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The Heterocysts of *Nostoc punctiforme*

From Proteomics to Energy Transfer

TANAI CARDONA



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Abstract

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The aim of this thesis is to provide a thorough characterization of the photosynthetic machinery from the heterocysts of *Nostoc punctiforme* strain ATCC 29133. In this thesis I describe the protocols I have optimized for the isolation of thylakoids from vegetative cells, the purification of heterocysts and the isolation of thylakoids from the purified heterocysts. The composition of the thylakoid membranes was studied by two dimensional electrophoresis and mass-spectrometry. Further insight into the functionality of the photosynthetic complexes was obtained by EPR, electron transport measurements through Photosystem II (PSII), and fluorescence spectroscopy. The proteome of the heterocysts thylakoids compared to that of the vegetative cell was found to be dominated by Photosystem I (PSI) and ATP-synthase complexes, both essential for keeping high nitrogenase activities. Surprisingly, we found a significant amount of assembled monomeric PSII complexes in the heterocysts thylakoid membranes. We measured *in vitro* light-driven electron transfer from PSII in heterocysts using an artificial electron donor, suggesting that under certain circumstances heterocysts might activate PSII. Parallel to my main research I also worked in a collaboration to elucidate the total proteome of *Nostoc* sp. strain 7120 and *Nostoc punctiforme* using quantitative shotgun proteomics. Several hundred proteins were quantified for both species. It was possible to trace the detailed changes that occurred in the energy and nitrogen metabolism of a heterocyst after differentiation. Moreover, the presence of PSII proteins identified in our membrane proteome was also confirmed and extended. Lastly, I studied how the heterocysts are capable of responding to variations in light quality as compared to vegetative cells. Using 77 K fluorescence spectroscopy on heterocysts and vegetative cells previously illuminated with light at specific wavelengths, I was able to demonstrate that heterocysts still possess a possibly modified but functional antenna system, capable of harvesting light and transferring energy preferentially to PSI. The characterization of the membrane and total proteome permitted to draw a more comprehensive and integrated picture of the interplay between the distinct metabolic processes that are carried out in each cell type at the same time; from oxygenic photosynthesis and carbon fixation in the vegetative cells to the anoxygenic cyclic photophosphorylation essential to power nitrogen assimilation in the heterocysts.

Keywords: *Nostoc punctiforme*, heterocyst, photosynthesis, thylakoid, isolation, proteomics, photosystem, energy transfer, hydrogen

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Life is beautiful

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I Cardona T, Battchikova N, Agervald Å, Zhang P, Nagel E, Aro EM, Styring S, Lindblad P, and Magnuson A. (2007) Isolation and characterization of thylakoid membranes from the filamentous cyanobacterium *Nostoc punctiforme*. *Physiologia Plantarum* 131: 622-634.
- II Cardona T, Battchikova N, Zhang P, Stensjö K, Aro EM, Lindblad P, and Magnuson A. (2009) Electron transfer protein complexes in the thylakoid membranes of purified heterocysts from the cyanobacterium *Nostoc punctiforme*. *Biochimica et Biophysica Acta - Bioenergetics* 178: 252-263.
- III Ow SY, Cardona T, Taton A, Magnuson A, Lindblad P, Stensjö K, and Wright P. (2008) Quantitative shotgun proteomics of enriched heterocyst from *Nostoc* sp. PCC 7120 using 8-plex isobaric peptide tags. *Journal of Proteome Research* 7: 1615-1628.
- IV Ow SY, Noirel J, Cardona T, Taton A, Lindblad P, Stensjö K, and Wright P. (2009) Quantitative overview of N₂ fixation in *Nostoc punctiforme* ATCC 29133 through cellular enrichments and iTRAQ shotgun proteomics. *Journal of Proteome Research* 8: 187-198.
- V Cardona T and Magnuson A. (2009) Excitation energy transfer to Photosystem I in heterocysts and vegetative cells of *Nostoc punctiforme*. Manuscript.

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Abbreviations

APC	Allophycocyanin
ATP	Adenosine triphosphate
BN	Blue native
BPG	1,3-Bisphosphoglycerate
Chl- <i>a</i>	Chlorophyll <i>a</i>
Cyt	Cytochrome
DCPIP	2,6-Dichlorophenolindophenol
DPC	Diphenylcarbazide
EPR	Electron paramagnetic resonance
F6P	Fructose-6-phosphate
FBP	Fructose-1,6-bisphosphate
FNR	Ferredoxin:NADP ⁺ oxidoreductase
G6F	Glucose-6-phosphate
GAP	Glyceraldehyde-3-phosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
iTRAQ	Isobaric tags for relative and absolute quantitation
L _{CM}	ApcE or terminal emitter
NAD(H)	Nicotinamide adenine dinucleotide
NADP(H)	Nicotinamide adenine dinucleotide phosphate
NDH-1	NADH:quinone oxidoreductase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phycobilisome
PC	Phycocyanin
PE	Phycoerythrin
PEP	Phosphoenolpyruvate
PQ	Plastoquinone
PSI	Photosystem I
PSII	Photosystem II
R5P	Ribose-5-phosphate
Ru5P	Ribulose-5-phosphate
Rubisco	Ribulose-1,5-bisphosphate carboxylase oxygenase
SDS	Sodium dodecyl sulfate

1 Preface

This thesis is my contribution towards the ideal of a modern civilization powered by the inexhaustible energy from the sun and the plentiful water molecules from the oceans; a clean civilization that thrives on the abundance of its resources and brings well-being and progress to our seven billion members' community. It is a minuscule contribution compared to the extent of the ideal, yet it is the materialized form of all my efforts as a doctoral student, it is the fulfillment of a child's dream of becoming a scientist, and the beginning of new and bigger dreams—for that I am proud and incredibly excited. It is also under the light of such an ideal that this work finds meaning and direction, and that I personally find the determination, courage, and delight to keep on venturing forth the immensity of science.

