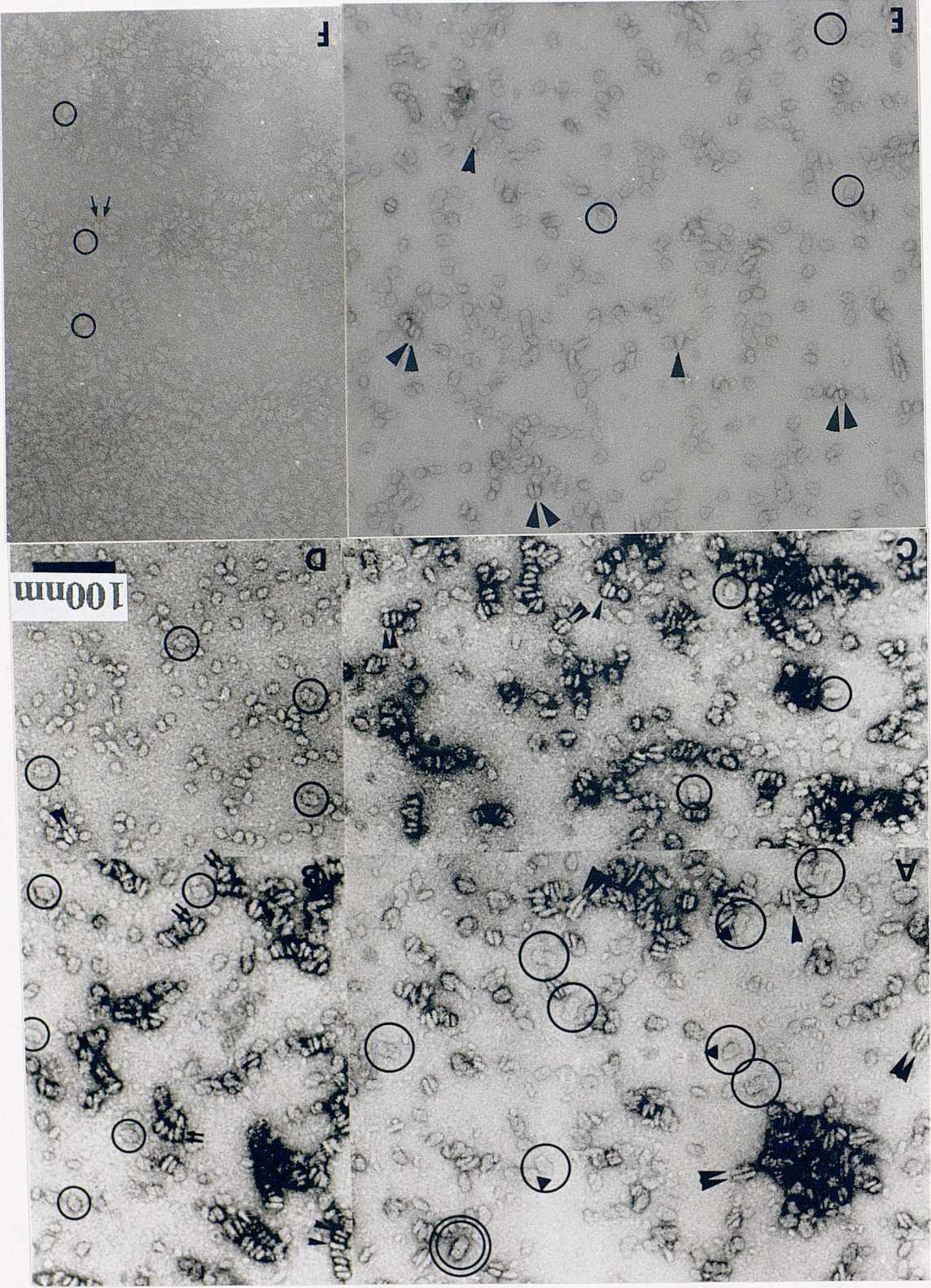


Figure 6.1 Electron micrographs of PSII preparations. (A) EM of the PSII-LHCII complexes isolated in Fraction 4 (Figure 5.19). Top-views of a large PSII-LHCII complex have been indicated by a single circle. A top-view projection resulting from two stacked PSII-LHCII complexes is recognisable from a thicker stain embedding pattern and has been indicated by a double circle. In side-view position, these PSII-LHCII complexes often aggregate into pairs (arrowheads). Sometimes a specific part of the PSII-LHCII complex was dissected (marked by a triangle); (B) EM of dimeric *Synechococcus* (CP47, CP43, D2, D1, cytb559 and the 33kDa subunit) PSII core complexes. Top-views are indicated by circles. In side-view position (long arrows) two protrusions are often visible (short arrows); (C) EM of dimeric spinach (CP47, CP43, D2, D1, cytb559 and the 33kDa subunit) PSII core complexes. Top-views are indicated by circles and side-views by arrows; (D) EM of dimeric Tris-washed *Synechococcus* PSII cores. These particles consisted of CP47, CP43, D2, D1 and cytb559. Top-views are indicated by circles and side-views by arrows (E) EM of dimeric Tris-washed spinach PSII cores. These particles consisted of CP47, CP43, D2, D1 and cytb559. Top-views are indicated by circles and side-views by arrows. (F) EM of sucrose gradient purified monomers. Top-views are circled. All complexes were negatively-stained with a 2% uranyl acetate solution.

6.2.2 Image Enhancement analysis of sucrose density gradient purified core monomers and dimers

Negatively-stained sucrose gradient purified monomeric and dimeric spinach (CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic polypeptide) core complexes were circled in Figure 6.1C and 6.1F respectively. 300 of the circled dimer preparation particles (Figure 6.1C) were subjected to single particle analysis. The averaged image, which showed two-fold symmetry about its central hole is shown in Figure 6.2A. Four major regions of electron density are seen to surround a central hole. This complex shows antiparallel, rotation symmetry around this central hole, as might be expected from a dimeric complex. Figure 6.2B shows the image of a monomeric core complex. The image of this monomer (average of 100 monomers) shows it to have an almost identical electron density pattern to that obtained from one half of the dimeric complex image shown in Figure 6.2B. This point is emphasised in Figure 6.2C, in which this monomer image is superimposed upon the outline of a dimer image. One further point can be gleaned from the monomer image; a criticism levelled at the single particle analysis technique is that it can be used to enforce symmetry where there is none. At the resolution of the images, there is clearly no symmetry in the monomer, shown in Figure 6.2B, and so it is concluded that the image enhancement process used, identified true and not pseudosymmetry in the dimeric spinach cores.



Figure 6.2. Image enhancement analysis of sucrose density gradient purified core monomers and dimers. (A) Average of 300 of the circled particles (top-views) seen in sucrose gradient purified dimer preparations (in Figure 6.1C), after imposing two-fold symmetry. (B) Average of 100 of the circled particles (top-views) seen in sucrose gradient purified monomer preparations (in Figure 6.1F). (C) Image of the monomeric PSII complex shown in (B) superimposed upon the outline of the dimeric core depicted in (A).

6.2.3 Classification of side-views of dimeric *Synechococcus* (CP47, CP43, D2, D1, cytb559 and 33kDa extrinsic polypeptide) complexes

Figure 6.1B shows an electron micrograph of dimeric PSII core complexes (CP47, CP43, D2, D1, cytb559 and 33kDa extrinsic polypeptide and associated low molecular weight subunits). Side-views of these complexes were marked with long arrows in Figure 6.1B. These side-views were subjected to single particle analysis and the best data was obtained with particles isolated from *Synechococcus* (Bald, Kruip & Rögner, unpublished data), although similar analyses were carried out with dimers isolated from spinach (not shown). During the analysis 443 particles were classified, and separated into distinct groups of best fit. The averaged images of the various *Synechococcus* core groups are shown in Figures 6.3A-F. The number of projections averaged to produce the images shown in Figures 6.3A-F are provided in the figure legend. Figure 6.1B showed that most of the analysed projections had a neighbour, but as the upper dimer was focused on during alignment, the lower ones appear to be poorly aligned in the images presented (Figure 6.3).

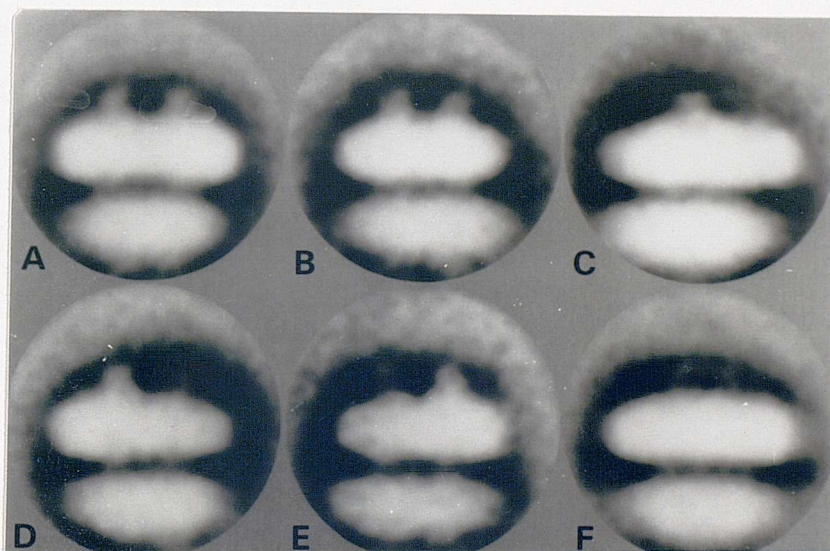


Figure 6.3 A classification of 443 side-view projections of the dimeric *Synechococcus* PSII core complexes shown in Figure 6.1B. The circular mask on the images has a diameter of 26.4nm. *Synechococcus* PSII core (CP47, CP43, D2, D1, cytb559, the 33kDa extrinsic polypeptide and associated low molecular weight subunits) dimer side-views were subjected to single particle analysis. The particles were grouped into classes of best fit. The number of particle images averaged to produce **Figures 6.3 A-F** are as follows: **A** (103), **B** (42), **C** (48), **D** (51), **E** (38) and **F** (125). Note: most of the analysed projections had a neighbour (Figure 6.1B), but as the alignment was only focused on the upper dimers, the lower ones appear to be poorly aligned.

Figure 6.3A shows two protrusions which are 6.3nm apart. The protrusions are shown to be closer to one another and in overlap position in Figures 6.3B and 6.3C, respectively. In Figures 6.3D and 6.3E the protrusions are separate, but suffer from very uneven stain embedding. One of the

protrusions is either completely absent or extremely unfocused in Figure 6.3F. Unfortunately, the number of side-views present in electron micrographs of spinach particles (not shown) and *Synechococcus* particles was not large enough to allow good statistical analysis, but visual examination shows very similar protrusions to exist. However, more information was obtained from the top-view projections in Section 6.2.4.

6.2.4 Location of the 33kDa subunits in dimeric PSII core complexes

In negatively-stained dimeric PSII cores isolated from spinach PSII, a large number of top-views with a small central stain bar can be seen (Figure 6.1C). 969 top-view projections of this type were selected and repeatedly aligned on various references. Next, the images were treated by multivariate statistics and subsequent classification. The classification showed all projections to be similar, with the same type of handedness, which indicates a strong preference in attachment to the carbon support. All of the averaged images had clear two-fold rotational symmetry. As a result, two-fold symmetry was imposed on the average of the best 300 top-view images (Figure 6.4A). Averaging 150 top-views of dimeric *Synechococcus* PSII core preparations of the same subunit composition, yielded similar results (Figure 6.4B).

Tris-washing was shown to specifically remove the 33kDa extrinsic polypeptide from spinach (CP47, CP43, D2, D1, cytb559 and 33kDa extrinsic polypeptide) dimers (Figure 5.15). Similar results were obtained for *Synechococcus* dimers subjected to the same treatment (Bald, Kruip & Rögner, unpublished results). Figures 6.4D and 6.4E show top-view contours of spinach and *Synechococcus* PSII core dimers after Tris-washing, respectively. The contours of the spinach dimers before Tris-washing (Figure 6.4A) were very similar to the corresponding (Figure 6.4B) *Synechococcus* complexes. After Tris-washing, the contours of the two dimer types still (Figure 6.4D) showed great similarity to the corresponding *Synechococcus* cores (Figure 6.4E). As a result, all the Tris-washed spinach and *Synechococcus* images were averaged and subtracted from the mean of all the non-Tris-washed spinach and *Synechococcus* cores. In this way, the density corresponding to the 33kDa subunit was identified. The three lowest contour lines marked in Figure 6.4F, outline the difference induced by Tris-washing. These contour lines correspond to the area of density altered by the removal the 33kDa subunits. The overall dimensions of the four top-views (Figures 6.4A, B, D and E) do not differ very much. The overall length (measured along the axis running vertically through the projection) varies between 20 and 21nm, with a mean of 20.6 nm. The width of these complexes was calculated to be 13.1nm. Correcting these values for a detergent layer of 1.7nm thickness (Dekker *et al.*, 1988), yields dimensions of about 17.2 x 9.7nm. From the side-views shown in Figure 6.4C, the height of the complex across the plane of the membrane, including the protruding tips, is calculated to be 9.1nm.

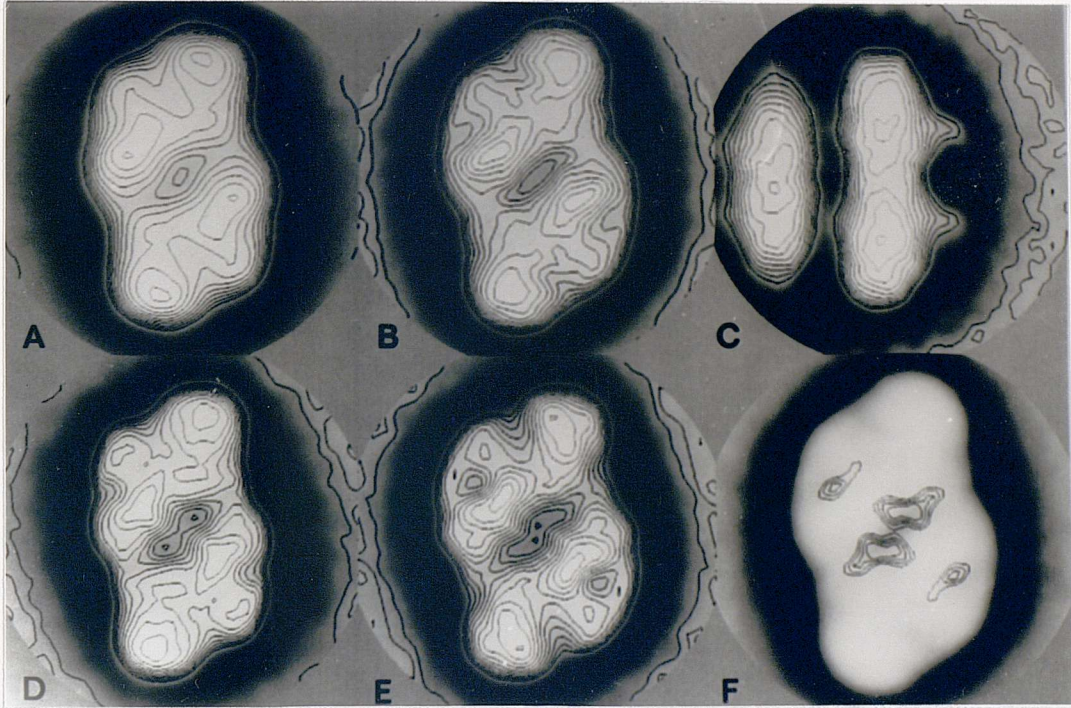


Figure 6.4. Single particle analysis of top and side-view projections of dimeric spinach and *Synechococcus* PSII complexes before and after Tris-washing. All images are shown in their two-fold symmetry version. The circular mask on the images has a diameter of 26.4 nm. (A) Average of 300 spinach PSII core dimer top-views before Tris-washing; (B) Average of 150 *Synechococcus* PSII core dimer top-views before Tris-washing; (C) Average of 85 *Synechococcus* PSII core dimer side-views before Tris-washing; (D) Average of 160 spinach PSII core dimer top-views after Tris-washing; (E) Average of 70 *Synechococcus* PSII core dimer top-views after Tris-washing; (F) Difference image of images A+B minus D+E. The areas showing the largest difference have been contoured, the contour lines of these regions were imposed on the image shown in B.

6.2.5 Comparison of PSII cores images with those obtained from the particles identified in PSII-LHCII complex preparations

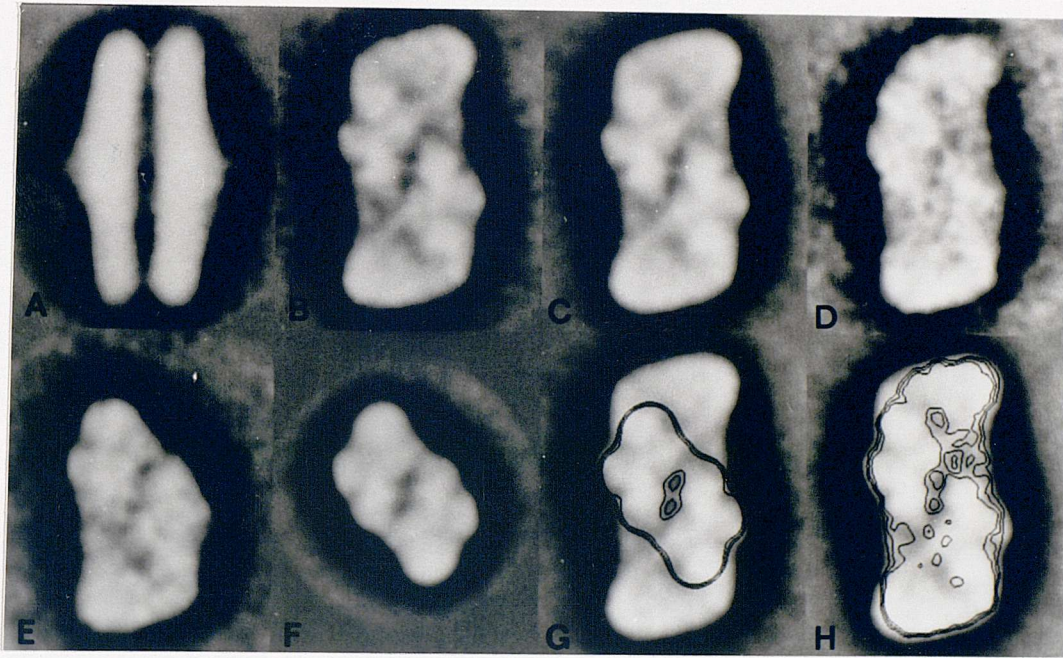
Figure 6.1A shows an electron micrograph of a sucrose gradient purified fraction (Figure 5.19, Fraction 4), which contained PSII-LHCII complexes (CP47, CP43, D2, D1, cytb559, the 33kDa extrinsic polypeptide, CP24, CP26, CP29, LHCII and associated low molecular weight subunits). A number of distinct particle types were visible in top-view orientation. The first is marked by a single circle, the second by a triangle (Figure 6.1A). Figure 6.5A shows the sum of 80 side-view projections of the circled particle. As was the case with the previously analysed core particles, the circled particles could be seen to aggregate into pairs. The two neighbours, forming one of these pairs, are marked by arrows in Figure 6.1A and are clearly visible in Figure 6.5A. Each of the two complexes has a central protrusion, when viewed in this side-view position. No other side-view classes, such as those seen in Figure 6.3, were detected. In this side-view position each neighbour has two-fold symmetry suggesting that each may be a dimeric complex.

Figure 6.5B shows the top-view projection obtained by averaging 200 of the particles circled in Figure 6.1A. The particles have a central hole or cavity and show two-fold rotational symmetry around it. The two-fold symmetry and the fact that it contained both PSII and LHCII, led to these particles being called dimeric PSII-LHCII supercomplexes and they will be referred to as such in the text. The contour map, shown in Figure 6.5C, was obtained by imposing two-fold symmetry on the analysis, given that the PSII-LHCII particle showed this organisation. Figure 6.5F shows the image of a dimeric (CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic polypeptide) PSII complex. The contours of this complex are strikingly similar to the central region of the dimeric PSII-LHCII supercomplex shown in Figure 6.5C and are superimposed on it in Figure 6.5G.

During the single particle analysis of the complexes, marked with a single circle in Figure 6.1A, a subset of the particles were shown to be lacking a small piece from the top left hand corner. The average of 45 such particles is shown in Figure 6.5D. The lower three contour lines of the complex (Figure 6.5D) are superimposed onto the PSII-LHCII supercomplex (Figure 6.5B) in Figure 6.4H to show the missing piece more clearly.

Figure 6.5E shows the enhanced image obtained as a result of averaging 75 of the particles marked with a triangle in Figure 6.1A. As Figure 6.5G shows the location of the dimeric PSII (CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic polypeptide) core complex, the piece of the PSII supercomplex (Figure 6.5C), which is missing in Figure 6.5E, is suggested to consist only of the chlorophyll a/b binding proteins CP24, CP26, CP29 and LHCII.

Top-view dimensions of the PSII-LHCII supercomplex (shown in Figure 6.5B) are 30.2 x 15.7 nm. If corrected for detergent, these values become about 26.8 nm and 12.3 nm, respectively. From the side-views, the edges of the particle were measured to have a thickness of 6nm, which is compatible to that of the thylakoid membrane. Including the central protrusion, the thickness of the particle was measured to be 8.8-9.3 nm. The latter value is similar to the thickness of the *Synechococcus* PSII core particles (9.1nm), which were shown in Figure 6.3C.



10nm

Figure 6.5 Single particle analysis of top- and side-view projections of the complexes detected in Figure 6.1A (A) a sum of 80 particle side-views, marked with arrows in Figure 6.1A; (B) a sum of 200 top-views, marked with a single circle in Figure 6.1A, analysed without imposing symmetry; (C) the two-fold symmetrically averaged image of B; (D) a sum of 45 projections of a class of particles lacking a small fragment present in (C); (E) a sum of 75 projections of the PSII-LHCII supercomplex, lacking one set of chlorophyll a/b binding proteins from its outer tip (marked with triangle in Figure 6.1A); (F) Top-view image of the dimeric spinach PSII (CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic polypeptide) core complex shown in Figure 6.4A; (G) Image shown in B with lowest three contour lines of PSII dimer (F) superimposed; (H) Image shown in B with lowest three contour lines of a particle lacking the small fragment identified in D.

6.3 Discussion

6.3.1 The dimeric nature of isolated PSII core complexes

Monomeric and dimeric PSII cores consisting of CP47, CP43, D2, D1, cytb559, the 33kDa extrinsic polypeptide and associated low molecular weight subunits, were isolated from spinach (Section 5.1). Dimeric complexes, having the same subunit composition, were isolated from *Synechococcus* by our collaborators (Bald, Kruij & Rögner). The top-views of such spinach monomers and dimers are shown in Figure 6.2A and B respectively. Figure 6.2C shows the image of a monomeric complex of this type superimposed upon the outline of a dimer. These results show that a monomeric particle is half the size of the dimer, and that the former has the same contours as each of the monomeric complexes composing the dimer. These results not only confirm the size exclusion analysis of these particles presented in Chapter 5, but show that the single particle analysis has accurately distinguished the electron density profile of the monomeric components forming a dimer. The fact that the monomeric complex shows no symmetry at this resolution, serves to confirm the conclusion that the symmetry detected in the dimer is true and not pseudo-symmetry. This is an important point as the presence of two sets of similarly sized subunits (i.e. CP47 and CP43, as well as D1 and D2) could result in pseudosymmetry being observed in a monomeric complex. Furthermore, this result shows that whereas the dimer has a central hole or cavity, the monomer does not. The similarity of the dimer image to those obtained by Bassi *et al.*, (1989) and Lyon *et al.*, (1993) supports the view that their ordered arrays consist of dimeric core complexes. It is difficult to envisage that the image studied by Holzenburg *et al.*, (1993) is that of a monomeric core since, like the dimers reported here and elsewhere (Bassi *et al.*, 1989; Lyon *et al.*, 1993; Santini *et al.*, 1994), its top view projection shows a central cavity (Figure 6.2A). Furthermore the top view projection of the dimeric core complexes characterised in this chapter are seen to have dimensions of 17 x 10nm, a value similar to that of the PSII core complexes (18 x 10nm) reported by Holzenburg *et al.*, (1993).

The top-view images of the dimeric spinach and *Synechococcus* complexes are shown in Figure 6.4A and 6.4B respectively. Both particles were first analysed without imposing symmetry. Both have four prominent regions of density surrounding a central hole. Furthermore, both had a two-fold symmetry around this central hole confirming the similarity in structure. The similarity in size and shape of the two dimers further suggests that the spinach PSII cores are indeed free of any CP24, CP26, CP29 and LHCII contamination, as cyanobacteria lack these subunits; having instead phycobiliproteins.