The main objective of this book is to provide a thorough characterization of the photosynthetic machinery from *Nostoc punctiforme*; a heterocyst forming filamentous cyanobacterium with potential for the production of hydrogen. It starts by describing in detail the protocols that I have optimized for the isolation of thylakoid membranes from vegetative cells, the purification of heterocysts, and the isolation of thylakoids from the purified heterocysts; I have schematized the procedures step-by-step so that other students and researchers can quickly access them for their own sample preparation. A second part of this thesis discusses and compares the composition and properties of the photosynthetic machinery from both types of cells; it is based on the results obtained from a variety of techniques that includes membrane proteomics and spectroscopy. At this stage it would be possible to draw a more complete picture of the distinct bioenergetic processes that are carried out within the vegetative cell and the heterocyst thylakoids. There is also a third section which focuses on the results from quantitative shotgun proteomics performed in total protein extracts from filaments and purified heterocysts. This section not only deals with data that concerns the photosynthetic apparatus, but it places it in the context of each cell type's particular metabolism and it allows a better understanding of their interrelationship. Finally in the last part, I center my attention on a series of experiments intended to give a dynamic perspective of the heterocyst's photosynthetic machinery by testing its responsiveness to varying light color and studying the consequent changes in the distribution of energy transfer by fluorescence spectroscopy. I expect that the knowledge acquired here may serve as a part

of the foundations where to build upon in the development of a powerful organism for the biological production of energy.

The research and accomplishments in this thesis would not have been possible without the guidance and insight of my supervisor Dr. Ann Magnuson; the fruitful collaboration with the Plant Physiology and Molecular Biology Laboratory from Turku University in Finland directed by Professor Eva-Mari Aro; the mutual work with Dr. Karin Stensjö at our department and her collaboration with the Biological and Environmental Systems Group at The University of Sheffield in the United Kingdom directed by Professor Phillip C. Wright.

2 Introduction

In this section I shall mention a few general words about some of the aspects that most concern this thesis: cyanobacteria, photosynthesis and the photosynthetic apparatus. I hope they will refresh the mind of the reader if there is a background in biology or biochemistry; if there is not, to browse an undergraduate biochemistry book while reading this thesis could come in handy. Immediately after, I shall also introduce the main character of the thesis: *Nostoc punctiforme* and its heterocysts.

2.1 Oxygenic photosynthesis

Among Earth's plethora of life forms, cyanobacteria are unique for their capacity to convert sunlight into chemical energy by decomposing water molecules; extracting their electrons, and releasing protons and elemental O_2 . The process is known as oxygenic photosynthesis and it is the main energy input into the biosphere: the electrons from water will be used to generate NADPH, and the protons will contribute to the electrochemical potential required for the formation of ATP; both molecules will power CO_2 fixation and the entire cell metabolism. O_2 on the contrary, is a byproduct of the reaction.

The origin of cyanobacteria and—as a matter of fact, the evolution of oxygenic photosynthesis are very ancient events in the history of the planet. Rosing and Frei (2004) provided evidence of the presence of O_2 and CO_2 fixation as far back in time as 3.7 billion years ago. More geological evidence for the presence of O_2 by 3.5 billion years ago has been reported recently by Hoashi et al. (2009), and it was undoubtedly widespread by a major oxygenation event that happened ca 2.4 billion years ago (Watanabe et al., 2009). Oxygenic photosynthesis had a tremendous impact on the destiny of Earth, leading to the accumulation of O_2 in the atmosphere, the formation of the ozone layer, and to the evolution of complex eukaryotic life based on respiration.

Oxygenic photosynthesis can be divided into two distinct phases: a light dependent one, also known as 'light reactions' (or photophosphorylation), and a light independent one, commonly referred as 'dark reactions'. The light reactions require the presence of two sequential photosystems, one that generates a strong oxidant capable of oxidizing water, denominated Photo-

system II (PSII) and one that generates a strong reductant necessary for the reduction of NADP^+ , denominated Photosystem I (PSI). The two photosystems are linked by the Cytochrome (Cyt) b_6f complex which receives electrons from PSII and shuttles them to PSI. The electron donation from water to NADP^+ is coupled to the formation of a proton gradient necessary for ATP synthesis. The second phase is independent of light and it is devoted to the fixation of CO_2 into carbohydrates at the expense of ATP and NADPH molecules. The process is known as the Calvin cycle and it starts by the binding of CO_2 to ribulose biphosphate, the reaction is catalyzed by ribulose biphosphate carboxylase (Rubisco).

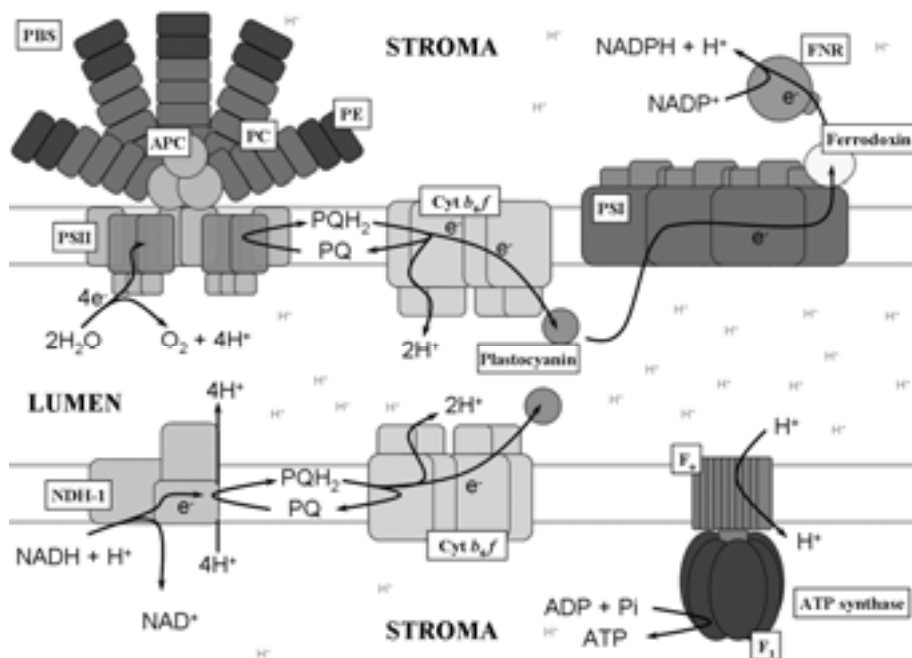


Figure 1. Schematic representation of the thylakoid membrane and its components.

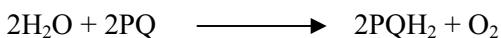
The light reaction components are multisubunit integral membrane protein complexes located within a membrane system called thylakoids. The thylakoid membranes form a distinct continuous enclosed compartment separated from the cytosol (Mullineaux, 2008), the space formed within the thylakoid membranes is called the lumen, depicted in Figure 1. During photosynthesis protons are translocated from the cytoplasm (or stroma) into the lumen, the higher concentration of protons in the lumen compared to the stroma generates the proton motive force or electrochemical potential for the synthesis of ATP at the cytoplasmic side. Since the cyanobacterial thylakoid membranes and its components play a central role in this thesis, I will proceed to de-

scribe a little bit more in detail each one of the complexes that powers photosynthesis.

2.1.1 Photosystem II

PSII catalyses the light-driven oxidation of two water molecules, which leads to the transfer of four electrons to two plastoquinone (PQ) molecules, and the four protons are released into the lumen contributing to the build-up of the electrochemical potential to drive the synthesis of ATP. The electrons are received by a mobile PQ that, once reduced, swims away from PSII into the lipid bilayer, then docks and donates its electrons to the Cyt b_6f complex (see Figure 1). Water is oxidized at an inorganic metal cluster made of four Mn atoms and one Ca, connected by oxygen bridges. There are several crystal structures ranging from 3.8 to 2.9 Å resolution (Zouni et al., 2001; Kamiya et al., 2003; Ferreira et al., 2004; Loll et al., 2005; and Guskov et al., 2009), and an attempt to crystallize a eukaryotic PSII from a red alga has been initialized by Adachi et al. (2009). PSII is suggested to be a dimer *in vivo*, although recent evidence challenges this view (Takahashi et al., 2009). There are at least 17 subunits in the cyanobacterial PSII: D1 and D2, making the reaction center and holding most of the redox cofactors such as the Mn_4Ca , PQ, and the redox active Chlorophyll-*a* (Chl-*a*) molecules (see Figure 2); CP43 and CP47 are the reaction center antenna proteins carrying most of the light harvesting Chl-*a* molecules; the Cyt b_{559} carrying a redox active heme cofactor; and a few small subunits which have been implicated in providing stability to the entire protein complex. There are in addition three extrinsic proteins involved in the stabilization of the Mn_4Ca cluster, located at the lumen side of PSII; namely, PsbO, PsbU, and the redox inactive Cyt c_{550} or PsbV. As determined by the highest resolution crystal structure (Guskov et al., 2009), there are in total 35 Chl-*a*, 2 pheophytins (Pheo), 2 PQ molecules, 1 non-heme iron, 12 carotenoids, 2 hemes, and 25 lipid molecules. Recent crystallographic data suggest that there are 2 Cl^- anions in the vicinity to the Mn_4Ca cluster (Kawakami et al., 2009).