6.3.2 Structure of the PSII-LHCII supercomplex

PSII-LHCII supercomplexes were purified by sucrose gradient centrifugation (Figure 5.19, Fraction 4). Apart from the PSII core components (CP47, CP43, D2, D1, cytb559 and the 33kDa

extrinsic polypeptide and associated low molecular weight subunits), these complexes were also associated with CP24, CP26, CP29 and LHCII (Figure 5.20). Figure 6.1A shows that such preparations contained a number of different particle types. The largest of these is marked by a single circle in Figure 6.1A. These circled complexes, being the largest in the preparation are thought to be the particles composing the 724kDa HPLC size exclusion peak (shown in Figure 5.22). The dimension (26.8 x 12.3 x 9.1nm), molecular weight and subunit composition of these particles, suggested that they were PSII-LHCII supercomplexes. The estimated molecular weights of these particles suggest that two LHCII trimers could be accommodated within them. Figure 6.5B shows the image enhanced top-view projections of these putative PSII-LHCII supercomplexes. They have a central hole and show a two-fold rotational symmetry around an axis passing through it. Imposing two-fold symmetry yields the contour lines shown in Figure 6.5C. The first striking feature of this contour map is that the central region has great similarity to the spinach and *Synechococcus* PSII core dimers (CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic polypeptide) shown in Figures 6.4A and B. For ease of comparison, the image of the PSII core dimer (Figure 6.4A) is shown once again in Figure 6.5F and its lower three contour lines are superimposed onto the putative PSII-LHCII supercomplex in Figure 6.5G. As the PSII core complex appears to be free of CP24, CP26, CP29 and LHCII contamination, the regions flanking the contoured region must contain these chlorophyll a/b binding proteins.

Bassi & Dainese, (1992) isolated discrete CP29-CP24-LHCII trimer complexes. These particles were shown to be more tightly associated with one another than with PSII. Figure 6.1A identified a number of PSII-LHCII supercomplexes from which tips had become dissociated. Figure 6.4E shows an enhanced image of such particles. The contours of these particles confirm that one of the chlorophyll a/b protein enriched tips (CP24, CP26, CP29 and LHCII) was missing in these particles. This is in agreement with the results of Bassi & Dainese, (1992).

A number of particles differing, from the PSII-LHCII supercomplex, in that they lacked one small segment at the top left corner, were also identified by single particle analysis (Figure 6.5D). Figure 6.4H shows the lower three contours of this complex superimposed upon the PSII-LHCII supercomplex. From its size, it is tempting to speculate that the missing piece is one of the CP proteins.

It has been suggested that the CP24, CP26 and CP29 link trimeric LHCII to the PSII core complex (Peter & Thornber, 1991b; Bassi & Dainese, 1992; see Janssen, 1994). This being the case the trimeric LHCII complex should form the outermost tips of the supercomplex. To check this proposal, the electron density profile of trimeric LHCII, reported by Wang & Kühlbrandt, (1991) was superimposed onto the PSII-LHCII supercomplex image (Figure 6.5). The result obtained shows that the electron density profile of a trimeric LHCII complex fits well into the position marked 9 in Figure 6.6B.

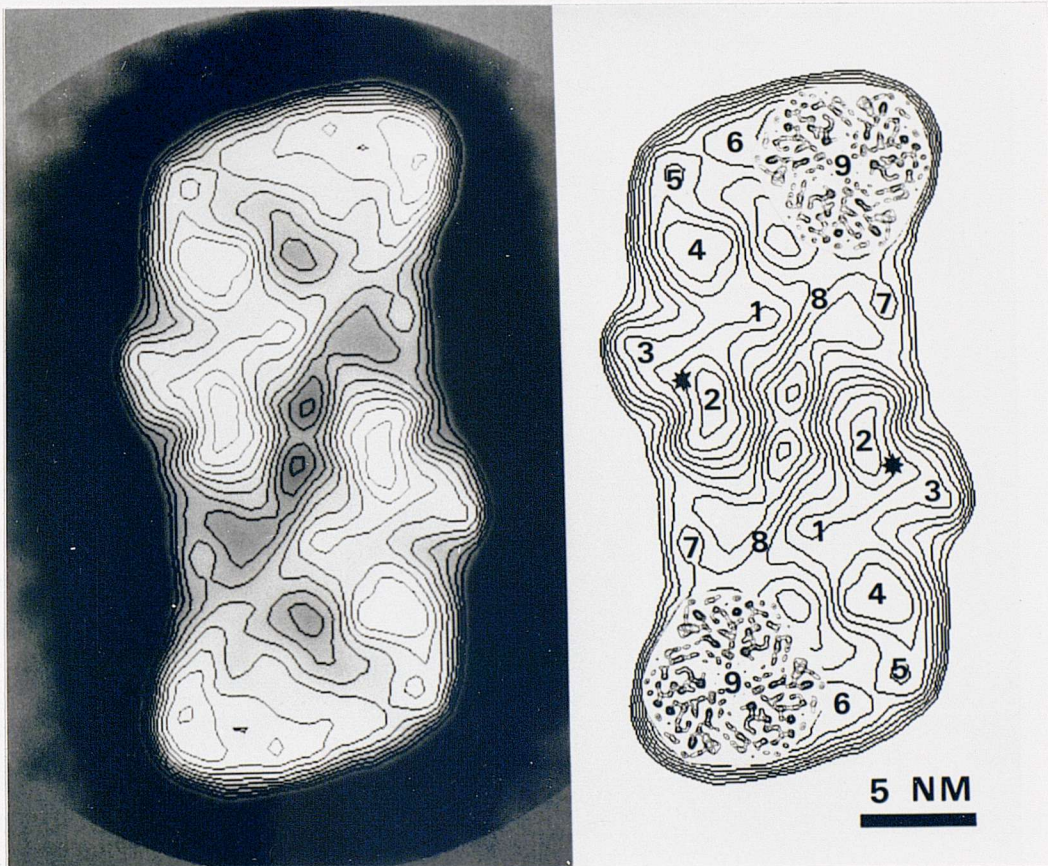


Figure 6.6 The position of two LHCII trimers within the PSII-LHCII supercomplex. (A) Image showing two-fold symmetry of the PSII-LHCII complex. (B) Numerical labelling of protein densities. Densities 1-4 were labelled according to Lyon *et al.*, (1993). The * indicates the position of the 33kDa subunit as determined by the Tris-washing experiments described in Figure 6.4. The electron density profiles of two trimeric LHCII complexes reported by Wang & Kühlbrandt, (1991) were superimposed onto the two tips of the PSII-LHCII supercomplex, marked 9.

Although, the overall stoichiometry of LHCII trimers to the dimeric complex is expected to be 8 LHCII trimers:1 PSII dimer in the thylakoid membrane (Peter & Thornber, 1991b), it is suggested to be 2 LHCII trimers:1 PSII dimer in our isolated supercomplex. Thus, the LHCII associated with the isolated supercomplex is thought to represent a tightly bound form of this antenna protein while other more weakly associated LHCII complexes are lost during the isolation procedure. This differentiation between tightly and loosely bound pools of LHCII has been postulated previously (Spangfort & Andersson, 1989). Fraction 4, which contained the PSII-LHCII supercomplexes analysed in this chapter, was subjected to reverse phase HPLC size exclusion analysis (Section 5.4) and found to contain 75 chlorophyll a, 32 chlorophyll b and 13 β -carotene molecules, for every 2 molecules of pheophytin. As a dimeric PSII-LHCII supercomplex would be associated with 4 pheophytin molecules (Nanba & Satoh, 1987; Gounaris *et al.*, 1990; Kobayashi *et al.*, 1990), these ratios are corrected to 150 chlorophyll a, 64 chlorophyll b and 26 molecules of β -carotene per 4 molecules of pheophytin.

A PSII-LHCII complex containing two subunits of CP47, CP43, D2, D1, cytb559, the 33kDa extrinsic polypeptide, CP29, CP26, CP24, as well as two LHCII trimer complexes (see Figure 6.7), should (according to the results of Nanba & Satoh, (1987), Gounaris *et al.*, (1990), Kobayashi *et al.*, (1990), Barbato *et al.*, (1991a) and Thornber *et al.*, (1993)) theoretically bind 158 chlorophyll a and 66 molecules of chlorophyll b. The theoretical pigment composition of such a complex is very similar to the values obtained for the PSII-LHCII supercomplex (150 chlorophyll a: 64 Chlorophyll b) and thus supports the hypothesis that the PSII-LHCII supercomplexes (Figure 6.7) analysed in this chapter are associated with 2 LHCII trimers per dimeric PSII core complex.

The results presented in this chapter not only suggest the relative positions of two LHCII trimer to a dimeric PSII core, but may indicate that the PSII-LHCII supercomplex can occur as a dimer *in vivo*. This conclusion is reached as monomeric PSII core particles, still associated with LHCII were never observed in any of our electron micrographs. Moreover, the dimeric core structure is maintained even when half the population of the chlorophyll a/b binding proteins is removed from one side of the supercomplex (Figure 6.5E). It could however be argued that the dimeric complexes analysed in this chapter are the product of solubilisation induced aggregation. Although this seems unlikely in view of the mild solubilisation conditions used to obtain the PSII-LHCII supercomplexes, it would be useful to supplement such preparations with lipid and to determine whether this treatment results in dimer dissociation. It should however be noted that the possibility that PSII may exist as a dimer *in vivo* has been suggested previously. One of the first reports of an isolated PSII dimer stems from work on *Synechococcus elongatus* (Rögner *et al.*, 1987) and suggested that PSII might exist as a dimer *in vivo* (Dekker *et al.*, 1988). Since then a number of studies, especially with PSII from higher plants, have come to the same conclusion (Peter & Thornber, 1991a; Bassi & Dainese, 1992). The size and shape of our dimeric complexes agree with the data obtained from ordered two-dimensional arrays of spinach PSII (Bassi *et al.*, 1989; Lyon *et al.*, 1993; Santini *et al.*, 1994) and extend them by showing high homology to the cyanobacterial structure. Furthermore, the possibility that PSII dimers are the product of fortuitous aggregation has been addressed by Santini *et al.*, (1994). The authors isolated PSII

complexes from both the granal stacks and stromal lamellae. PSII complexes isolated from granal stacks appeared to be predominantly dimeric while those obtained from the stromal lamellae were mainly monomeric. Detergent treatments did not appear to result in their inter conversion, suggesting that the dimers they had isolated were not the product of fortuitous aggregation

The unusual shape of the PSII-LHCII supercomplex (Figure 6.6) could conceivably facilitate the formation of arrays in the granal stacks of thylakoids, in which PSII is highly enriched *in vivo*. The clustering of PSII is probably essential for efficient photosynthesis as the slower energy transfer processes of PSII results in a need for spatial separation between it and PSI, which is able to drive faster rates of charge separation (Trissl & Wilhelm, 1993). In cyanobacteria, such PSII clustering is observed in these organisms. PSII complexes aggregate into rows on top of which hemi-discoidal phycobilisomes are attached (Mörschel & Schatz, 1987; Dekker *et al.*, 1988). In plants, it seems that a similar organisation of PSII occurs with tightly bound LHCII antenna, located at the periphery of the dimeric PSII core (Peter & Thornber, 1991b; Bassi & Dainese, 1992). This similarity in the basic organisation of PSII, lends support to the concept of a common evolutionary origin for chloroplasts, with ancestral chloroplasts containing both phycobilisomes and LHC's (Wolfe *et al.*, 1994; Bryant, 1992).

In view of the findings of Santini *et al.*, (1994) which suggest that PSII is predominantly in a dimeric state in the granal stacks and monomeric in stromal lamellae, it is interesting to study the PSII monomer-dimer inter conversion model (Figure 6.7) suggested by Barbato *et al.*, (1992a). Barbato *et al.*, (1992a) suggested that intact PSII dimers monomerised as part of the photoinhibition/repair strategy depicted in Figure 6.7. This figure is a summary of the results presented below.

Illuminating donor side inhibited thylakoids ($4500 \text{ mE}^{-2} \cdot \text{s}^{-1}$ for 2.5min) caused dimeric PSII to monomerise (Barbato *et al.*, 1992a). D1 breakdown was only observed after a 5 min illumination period, leading the authors to conclude that monomerisation preceded subunit damage, making it one of the first steps in the photoinhibitory process. Whether monomerisation is a protective mechanism or the first stage of damage remains to be determined.

Monomerisation does not appear to be controlled by a phosphorylation - dephosphorylation switch, as thylakoids were extensively dark adapted before their use, and no ATP was added during the trials (Barbato *et al.*, 1992a). As CP43 was released from PSII cores during photoinhibition (Barbato *et al.*, 1992a), but was thought to play a role in defining the site of D1 cleavage (Barbato *et al.*, 1992b), the scheme shows D1 damage to precede CP43 release. Barbato *et al.*, (1992a) suggests that if CP43 release precedes D1 damage, a 24kDa C-terminal D1 fragment, such as that seen in CP43-less PSII cores, would have been expected (Barbato *et al.*, 1991b) (Barbato *et al.*, 1992b). As no such 24kDa C-terminal fragments were observed in photoinhibited thylakoids (Barbato *et al.*, 1992a), it was suggested that D1 cleavage occurs before the release of the CP43 subunits.

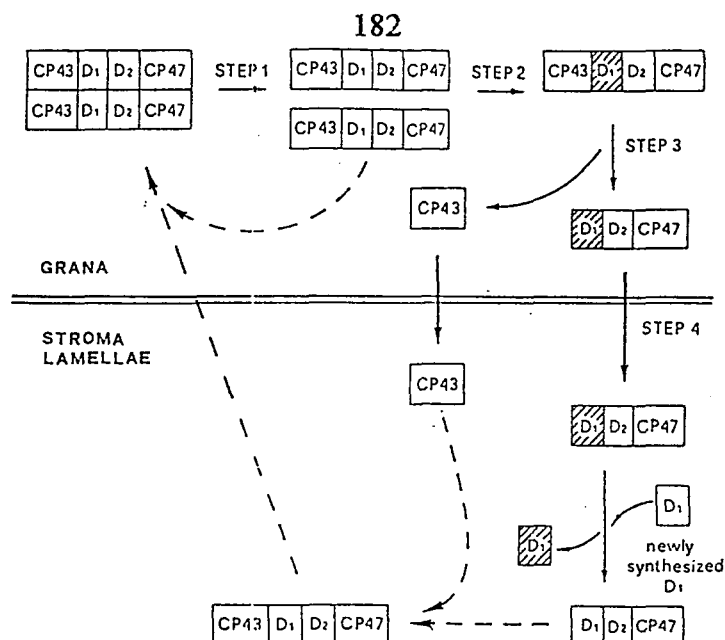


Figure 6.7 Photoinhibition and repair cycle of Barbato *et al.*, (1992a). Solid lines are stages shown experimentally. Dashed lines suggest possible addition steps in the process.

Whether or not CP47, D2 and D1 migrate to the stroma in an organised complex, or whether these subunits dissociate prior to migration remains unclear. As CP47 was at least in part associated with D1 and D2, (Figure 6.7) shows that these subunits migrate to the stroma in an organised complex. However, the presence of non-stoichiometric amounts of various PSII proteins in the stroma lamellae after photoinhibition has led to an alternative hypothesis proposing that core dissociation takes place in the grana (Mattoo & Edelman, 1987; Ghirardi *et al.*, 1990). As newly synthesised D1 is found predominantly in the stroma (Adir *et al.*, 1990), Figure 6.7 shows the damaged CP47, D2, D1 complexes migrating to the stroma, for repair. The damaged D1 is replaced with newly synthesised intact D1 before the repaired CP47, D2, D1 complexes re-associate with CP43 and migrate back to the grana. The last stage shows the repaired monomers to reassociate into dimeric complexes.

From the above studies, it is apparent that there is evidence for the existence of both monomeric and dimeric PSII core complexes *in vivo* and that dark adapted granal stacks contain predominantly dimeric PSII core complexes (Barbato *et al.*, 1992a; Santini *et al.*, 1994). If this is indeed the case, then one question which must be addressed is why the HTGP and OG cores, which were isolated from granal stacks contain predominantly monomeric PSII core complexes. One possibility is that the exposure of BBYs to high ionic strengths, results in the monomerisation of the membrane embedded dimeric core complexes, as is explained below.

High ionic strength conditions (43mM $MgCl_2$) are commonly employed at the BBY (grana stacks) solubilisation stage as they facilitate the precipitation of LHCII, allowing the PSII cores to be harvested from the supernatant after centrifugation (Ghanotakis & Yocum, 1986; Ghanotakis *et al.*, 1987a; Ghanotakis *et al.*, 1987b; De Las Rivas *et al.*, 1992a). Although, this procedure is effective at precipitating LHCII, the exposure of BBYs to such high ionic strengths could explain the high degree of dimer dissociation. To explain such dimer dissociation the results of Kruij *et al.*, (1994) will be

compared with published reports on the isolation procedures used, to purify monomeric and dimeric PSII complexes.

Some membrane protein oligomers are susceptible to salt-induced dissociation, while still embedded in the lipid bilayer, from which they are to be extracted. Trimeric PSI is one such example. If the *Synechocystis* PCC 6803 thylakoid membranes, from which PSI trimers were isolated, were exposed to low salt concentrations (20mM Mes pH 6.5, 500mM mannitol and 10mM CaCl_2) during isolation, 75% of the isolated complexes were in the trimeric state. The addition of 50mM MgSO_4 to this solubilisation mixture, lowered the trimer yield to 50%. Trimer yield was reduced to 25% if thylakoids were incubated in the presence of 160mM MgSO_4 , during the membrane solubilisation step (Kruip *et al.*, 1994). Once isolated, however, the trimers were stable in MgSO_4 concentrations as high as 500mM. These observations are explained as follows: Prior to solubilisation, trimeric PSI complexes are embedded in the thylakoid membrane. Electrostatic and van der Waals forces are both involved in holding together the three monomers composing it (Kruip *et al.*, 1994). The van der Waals forces, are short range attractive forces, which occur predominantly in the membrane spanning region of the protein. They can take place between adjacent monomers. However, such monomer:monomer interactions are reduced, if replaced by monomer:lipid interactions. Under these conditions the strength of electrostatic interactions between adjacent monomers may be able to tip the balance between monomer and trimer formation.

If the buffer in which PSI is solubilised from the membrane, has a high salt concentration, it follows that the dielectric constant of the aqueous phase will also be high. A solution having a high dielectric constant is able to shield charge interactions between too spatially separated ions or polyionic components (eg. monomeric PSI complex). Under these conditions, the electrostatic attraction between adjacent monomers, forming a trimer, are reduced and so could induce PSI to monomerise. As isolated trimers were shown to be stable at concentrations as high as 500mM MgSO_4 , electrostatic interactions can no longer be the dominant attractive force controlling trimer stability. This is thought to be because the hydrophobic regions of adjacent monomers, within such a delipidated trimer, are no longer able to undergo lipid-protein interactions to the same extent. As a result, the van der Waals forces between monomers are more difficult to break making the detergent solubilised trimer less susceptible to salt induced dissociation, than its membrane embedded counterpart (see Chapter 7).

It is possible to draw theoretical parallels between the factors affecting the isolation of trimeric PSI and dimeric PSII. OG cores are isolated by solubilising BBY-type membranes with octyl glucoside in the presence of 43mM Mes pH6.0, 1.0M sucrose, 10mM NaCl and 43mM MgCl_2 . As 50% of PSI trimers dissociated into monomers, when thylakoid membranes were solubilised in the presence of 50mM MgSO_4 , it is quite possible that PSII might also dissociate into monomers in the presence of 43mM MgCl_2 . To test this hypothesis, the ionic strengths of BBY solubilisation buffers used to isolate core particles of known oligomeric state (Rögner *et al.*, 1987; Barbato *et al.*, 1989; Haag *et al.*, 1990), were compared with the results obtained in Chapters 3, 4 and 5. Table 6.1 lists ionic compositions and strengths (excluding buffers) of BBY solubilisation buffers. The preparation of Barbato *et al.*, (1989) is

included as the two sucrose gradient purified cores reported, correspond to monomeric and dimeric complexes characterised in Section 5.2.

Table 6.1 shows that Rögner *et al.*, (1987) used a solubilisation buffer having an ionic strength of 66mM. The authors reported the isolation of predominantly dimeric PSII from *Synechococcus*, supporting the hypothesis that such conditions would favour dimer isolation. The HTGP and OG cores were exposed to a solubilisation buffer, having an ionic strength of 294mM, and yielded predominantly monomeric PSII complexes with only small amounts of dimer (Figure 3.10 and Figure 4.8). Raising the ionic strength to 760mM resulted in the isolation of monomeric PSII core complexes (Haag *et al.*, 1990). So far, the results fit the salt-induced dimer dissociation hypothesis. Furthermore, they follow a classical salting-in mechanism, as monomers are more solvated than their dimeric counterparts. Salting-in is the process whereby the addition of salt to a mixture of protein and water, increases the solubility of the protein. This increased solubility is precisely because the dielectric constant is raised by the addition of salt, and so screens monomer:monomer interactions. This screening effect may result in dimer dissociation.

At high solute concentrations (1M NaCl and 0.4M sucrose), such as those used at the BBY solubilisation stage (Barbato *et al.*, 1989), the dimer dissociating effect of the solutions high dielectric constant is counteracted by the number of waters of solvation available per PSII monomer. Under these conditions, sucrose and salt compete for the water molecules solvating PSII complexes embedded in the thylakoid membrane. As a result, it is energetically favourable that PSII cores aggregate into dimers having a lower degree of solvation. This seems to explain why Barbato *et al.*, (1989) was able to isolate both monomers and dimers, at an ionic strength of 2060mM, whereas Haag *et al.*, (1990) isolated monomeric PSII.

Table 6.1 A review of the ionic strength contributions of the ions present in the buffers used to solubilise PSII from the thylakoid membrane

		Rögner <i>et al.</i> , (1987)	BBY solubilisation (Figure 5.19)	OG and HTGP cores	Haag <i>et al.</i> , (1990)	Barbato <i>et al.</i> , (1989)
Ion concentration (mM)	K ⁺	4	0	0	0	0
	Na ⁺	0	20	18	350	1000
	Ca ²⁺	0	5	0	10	10
	Mg ²⁺	10	0	43	0	0
	Cl ⁻	20	20	104	370	1020
	HPO ₄ ⁻	2	0	0	0	0
	HCO ₃ ⁻	0	10	0	0	0
Ionic strength (mM)	K ⁺	4	0	0	0	0
	Na ⁺	0	20	18	350	1000
	Ca ²⁺	0	20	0	40	40
	Mg ²⁺	40	0	172	0	0
	Cl ⁻	20	20	104	370	1020
	HPO ₄ ⁻	2	10	0	0	0
	HCO ₃ ⁻	0	10	0	0	0
	Total	66	80	294	760	2060
Oligomeric state		Mainly dimer, some monomer	Mainly dimer, some monomer	Mainly monomer, some dimer	Pure monomer	Mainly monomer, some dimer

From the above discussion, it can be seen that there is then considerable evidence to support the view that PSII cores can exist as dimers *in vivo* and that the fact that they are present in OG and HTGP cores at far lower concentrations than their monomeric counterparts, may be due to their salt-induced dissociation during the BBY solubilisation stage. The results of Santini *et al.*, (1994) and Barbato *et al.*, (1992a) do however also support the view that thylakoids contain a monomeric form of PSII. Indeed, Barbato *et al.*, (1992a) suggest that light-induced PSII monomerisation takes place before photoinhibition induced D1 damage is detected (Figure 6.7). It is therefore interesting to speculate that PSII may monomerise as part of a protective mechanism and that both the aggregation states of PSII are active *in vivo*.

6.3.3 Location of the 33kDa subunit

Having located the dimeric PSII core and proposed the position of the two LHCII trimers within the PSII-LHCII complex, we attempted to locate the other subunits. As the 33kDa extrinsic polypeptide is the easiest to remove, its localisation was the first to be tried.

The main type of side-view projection of the dimeric *Synechococcus* PSII core complexes (CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic polypeptide) show two protrusions, which, in projection are 6.3 nm apart (Figures 6.3A and 6.4C). The other side-views (Figures 6.3B-6.3F) are obtained as a result of the orientation in which particles bind to the carbon support. Depending on the

particle orientation the protrusions can be seen to be closer (Figure 6.3B) or in overlap position (Figure 6.3C). As the overlap view gives by far the longest projection, it is suggested that the two protrusions are lying roughly superimposed in this type of projection, thus exaggerating its size. As the thickness of the particles was 9.1nm (with protrusion) and 6nm (without protrusion), these protrusions were shown to extend 3.1nm from the surface of the rest of the core complex. Dekker *et al.*, (1988) identified similar projections and showed them to be removed by Tris-washing. Thus, it is concluded that these protrusions are two 33kDa subunits, one bound to each monomeric segment of a dimeric PSII core.

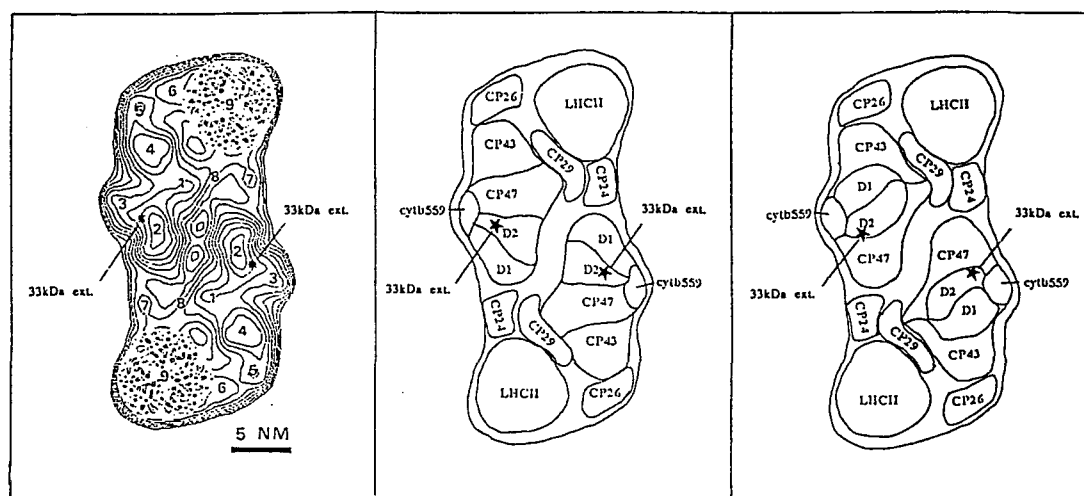
Side-views of the PSII-LHCII supercomplex yielded a single image class in which the protrusions were in the overlap position right at the centre of the projected view. This result suggests that, as the LHCII complexes are lying on their longest side when viewed in the side-view position, the overlapped protrusions lie above and below the central hole and so correspond to the areas marked as density 2 in Figure 6.8A. As the side-views of the PSII cores showed the two protrusions to be 6.3 nm apart, and the two regions marked 2 in Figure 6.8A are also approximately separated by this distance, it is indeed possible that these regions correspond to the position of the 33kDa extrinsic polypeptide.

Tris-washing specifically removes the 33kDa extrinsic polypeptide. To test the hypothesis that regions 2 on two adjacent monomers within a dimer, correspond to the positions of the 33kDa extrinsic polypeptides, Tris-washed and non Tris-washed PSII core top-view images were compared. Figures 6.4A and 6.4B show top-views of spinach and *Synechococcus* PSII (CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic polypeptide) core dimers, respectively. Figures 6.4D and 6.4E show top-view contours of spinach and *Synechococcus* PSII Tris-washed core dimers. The contours of the spinach and *Synechococcus* dimers were very similar. The contours of the Tris-washed spinach dimers also showed great similarity to the corresponding *Synechococcus* cores. As a result all the Tris-washed spinach and *Synechococcus* images were averaged and subtracted from the mean of all the non Tris-washed spinach and *Synechococcus* cores. In this way, the density corresponding to the 33kDa subunit was identified. The three lowest contour lines marked in Figure 6.4F outline the difference observed on Tris-washing and correspond to the putative binding position of the 33kDa subunits. This density does indeed overlap region 2 (Figure 6.6A) and extends towards the central hole. It is therefore concluded that the 33kDa subunit forms part of the protein density in region 2 (Figure 6.6A).

6.3.4 A model of the subunit composition of the PSII-LHCII supercomplex.

Nine areas of protein density are marked in Figure 6.8A. The protein densities within the PSII core particles are marked (1-4) according to Lyon *et al.*, (1993). The 33kDa extrinsic subunit was suggested to contribute to density 2 of each monomeric core complex. The densities marked 9 are thought to correspond to the two LHCII trimers. Thus, it is suggested that densities 5-8 are composed of CP24, CP26 and CP29. Figure 6.8B and C present two models of the PSII-LHCII supercomplex topology. Both models incorporate information obtained from cross-linking studies, data collected from ordered two-dimensional arrays, as well as the results presented in this chapter. The validity of

each model is discussed below. Clearly as two possible models are presented, a number of unanswered questions remain. However, these models do provide a framework for future studies.



**A. Numbering of
protein densities**

B. MODEL 1

C. MODEL 2

Figure 6.8 Subunit topology of the PSII-LHCII supercomplex. (A) Numerical labelling of the PSII-LHCII supercomplex protein densities according to Lyon *et al.*, (1993). (B) Shows a model (1) of proposed subunit composition. (C) Shows a second model (2) of proposed subunit composition.

Ordered two-dimensional arrays of CP47, D1, D2, cytb559 complexes were reported by Dekker *et al.*, (1990). Dekker *et al.*, (1989) clearly show CP47, D1, D2, cytb559 complexes to be highly purified and free of LHCII. Figure 6.9 shows the topology of this complex. As the transmembrane regions of this complex are embedded in the membrane, the prominent density must correspond to an extrinsic portion of the complex.

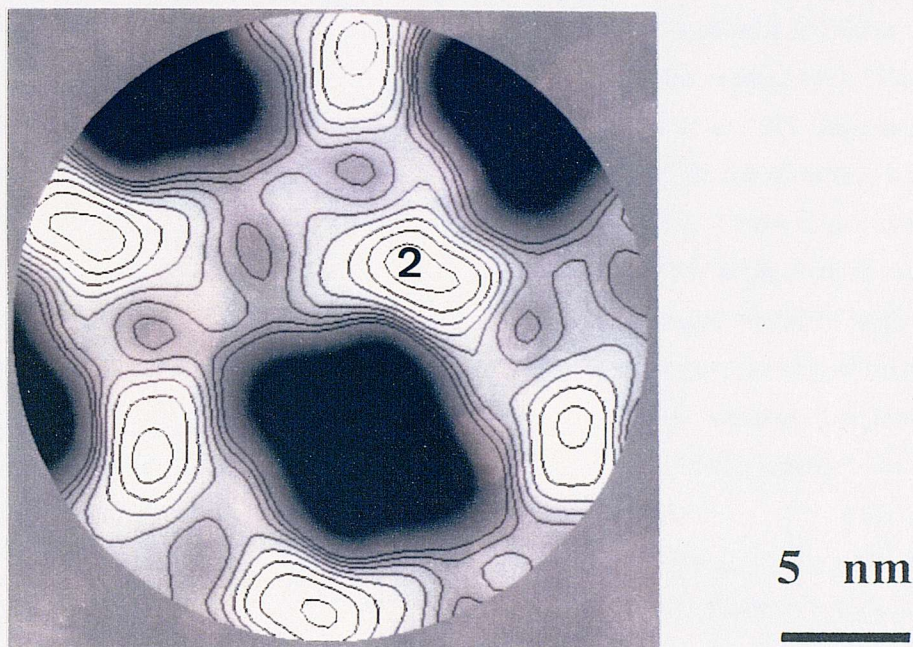


Figure 6.9 Topology of CP47-D1-D2-cytb559 complexes.

This figure is taken from Dekker *et al.*, (1990).

D1, D2 and cytb559 only have short extrinsic segments. The stromally exposed loops of CP47 are much shorter than those exposed to the luminal side of the membrane. Thus, it seems likely that density 2 (Figure 6.9) is predominantly composed of the latter. Furthermore, loop E of CP47 (Figure 1.13) is by far the longest of these luminal CP47 loops and so is likely to account for most of the protein density marked 2 (Figure 6.8). This density (density 2, Figure 6.9) is similar in shape to the density marked 2 in Figure 6.8A. The CP47, D1, D2, cytb559 complex differs from the Tris-washed dimers (Figure 6.3D), in that it lacks the CP43 subunit. It is suggested that density 4 (Figure 6.8A) is reduced in the absence of CP43. As a result, density 4 has been labelled as CP43 in the two subunit models provided in Figures 6.8B and C. Although loop E of CP47 is marked in region 2, the positions of the transmembrane helices of this subunit are less clear. Moskalenko *et al.*, (1992) subjected a CP47, D1, D2, cytb559 complex to cross-linking studies. D1, D2 and the α - β subunits of cytb559 cross-linked with each other and so have been placed in close contact in both models. CP47 only cross-linked with D2, and so the two subunits were placed next to one another in models 1 and 2. However, models 1 and 2 differ in the orientation of the CP47, D1, D2, cytb559 complex. Model 1 places the bulk of CP47 between CP43 and D2, while model 2 locates it near the outer edge of each monomeric

PSII core complex. Subjecting monomeric CP47, CP43, D1, D2, cytb559 complexes to cross-linking studies, may provide the evidence required to identify which of these two models corresponds more closely to the subunit organisation of PSII *in vivo*. In whichever orientation CP47 is placed, its luminal loop E (Figure 1.13) could extend over D2 to form the protein density region 2. Loop E of CP47 has been shown to be in close contact with the 33kDa polypeptide in Figures 6.6B and C and by Bricker *et al.*, (1988), Odom & Bricker, (1992), Frankel & Bricker, (1992), Putnam-Evans & Bricker, (1992) and Haag *et al.*, (1993). As the 33kDa subunit is also reported to be in close contact with CP43 (Isogai *et al.*, 1985; Garstka & Kaniuga, 1991; Enami *et al.*, 1992), cytb559 (Tae *et al.*, 1988; Takahashi & Asada, 1991), as well as the PSII reaction centre (Gounaris *et al.*, 1988), both models allow for these contacts, though it must be said that they are easier to accommodate in model 2. However, as the two models only map the approximate location of the PSII subunits and not the detailed architecture, a final conclusion is premature. It should also be noted that the low molecular weight subunits associated with these complexes have not been characterised in detail. The *psbI* gene product is likely to be part of the supercomplex, being closely associated with the D1, D2, cytb559 subunits of the reaction centre (Webber *et al.*, 1989b). Interestingly, Enami *et al.*, (1992) reported the 33kDa subunit to be within 11Å (1.1nm) of the *psbI* gene product.

The position of CP29, CP26 and CP24 cannot be proved using the available data. CP29 (Rigoni *et al.*, 1992; Dainese *et al.*, 1992a) and CP26 (Dainese *et al.*, 1992a) have both been shown to be closely associated with CP47. Dainese *et al.*, (1992a) also showed both CP29 and CP26 to cross-link with CP43. CP29 was placed tentatively near density 8, as Bassi & Dainese, (1992) showed that CP29/CP24 were in contact with one another. The authors isolated a CP29, CP24, LHCII trimer complex, which dissociated into an LHCII trimer and a CP29-CP24 unit after phosphorylation. If CP26 were placed near density 8 and CP29 near density 6, a CP29, CP24 interaction would be unlikely. Furthermore, from the results of Bassi & Dainese, (1992) it seems that CP26 is not tightly bound to CP24, CP29 or trimeric LHCII. As Figures 6.5D and H identify dimeric PSII-LHCII supercomplexes, lacking a fragment near density 6 (Figure 6.7A), CP26 is tentatively assigned to this location. The one piece of evidence, which neither model shows is the interaction between CP26 and CP47 (Dainese *et al.*, 1992a). Although, the models presented in Figure 6.7 provide a framework for future studies, higher resolution images will be required to confirm their validity.

6.4 Conclusion

Single particle analysis studies of monomeric and dimeric cores consisting of CP47, CP43, D2, D1, cytb559, the 33kDa extrinsic polypeptide and associated low molecular weight subunits were presented. The images obtained from dimeric cores were very similar to those obtained by Bassi *et al.*, (1989), Lyon *et al.*, (1993) and Santini *et al.*, (1994). It is therefore suggested that their ordered arrays consisted of dimeric PSII particles, as concluded by the authors themselves. PSII-LHCII supercomplexes consisting of CP47, CP43, D2, D1, cytb559, the 33kDa extrinsic subunit as well as CP24, CP26 and CP29 and LHCII were also subjected to single particle analysis. Electron densities of this supercomplex were compared with those of a dimeric PSII (CP47, CP43, D1, D2, cytb559 and 33kDa extrinsic polypeptide) complex. In this way, the centre of the supercomplex was shown to consist of a PSII dimer. Comparison of the contour map of trimeric LHCII reported by Wang & Kühlbrandt, (1991) with that of the supercomplex, suggest that trimeric LHCII may form the tips of the latter. Four protein densities, lying between PSII and LHCII in the supercomplex have been identified and are thought to correspond to CP29, CP26 and CP24. By comparing the electron densities of dimeric PSII cores (CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic subunit) with those of a core consisting of CP47, CP43, D2, D1 and cytb559 has allowed the localisation of the 33kDa extrinsic polypeptide.

Although the identification of dimeric PSII cores opens up the possibility that such dimers exist *in vivo*, a conclusion on this subject awaits their detection in intact thylakoid membranes.

Although, cross-linking experiments on monomeric CP47, CP43, D1, D2, cytb559 cores and the PSII-LHCII supercomplex would help to identify the positions of individual subunits, highly ordered two-dimensional or three-dimensional crystals will ultimately be required to complete these structural studies.

Chapter 7 Initial two-dimensional crystallisation trials of PSII

This chapter was written, not with the intent of being a definitive study, but rather as a collection of experiments which provide useful information for future two-dimensional crystallisation trials of PSII. The introduction outlines the method of producing, and factors controlling, two-dimensional crystallisation. Particular emphasis will be placed on protein and lipid structure, the importance of detergent choice, as well as discussing temperature and pH effects. Initial two-dimensional crystallisation experiments are presented together with studies on PSII thermostability and lipid content.

7.1 Introduction to two-dimensional membrane protein crystallisation

The membrane protein class includes hormone and other signal receptors, ion channels and energy transducers, all of which are vital for cell viability and many of which are being subjected to intense structural research. The amphipathic nature of membrane proteins, prevented researchers from growing crystals of any proteins in this class until the Nobel prize winning breakthrough, which resolved the structure of *Rhodospseudomonas viridis* photosynthetic reaction centres at a resolution of 3Å (Deisenhofer *et al.*, 1984; Deisenhofer *et al.*, 1985). This result was essentially achieved by protecting the hydrophobic region of the membrane protein with detergent and inducing the resultant complex to pack into ordered three-dimensional lattices, by slowly removing the excess water, using traditional vapour diffusion techniques.

In the race to develop membrane protein crystallisation techniques, capable of yielding high resolution structural data, a fundamentally different approach was being taken by other pioneering research groups. Henderson & Unwin, (1975) showed that the membrane protein bacteriorhodopsin naturally packed into ordered two-dimensional arrays *in vivo* in the plasma membrane of *Halobacterium halobium*. When placed in the path of an electron beam, these ordered arrays diffracted electrons to 7Å. Subsequent improvements in both sample preparation and analysis allowed the structure of these membranes to be determined at a resolution of 2.8Å (Baldwin & Henderson, 1984; Baldwin *et al.*, 1988).

With the realisation that two-dimensional crystals could yield structural information of this resolution, other membrane proteins were induced to form ordered two-dimensional arrays, simply by solubilising away excess lipid from purified lipid bilayers that contained high concentrations of the protein complex of interest. In this way a whole series of two-dimensional arrays were obtained, but none yielded high resolution structural data. The reasons for this are most likely to be the presence of impurities and variability between preparations (reviewed in Kühlbrandt, 1992).

To overcome this, membrane proteins were first purified, before being incorporated into a pure lipid bilayer. Indeed, it is now almost universally accepted that the best method of obtaining highly ordered two-dimensional arrays is to dialyse detergent out of a mixture of lipid, detergent and highly purified protein. Two bacterial porins (OmpF and PhoE) were crystallised in this way and

diffracted electrons to a 3.2Å and 2.8Å resolution, respectively (Sass *et al.*, 1989; Jap *et al.*, 1991). A 2.9Å electron diffraction data set has also been obtained for pea light-harvesting complex II, using this procedure (Wang & Kühlbrandt, 1991). To appreciate the underlying principles controlling the formation of such crystals, the general method used to produce them will be introduced and related to the parameters affecting their development.

7.1.1 Two-dimensional crystallisation of detergent solubilised membrane proteins

One of the beauties of growing two-dimensional crystals using the detergent dialysis method is its theoretical simplicity. Furthermore, two-dimensional crystals, where the protein is embedded in a membrane-like bilayer, resemble the *in vivo* environment much more closely than the detergent solubilising conditions required for three-dimensional crystallisation. To evaluate the potential of this approach, for yielding high resolution structural data of PSII, the basic principles of the method will be introduced together with a more detailed study of the parameters controlling crystal development.

The starting materials required for any two-dimensional crystallisation trial of this type are highly purified membrane protein, a pure bilayer-forming lipid or lipid mixture and a suitable detergent. The purified lipid is dissolved in chloroform, aliquoted into an Eppendorf and dried under nitrogen, before being mixed with a suitable buffer-containing detergent. A 2-3min sonication step allows the rapid formation of lipid-detergent mixed micelles. Detergent-solubilised membrane protein is added before the resultant protein/lipid/detergent mixture is dispensed into a dialysis vessel. The dialysis membrane, which typically has a 12-14kDa pore size, allows the selective diffusion of detergent into the surrounding detergent free buffer. As the detergent is dialysed out of the system, both the lipid and the protein are forced to aggregate. If the lipid:protein ratio is high, the bilayer sheets and vesicles that form, will contain protein complexes which are not lipid limited and so will not be forced to arrange themselves into organised arrays. As the lipid:protein ratio is lowered, protein complexes become more tightly packed and ordered arrays start to form. If the lipid:protein ratio is lowered still further, there comes a point when there is insufficient lipid to protect the membrane spanning region of the protein from coming into contact with the aqueous phase. At this point, it becomes energetically favourable for the exposed membrane spanning regions to associate with one another and form disordered precipitates.

Lipid bilayers and two-dimensional crystals have been shown to develop spontaneously many times using such dialysis trials (reviewed in Kühlbrandt, 1992). This spontaneity is, in itself, a manifestation of the hydrophobic effect described in Tanford, (1980) and Sharp, (1991). To explain the energetics of two-dimensional crystal formation, the free energy of the system must be considered.

As with any chemical reaction, the process of crystallisation will only proceed spontaneously if the free energy of the system is reduced in the process. The dialysis-driven detergent removal forces the components in the crystallisation mixture to rearrange themselves in order to maintain the lowest possible free energy level.

Detergents are classed as amphipathic molecules as they have both a hydrophilic and hydrophobic region. In their absence, membrane proteins in aqueous solution precipitate. Above the critical micelle concentration (cmc), detergent monomers aggregate to form spheres (micelles), with the hydrophobic tails forming the centre while the polar heads are in contact with the aqueous phase. Detergents solubilise membrane proteins by forming a belt around the membrane spanning region, thus preventing the hydrophobic section from coming in contact with the aqueous phase. The hydrophobic portion of the detergent is in contact with the protein and the polar head group is in contact with the aqueous phase. As a result detergent-protein complex solvation becomes energetically favourable.

Solubilised membrane proteins are incubated with lipid during two-dimensional crystallisation trials. As dialysis proceeds, there comes a point when there is insufficient detergent in the dialysate to protect the hydrophobic region of the protein from exposure to water. The membrane spanning regions prevent contact with water, by forming a lipid:protein complex, in which the lipids replace the detergent. The use of bilayer-forming lipids induces sheet formation. Electron microscopy shows that these bilayers form and grow as the dialysis proceeds, indicating that the process is favourable in free energy terms. In order to understand the energetics of protein solvation and two-dimensional crystallisation in more detail, the Gibb's free energy function must be brought into play (Equation.7.1).

$$\Delta G = H - T\Delta S$$

Equation 7.1

where ΔG =Free energy change (J/mol), H =Enthalpy change (J/mol), T =Temperature(K), S =Change in entropy ($J.K^{-1}.mol^{-1}$) = measure of the change of disorder.

As a reaction is only spontaneous when ΔG is negative, there must either be a reduction in enthalpy (H) or an increase in the entropy (S). Membrane proteins are not readily soluble in water, whereas detergent-protein complexes dissolve spontaneously. The insolubility of membrane proteins is caused by the hydrophobic region, which disrupts the hydrogen-bonding structure of the solvent. This causes a substantial increase in enthalpy. Once inserted, the water molecules in contact with the hydrophobic membrane proteins are unable to form hydrogen bonds with the non-polar groups at the surface. An increase in enthalpy and hence in free energy, can only be prevented by reducing the hydrophobic surface area and so the protein precipitates. In contrast, detergent solubilised membrane proteins do not have any exposed hydrophobic regions and dissolve spontaneously. In this case the polar detergent surface is able to form hydrogen bonds with the solvating water molecules, so preventing an increase in the enthalpy of the system.

During two-dimensional crystallisation, lipids and proteins spontaneously aggregate into bilayers as the detergent is dialysed out of solution. In part this reaction occurs to prevent an increase in enthalpy, caused by contact between hydrophobic regions and the aqueous phase.

The two-dimensional crystallisation process is also driven by an increase in entropy (S). At first it seems paradoxical that the disorder of the system must increase, as ordered two-dimensional crystals start to form. This is explained by the fact that the total disorder of the system is the sum of

the disorder of all its constituent components (water, protein, detergent, lipid and additives). If the disorder of one component is large enough, this increase in entropy may be sufficient to allow the ordering of the other components without an increase in the free energy of the system. At the start of crystallisation, each protein-detergent complex and each lipid-detergent mixed micelle is surrounded by an ordered water cage (solvation layer). The number of water molecules involved in solvation are proportional to the sum of the protein-detergent complex surface areas. As the lipid and protein monomers pack into ordered two-dimensional crystals, their surface area is decreased, causing a reduction in the number of water molecules present in the ordered solvation layer. This causes a net increase in bulk water disorder and hence a reduction in free energy of the system. Taken separately, the free energy of lipid and protein increases, as the ordered two-dimensional arrays grow. This increase in free energy is more than compensated for (in free energy terms) by the increase in disorder of water molecules and it is this which drives crystal growth. Indeed, the reduction in free energy of the system is sufficient to compensate for the steric and electrostatic repulsions between tightly packed protein complexes in the forming lipid bilayer (Cevc & Marsh, 1987).

7.1.2 Protein

7.1.2.1 Protein purity

Protein purity is a crucial factor affecting the order of packing within a two-dimensional crystal. Any hydrophobic impurity may become inserted into a developing two-dimensional crystal and in so doing will disrupt the order of developing arrays. Polar impurities could affect crystal formation, by altering the surface charge of the protein or by causing protein damage. The effects of polar and hydrophobic impurities on two-dimensional crystal packing have not been studied in detail.

7.1.2.2 Protein concentration

Optimal protein concentrations for growing two-dimensional crystals of most membrane proteins are of the order of $1\text{--}2\text{mg.ml}^{-1}$ (Sass *et al.*, 1989; Jap *et al.*, 1991; Wang & Kühlbrandt, 1991; Kühlbrandt, 1992). This is distinctly lower than the $10\text{--}30\text{mg.ml}^{-1}$ protein concentration range commonly used for three-dimensional crystallisation trials. Higher protein concentrations used in three-dimensional crystallisation trials place a limitation on the number of water molecules available for protein-detergent complex solvation, thus forcing them to aggregate into an ordered crystal or a precipitate. A similar limitation of the waters of solvation in two-dimensional crystallisation trials is not desirable as it would force any bilayers formed, into aggregation states, having a smaller surface area (Section 7.1.5). Such aggregates could form a number of phases, one of which is a stacked bilayer structure (Figure 7.1). Such changes introduce another level of complexity, which only complicate later structural analyses and so are not desirable.