The general catalytic reaction of PSII can be summarized by the following reaction:



Briefly, light is harvested by the antenna proteins CP43 and CP47 and funneled into the primary electron donor within D1 and D2, which is formed of four coupled Chl-*a* molecules. It has a maximum absorption at 680 nm, thus it is referred as P680. Excitation energy will be then transferred to P680 triggering electron transfer and charge separation; electrons will be shuttled from a Chl-*a* molecule to Pheo, a bound PQ, known as Q_A and finally to the mobile PQ known as Q_B (see Figure 2). One Q_B molecule must be reduced

twice within PSII in order to be released into the lipid bilayer. The electron whole formed, $P680^+$, will be quickly filled with an electron coming from a redox active tyrosine, Y_Z , forming the tyrosine radical $Y_Z^\bullet$ and located within the D1 protein; it will in turn receive an electron from the Mn_4Ca cluster. The process is repeated four times, so that the cluster accumulates four positives charges necessary for the oxidation of two water molecules. The electron donation from the Mn_4Ca cluster to the acceptor side is coupled with the release of four protons to the lumen. Within PSII there is also a second redox active tyrosine, called Y_D ; it is located in the D2 subunit and it is homologous to Y_Z as a consequence of an ancient gene duplication. Y_D can also transfer electrons to $P680^+$, and it is proposed to aid in the assembly of the Mn_4Ca cluster (Ananyev et al., 2002) and in the tuning of $P680$ redox potentials (Szczpaniak et al., 2008).

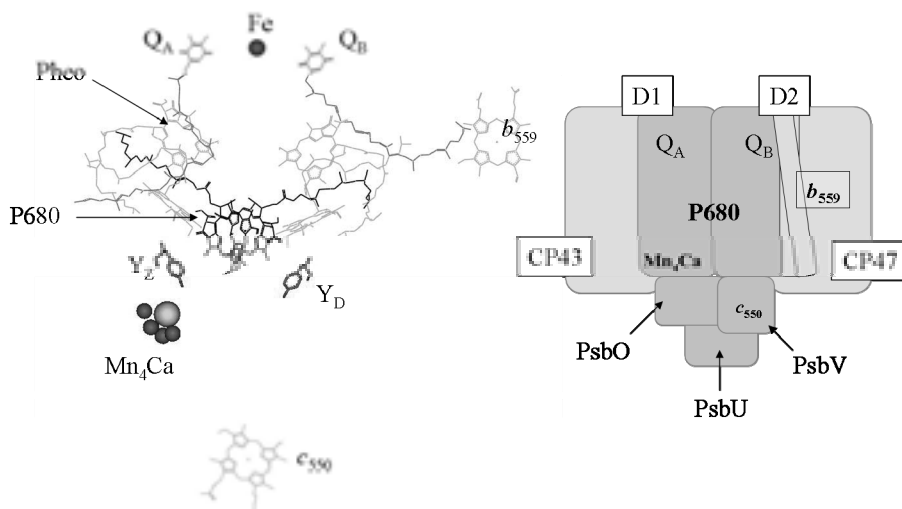


Figure 2. Photosystem II. The redox cofactors are depicted to the left (see text for abbreviations). To the right, a schematic representation of the PSII subunits.

2.1.2 Cytochrome b_6f complex

The high resolution crystallographic structures for the Cyt b_6f complex have been solved for the heterocystous cyanobacteria *Mastigocladus laminosus* (Kurusu et al., 2003) and *Nostoc* sp. PCC 7120 (Baniulis et al., 2009) both to 3.0 Å, and for the green alga *Chlamydomonas reinhardtii* at 3.1 Å (Stroebel et al., 2003). The function of the Cyt b_6f complex is to receive the electrons from PQH_2 generated in PSII and transfer them to the soluble electron carriers, plastocyanin or Cyt c_6 , via a high-potential electron transfer chain within the complex; with the concomitant release of two protons to the lumen of the thylakoid membrane. The soluble electron carrier will donate their electrons

to PSI. The Cyt b_6f complex is made of four main components a Cyt f , a Cyt b_6 , the Rieske iron-sulfur (FeS) protein, and the subunit IV; in addition to four other small subunits denominated PetG, PetL, PetM and PetN. The functional form of the Cyt b_6f complex is the dimer; a monomer contains four hemes, an $[\text{Fe}_2\text{S}_2]$ cluster, one Chl- a , one β -carotene, a PQ and a few lipids (Figure 3).

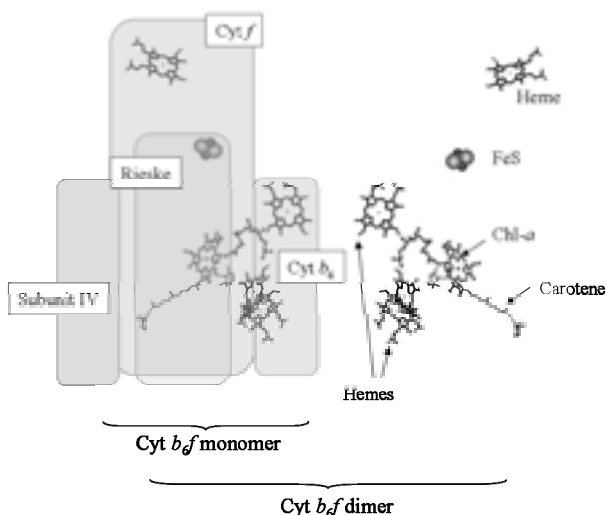


Figure 3. The Cytochrome b_6f complex. Schematic representation of cofactors and subunit composition.