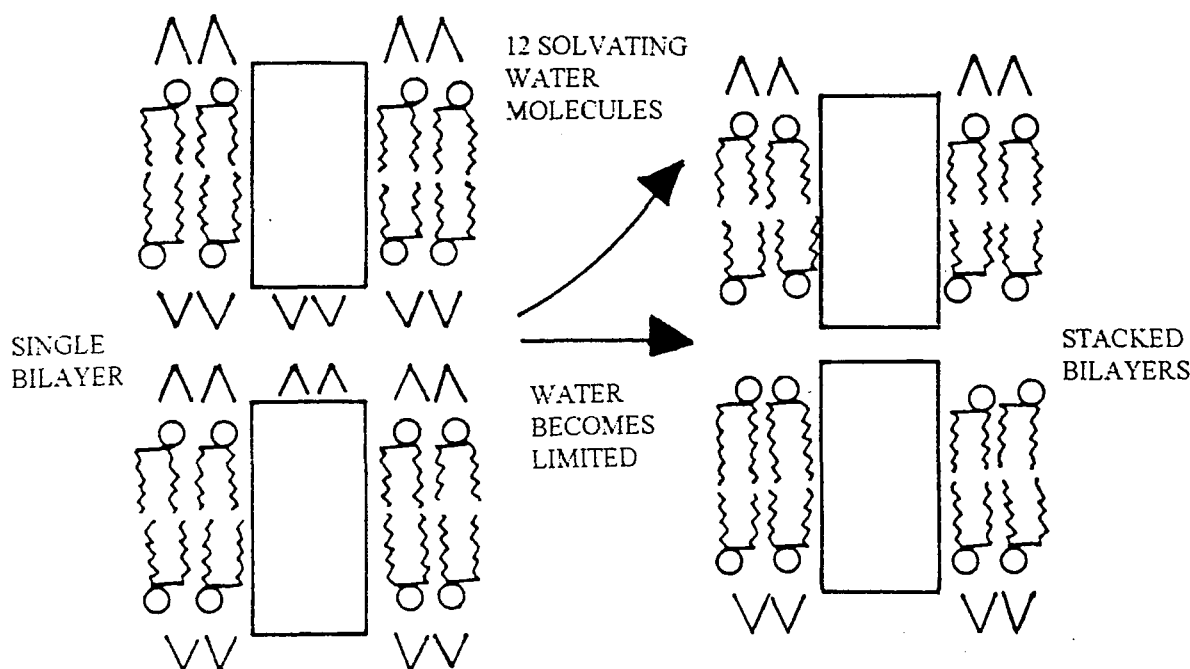


Figure 7.1 Diagram showing the driving force of bilayer aggregation at high protein concentrations. Membrane proteins and the bilayer forming lipids in which they are embedded tend to be hydrated in aqueous solution. In this figure, the waters of hydration are represented as ($\wedge$). At high protein concentrations, water may become limiting and result in the stacking lipid-protein bilayers as shown.

7.1.2.3 Electrostatic interactions of proteins

The electrostatic properties of membrane proteins will affect their ability to pack into ordered arrays. Charges of the same polarity on the hydrophilic surfaces of adjacent protein monomers will repel each other, reducing both packing density and stability of the developing lattice. The majority of complexes crystallised to date, have been isolated from membranes in which they were highly concentrated in the first place. Their close association *in vivo* is a good indication that the surface charges they carry do not prevent their close association (Kühlbrandt, 1992). Examples of two-dimensional crystals, grown from membrane proteins isolated from membranes in which they were already closely associated, include the outer membrane bacterial porins OmpF (Dorset *et al.*, 1983) and PhoE (Jap *et al.*, 1990), as well as maltoporin (LamB) (Lepault *et al.*, 1988), PSI reaction centres (Ford *et al.*, 1990), bacterial reaction centres (Miller & Jacob, 1983), NADH:ubiquinone reductase (Complex I) (Leonard, 1987), cytochrome reductase (Hovmöller *et al.*, 1983), bacteriorhodopsin (Michel *et al.*, 1980) and LHCI (Wang & Kühlbrandt, 1991). Many membrane proteins are not present at such high concentrations *in vivo* and so do not have the same steric or charge restraints placed on the more closely associated complexes. As a result, they may have structures, that are unable to pack into

ordered arrays. The only highly diffracting crystals that have been grown from complexes isolated from membranes containing low densities of the protein in question, were of CaATPase (Stokes & Green, 1990; reviewed in Kühlbrandt, 1992).

The second similarity between the proteins yielding the most highly diffracting crystals, PhoE bacterial porin (2.8Å)(Jap *et al.*, 1990), LHCII (2.9Å)(Wang & Kühlbrandt, 1991), OmpF bacterial porin (3.5Å)(Dorset *et al.*, 1983) and bacteriorhodopsin (3.5Å)(Michel *et al.*, 1980), is that all four have very short extra-membranous loops, which do not protrude much beyond the membrane surface. Crystals of multi-subunit complexes with large extra-membranous domains, such as the complex of the mitochondrial respiratory chain, have tended to yield less well-ordered crystals (reviewed in Kühlbrandt, 1992). This may be because large hydrophilic regions reduce the packing order, by introducing additional electrostatic and steric repulsion forces into the equation. The repelling forces between adjacent hydrophilic domains can be partially overcome by the use of suitable biochemical reagents. N-hydroxy-succinamide and maleimides can be used to react, both with free amino acid groups of lysine and the N-termini of individual subunits, thereby substantially reducing repulsion between positively charged groups. Removal of the more highly charged extrinsic subunits altogether, may also aid the two-dimensional crystallisation process (Kühlbrandt, 1992).

Electron diffraction resolution is not only dependent on the packing order of the crystal, but also on the number of repeating units within it. As the size of a given complex increases, the crystals grown from it must have proportionally larger dimensions if diffraction resolution is to be maintained. This is not always easy as larger sheets are easily damaged.

7.1.3 Lipids

One distinct advantage of two-dimensional crystallisation over three-dimensional crystallisation is that proteins do not have to be purified of their lipid to the same extent (Kühlbrandt, 1992). Lipid heterogeneity is a serious problem in three-dimensional crystallisation trials. Their complete removal requires the use of very high detergent concentrations, which can be damaging to the protein (Michel, 1991). In contrast, the presence of lipid is a pre-requisite for the formation of two-dimensional crystals and their complete removal is not only unnecessary, but undesirable (Kühlbrandt, 1992). Additional purified lipid or lipid mixtures, are usually added. Not all lipids are useful for two-dimensional crystallisation and so the required lipid characteristics will be presented in this section.

Lipids can be completely hydrophobic (neutral lipids) or amphipathic (polar lipids) in nature. As bilayer forming lipids belong to the latter group, only properties of the polar lipids will be discussed in this chapter.

Amphipathic molecules are defined as structures having both a hydrophobic and a hydrophilic region. Polar lipids consist of two fatty acid chains, which can vary in saturation and are esterified to a polar head group, which determines the class to which the lipid is grouped. Some common lipid classes include: phosphatidyl choline, phosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidic acid. Table 7.1 shows the head group structures of these lipid classes.

In aqueous solution it is energetically favourable for lipid monomers to form aggregates. A uniform suspension of lipid monomers in an aqueous solution would have a far higher free energy level, than that observed on their aggregation, because of increase in enthalpy. The insertion of a lipid monomer into water causes the disruption of the solvent hydrogen-bonding network and results in an increase in the enthalpy of the system. The aggregate shape, is to a large extent, controlled by the head to tail size ratio, as shown in Figure 7.2.

Figure 7.2 shows that, if flat two-dimensional crystals are to be obtained, pure bilayer forming lipids or lipid mixtures are required. Lipid bilayers are able to exist in two temperature-dependent phases. At low temperatures, the lipid monomers become closely associated, pack into a crystal-like lattice and are held in position by short range van der Waals forces. In this phase the bilayers are referred to as crystalline. The strong van der Waals forces observed in this phase make it rigid and so do not allow the free movement of any proteins embedded in it. Such movement is necessary if the membrane proteins are to arrange themselves into ordered two-dimensional arrays. On heating the bilayer lipids in the crystalline phase, the vibrational energy stored in each monomer increases to the point where the attracting van der Waals forces between monomers are overpowered. At this point the crystalline bilayer melts forming a more fluid, liquid-crystalline phase, in which embedded membrane proteins can move freely. X-ray diffraction can be used to study this phase transition (Luzzatti *et al.*, 1968; Tardieu *et al.*, 1973) as can calorimetry (Byrne & Chapman, 1964; Chapman & Collin, 1965). Figure 7.3 depicts the structural changes observed during the crystalline/liquid-crystalline transition of a bilayer. The transition temperature is dependent on both the properties of the head and tail groups.

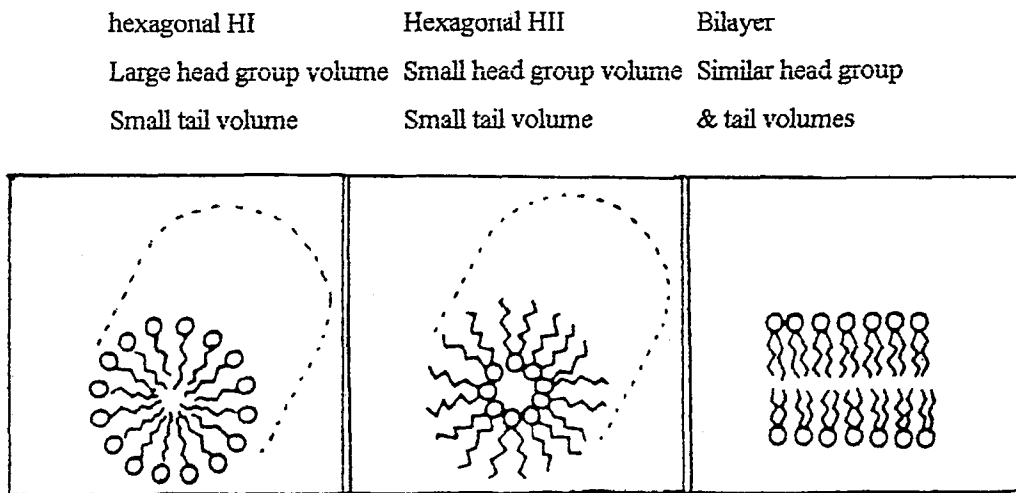


Figure 7.2 The effect of lipid head and tail volume ratio on aggregation. Figure taken from Gounaris, (1983).

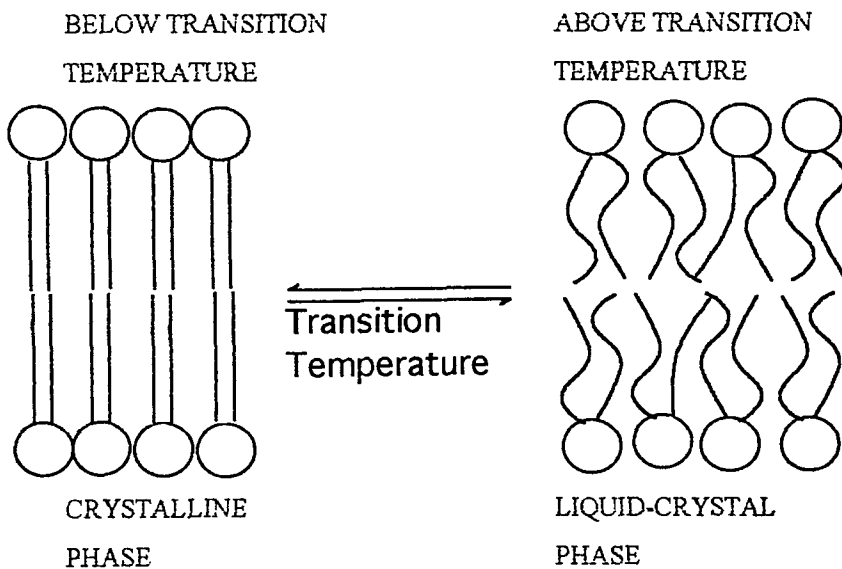


Figure 7.3 The crystal/liquid-crystal transition of bilayer forming lipids

7.1.3.1 Lipid head groups

sheet

The final packing order of a forming lipid¹ is dependent on the balance of attractive and repulsive forces of adjacent head groups and hydrocarbon chains. The structure and charge properties of some of the common lipid head groups are given in Table 7.1. The polarity of the head groups shown below are due to the presence of oxygen, nitrogen, phosphorus or hydroxyl groups and are thought to interact with neighbouring head groups by means of hydrogen-bonding, steric and electrostatic interactions.

Table 7.1 The structure and charge properties of some common polar lipids

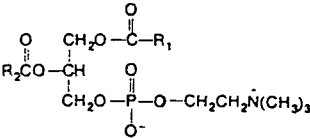
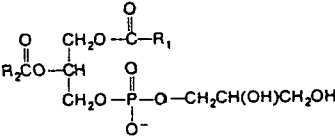
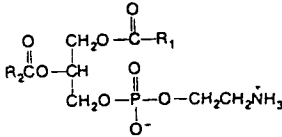
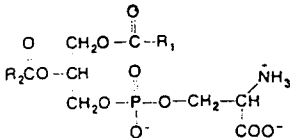
Table of Head groups	Head group Structure	Charge properties
Phosphatidyl choline		Zwitterionic
Phosphatidyl glycerol		negative charge
Phosphatidyl ethanolamine		Zwitterionic
Phosphatidyl serine		Zwitterionic

Table 7.1 adapted from Zubay, (1983) and Gounaris, (1983).

7.1.3.2 The hydrophobic tail

Hydrophobic lipid chains can be either saturated or unsaturated fatty acids. Fully saturated hydrocarbon chains are flexible, as their movement is not restricted by the presence of double or triple bonds and so are able to undergo close association with adjacent fatty acids. Van der Waals forces, the main attractive forces between them, arise from electronic coupling and only act over short distances. The presence of unsaturated bonds or bulky side chains introduces steric interference factors, which prevent tight monomer packing, causing a concomitant reduction of the van der Waals attraction between neighbouring lipid molecules. This saturation-dependent reduction in the van der Waals attraction, results in a reduction in the crystal/liquid-crystal transition temperature. The transition temperature of distearyl phosphatidyl choline (C18) is 55°C, while the introduction of a single double bond (dioleyl phosphatidyl choline) causes it to drop to -22°C (Table 7.2). Table 7.2 shows that the melting temperature of a lipid increases with chain length. This is a result of the number of van der Waals interactions being proportional to the length of adjacent hydrocarbon chains.

Table 7.2 The effect of chain length and hydrocarbon chain saturation on the crystal/liquid-crystalline phase transition temperature of some phospholipid bilayers at pH7 and an ionic strength of 0.1.

Chain length	No. of C=C double bonds	Head group			
		Phosphatidyl choline	Phosphatidyl glycerol	Phosphatidyl ethanolamine	Phosphatidyl serine
Dilaureyl (C12)	0	-1	-2	30.5	13
Dimyristoyl (C14)	0	23	24	49.5	36
Dipalmityl (C16)	0	41.5	41.5	64	52
Distearoyl (C18)	0	55	54.5	74	68
Dioleyl (C18)	1	-22			

Table 7.2 adapted from Cevc & Marsh, (1987).

As Table 7.2 shows, the transition temperatures of the above lipids are dependent on both the head and tail group structure. Many proteins are not stable at the high temperatures required to maintain the saturated lipids in a liquid-crystalline phase. Dimyristoyl phosphatidyl choline (DMPC), which has the relatively low crystal/liquid-crystal transition temperature of 23°C, has proved useful in the crystallisation of the bacterial OmpF porin (Dorset *et al.*, 1983) and bacterial PhoE porin (reviewed in Kühlbrandt, 1992). It must, however, be considered that the stability of a membrane protein is affected by the fatty acid chain length, as this controls the extent to which the membrane spanning region of the protein is protected against coming into contact with the aqueous phase. Thylakoid lipids contain almost exclusively C16 and C18 fatty acids (see Barber, 1987b). Using any of the saturated C16 or C18 lipids (listed in Table 7.2) would require the use of incubation temperatures greater than 40°C. This is clearly very high and likely to cause protein denaturation. Thylakoid membranes are known to be largely unsaturated and fluid at much lower temperatures, even though the fatty acid chains are C16 and C18 in length. This makes them possible candidates for use in two-dimensional crystallisation trials. Their use would have the additional advantage that specific protein:lipid interactions, which may be important in protecting protein structure (Marsh, 1990), can be incorporated into the experiment. To determine the possibilities of using thylakoid lipids in two-dimensional crystallisation trials, their structures are presented in the next section.

7.1.3.3 Thylakoid lipid composition

Thylakoid membranes consist of five polar lipid types. Phosphatidyl glycerol and phosphatidyl choline were only found to be present in small amounts (Wintermans, 1960). A more detailed analysis of *Vicia faba* thylakoid membranes identified monogalactosyldiacyl glycerol (MGDG), digalactosyldiacyl glycerol (DGDG), sulphoquinovosyldiacylglycerol, phosphatidyl glycerol and phosphatidyl choline to be present (Gounaris, 1983). All of them except MGDG are bilayer forming lipids. Above -30°C , MGDG is in the liquid-crystalline phase (Shipley *et al.*, 1973) and forms hexagonal type II structures (Figure 7.3)(Rivas & Luzzatti, 1969; Shipley *et al.*, 1973; Sen *et al.*, 1981; Gounaris *et al.*, 1983b). Below this temperature MGDG prefers a lamellar organisation. Despite the fact that MGDG is the most abundant thylakoid lipid, there is no evidence to suggest that this membrane, contains non-bilayer structures under normal conditions (Gounaris *et al.*, 1983c). Non-bilayer structures have only been observed in thylakoid membranes under extreme conditions, such as heat stress (Gounaris *et al.*, 1984) or protein denaturation (Machold *et al.*, 1977).

The function of MGDG in the thylakoid is not clearly understood as the membrane has a bilayer structure. However, it has been suggested that its shape makes it ideal for packing large multi-peptide complexes (Murphy, 1982). Total thylakoid extracts lose their bilayer configuration if the pH is lowered or as the cation levels are raised (Gounaris *et al.*, 1983a).

The head group structures of these thylakoid lipids are given in Table 7.3, together with their charge properties and the percentage proportions in which they occur in *Vicia faba* thylakoid membranes. It should be noted that in all cases the lipid head groups are linked to glycerol at the 3-sn position.

Table 7.3 Thylakoid polar lipid head group structure and properties

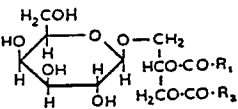
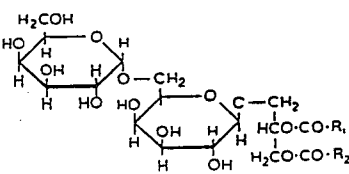
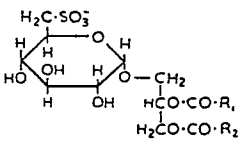
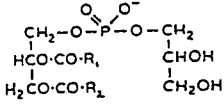
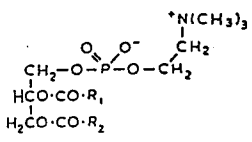
Table of Head groups	Head group Structure	Charge properties	Charge at physiological pH	Percentage of total <i>Vicia faba</i> thylakoid lipid (%)
Monogalactosyl diacyl glycerol		neutral		50.7
Digalactosyldiacyl glycerol		neutral		27.4
Sulpholipid		charged		14.4
Phosphatidyl glycerol		charged		5.1
Phosphatidyl choline		Zwitterionic	-ve	2.4

Table 7.3 adapted from Gounaris, (1983).

Analysis of the fatty acid units isolated from *Vicia faba* thylakoid lipids showed there to be considerable heterogeneity in the degree of saturation. In all cases the fatty acid chains contained 16 or 18 carbon atoms, as is shown in the Table 7.4.

Table 7.4 Fatty acid composition of polar thylakoid lipid classes of broad bean (*Vicia faba*) thylakoid membranes

Lipid class	Fatty acid (mole %)					
	16:0	16:1	18:0	18:1	18:2	18:3
Monogalactosyldiacyl glycerol	4.1	-	1.1	3.3	10.7	80.7
Digalactosyldiacyl glycerol	6.9	-	2.0	2.2	7.2	81.7
Sulpholipid	29.7	-	7.1	17.6	7.3	38.4
Phosphatidyl glycerol	21.8	5.0	5.3	15.0	22.5	30.3
Phosphatidyl choline	36.3	-	8.0	17.0	18.6	20.0

Table 7.4 was taken from Gounaris, (1983).

7.1.3.3.1 MGDG and DGDG

The structure of MGDG (1,2-diacyl-(β -D-galactopyranosyl (1'-3))-sn-glycerol) and DGDG (1,2-diacyl- α -D-galactopyranosyl-(1'-6')-(β -D-galactopyranosyl (1'-3))-sn-glycerol) were first defined by Carter *et al.*, (1956). MGDG and DGDG were found to be identical in composition in a number of different species (Sastry & Kates, 1964a; Sastry & Kates, 1964b; Roughan & Batt, 1969). In all cases they were found to be mainly associated with photosynthetic tissues. The fatty acids associated with both MGDG and DGDG isolated from different plant species fall into two categories, with the *Solanaceae*, *Brassicaceae* and some minor families being characterised by the presence of both C16:3 and C18:3 (Jamieson & Reid, 1971). In other species the galactolipid fatty acid composition (Table 7.4) was shown to be almost exclusively C18:3; the double bonds being of the *cis* configuration (Roughan, 1970; O'Brien & Benson, 1964). MGDG isolated from C18:3 plants was predominantly found to be associated with two linolenoyl (C18:3) moieties. In contrast, the DGDG head group was usually linked to either two linolenoyl chains or formed a 1-palmitoyl (C16:0)/2-linolenoyl (C18:3) species (Nishihara *et al.*, 1980). C16:3 plants contained a high proportion of hexa-decatrienoic (C16:3) fatty acid, esterified to the C-2 position, and were exclusively associated with the MGDG (Rullkötter *et al.*, 1975; Nishihara *et al.*, 1980). 80% of fatty acids present in MGDG and DGDG are of the C18:3 type.

Studies of the surface active properties of MGDG and DGDG have shown that these two galactolipids show tighter head group packing than phospholipids, binding identical fatty acid chains (Bishop *et al.*, 1980; Oldani *et al.*, 1975; Demel *et al.*, 1972; Sen *et al.*, 1981). This closer head group association is probably due to increased hydrogen bonding between neighbouring galactolipid than is observed between adjacent phospholipids. The similar charges, carried by adjacent phospholipids,

would be expected to induce electrostatic repulsion unless screened by oppositely charged ions in solution, which are able to form salt bridges.

7.1.3.3.2 Sulphoquinovosyldiacylglycerol

Sulphoquinovosyldiacylglycerol was first reported by Benson *et al.*, (1959). Its full name is 1,2-diacyl-6-sulpho- α -D-glucose. The head group structure of this lipid is given in Table 7.3. The sulphonic acid group gives this lipid its negative charge. In most plant species the lipid is composed mainly of palmitoyl (C16:0)/linolenoyl (C18:3) or palmitoyl (C16:0)/linoleoyl (18:2) (Nishihara *et al.*, 1980; Burns *et al.*, 1977) fatty acid chains. Palmitic acid (C16:0) is present in large amounts in sulphoquinovosyl diacylglycerol, isolated from *Vicia faba* (Gounaris *et al.*, 1983a).

The density of sulphoquinovosyldiacylglycerol monomer packing has been shown to increase as the degree of fatty acid saturation increases (Bishop *et al.*, 1980).

7.1.3.3.3 Phosphatidyl glycerol

Although phosphatidyl glycerol (1,2-diacyl-sn-glycerol-3-phosphoryl-l-sn-glycerol), isolated from photosynthetic tissues binds a range of C16 and C18 fatty acids (Table 7.4) it contains a fatty acid not found in any other thylakoid lipid. Trans-3-hexadecanoic acid (C16:1) was found esterified to the C2 position in spinach (Haverkate & van Deenen, 1965) and broad bean (Harwood, 1980). Palmitic acid (C16:0) is present in large amounts in phosphatidyl glycerol, isolated from *Vicia faba* (Gounaris *et al.*, 1983a). Phosphatidyl glycerol also contains the 3-trans-16:1 isomer, which is usually found esterified to the C-2 position. Most of the trans-acid-containing phosphatidyl glycerol molecules were associated with linolenic acid (C18:3) at the glycerol C1 position. This pattern of fatty acid association with glycerol is the reverse of that usually seen in phospholipids isolated from animals, where the unsaturated acids are mostly present in the C2 position (Harwood, 1980).

The density of phosphatidyl glycerol monomer packing has been shown to increase as the degree of fatty acid saturation increases (Bishop *et al.*, 1980).

7.1.3.3.4 Phosphatidyl choline

Phosphatidyl choline (1,2-diacyl-sn-3-phosphorylcholine) is only present in the thylakoid membrane in small amounts. Its head group is zwitterionic in nature, due to the quaternary ammonium group. A number of C16-C18 fatty acids make up the hydrophobic portion (Table 7.4). The most saturated fatty acids appear to be bound to the C-1 position (Devor & Mudd, 1971; Gurr & Brown, 1970). Thylakoid phosphatidyl choline was found to be similar in composition to that isolated from non-photosynthetic tissues (Nishihara & Kito, 1978; Nishihara *et al.*, 1980).

7.1.4 Detergents

During dialysis lipid-detergent mixed micelles and protein-detergent complexes are induced to merge into sheets. The detergent is then removed from the system altogether by dialysis. If detergent micelles are to fuse, they must interact strongly with one another. The slight increase in their hydrophobicity, makes them more likely to fuse with one another (Section 7.1.1). The interaction between detergent micelles is strongly temperature-dependent (Zulauf, 1990). Micelles of polyoxyethylene detergents, such as Triton X-100, interact more strongly as their temperature is increased (Bueren & Gropmann, 1971; Schönfeldt, 1976). This is because the ether oxygen groups, in polyoxyethylene-based detergents, become less hydrated as the temperature is increased, thus making micelle fusion more energetically favourable. In contrast, OG micelles merge more readily as the temperature is decreased.

The growth rate of two-dimensional crystals is dependent on the critical micelle concentration of the detergent. The higher the CMC, the more easily the detergent removal will be. The CMC value is a characteristic property of each detergent (Zulauf, 1990). Slow detergent removal seems to favour the development of ordered two-dimensional arrays (reviewed in Kühlbrandt, 1992).

7.1.5 Ionic strength and pH

Two-dimensional crystal formation can be improved by reducing electrostatic repulsion forces between the polar regions of a two-dimensional crystal. This can be achieved by keeping the pH close to the isoelectric point (Miller & Jacob, 1983) of the protein or by increasing the ionic strength of the solution (Ford *et al.*, 1990; Dekker *et al.*, 1990). The former reduces electrostatic repulsion between adjacent complexes, while the latter approach supports the formation of salt bridges. Both approaches will induce protein aggregation, by enhancing the hydrophilic interactions between adjacent polar domains. However, it must be noted that if the ionic strength is increased too much, membrane stacking can occur, as the repelling surface charges of two parallel membranes are attracted to oppositely charged ions in the solution between them (Kühlbrandt, 1984). Indeed, thylakoid membranes have been shown to undergo stacking, with increasing levels of cations added to screen surface negative charges (Barber & Chow, 1979). Such stacks are difficult to analyse and so conditions avoiding their formation are favoured.

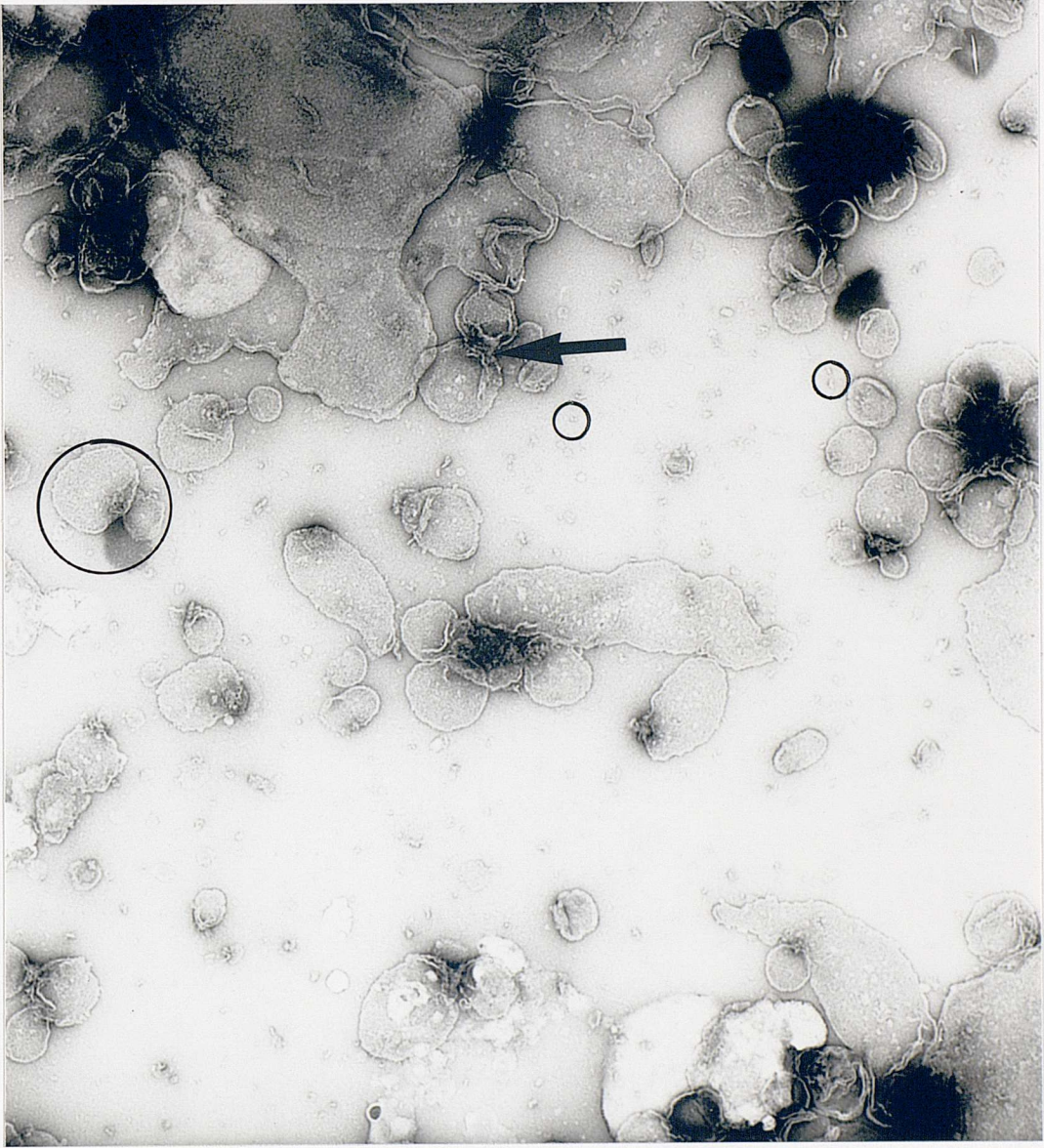
Glycerol can be used to disrupt the hydration shell surrounding detergent-protein complexes (Kühlbrandt, 1992). Merging of these complexes is increased because the disruption of the hydration shell results in a reduction of the solvents dielectric constant. This in turn allows stronger van der Waals attraction between adjacent protein-detergent complexes.

7.2 Results

7.2.1 The effect of lipid:protein ratio on the formation of pea HTGP core/DMPC sheets during initial two-dimensional crystallisation trials

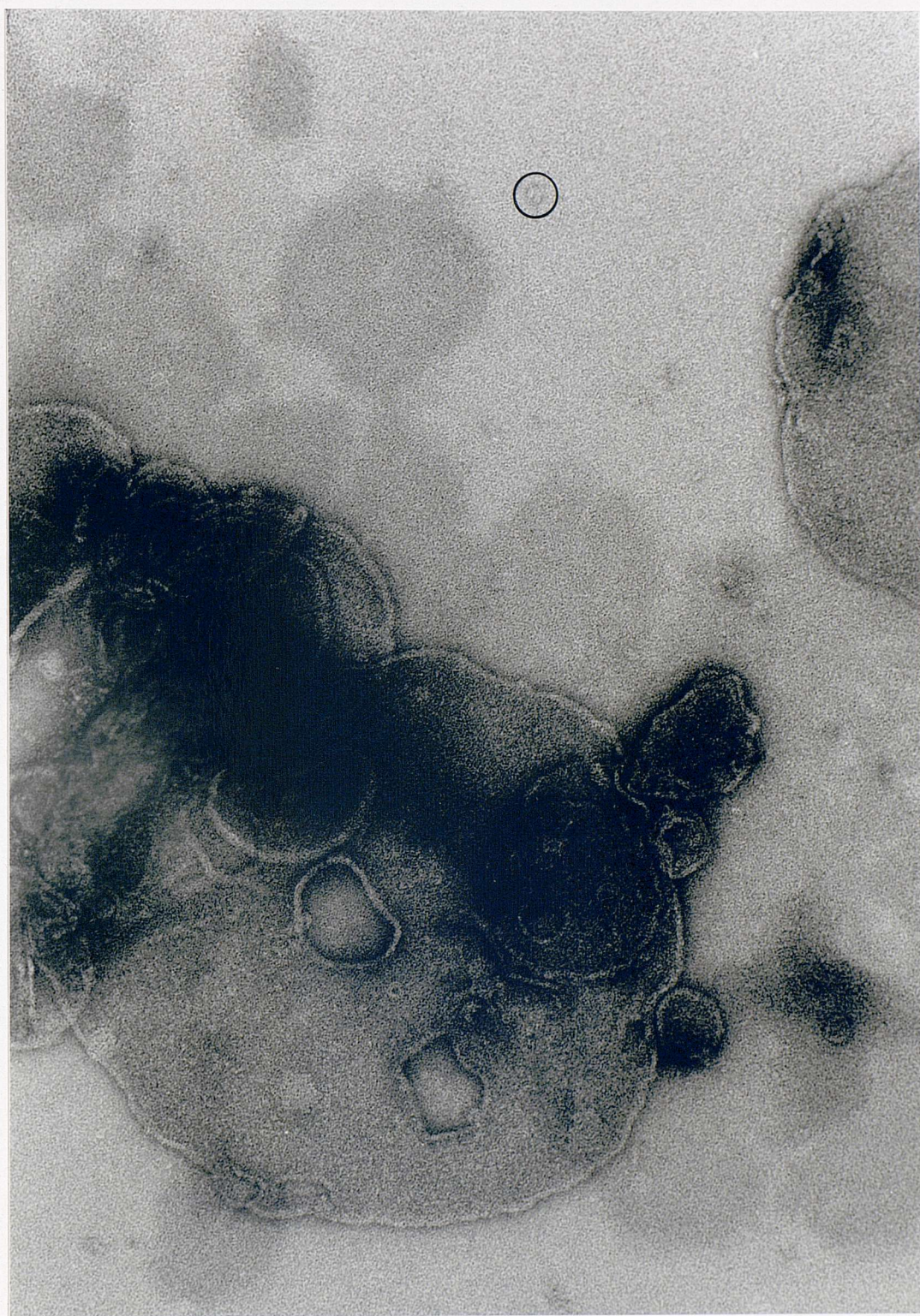
This experiment aimed to determine whether varying the ratio of DMPC:HTGP core during two-dimensional crystallisation trials, resulted in changes in the formation of HTGP core-DMPC sheets. PSII cores isolated from pea BBYs using HTGP were used in this experiment. They consisted of CP47, CP43, D2, D1, cytb559, the 33kDa extrinsic polypeptide, small amounts of CP29, CP26, LHCII and associated low molecular weight polypeptides. These cores were solubilised in 2mM DM and their spectra under these conditions are shown in Figure 7.8. DMPC was added to DM solubilised PSII to give molar DMPC:PSII core ratios of 5000:1, 1000:1 and 500:1. The samples were then dialysed (at 27°C for 24h) against 50mM Mes pH6.1, before being subjected to a 37°C heat shock (1h), to facilitate the formation of ordered PSII core arrays. Cloudiness was taken as an indication of lipid sheet formation. Figure 7.4 shows an electron micrograph of a sample having a DMPC:PSII core ratio of 5000:1. Collapsed vesicles, which have started fusing into sheets are visible. The light spots (approximately 20nm in diameter) in the vesicles and lipid sheets may be PSII core complexes. Discrete particles (Small circles, Figure 7.4), of similar size (20-40nm) are visible in the aqueous phase. A control sample lacking the PSII, would have to be analysed to confirm whether these particles and the light spots in the forming sheets and vesicles correspond to this complex.

All three trials were incubated for a further two days at 27°C, before being subjected to another 37°C heat shock (2h). At this stage, all three samples had a cloudy appearance. The sample containing a 5000:1 lipid:protein ratio showed sheet formation (Figure 7.5), but the increased incubation time did not appear to improve the packing order from that seen after one day (Figure 7.4). Similar sheets were found in the samples having a 1000:1 and 5000:1 lipid:protein ratio (Figures 7.6 and 7.7). The largest sheets were seen in the samples having a 500:1 lipid:protein ratio. In no case were ordered arrays detected.

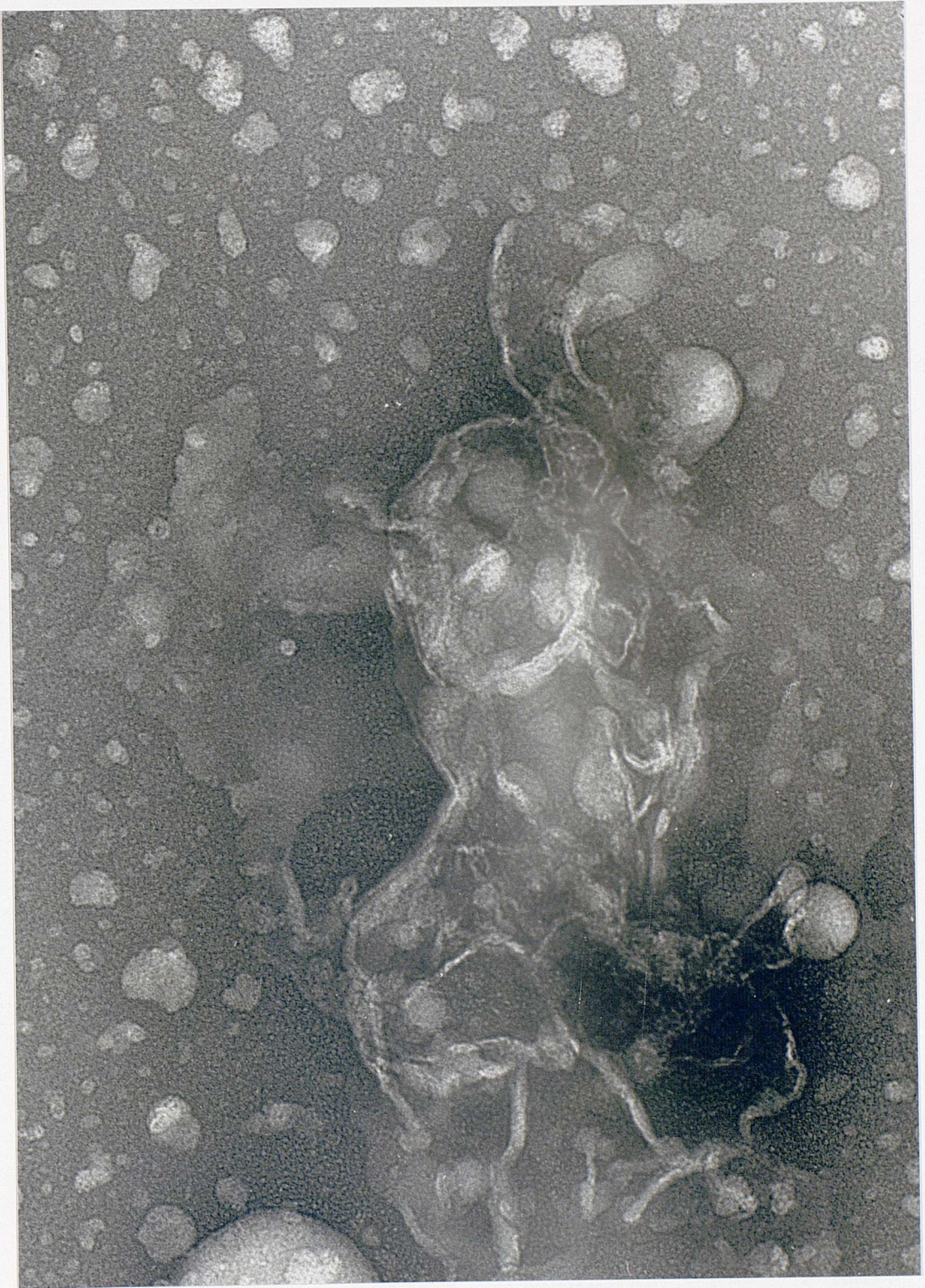


500nm

Figure 7.4 DMPC vesicles and bilayers in a sample containing a DMPC:pea HTGP core ratio of 5000:1 after dialysing for 24h at 27°C and 1h at 37°C. The particles marked by the small circles have a diameter of 20-40nm and may be detergent solubilised PSII core complexes. The larger circle indicates a collapsed vesicle possibly containing protein (light spots). Vesicles are seen to fuse into sheets (→).

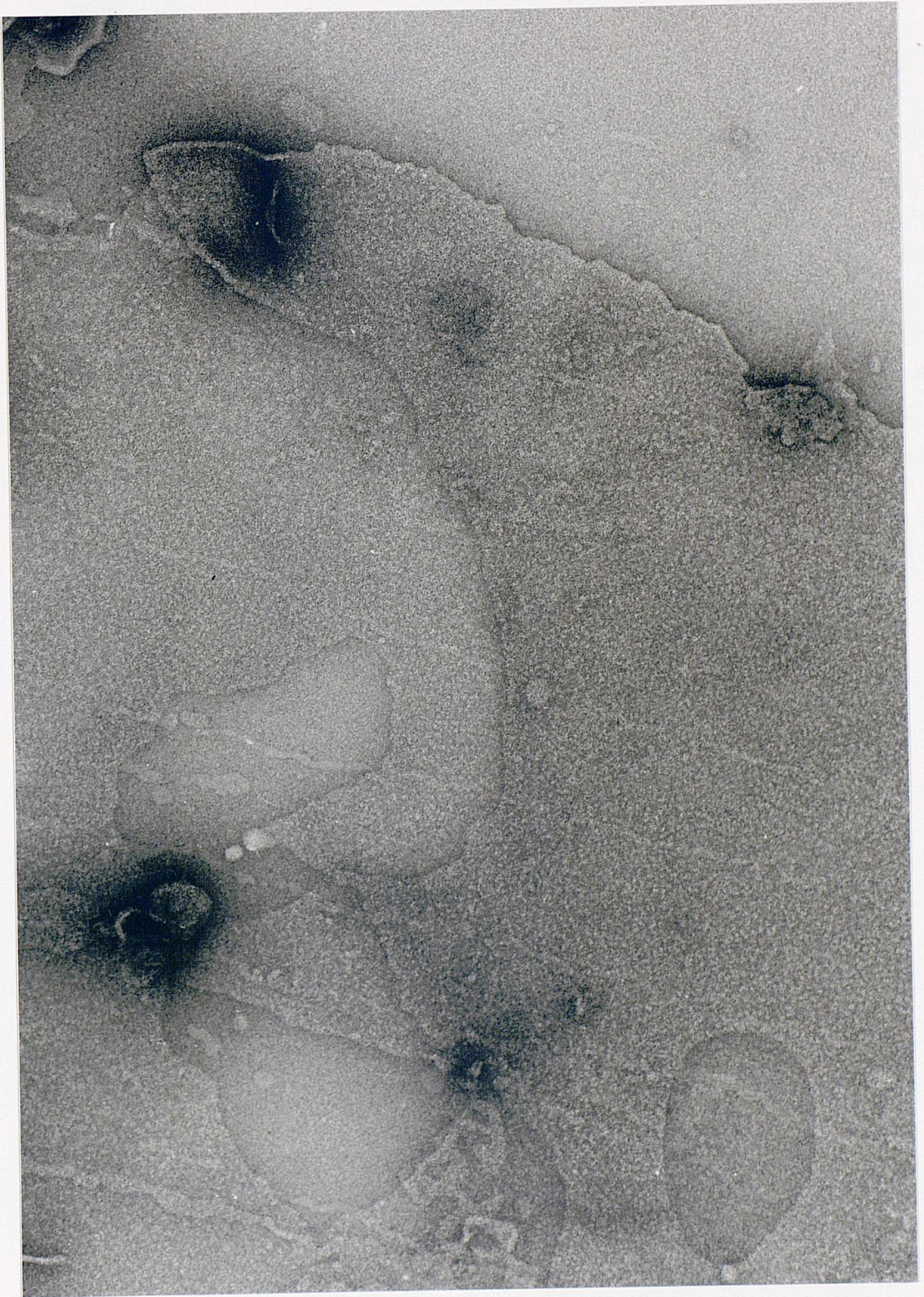


Figures 7.5 DMPC vesicles and bilayers in a sample containing a DMPC:pea HTGP core ratio of 5000:1, after dialysing for 72h at 27°C and 3h at 37°C. The circled particles measure approximately 20nm in their largest dimension, suggesting that they may be PSII core complexes. Although lipid sheets and vesicles are visible, the resolution of this electron micrograph is not sufficiently high to allow the detection of protein complexes within such membrane structures.



100nm

Figures 7.6 DMPC vesicles and bilayers in a sample containing a DMPC:pea HTGP core ratio of 1000:1, after dialysing for 72h at 27°C and 3h at 37°C. Although lipid sheets and vesicles are visible, the resolution of this electron micrograph is not sufficiently high to allow the detection of protein complexes within such membrane structures.



100nm

Figures 7.7 DMPC vesicles and bilayers in a sample containing a DMPC:pea HTGP core ratio of 500:1, after dialysing for 72h at 27°C and 3h at 37°C. Distinct lipid sheets are visible. It is possible that the light spots in these sheets are embedded membrane proteins.

7.2.2 Stability trials

The removal of detergent from a mixture of DM, DMPC and PSII cores supported protein/lipid bilayer formation (Section 7.2.1.). As DMPC has a transition temperature of 23°C, a temperature slightly above it (27°C) was used to maintain the liquid-crystalline phase. Prolonged incubation at such high temperatures may be enough to cause PSII degradation. To test this, pea HTGP cores were incubated at 4, 18 and 27°C for 11 days. Core spectra were measured at specific time points throughout the trial. Figure 7.8 shows the spectrum of an intact PSII core isolated from pea, according to De Las Rivas *et al.*, (1992a). These cores contained CP47, CP43, D1, D2, cytb559 and the 33kDa extrinsic subunit and small quantities of CP29 or LHCII. Their pigment composition was reported to be 38.4 chlorophyll a; 2.0 pheophytin:3.8 β -carotene: 1 cytb559. The 419nm and 437nm peaks correspond to the absorption maxima of pheophytin and chlorophyll a (De Las Rivas *et al.*, 1992a). The two pheophytin molecules are assumed to be associated with the D1-D2-cytb559 reaction centre complex (Kobayashi *et al.*, 1990; Gounaris *et al.*, 1990).

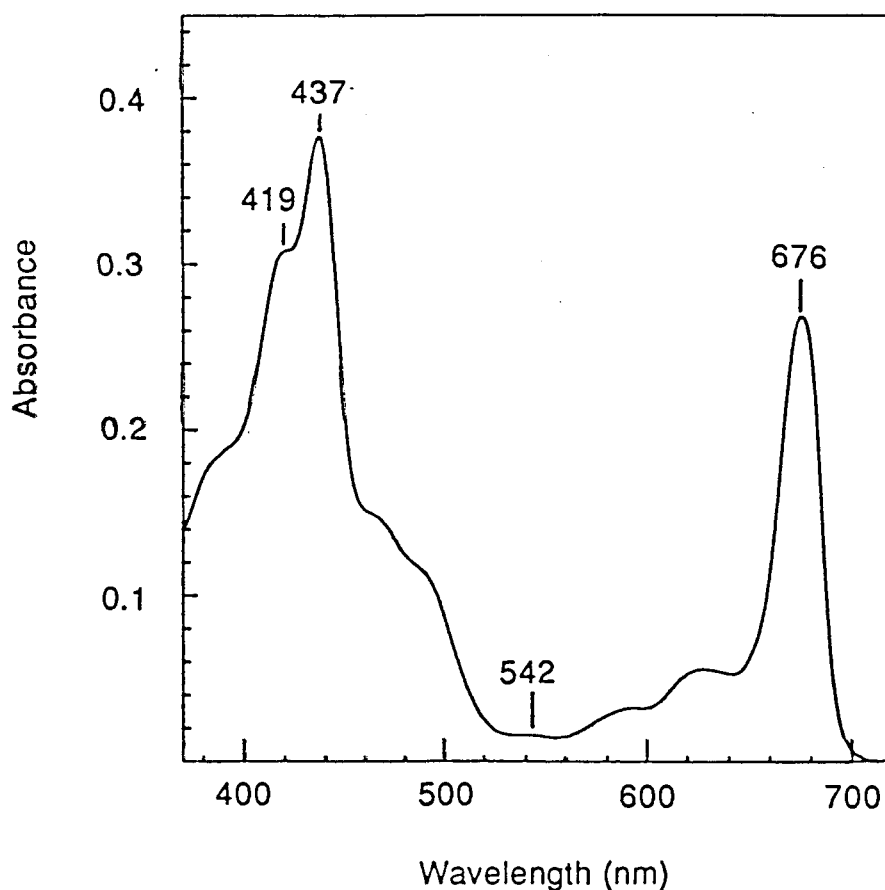


Figure 7.8 Spectrum of intact PSII core isolated from pea BBYs using HTGP.