2.1.3 Photosystem I

The next electron transport complex in oxygenic photosynthesis is PSI (Figure 4). PSI receives the electrons from plastocyanin and Cyt c_6 at the lumen and transfers them to ferredoxin at the stromal side. The PSI structure has been solved to 2.5 Å for the thermophilic cyanobacterium *Synechococcus elongatus* (Jordan et al., 2001) and for the plant *Pisum sativum* at 4.4 Å (Ben-Shem et al., 2003) with further improvement to 3.4 Å (Amunts et al., 2007). The cyanobacterial PSI functional form is the trimer, a monomer is made of at least 11 protein subunits, containing a total of 96 Chl- a , 2 phyloquinones, 3 $[\text{Fe}_4\text{S}_4]$ clusters, 22 carotenoids and 4 lipids. The major reaction center subunits, PsaA and PsaB, carry the majority of the Chl- a molecules, the 2 phyloquinones and one $[\text{Fe}_4\text{S}_4]$ cluster. There are three extrinsic proteins located at the stromal side, PsaC which contains the remaining $[\text{Fe}_4\text{S}_4]$ clusters, PsaD, and PsaE; together they provide a docking site for ferredoxin. The smaller subunits of PSI help in the coordination of a few Chl- a molecules and provide stabilization for the formation of trimers.

Contrary to PSII, the PSI absorption maximum is shifted 20 nm towards the red, thus the primary electron donor is known as P700. In this case, once P700 is excited an electron will be transferred to A_0 , a redox active Chl-*a* (Figure 4). The charge separated state will be rapidly stabilized by electron donation from A_0^- to A_1 (a phylloquinone) and then to the $[Fe_4S_4]$ clusters termed F_X , F_A , and F_B . Next, there will be electron transfer from the acceptor side of PSI to ferredoxin in the stroma. Ferredoxin donates electrons to ferredoxin:NADP⁺ oxidoreductase (FNR), which in turn will reduce NADP⁺ to NADPH to power all sorts of metabolic reactions in the cell.

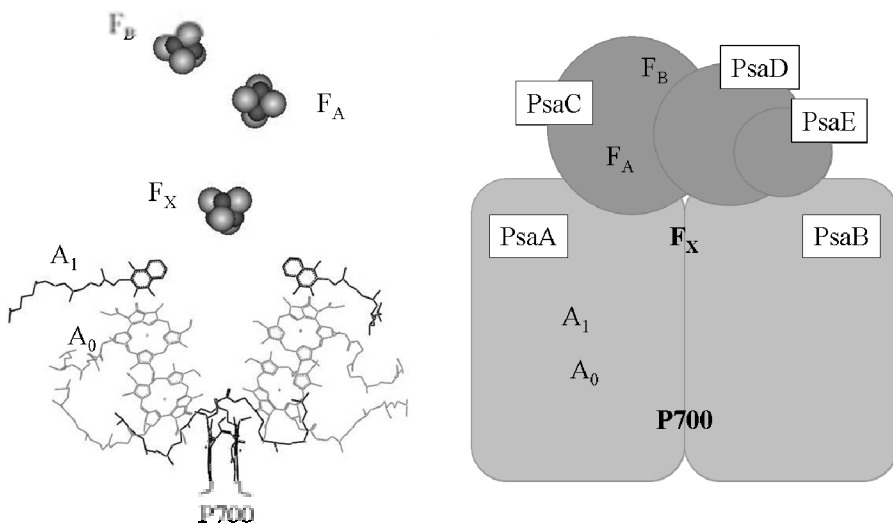


Figure 4. Photosystem I. The redox cofactors are depicted to the left (see text for abbreviations). To the right, a schematic representation of the PSI subunits.

Another property of PSI is the presence of very long wavelength Chl-*a* molecules, usually named as ‘far-red’ Chl-*a*. While most of the antenna Chl-*a* absorbs below 700 nm in order to form a downhill energy gradient to transfer the excitation energy to P700, PSI also harbors a few Chl-*a* molecules that can absorb wavelengths above 700 nm, some of them extending their absorption as far into the red as ca 740 nm (Karapetyan et al., 2006). This Chl-*a* will work as an energy trap competing with P700 for the available excitons. It has been proposed that the existence of such red Chl-*a* might help to enhance absorption under very low light intensities (Trissle, 1993), and also to help dissipate excess energy to avoid photoinhibition at high light intensities (Karapetyan et al., 1999).

2.1.4 Phycobilisomes

Light is the driving force of photosynthesis and in cyanobacteria there is a very efficient and dynamic antenna system in charge of harvesting light and funneling it to the photosystems. It is named the phycobilisome (PBS) and it is a water soluble antenna system reaching up to 3000 kDa in molecular weight and capable of bearing up to a thousand pigments (linear tetrapyrrole molecules or phycobilins). It is located at the stromal side of the thylakoid membrane (Bald et al., 1996). The PBS structure and protein composition varies among species; the most commonly found PBS is composed by two main parts, six rods connected to a protein core made of two to five cylinders. The most commonly found groups of phycobiliproteins are phycoerythrin (PE) and phycocyanin (PC) in the rods, and allophycocyanin (APC) at the core, with absorption maxima ranging from ca 550 nm (PE) up to 660 nm (APC). Within the APC core two main components have been identified absorbing at longer wavelengths; the phycobiliprotein ApcD, also referred as allophycocyanin B, α^B , or APC-B; and the linker protein ApcE, also called 'linker core-membrane', or L_{CM} for its role as a linker polypeptide between the PBS and the photosystems. ApcE besides a linker polypeptide domain also possesses a phycobiliprotein domain, carrying a phycobilin molecule that has an absorption maxima at ca 674 nm and emission at ca 680-683 nm (Gindt et al., 1994); it is suggested that ApcE facilitates the energy transfer to PSII (Zhao et al., 2005 and Guan et al., 2007). The crystal structure for the entire PBS has not been yet successfully accomplished, although about 20 different crystal structures exist for a few of its components (summarized in Adir, 2005). For a detailed description of the PBS structure and properties the reader is suggested to refer to Adir (2008).