Figure 7.8 shows the spectrum of PSII cores isolated from pea BBYs using HTGP, according to De Las Rivas *et al.*, (1992a). Pheophytin absorbs at 419 and 542nm. Chlorophyll a absorbs at 437 and 676nm in these core particles.

Figure 7.9 shows the spectral shift observed when cores are incubated at 4, 18 and 27°C, in a medium containing 0.3M sucrose. Single representative traces, taken from triplicate samples are shown for each time point to allow shift visualisation. The 417:435nm absorption ratio data, presented in Figure 7.11, are obtained from triplicate samples.

The increase in the 542nm peak size, which increases with the peak shift from 437 to 419 nm is indicative of pheophytinisation (the conversion of chlorophyll a to pheophytin), (Figure 7.9). Chlorophyll a was almost entirely converted to pheophytin after 11 days incubation at 27°C. Reducing the temperature to 18 and 4°C slows down the rate of pheophytinisation, with the least shift observed at the lowest temperature.

In order to try to combat the damage observed on incubating PSII cores at high temperatures for a prolonged period of time, samples were supplemented with 2.4M sucrose, so as to reduce heat induced protein unfolding. The spectra of samples incubated in the presence of 0.3M and 2.4 M sucrose, after an 11 day incubation, are compared in Figure 7.10.

At 27°C the rate of pheophytinisation is considerably reduced at high sucrose concentrations. A similar pattern emerged at 18 and 4°C, but became less pronounced as the peak shifts changed in the presence of 0.3M sucrose. The differences in pheophytinisation rate are shown in Figure 7.11, by plotting triplicate 419:437nm absorption ratios against time.

Increasing the sucrose concentration or decreasing the incubation temperature, reduced the 419:437 nm ratios from those obtained from samples incubated with 0.3M sucrose at 27°C.

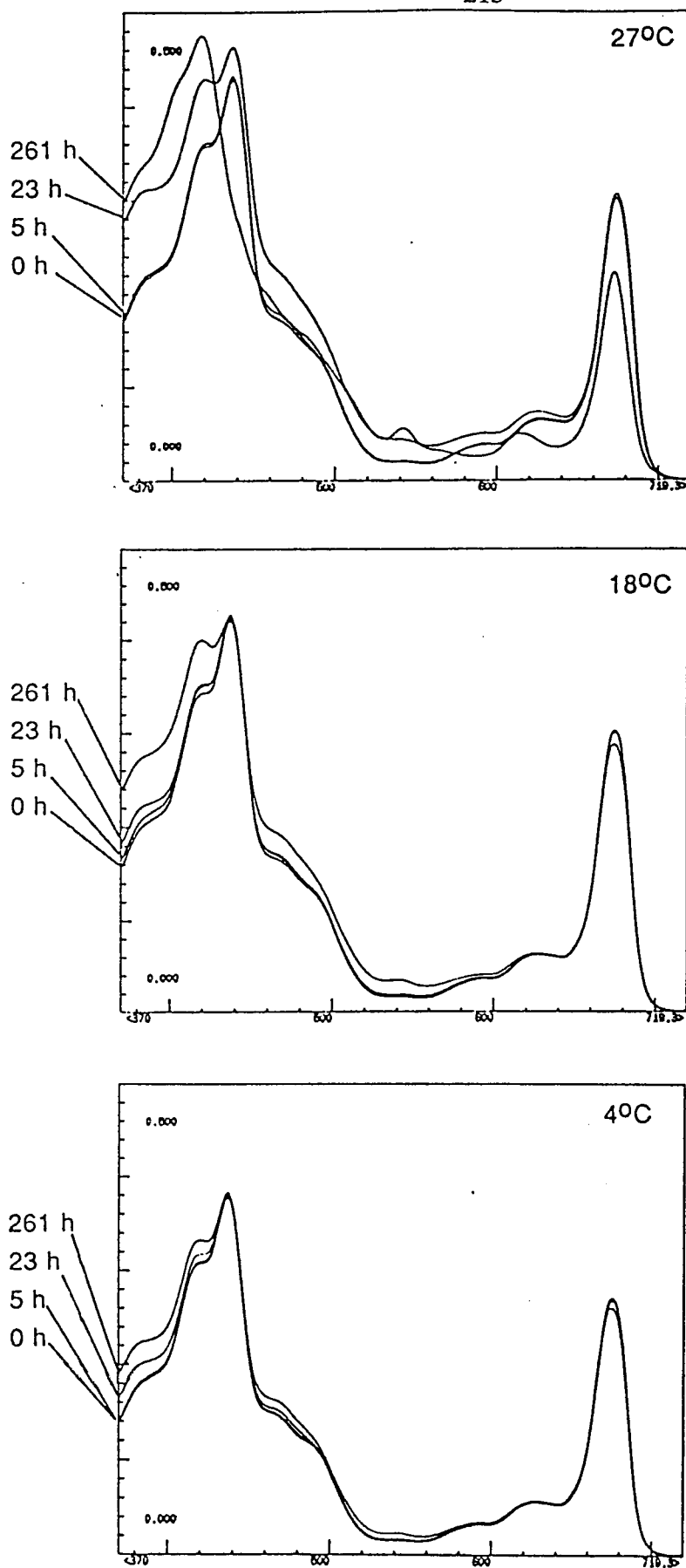


Figure 7.9 Changes in absorption spectra of pea HTGP cores during an 11 day incubation period, in the presence of 0.3M sucrose at 4, 18 and 27°C.

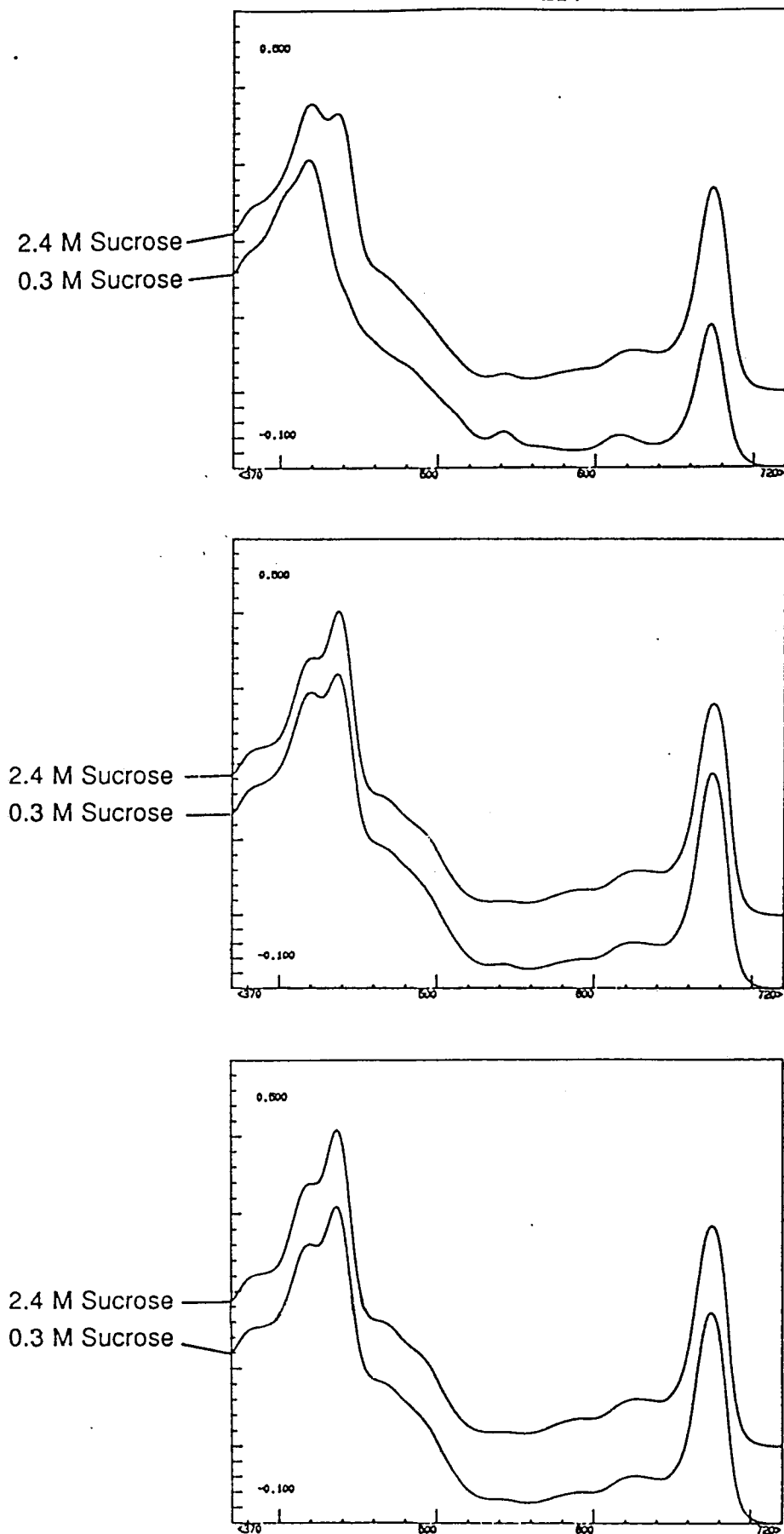


Figure 7.10 Spectra of pea HTGP cores after an 11 day incubation at 27, 18 and 4°C, in the presence of either 0.3 or 2.4M sucrose.

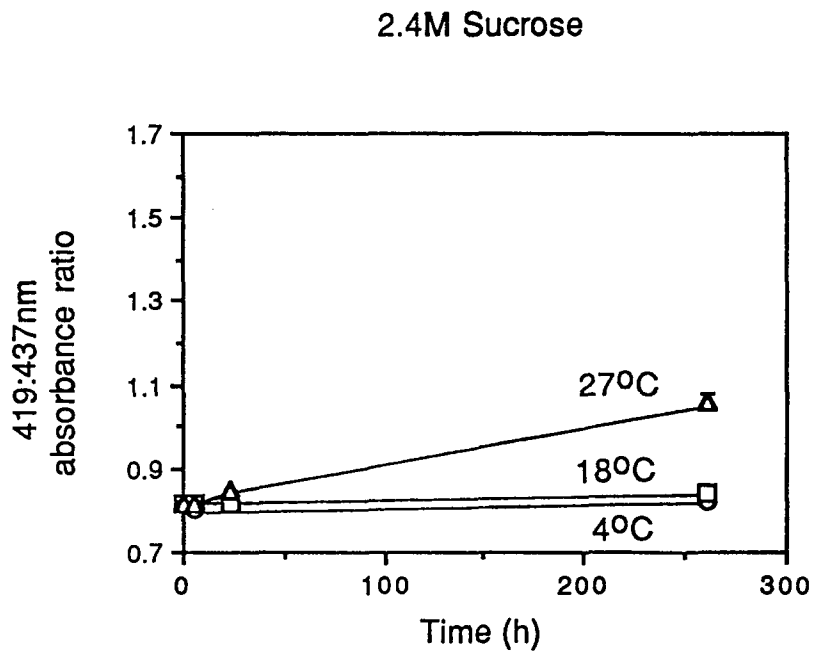
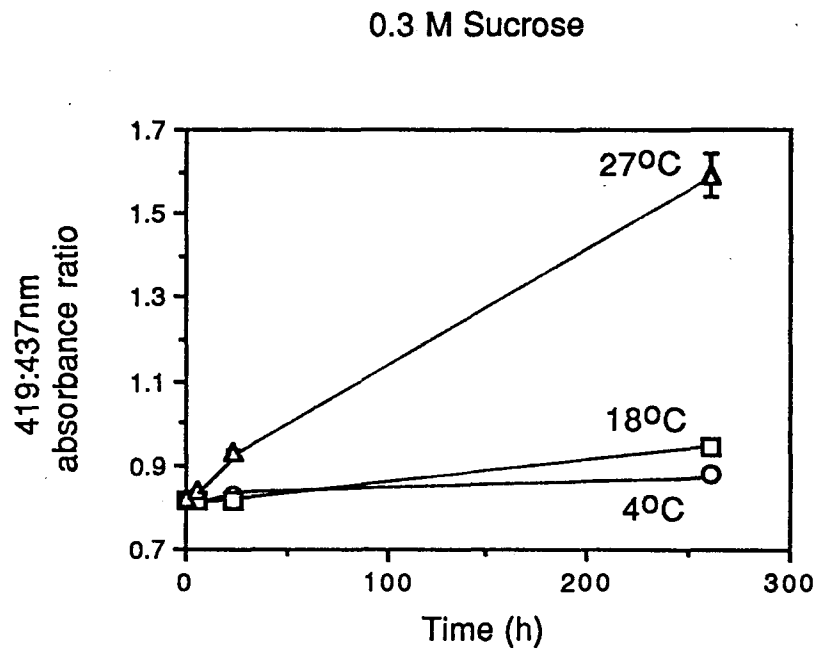


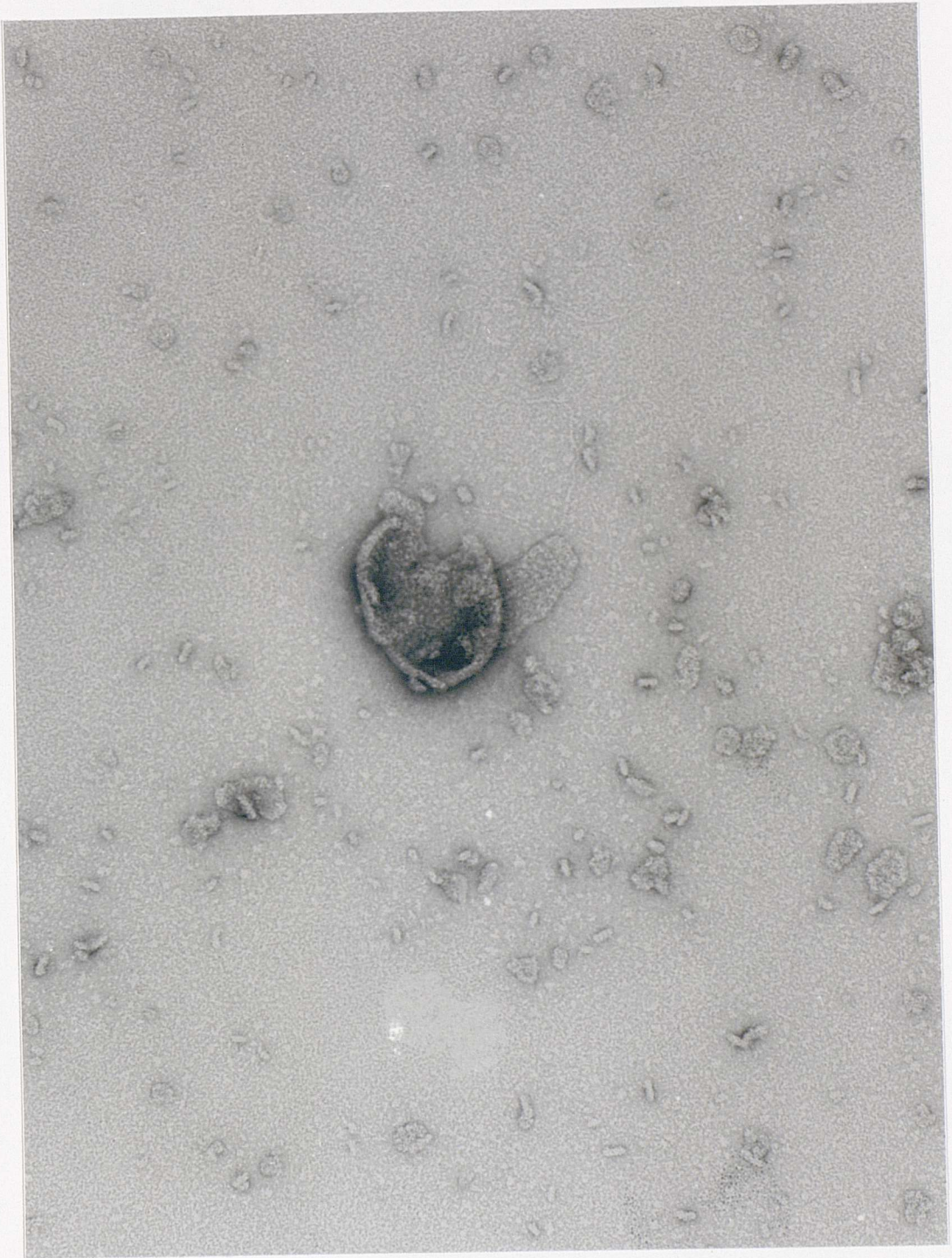
Figure 7.11 The change in 419:437nm absorption ratio of pea HTGP cores with time, in the presence of 0.3 and 2.4M sucrose.

7.2.3 Crude estimation of the amount of thylakoid lipid still associated with spinach HTGP and OG core preparations

The aim of this experiment was to determine whether PSII core preparations, isolated from spinach BBYs using HTGP (Chapter 3) and OG (Chapter 4), bound sufficient thylakoid lipid to allow sheet formation without supplementing with additional lipid. Figure 7.12 shows an electron micrograph of DM solubilised spinach HTGP cores (Chapter 3). Although, membrane-like fragments are visible, the presence of lipid can be more readily detected in the absence of detergent. Detergent removal was achieved by the addition of hydrophobic beads (Bio-beads SM7), which bind the hydrophobic portions of the detergent and so force the lipid and protein in the system to aggregate. If the lipid levels are low, the PSII cores are forced to precipitate, whereas the presence of large amounts of bilayer forming lipid support sheet formation in much the same way as the dialysis method discussed in the introduction to this chapter. Incubating the sample shown in Figure 7.12, with Bio-beads for 16h at 4°C, resulted in the formation of large sheets (Figure 7.13). This result shows that PSII cores isolated from spinach BBYs using HTGP, still contained too much thylakoid lipid to allow ordered two-dimensional crystal packing.

This experiment was repeated using DM solubilised spinach OG cores (Chapter 4). Prior to Bio-bead treatment (Figure 7.14), these PSII cores had a very similar appearance to those purified using HTGP (Figure 7.12). However, on detergent removal small two-dimensional crystals were obtained (Figure 7.15). Although the order was still irregular, the core particles could be seen to organise into rows. This result suggests that OG removed more thylakoid lipid than HTGP. The fact that ordering is observed, supports the view that the lipid still attached to the core is in the liquid-crystalline phase at 4°C and indicates the potential usefulness of thylakoid lipids in future two-dimensional crystallisation experiments.

Pure dimeric PSII (see Chapter 5), was also analysed using the Bio-bead detergent removal process. DM solubilised dimers are shown in Figure 6.1C. Detergent removal resulted in PSII core precipitation, which was difficult to photograph. This suggests that much of the lipid associated with OG solubilised cores, was stripped off during sucrose gradient purification of the PSII dimers.



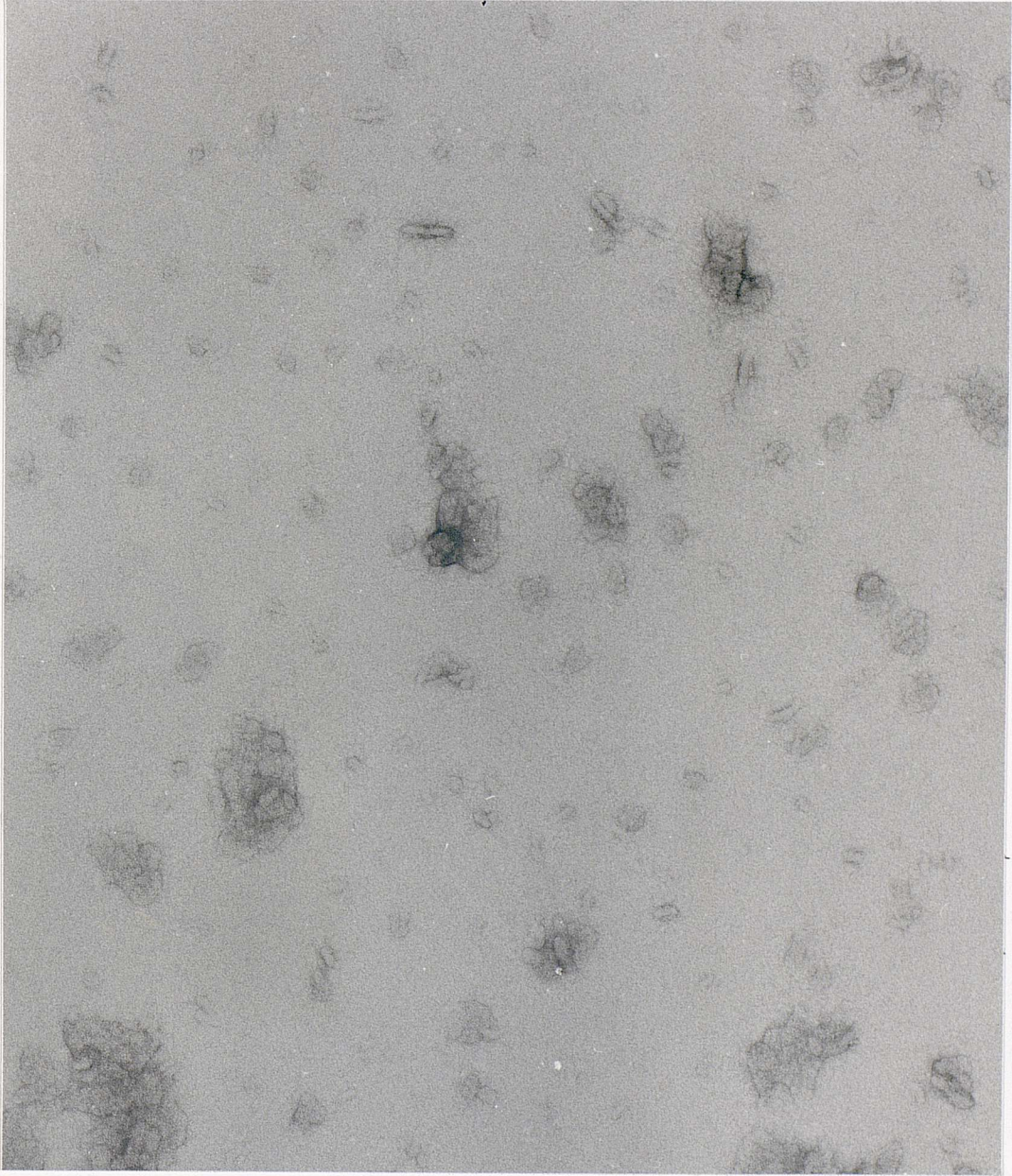
100nm

Figure 7.12 Electron micrograph of negatively stained HTGP cores. HTGP core preparations appear to contain some small membrane fragments as well as detergent solubilised complexes.



100nm

Figure 7.13 Electron micrograph of negatively stained detergent depleted HTGP cores. Depleting HTGP cores of detergent resulted in lipid sheet formation. The light spots in the membrane may represent membrane embedded protein complexes.



100nm

Figure 7.14 Electron micrograph of negatively stained OG cores.



200nm

Figure 7.15 Electron micrograph of negatively stained detergent depleted OG cores. Depleting OG cores resulted in lipid-protein bilayer formation and yielded some small two-dimensional crystals as shown above.

7.3 Discussion

To date three-dimensional crystallisation trials have not, as yet, yielded highly ordered three-dimensional crystals of PSII. This is, in part, due to the instability of the PSII complex (Adir *et al.*, 1992; Fotinou *et al.*, 1993) on isolation. Lipids associated with membrane proteins can cause reduced three-dimensional crystal quality (Michel, 1991), but the harsh detergent treatments required to remove them can damage membrane proteins. In contrast, the two-dimensional crystallisation approach does not require the use of lipid free PSII complexes; although highly homogeneous particles are a prerequisite if ordered arrays are to be obtained (Kühlbrandt, 1992).

Higher plant PSII complexes are embedded in the granal stacks of the thylakoid membrane, where they are found to be closely associated with one another. The fact that PSII complexes are able to undergo such tight packing *in vivo*, suggests that steric and electrostatic repulsion between individual cores is minimal. This is, in itself, an important finding as it suggests that purified PSII complexes can pack into ordered arrays in reconstituted bilayers. LHCII (Wang & Kühlbrandt, 1991), bacterial porins (Sass *et al.*, 1989; Jap *et al.*, 1991) and bacteriorhodopsin (Baldwin & Henderson, 1984; Baldwin *et al.*, 1988), were all isolated from membranes in which they were highly enriched and all yielded highly ordered crystals.