2.1.5 NADH:quinone oxidoreductase

Compared to PSII and PSI, remarkably little is known about the cyanobacterial NADH:quinone oxidoreductase (NDH-1); nevertheless, in the last decade some insight has been gained into the structure and function of this versatile complex thanks to genetics, proteomic studies (Zhang et al., 2004 and Herranen et al., 2004), and electron microscopy (Arteni et al., 2006 and Folea et al., 2008). NDH-1 is homologous to the respiratory Complex I in other prokaryotes and mitochondria. In cyanobacteria, NDH-1 is located not only in the cytoplasmic membrane but in the thylakoid membranes too, and it has been inherited by chloroplasts in the plant kingdom (Ogawa and Mi, 2007). It catalyses the transfer of two electrons from NADH to a PQ, coupled to the translocation of protons to the lumen of the thylakoids.

Structurally the NDH-1 is L shaped with the long arm spanning the membrane and a shorter hydrophilic extension. It bears a flavin mononucleotide cofactor (FMN) and several FeS clusters. To date no crystal structure exists

from the cyanobacterial NDH-1, whereas the hydrophilic domain of the Complex I from *Thermus thermophilus* has been solved at 3.3 Å (Sazanov et al., 2006). The protein composition can vary significantly, from 45 subunits in eukaryotes to 14 subunits in *Escherichia coli*. In cyanobacteria 11 homologue genes to other prokaryotic NDH-1 have been found. Interestingly, the three subunits “missing” are the ones carrying most of the cofactors and the NADH binding site. Which proteins are fulfilling their role in cyanobacteria have not yet been identified (Battchikova and Aro, 2007).

NDH-1 in cyanobacteria has been implicated to support at least three different functions: (i) respiration and heterotrophic growth, (ii) cyclic photophosphorylation through PSI, and (iii) inorganic carbon concentration and uptake. This diversity of function is reflected in the multiplicity of forms that have been found present in the thylakoid membrane of cyanobacteria: there is great variation in subunit composition and sizes ranging from a couple of hundreds up to a thousand kDa (Zhang et al., 2004; Herranen et al., 2004; Ma and Mi, 2008; and see Battchikova and Aro (2007) for a nice minireview on the subject).

2.1.6 ATP synthase

One of the most conserved membrane protein complexes in all three domains of life, ATP synthase will use the electrochemical potential stored as a proton gradient across the membrane to regenerate the ATP necessary to power all metabolic functions in life. The cyanobacterial ATP synthase is of the F_0F_1 type (different to P-type ATPases involved in Na^+K^+ or Ca^{2+} transport, for example); F_0 is the membrane spanning part making up the rotatory proton pumping motor domain. F_1 is located peripheral to F_0 towards the stromal side of the thylakoid membrane and it is an assembly of five different polypeptides where the ATP formation is carried out. The F_0 domain has a cylindrical shape made from multiple copies of the transmembrane subunit c, and a peripheral stalk made of subunits a, b and b' essential for the connection of F_0 to F_1 ; the latter of these subunits, b', is unique to cyanobacteria and chloroplasts (Claggett et al., 2009). Most prokaryotic F_0 are made of 10 or 11 subunits c, however this number might vary from organism to organism. The number depends on the metabolic demands, because the ratio of translocated protons to ATP formation will vary with the number of subunits c in the rotor: the F_0 of spinach is made of 14 subunits c (Seelert et al., 2000) while that one of the filamentous cyanobacterium *Spirulina platensis* has 15 (Pogoryelov et al., 2005).

The crystal structure for cyanobacterial ATP synthase has not yet been reported, although there is structural information available for the chloroplast one (Groth and Pohl, 2001 and Vollmar et al., 2009).

2.2 *Nostoc punctiforme* and heterocysts

Nostoc punctiforme strain PCC 73102 (identical to ATCC 29133, from here on simply referred as *Nostoc punctiforme*) is the target organism of this work. It is a N_2 fixing multicellular cyanobacterium found predominantly in terrestrial environments, either free living or in symbiosis with plants and fungi. It usually forms colonies of non-branching filaments and under nutrient rich conditions it will grow photoautotrophically: in this state the cells are said to be in a vegetative state. I will refer along the text to the autotrophic cells as the vegetative cells. *Nostoc punctiforme* is characterized by a great morphological and metabolical versatility (Meeks et al., 2001):

- It can live under continuous darkness heterotrophically if sugars are supplied.
- It shows complementary chromatic adaptation.
- It can differentiate vegetative filaments into motile gliding filaments, also known as hormogonia. These ones are the infectious form when establishing contact with a plant host or when conquering new territories.
- In the absence of phosphates or in periods of draught it can differentiate a vegetative cell into a resting spore, also known as akinete.

In addition to the above mentioned attributes, when *Nostoc punctiforme* faces compound nitrogen starvation, it is also able to differentiate a vegetative cell into a type of cell specialized in atmospheric N_2 fixation, the heterocyst (Figure 5). Approximately 5 to 10% of the cells in a filament will become a heterocyst and the differentiation process will induce drastic alterations in cell structure and metabolism, in order to provide an anaerobic environment to harbor the nitrogenase enzyme, which otherwise would be inactivated by O_2 . To achieve this, heterocysts must deplete the interior of the cell from O_2 ; this requires the inactivation of PSII (Wolk and Simon, 1969; Thomas, 1970; 1972). At the onset of nitrogen starvation there will also be a partial degradation of PBS (Wood and Haselkorn, 1980 and Baier et al., 2004). A three-layered coat is deposited over the cell wall to limit the rate of O_2 entry into the interior (Walsby, 1985 and 2007). Additionally, the O_2 level inside the differentiating cell must reach a low limit before transcription of nitrogenase genes is initialized, therefore respiration is enhanced (Fay, 1992). CO_2 fixation is stopped in the heterocysts; probably because the ATP and reductant demands required for N_2 fixation would not allow a heterocyst to sustain high Rubisco activities (Wolk, 1968 and Haselkorn, 1978). In consequence, the heterocysts are dependent on cyclic photophosphorylation for the generation of ATP, and the required reducing equivalents enter the electron transport chain via respiration: these are supplied by the neighboring vegetative cells in the form of sugars and are metabolized by the oxidative pentose phosphate pathway (Summers et al., 1995 and Böhme et

al., 1998). Besides the mentioned changes, evidence has been given that the thylakoid membranes from the vegetative cell may be degraded and rebuilt upon differentiation (Lang, 1965; Lang and Fay, 1971); the typical concentric and peripheral thylakoid membrane pattern of the vegetative cell is turned into a more convoluted pattern in the heterocysts. Towards the polar regions of the heterocyst, the so-called honeycomb structures appear; this lattice-like membrane system is unique to heterocyst and few investigations hint towards functions in O₂ scavenging and respiration. Oxidation of diaminobenzidine in the polar regions from the heterocysts of *Anabaena cylindrica* suggested the presence of hemoprotein oxidases (Murry et al., 1981) and mutants of some of the terminal respiratory oxidases in *Nostoc* sp. PCC 7120 failed to produce the honeycomb structures (Valladares et al., 2007) demonstrating that the honeycomb structures are respiratory sites.

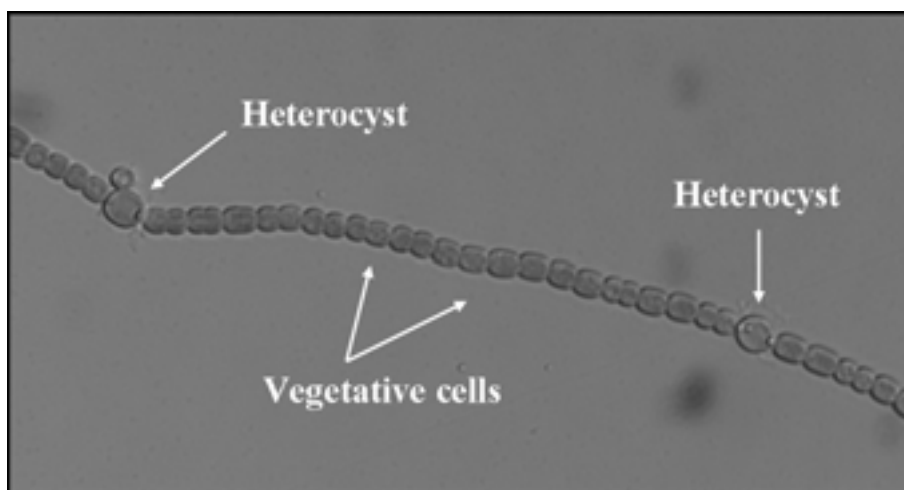


Figure 5. Heterocysts and vegetative cells in a filament of *Nostoc punctiforme*.

It is clear now that heterocysts are completely different from the parent vegetative cells. Even though there is a rough understanding of the processes that go on within the heterocysts, the full extent of the physiological changes are not understood and there is a considerable lack of direct observations; from the genetics of differentiation, to the metabolic interplay between the two types of cells, to the alterations in structure and composition of the photosynthetic machinery. One of the reasons could be the difficulty of studying cell type specific events as a consequence of the low yield of heterocysts within the filaments. In the next sections of this thesis I shall present my efforts to characterize the bioenergetic processes and components of *Nostoc punctiforme* and its heterocysts, in detail and with a variety of biochemical and biophysical methods. The goal is to hopefully gain a much better understanding of this fascinating organism.