Ordered two-dimensional PSII arrays, grown by delipidating the stacked grana regions (Bassi *et al.*, 1989; Holzenburg *et al.*, 1993; Lyon *et al.*, 1993), have only yielded a 17Å resolution map (Lyon *et al.*, 1993). The low resolution is probably partially due to the heterogeneity of the sample. Indeed, all three preparations contained a large number of polypeptides, many of which appeared not to be present in non-stoichiometric amounts, suggesting that they may consist of a heterogeneous population of PSII complexes.

Contamination and complex heterogeneity are problems inherent to the delipidation approach of growing two-dimensional crystals. To eliminate these, the experiments presented here were based on reconstituting protein-lipid bilayers from purified and detergent-solubilised cores. PSII cores isolated from pea according to the method of De Las Rivas *et al.*, (1992a), contained CP47, CP43, D1, D2, cytb559 and the 33kDa extrinsic subunit, but only very small quantities of CP29 or LHCII. Their high chlorophyll a:b ratio and subunit composition showed them to be both more homogeneous and smaller than those used by Bassi *et al.*, (1989), Holzenburg *et al.*, (1993) and Lyon *et al.*, (1993).

Ideally a saturated lipid, having a low crystal/liquid-crystal transition temperature would be mixed with the pure detergent-solubilised protein to allow ordered two-dimensional crystals to be formed. In addition, the acyl chain length of the lipid would be the same as that of the lipids with which the complex is associated *in vivo*. Optimal chain length match is best able to protect the membrane spanning region from coming into contact with the aqueous phase. Unfortunately, acyl chain length and saturation have to be balanced with the transition temperature of the lipid. Dimyristoyl phosphatidyl choline is commonly chosen to balance these factors (Sass *et al.*, 1989; Ford *et al.*, 1990; reviewed in Kühlbrandt, 1992). It consists of two saturated C14 fatty acids, attached to the phosphatidyl choline head group shown in Table 7.1. Although the acyl chain length (C14) is shorter

than that of any of the thylakoid lipids (C16 and C18), this is compensated for by the fact that it is saturated, making the lipid less susceptible to oxidation. Saturated C16 or C18 lipids cannot be used for the formation of bilayers as their transition temperatures are high enough to denature most proteins (Table 7.2). In addition, it should be noted that a 2min heat shock (55°C) induces the dissociation of the 33kDa extrinsic polypeptide from the rest of the complex (Chapman, unpublished results). As the 33kDa polypeptide is required to maintain oxygen evolution ability of higher plant PSII cores *in vitro*, the use of saturated C16 or C18 is not an option if the intact complex is to be crystallised.

Initial two-dimensional crystallisation trials using dimyristoyl phosphatidyl choline produced lipid-protein sheets, the first step towards the formation of ordered two-dimensional crystals. PSII cores (De Las Rivas *et al.*, 1992a) were not tightly packed into the bilayers formed using 5000:1, 1000:1 and 500:1 molar DMPC:PSII ratios (Figures 7.5-7.7). These results indicate that all three trials contained sufficient lipid to completely solubilise the cores. In the presence of excess lipid, cores were not forced to aggregate. However, it was noted that the largest sheets were found at the lowest DMPC concentrations.

The finding that sheets were only formed after incubating samples at 27°C for three days, was a concern as these conditions might cause the PSII complex to denature. To test this, a series of time course experiments were set up in which spectral shifts of DM solubilised cores (isolated according to De Las Rivas *et al.*, 1992a), incubated at 4, 18 and 27°C, were recorded. The spectra, which were measured in the presence of 0.3M sucrose (the starting concentration of a dialysis-type two-dimensional crystallisation trial), were measured over an 11 day time period (Figure 7.9). At 27°C, a clear peak shift from 437nm (absorption peak maximum of chlorophyll a) to 420nm (absorption maximum of pheophytin) was seen after 11 days. The simultaneous appearance of a peak at 542nm, is further confirmation that chlorophyll a was converted to pheophytin under these conditions.

The pheophytinisation process involves the loss of the Mg^{2+} ion from the porphyrin ring of chlorophyll a. Chlorophyll molecules are neutral lipids and are associated with the hydrophobic membrane spanning region of PSII. Mg^{2+} is a very highly charged ion and its release from the porphyrin ring is only likely to occur if it comes into contact with the polar aqueous phase or with an anion, within the hydrophobic region. Both scenarios are likely to require considerable conformational change. If the structure of intact PSII is to be elucidated, such structural changes are to be avoided. In part these changes could be reduced by lowering the temperature (Figure 7.9).

Protein unfolding and the dissociation of the 33kDa polypeptide from the complex may be the result of increased solvation at high temperatures. To test whether the observed spectral shifts might be due to such temperature-induced conformational changes, cores were incubated in the presence of 2.4M sucrose. Sucrose is a compatible co-solute. At high concentrations it competes for the water molecules, solvating the hydrophilic regions of the protein. In the absence of a co-solute, the degree of solvation is high, thus reducing van der Waals and electrostatic attractions, as well as the salt bridging between adjacent hydrophilic regions. Reduced attraction between adjacent hydrophilic loops, makes the whole complex more flexible. In doing so the ΔG of the unfolded state increases, making the

formation of the latter less energetically favourable. Sucrose, has been shown to stabilise PSII at high temperatures, as have other compatible co-solutes (Williams & Gounaris, 1992). Similar stabilisation of reaction centres was not achieved by this method and the authors concluded that increased core stability was due to improved binding of the 33kDa subunit.

The addition of 2.4M sucrose reduced the spectral shift rate (Figures 7.10 and 7.11) and could indicate the involvement of a temperature dependent conformation change step. The results presented here are too preliminary to assess the exact mechanism of the pigment damage, but do correlate well with those of Williams & Gounaris, (1992). If the temperature does indeed cause the loss of the 33kDa polypeptide from the core, the addition of lipid would not be expected to increase core stability at 27°C.

Faced with this problem there are two possible approaches to future two-dimensional crystallisation trials. The first involves the purification of thermostable PSII cores. Although PSII cores have been isolated from thermophilic organisms, procedures are laborious and yields usually low (Rögner, personal communication). Furthermore, there is little information available on the stability of detergent solubilised PSII cores, isolated from thermophilic organisms. However, isolated *Synechococcus vulcanus* (Shen *et al.*, 1992) membranes, containing oxygen-evolving PSII, are able to maintain oxygen evolution at high temperatures. Pure dimeric PSII cores have been isolated from *Synechococcus sp.* (Rögner *et al.*, 1987) and this approach may prove to be useful for the isolation of thermostable cores from *Synechococcus vulcanus*.

In my work, a second approach was taken. Instead of focusing on obtaining thermostable PSII cores, the usefulness of thylakoid lipids for two-dimensional crystallisation trials was investigated. Thylakoid lipids are to a large extent unsaturated (Table 7.4) and so are susceptible to oxidation, but they do have the advantage of being in the liquid-crystal phase at 4°C (see Barber, 1987b). The use of thylakoid lipids in two-dimensional crystallisation trials has the additional advantage that specific lipid-protein interactions found *in vivo*, can be satisfied *in vitro*. The main problem of using thylakoid lipids, is their susceptibility to oxidation. Hydroxyl radicals (OH[•]) are well known to stimulate lipid peroxidation. Mannitol and formate are both effective OH[•] scavengers. Superoxide radicals (O₂^{•-}) are insufficiently reactive to abstract H from lipids and its charged nature would prevent it from entering the hydrophobic portion of the membrane. The protonated form of O₂^{•-} (HO₂[•]) is more reactive and is able to oxidise linoleic acid by abstracting H. Histidine reacts with OH[•], singlet oxygen (¹O₂) and with superoxide (O₂^{•-}). Lipid peroxidation is reviewed in detail in Halliwell & Gutteridge, (1991).

Section 7.2.3 describes an initial study to determine whether HTGP and OG cores bind thylakoid lipid and if so, whether the amount is sufficient to support bilayer formation. In the presence of detergent, membrane fragments can be seen in both preparations (Figures 7.12 and 7.14). To test whether the lipid present was sufficient to support bilayer formation, the samples were then depleted of detergent to induce lipid-protein sheet formation. PSII cores, purified using HTGP (Chapter 3), contained sufficient lipid to allow bilayer formation (Figure 7.13). The cores did not form ordered arrays, suggesting that the lipid concentration was high enough not to force the complex into close

association. In contrast, PSII cores purified using OG (Chapter 4) formed slightly ordered, if somewhat small, arrays on detergent removal. This suggests that the OG treatment was more efficient at stripping lipid away from the PSII core complex than HTGP. Analysis of the lipid content of the latter core types, would provide information about the lipids most closely associated with these PSII complexes and would give a rough indication of the lipid:protein ratio required to obtain two-dimensional arrays.

Detergent removal resulted in the precipitation of the sucrose gradient-purified monomeric and dimeric PSII cores (Section 5.2), indicating that most of the thylakoid lipids associated with the other OG and HTGP cores were removed during the sucrose gradient purification step. Mixing detergent solubilised PSII dimers (CP47, CP43, D2, D1, cytb559, the 33kDa extrinsic polypeptide and associated low molecular weight subunits) with purified thylakoid lipids and subjecting the mixture to controlled detergent removal, seems to be a logical next step in the search for conditions yielding highly ordered two-dimensional crystals of PSII.

7.4 Conclusion

Highly purified dimeric PSII core complexes, consisting of the minimal number of subunits required for *in vitro* oxygen evolution (CP47, CP43, D2, D1, cytb559, the 33kDa extrinsic polypeptide and associated low molecular weight subunits) have been isolated. HTGP and OG cores have been shown to organise into two-dimensional arrays, on detergent removal in the presence of thylakoid lipids. Supplementing the pure PSII dimers, which do not appear to be associated with much lipid, with increasing amounts of DGDG or purified thylakoid lipid should allow the formation of ordered two-dimensional arrays. Purified DGDG and thylakoid lipid extracts are in a liquid-crystalline state at 4°C and so appear to be useful two-dimensional crystallisation trials at this temperature. The results show that these experiments can be done at 4°C, as all the thylakoid lipids are in their liquid-crystalline phase at this temperature. As thylakoid lipids are not saturated, antioxidants such as histidine should be included in such crystallisation trials.

Chapter 8 Three-dimensional crystallisation trials of PSII

8.1 Introduction

8.1.1 General introduction

In 1982 Michel reported three-dimensional crystals of the reaction centre of *Rhodospseudomonas viridis*. As a consequence of obtaining these crystals, it was possible to determine the atomic structure of this reaction centre at a resolution of 2.5Å (Deisenhofer *et al.*, 1984; Deisenhofer *et al.*, 1985; Michel *et al.*, 1986). This achievement was awarded the Nobel Prize in 1985. Analysis of the X-ray diffraction patterns of highly ordered crystals of the reaction centre of *Rhodobacter sphaeroides*, allowed the atomic structure of this complex to be determined at a resolution of 2.8Å (Allen *et al.*, 1987). X-ray diffraction analysis of three-dimensional PSI crystals has allowed the elucidation of its structure at 6Å (Krause *et al.*, 1993). The resolution of this structure was sufficiently high to allow the positions of the 4Fe-4S clusters (F_X , F_A and F_B), as well as 28 α -helices and 45 chlorophyll *a* molecules to be determined. Although, no three-dimensional PSII crystals had been reported at the start of the work presented in this thesis, some successes have been reported since. In 1992, Adir and co-workers reported crystals of oxygen-evolving core particles of unspecified subunit composition, which were able to diffract X-rays to a resolution of 7Å. As 6Å resolution X-ray diffraction patterns had proved sufficient to locate the iron sulphur centres in PSI, the 7Å diffraction pattern reported by Adir *et al.*, (1992) was tantalisingly close to allowing the identification of the Mn cluster location. The authors concluded that the relatively poor order of these crystals was at least in part caused by the heterogeneity of the preparation. Furthermore, the heterogeneity appeared to be related to subunit damage. Interestingly, the damaged PSII complexes, reported by Adir *et al.*, (1992), were purified from an isolated monomeric core complex. Chapter 5 shows that in my hands PSII core monomers are more unstable than their dimeric counterparts. In addition, Fotinou *et al.*, (1993) found that CP47, CP43, D2, D1, cytb559 complexes underwent subunit damage, even after they had packed into ordered crystal lattices. The results of Adir *et al.*, (1992) and Fotinou *et al.*, (1993) clearly demonstrate that PSII subunit stability directly affects crystal quality.

At the start of the crystallisation trials presented in this chapter, the highly purified and intact dimeric (CP47, CP43, D2, D1, cytb559, the 33kDa extrinsic subunit and associated extrinsic polypeptides) core complexes characterised in Chapter 5, were not available. As a result, crystallisation trials were set up using less pure core complexes (HTGP, OG and sucrose gradient purified PSII core monomers), as well as PSII reaction centres (D2, D1, cytb559 and *psbI* gene product), RC-47 (CP47, D2, D1, cytb559 and *psbI* gene product) and CP43. Using this approach it was possible to identify conditions, which might be suitable for future crystallisation trials using more homogeneous, PSII dimer stocks. The results of these trials are presented in Section 8.2.

Section 8.1.2 outlines some of the factors involved in vapour diffusion induced membrane protein crystal formation.

8.1.2 Membrane Protein solubility

The crystallisation trials presented were mostly set up using the automated batch system developed at Imperial College (Chayen *et al.*, 1990)(Section 2.10). Using this system, proteins, precipitants, detergents, buffers and additives can be dispensed into oil-filled trays and left to evaporate slowly. As the droplets evaporate, the solute concentration is raised to its saturation level, thereby inducing protein crystallisation or precipitation.

At the starting point of any protein crystallisation trial, fully solubilised protein is required (McPherson, 1991). Membrane proteins do not dissolve in water because of the hydrophobic nature of their membrane spanning regions. Their insolubility is due to two reasons: firstly, the strong van der Waals attraction between the membrane spanning regions of individual protein subunits and complexes, makes their aggregated state energetically favourable, when in contact with the aqueous phase. Secondly, the surface tension between the hydrophobic (transmembrane region) and hydrophilic (aqueous phase) makes their interaction energetically unfavourable. As a result, the proteins aggregate, to reduce the surface area of the transmembrane regions in contact with the aqueous phase.

Detergents are used to solubilise membrane proteins. They are amphiphiles, and thus by definition, have a hydrophilic and a hydrophobic end. When mixed with isolated membrane proteins in an aqueous solution, detergents form a belt around the transmembrane region (Michel, 1991). The non-polar ends of the detergent molecules are in contact with the transmembrane region and the charged head groups interact with the aqueous phase. As the transmembrane helices of a protein, protected by detergent, are no longer in contact with water, a protein:detergent complex has a lower surface tension than a membrane protein in the absence of detergent. Van der Waals attractions between adjacent detergent solubilised membrane proteins, in close proximity to one another, are also shielded by the hydrated detergent belt, and so prevent precipitation.

At the borderline of their solubilities, discrete membrane protein-detergent complexes can be considered to be small particles dispersed in water. As such, they have considerable similarity to colloidal solutions. As will be discussed below colloids are kinetically, but not thermodynamically stable. To explain this the Gibb's free energy function must be brought into play (see Equation 7.1, Section 7.1.1). As with any chemical reaction, the process of crystallisation occurs spontaneously only if the free energy of the system is reduced in the process. The fact that neither precipitate or crystals are immediately observed in solubilised crystallisation mixtures suggests that an energy barrier must be overcome before aggregation is able to proceed (McPherson, 1991). Let us consider the spontaneous aggregation phase observed after the initial energy barrier has been overcome. For aggregation to precede spontaneously in a saturated solution, ΔG must be negative and so the enthalpy (H) must either be reduced, or entropy (disorder) increased (Equation 7.1, Section 7.1.1). At first, it seems contradictory that the disorder of the system can be increased by ordering the protein-detergent units into a crystal lattice or precipitate. The reason is that the disorder of the system is the sum of the disorder of the protein-detergent units and the aqueous phase (Atkins, 1986). As crystals grow the

increased order of the packing protein-detergent units is more than compensated (in reduction of free energy terms) by the increase in disorder of water molecules. This is because each protein-detergent unit is coated by an organised water cage (solvation layer). As these protein-detergent units aggregate into a precipitate or crystal lattice, a large proportion of the solvating water molecules are displaced, releasing them into the disordered bulk water phase. This accounts for the overall entropy increase required to drive the crystallisation process forward.

As water evaporates from a vapour diffusion system, as in the micro-batch system, used in our experiments, the solubilising forces are overcome by the attractive forces. This corresponds to the nucleation phase. To understand what these attractive and solubilising forces are and how the free energy level of the system is increased, to overcome the free energy barrier described, colloid stability theory proves useful.

8.1.3 Attracting Forces

Let us consider protein-detergent complexes as individual colloid particles. Colloids have a very large surface area and are thermodynamically unstable because each of the monomers is surrounded by an ordered water cage (Section 8.1.2). The aggregation of discrete protein-detergent complexes into a disordered precipitate, or ordered crystal lattice, would lead to a reduction in the number of water molecules associated with its ordered solvation layer, and so result in an increase in entropy. Indeed, van der Waals forces would also be expected to aid aggregation. The energy of interaction between two individual atoms varies with their separation as $1/R_a^6$ (van der Waals dispersion energy), where R_a is the distance separating two atoms (Atkins, 1986). Clearly, these interactions decrease extremely rapidly with an increase in distance and would only take effect when the protein-detergent complexes are in close contact with one another (i.e. at saturated protein-detergent complex concentration). However, the sum of all the pair-wise van der Waals interactions describes the total interaction forces (i.e. van der Waals forces) between two separated colloid particles. The interaction between whole colloid particles is reduced as a function of $1/R_c^2$, where R_c is the distance between the centre of the separated colloid particles. This means that the van der Waals dispersion energy interaction is much greater for protein-detergent micelles than for individual atoms. It also means that, the larger the detergent solubilised membrane protein units are, the stronger the attractive van der Waals energy between them will be.

Judging by the factors discussed so far, monomer units would be expected to aggregate immediately without having to overcome an energy barrier. Indeed hydrophobic complexes precipitate easily, making it difficult to achieve slow, ordered aggregation. As aggregation is not immediately observed these attracting forces must be opposed by solubilising forces.

8.1.4 Solubilising forces

The more polar a molecule is the more soluble it is in aqueous media. Water is an excellent solvent due to its extremely high dielectric constant. This constant is a measure of the ability of a

solvent to block charge interactions between electrostatically charged species dissolved in it. Protein-detergent units can be considered to be polyionic species. The ionic and polar groups on their surfaces are shielded by an oppositely charged ionic atmosphere. Because of the strong charge shielding by water, the electrostatic and van der Waals interactions between adjacent protein-detergent complexes, can only act on each other once the particles are in close proximity to one another. Vapour diffusion systems work, in that slow removal of water gradually concentrates the protein-detergent units to such an extent that the long range electrostatic forces and attractive van der Waals forces come into play (Michel, 1991). Where monomers have regions of opposite charge, the attractive van der Waals forces are likely to be complemented by strong electrostatic attraction. If all protein-detergent units are similarly charged, the attractive van der Waals forces and repelling electrostatic forces will act antagonistically, preventing ordered packing and will probably result in precipitation, when their solubility limit is exceeded. The actual interactions observed are entirely dependent on the conditions being used. For example, the degree of electrostatic repulsion and attraction is affected by the concentration and valency of the counter ions in solution (Barber, 1982a & b). If the packing of protein-detergent units is only favoured in one dimension, precipitation will be likely to occur. Two-dimensional aggregation can lead to the formation of sheets, which can pack into plates or roll up into needle-shaped structures (Michel, 1991). Attraction of monomers in all three dimensions can give a whole range of crystal shapes, depending largely on the unit cell shape. Needle-like crystals can be true three-dimensional crystals rather than rolled up sheets, their shape being controlled by faster growth in one dimension than in the other two. The conditions which yielded crystals are reported and discussed in Section 8.2

8.2 Results and Discussion

8.2.1 Initial crystallisation screens of a number of different PSII particle preparations.

The initial aim of the work presented in this chapter was to use PSII complexes, more readily available than the sucrose gradient purified dimer (Chapter 5), to screen a large number of crystallisation conditions. In this way, it was hoped that conditions could be identified, which might be potentially useful for crystallisation trials, of more highly purified PSII complexes. To achieve this, PSII reaction centres (D1, D2, cytb559 and psbI gene product), RC-47 (CP47, D1, D2, cytb559 and associated low molecular weight subunits), purified CP43 and the HTGP cores (CP47, CP43, D2, D1, cytb559, the 33kDa extrinsic polypeptide, CP29/CP26 and associated low molecular weight subunits) were first solubilised in 8mM DM/20mM Bis-Tris-propane pH6.3. The concentrations of the DM solubilised stock solutions were 10mg.ml⁻¹ (reaction centres), 2mg.ml⁻¹ (RC-47), 7.4mg.ml⁻¹ (CP43) and 5.9mg.ml⁻¹ (HTGP cores) protein, respectively. As these stock solutions were used for the first of my crystallisation screens, their respective concentrations do not follow any particular planned pattern, but simply what was available at the time of the trials. 1µl of each stock solution was added to 1µl of the crystallisation stocks listed in Table 2.7.

Of all the PSII particles tested only the purified CP43 subunit yielded any crystals at all. Fine green needle-like crystals were obtained by mixing 1 μ l of the DM solubilised CP43 with an equal volume of 15% PEG4000/0.1M Mes pH 6.1. Later trials yielded, CP43 crystals 20 μ m in diameter under the conditions described in Figure 8.1. As these needles were so small, it was impossible to determine whether they were truly three-dimensional crystals or rolled up two-dimensional sheets, but the result did provide a handle for oxygen-evolving core crystallisation screens.

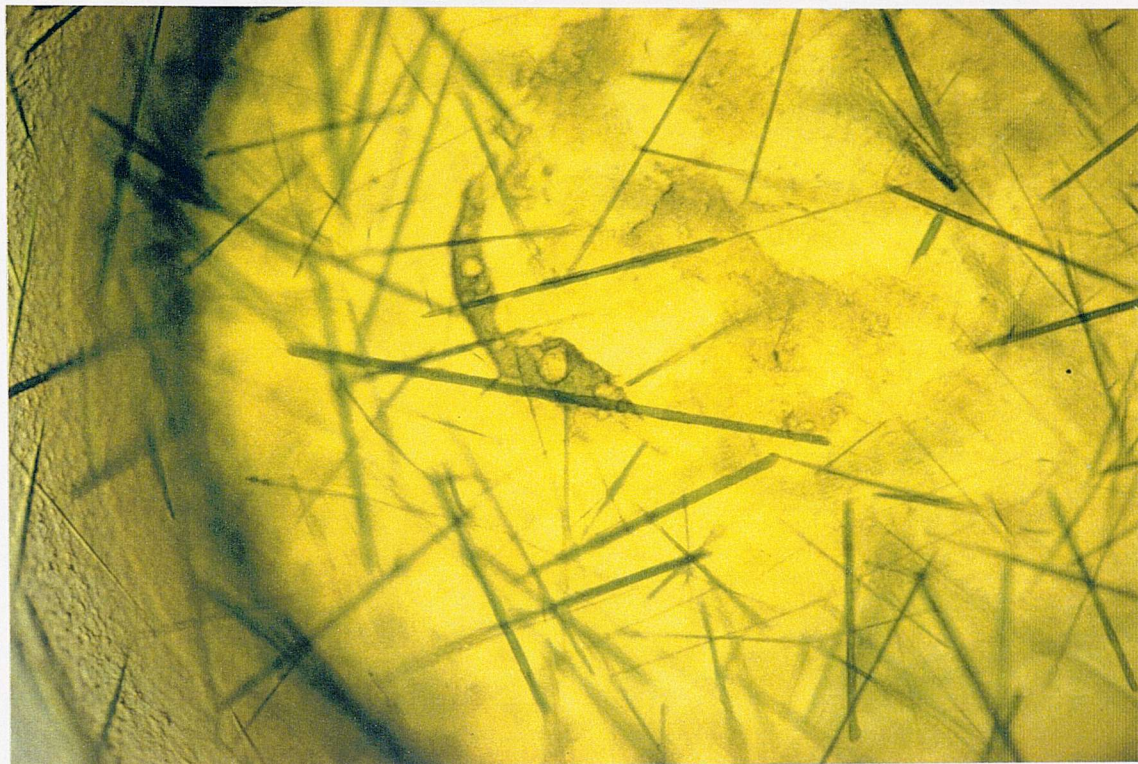


Figure 8.1 Crystals of CP43 Fine green needle-like crystals of CP43, having a diameter of 20 μ m, were obtained at 5.5mg.ml⁻¹ protein, 4% PEG4000, pH 6.1 at 4°C after about 14 days.

8.2.2 Crystallisation trials of oxygen-evolving core complexes using PEG4000

CP43 is a relatively hydrophilic membrane protein and an intrinsic part of all oxygen-evolving PSII core particles (Table 3.1). As electrostatic interactions between the hydrophilic groups of adjacent protein-detergent units are likely to play an important role in crystal formation, it seemed feasible that conditions which supported the crystallisation of CP43 might also support the crystallisation of oxygen-evolving cores containing this subunit. One critical difference between the CP43 and HTGP core crystallisation conditions described in Section 8.2.1, was the protein concentration. The final protein concentration used in the CP43 crystallisation trials was 3.7mg.ml^{-1} (i.e. after mixing with crystallisation stocks (Section 2.10), while that of the HTGP cores was 2.95mg.ml^{-1} . As the latter value was low, the protein concentration of HTGP cores was raised in subsequent trials. Crystallisation mixtures containing 5.5% PEG4000, 10mg.ml^{-1} protein, 50mM Mes pH6.1, 2mM DM yielded crystals such as those shown in Figure 8.2. Crystals of this type were obtained over quite a large PEG4000 (4-7.5%) and protein ($7\text{-}12\text{mg.ml}^{-1}$) concentration range. The incubation time required for maximum size to be obtained depended on the conditions used, but varied between 7 and 14 days at both 4 and 18°C . The thickest crystals obtained were $30\text{-}40\mu\text{m}$ in diameter. To test whether impurities in the HTGP core preparations were reducing the observed growth rates in any of the three dimensions, OG and sucrose gradient purified monomer cores were also screened under these conditions, but yielded virtually identical results.

The results suggested that there might be a fundamental factor other than protein purity which was slowing crystal growth in at least one of the three dimensions (Note: if needles are rolled up sheets then they have grown in two dimensions). Electrostatic repulsion, between specific surfaces of the protein-detergent units, seemed to be an obvious reason why crystal growth might be limited. Thus, if protein-detergent units were similarly charged at critical positions, their close approach could be prevented resulting in needles growing in the hydrophobic plane. Such repulsion might be reduced by altering the pH of the crystallisation medium. As the functional integrity of PSII is compromised at a $\text{pH} < 6$ or > 7 , this approach could not be taken.

However, it is well established that colloidal particles, which carry the same polarity of charge, can be flocculated by ions of opposite charge. The higher the valency of these ions, the more effective they act as flocculating agents (see Barber, 1982a; Atkins, 1986). Thus trivalents are more effective flocculants than divalents, which in turn are more efficient than monovalents. The reason for this is that the higher the charge density of the ions the more effective they are at screening the repulsion between the colloid particles (Barber, 1982a; Atkins, 1986), it was therefore thought possible that the addition of a given salt could aid crystal growth in one or two dimensions by screening electrostatic repulsions. The addition of KCl (150mM) allowed the rod thickness to increase from approximately $30\mu\text{m}$ in its absence to $50\mu\text{m}$ in its presence (data not shown).

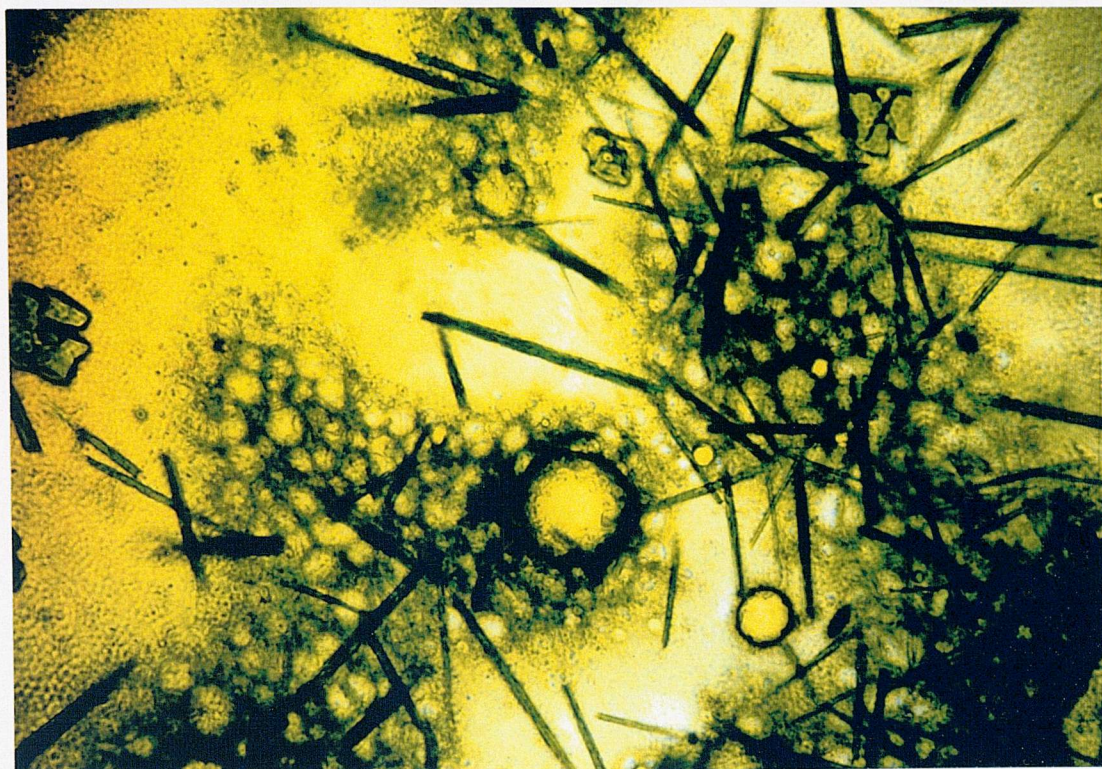


Figure 8.2 Spinach cores prepared by HTGP solubilisation Crystals were obtained after an incubation period of 14 days at 18°C. Crystal diameters grew to approximately 30-40µm. The crystallisation mixture consisted of 5.5% PEG4000, 10mg.ml⁻¹ protein, 50mM Mes pH6.1.

As might be expected for a divalent metal salt, MgCl₂ had a more dramatic effect on HTGP core crystal morphology. At first needles grew in the control (7% PEG4000, 9mg.ml⁻¹, 50mM MES pH6.1), as well as in the solutions containing an addition of MgCl₂ (40, 60, 80 and 100mM). However, after about 10 days the needles in the samples containing MgCl₂ dissolve, probably because of the competitive growth of more three-dimensional crystals and aggregates, shown in Figure 8.3. Figure 8.3 shows that many of the green crystals/aggregates are irregular in shape, but closer examination shows some, near the centre of the picture, to have a hexagonal appearance. These hexagonal "crystals" had a diameter of 30µm and were thus too small for X-ray diffraction analysis.

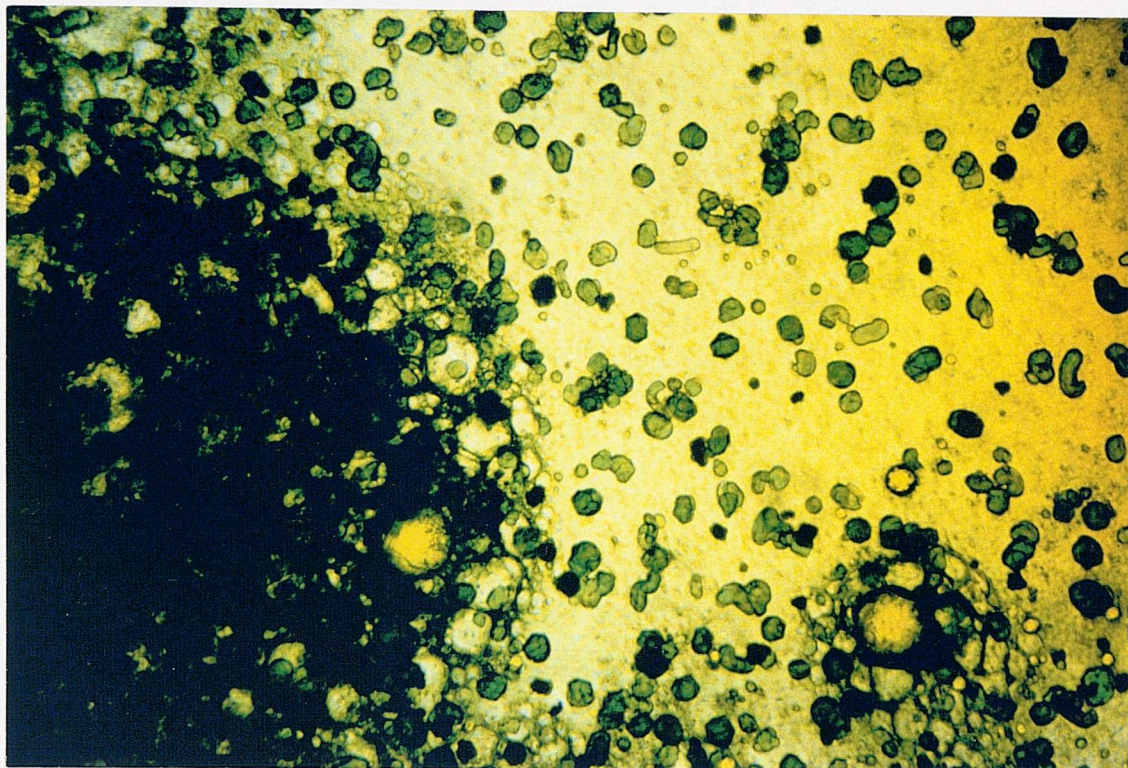


Figure 8.3 HTGP core crystals grown in the presence of MgCl_2 Core crystals were obtained after 10 days of incubation at 18°C . The crystallisation mixture contained 7% PEG4000, $9\text{mg}\cdot\text{ml}^{-1}$ protein, 50mM Mes pH6.1, 40mM MgCl_2 .

More ordered crystals often take longer to nucleate than less ordered crystal forms, but once they do, they tend to grow more rapidly being the more thermodynamically stable state due to the entropy term of the Gibb's free energy function. This might explain the growth of the crystal form shown in Figure 8.3, at the cost of the dissolution of the needle form.

All of the conditions which yielded crystals of CP43 and PSII cores, used polyethylene glycol (PEG) as a precipitant. PEG is a polymer containing regularly spaced ether oxygens and ethylene groups. On addition to an aqueous solution, these groups are thought to interact with water molecules to form a PEG-water network (McPherson, 1985). As a result, crystallisation trials in which PEG is used as a precipitant often result in the formation of a PEG-water phase, as well as a protein rich phase (McPherson, 1985). Figures 8.1 shows such phase separation. The clear region is likely to be PEG-rich. The green (bubbly) region appears to correspond to a phase rich in CP43/DM complexes. All the crystals obtained from CP43, HTGP, OG or sucrose gradient purified monomer cores grew from the protein/detergent rich phases.

Like salt, PEG is thought to aid crystallisation by competing with the detergent solubilised protein, for solvating water molecules. When water becomes limiting the protein-detergent complexes are forced to aggregate into crystals or precipitate. One fundamental difference between salts and PEG is that the former are ionic, while the latter is a polar molecule, carrying no net charge. The addition of a salt to water is thought to increase the dielectric constant far more than a PEG solution of similar concentration (McPherson, 1985). Under the conditions described in Figure 8.3 (HTGP cores (9mg.ml^{-1} protein), 7% PEG, 50mM Mes pH 6.1, 40mM MgCl_2), PEG probably dehydrates the protein-detergent complex, without greatly increasing the dielectric constant of the crystallisation mix, while the MgCl_2 may provide sufficient counter ions to prevent electrostatic repulsion between protein detergent units. However, this conclusion may be premature.

8.2.3 Identification of alternative crystallisation conditions

It is, of course, important to identify other crystallisation conditions. An initial trial using conditions published by Adir *et al* (1992), but reducing the pH to 6.1 yielded what appeared to be green aggregates or irregularly shaped crystals (Figure 8.4). In this experiment, our conditions also differed from those used by Adir and colleagues in that we used larger core particles (OG cores).

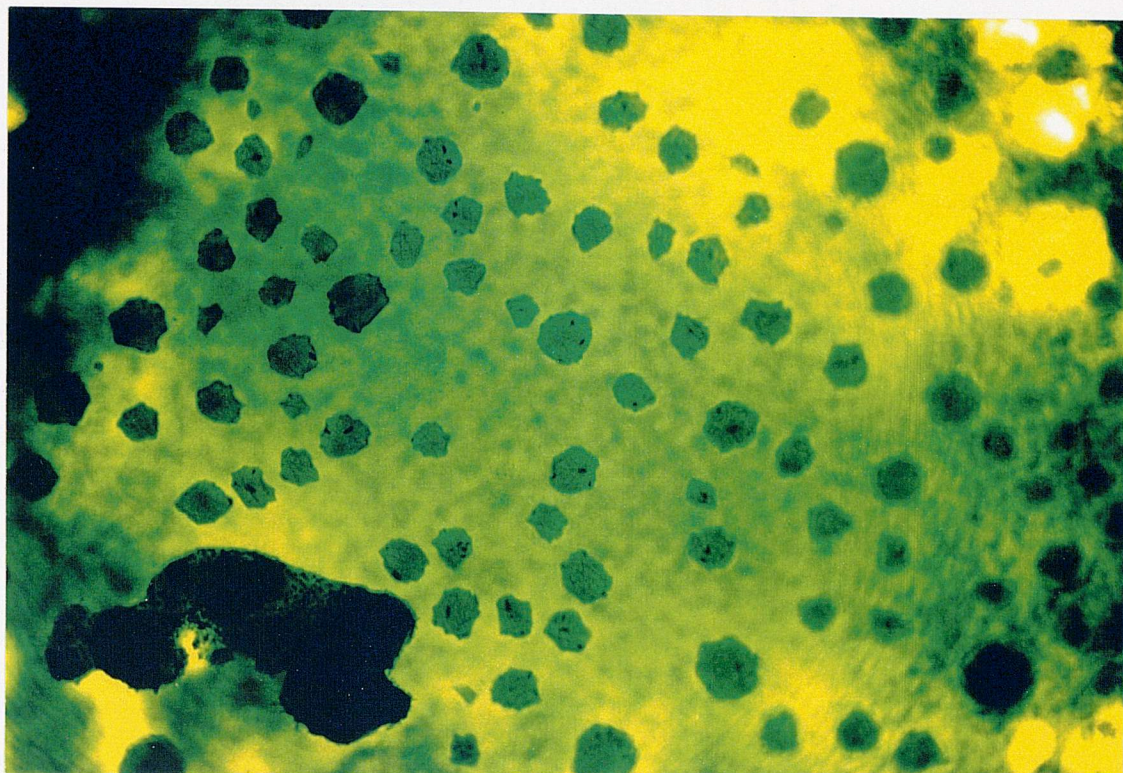


Figure 8.4 Aggregates obtained using a method modified from Adir *et al.*, (1992). The droplet solution (20 μ l) contained (2mg.ml⁻¹ chlorophyll of OG cores (i.e. approximately 17mg.ml⁻¹ protein), 4% PEG4000, 1% DM, 1% HTGP, 1% heptane triol, 50mM Bis-Tris pH6.1). The surrounding 1ml of well solution contained 10% PEG4000, 0.1M NaCl in 50mM Bis-Tris pH6.1. Crystals were observed after an incubation period of about 14 days at 4°C in sitting drop trays.

8.3 Conclusion

Needle-like crystals of DM solubilised CP43, HTGP cores, OG cores and monomeric (CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic polypeptide) PSII core complexes were obtained using PEG4000 as a precipitant. In the presence of PEG4000 and MgCl₂, small hexagonal crystals/aggregates were obtained using HTGP cores. Subjecting OG cores to similar crystallisation conditions to those reported by Adir *et al.*, (1992) resulted in the formation of distinct green aggregates, but no crystals. Having identified conditions yielding HTGP, OG and PSII monomer core crystals and aggregates, it seems logical to repeat these experiments, using the sucrose gradient purified dimeric PSII core complexes consisting of CP47, CP43, D2, D1, cytb559 and the 33kDa extrinsic subunit.

Chapter 9 Final conclusion and future work

One of the most important goals in photosynthesis research is to elucidate the structure of PSII. Prior to the work presented in this thesis a great deal of biochemical knowledge in the form of gene and protein sequence data, PSII core subunit and pigment composition, as well as D1 breakdown patterns, has been accumulated. Much of this information has already been drawn on in the search to determine the atomic structure of PSII. This thesis has concentrated on the development of isolation procedures yielding pure, undamaged and active PSII core complexes for the purpose of crystallisation. Some of the more promising complexes were then used for single particle analysis and initial crystallisation experiments.

In this thesis I have described the isolation and characterisation of highly purified monomeric (230-240kDa) and dimeric (445-540kDa) core complexes consisting of CP47, CP43, D2, D1, cytb559, the 33kDa extrinsic polypeptide and associated low molecular weight subunits. The oxygen evolution rates supported by the dimeric complexes were almost twice as high (on the basis of chlorophyll concentration) as those of their monomeric counterparts. Western blot analysis showed the dimeric core complexes to be associated with lower levels of both D1 and D2 breakdown fragments. Taken together these results indicate that the dimeric cores are considerably more active and far less damaged than their monomeric counterparts. To my knowledge, these oxygen-evolving dimers are the first to have been isolated from higher plant thylakoids, although PSII core dimers have been purified from cyanobacteria (Rögner *et al.*, 1987). The comparison of 25Å resolution projection images of such higher plant and cyanobacterial complexes (Chapter 6) showed them to be very similar in structure. In top view projection, both dimers had dimensions of 17.2 x 9.7nm and contained four regions of prominent protein density which surrounded a central hole or cavity. Using single particle analysis, monomeric PSII core complexes were shown to correspond in size and topology, to half of the dimeric PSII core. At a resolution of about 25Å PSII core monomers did not appear to contain a central hole or cavity and showed neither symmetry nor pseudosymmetry.

The projection images of the dimeric cores characterised in this thesis have striking similarity to the structural maps of PSII complexes reported by Bassi *et al.*, (1989), Holzenburg *et al.*, (1993), Lyon *et al.*, (1993) and Santini *et al.*, (1993). Each of these projection maps identified four regions of prominent density surrounding a central hole or cavity. Furthermore, the complexes of Bassi *et al.*, (1989), Lyon *et al.*, (1993) and Santini *et al.*, (1994) showed two-fold rotational symmetry around an axis passing through this cavity. From these results, Bassi *et al.*, (1989), Lyon *et al.*, (1993) and Santini *et al.*, (1994) concluded that the ordered two-dimensional arrays yielding the images they had reported, consisted of dimeric PSII cores. In contrast, Holzenburg *et al.*, (1993) suggested that their ordered arrays consisted of monomeric PSII core complexes. In view of the finding that the monomeric core complexes which we analysed did not appear to contain a central hole or cavity nor did they show any pseudosymmetry, the conclusions of Holzenburg *et al.*, (1993) should perhaps be reconsidered. However, a final conclusion will have to await higher resolution structural studies, as the preparations

of Holzenburg *et al.*, (1993) contain chlorophyll a/b binding proteins which probably contribute to some of the observed regions of density.

Sucrose density gradient centrifugation of DM-solubilised BBYs yielded a PSII-LHCII supercomplex containing a central dimeric PSII core, flanked by two chlorophyll a/b binding protein complexes consisting of CP24, CP26, CP29 and trimeric LHCII. From the work presented in this thesis it is proposed that these PSII-LHCII supercomplexes may represent the *in vivo* organisation of PSII, CP24, CP26, CP29 and trimeric LHCII, though this awaits confirmation by the direct detection of such complexes within the thylakoid membrane. The possibility that the dimeric PSII cores and PSII-LHCII supercomplexes characterised in this thesis are the product of fortuitous aggregation must be considered. Such fortuitous aggregation seems unlikely considering the structural similarity of the higher plant and cyanobacterial dimers to one another (Rögner *et al.*, 1987; Dekker *et al.*, 1988; Boekema *et al.*, 1994) and to the images obtained from ordered arrays of dimeric PSII core complexes (Bassi *et al.*, 1989; Lyon *et al.*, 1993; Santini *et al.*, 1994). The ordered arrays of dimeric PSII core complexes reported by Bassi *et al.*, (1989), Lyon *et al.*, (1993) and Santini *et al.*, (1994) were obtained by subjecting granal stacks to a mild delipidation step and so it seems likely that the dimeric core complexes they contain reflect the existence of dimers *in vivo*. To support this view, Santini *et al.*, (1994) showed that the PSII dimers they had isolated from granal stacks were not the result of fortuitous or purification-induced aggregation. Interestingly, the authors also showed that LHCII was predominantly associated with dimeric PSII, a conclusion also reached from our single particle analysis.

One possible way of testing whether the dimeric PSII core and PSII-LHCII supercomplexes I have isolated are the product of fortuitous aggregation or are structural units in their own right, would be to supplement such preparations with lipid. Random aggregates might be expected to dissociate on becoming embedded in a lipid matrix, whereas a structural dimer would not. Such lipid:protein reconstitution studies are now underway and are also interesting as they could potentially yield two-dimensional crystals.

The comparison of dimeric PSII core complexes and their Tris-washed counterparts allowed the positions of two 33kDa subunits to be identified. The structural information gained from the single particle analyses presented in this thesis together with information obtained from previously published structural studies allowed me to propose two possible models for the subunit organisation of the PSII-LHCII supercomplex characterised in Chapters 5 and 6. The fact that two subunit organisation models could be proposed from the available information, serves to illustrate how little we know about the subunit organisation of this complex, let alone its atomic structure. In the future we hope to locate individual subunits by combining Fab fragment labelling techniques with cryo-electron microscopy. Fab fragments, which contain the antigen recognising portion of an antibody, can be purified from suitable antibody preparations and used to label individual subunits within PSII preparations such as the PSII-LHCII supercomplex. Subjecting labelled and unlabelled PSII preparations to cryo-electron microscopy and subsequent single particle analysis should allow the locations of individual subunits to be identified.

Ultimately ordered two- or three-dimensional crystals will have to be obtained if the atomic structure of PSII is to be elucidated. The pure and active nature of the dimeric PSII core complexes make them useful candidates for further crystallisation trials. Subjecting these PSII core particles to the crystallisation conditions yielding three-dimensional crystals of the less pure HTGP and OG cores (Chapter 8), may well improve crystal quality. There is however still room to improve the purification procedure yielding dimeric cores. Both the D1 and D2 subunits of these dimers were slightly damaged during the sucrose gradient purification step. The size of the D1 and D2 breakdown fragments suggest that subunit proteolysis may have occurred on the acceptor side of PSII (Greenberg *et al.*, 1987; De Las Rivas *et al.*, 1992b; De Las Rivas *et al.*, 1993; Cánovas & Barber, 1993; Andersson *et al.*, 1994; Friso *et al.*, 1994; Ponticos *et al.*, 1994). Trebst *et al.*, (1994) have suggested that acceptor side damage can occur near the Q_A and Q_B sites when these sites are unoccupied. It is therefore possible that the D1 and D2 subunits of PSII core complexes could be protected against subunit damage if isolated in the presence of Q_A and Q_B analogues. Free radical scavengers and protease inhibitors may also be employed in the future to improve the quality of these PSII core preparations still further. In this respect it would be interesting to identify the D1 and D2 cleavage sites by subjecting the C-terminal fragments of these subunits to N-terminal sequence analysis.

The low molecular weight subunits associated with the various PSII core preparations described in this thesis, were not subjected to detailed characterisation. It has been proposed that a low molecular weight subunit, PsaL, may be involved in determining whether PSI exists as a monomeric or trimeric complex (Chitnis & Chitnis, 1993). It would therefore be interesting to subject monomeric and dimeric PSII core complexes to a detailed analysis of their low molecular weight subunit composition, to determine whether a similar pattern is observed in PSII. Characterisation of these subunits would also indicate whether the high levels of D1 and D2 subunit damage observed in the monomeric core preparations could be related to the loss of any specific low molecular weight subunits.

Although the dimeric PSII core preparations are already active and homogeneous enough to warrant both two- and three- dimensional crystallisation trials it may be beneficial to isolate similar cores from thermophilic cyanobacteria, such as *Synechococcus* sp. It is likely that PSII core complexes isolated from such thermophiles are more stable than those isolated from higher plants. The thermostability would be particularly useful in the context of two-dimensional crystallisation trials, as unsaturated thylakoid lipids could be replaced with saturated lipids having a higher crystal:liquid-crystal transition temperature, but which are less sensitive to oxidative damage.

There is clearly much work to be done if the high resolution structure of PSII is to be obtained. I hope that the work presented in this thesis contributes towards the isolation of active and pure PSII core complexes and the identification of the crystallisation conditions that yield highly ordered crystals, be they two- or three-dimensional.

Chapter 10 References

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