3 Isolation of thylakoid membranes

A thylakoid preparation can be a tedious, difficult, time consuming, and exhausting job. The following protocols were optimized for a rapid, uncomplicated, yet efficient sample preparation that could be completed in less than a day of work. It is of crucial importance to keep the time of isolation as compact as possible in order to preserve the activity and intactness of the thylakoid membranes (von Jagow et al., 2003). It is also convenient for the researcher so that he or she is able to measure O₂ evolution, electron transfer, protein concentration, Chl-*a* concentration, or any other type of measurement immediately after the preparation is finished, before the samples are stored at -80°C, and with a fresh and clear mind. In addition, such protocols are easy to memorize, diminishing the chance of making mistakes and improving the reproducibility. I can almost guarantee that the interested researcher would succeed in preparing suitable samples after the very first try.

The protocols were optimized for a starting volume of cultures ranging from six to ten liters with a final concentration of cells approximately 5 µg Chl-*a* ml⁻¹ after seven days of growth. Keeping this in mind, a thylakoid preparation from vegetative cells should be completed in approximately five to six hours. The purification of heterocysts should be completed in approximately three to four hours and the thylakoid preparation from heterocysts should take no longer than three hours: thus, the purification of heterocysts and the isolation of thylakoids can be completed during the same day and still leave enough time for some extra measurements. The isolations should preferably be done under dim green light or complete darkness when appropriate, in a cold room at a temperature of 4°C, and all the instruments (e.g disruption vessel, rotors, centrifuges) should be also refrigerated before the start of the experiment.

Paper I and Paper II describe and discuss extensively the thylakoid isolation protocols; therefore, I will limit myself to point out a few aspects of the protocols that I consider the reader should know for a more conscious and successful preparation. I have also schematized the entire procedures, so that this thesis can be used as a laboratory guide, if thus desired. For more details on recommended volumes and concentrations for each step, I kindly advice the reader to refer to the mentioned papers included at the end of this thesis.

The recipes for the buffer solutions are listed in Table 1. The disruption buffer and thylakoid washing buffer in Paper I are identical to buffer solu-

tion B and C in Paper II, respectively; the lysis buffer mentioned in this thesis is the same as buffer solution A in Paper II.

Table 1. *Buffer recipes for the isolation of thylakoids and purification of heterocysts.*

	Cell mM	Disruption mM	Thylakoid mM	Lysis mM
MES/NaOH	10	10	10	-
HEPES/NaOH	-	-	-	50
Sucrose	800	800	800	400
CaCl ₂	5	5	20	-
MgCl ₂	5	5	20	-
NaCl	-	-	-	10
EDTA	10	-	10	10
Benzamidine	-	1	-	-
PMSF	-	1	-	-
pH	6.35	6.35	6.35	7.2

3.1 Isolation of thylakoids from vegetative cells

The heart of this protocol is the cell rupture by N₂ pressurization and decompression with a Parr cell disruption vessel (Parr Instrument Company, Illinois, USA). The principle is similar to that of Yeda presses, where the cells are filled with N₂ at very high pressures within a stainless steel vessel, when the pressure is released the gas forms bubbles stretching the cells from the interior out until they ‘explode’, liberating all their contents into the solution. This method has several advantages in comparison with other commonly implemented cell disruption techniques (e.g. sonication, glass beads, French press): (i) it neither causes heat damage nor mechanical or chemical stress to the sample; (ii) it is very convenient because the extent of disruption is independent of the sample volume or concentration, even down to less than 1 ml sample; (iii) besides, it is safe, quick, and easy to learn (see the manufacturers website for details and specifications at www.parrinst.com). Notice that a Parr cell disruption vessel is different to a French press which does not use gas decompression; instead the latter uses a plunger or piston to put the high pressures onto the liquids (French and Milner, 1955).

Once the filaments have been harvested and the growing media washed away (see Figure 6), it is recommended to incubate the filaments for at least an hour in the disruption buffer containing the protease inhibitors benzamidine and PMSF; this will improve the yield and facilitate the disruption of the cells. During the cell disruption step, and after the pressure has been raised to 150 bar it is important to wait about 5 minutes before opening the valve of the Parr vessel, to allow the N₂ gas to equilibrate in the buffer and within the cells homogenously. When opening the valve and extruding the homogenate, the pressure within the vessel will drop rapidly; once the pres-

sure has dropped around 30-50 bar the release should be stopped, the valve closed, and the pressure restored to 150 bar. It must be done as many times as necessary until the vessel is empty and the entire sample has been recovered, this simple 'trick' is essential to maximize cell rupture and the final yield of thylakoids. The final steps of the protocol consist in separating the thylakoids from the cell debris by centrifugation and separation of the thylakoids from the cytoplasmic material and PBS by ultracentrifugation. The final precipitate is resuspended in the thylakoid washing buffer to the desired concentration, used immediately for experimentation, or stored at -80°C. The isolated thylakoid membranes with this method have an O₂ evolution of 112 ± 28 µmol O₂ mg⁻¹ Chl-*a* h⁻¹ with up to ca 80% of the PSII retaining water splitting activity, as assessed by light induced DCPIP reduction (Paper I).

3.2 Purification of heterocysts

The development of this protocol was aimed to preserve the integrity of the heterocysts and for purities higher than 90%. The first heterocyst purifications were established by Fay and Walsby (1966) using a French press treatment, a comprehensive comparison of different methods for purification was published by Fay and Lang (1971). In these studies the extent of the damage was inspected by electron microscopy ultrastructural analysis. Later methods combined a short incubation of the filaments in lysozyme-containing buffer previous to disruption with low pressure passages through a Yeda press (Tel-Or and Stewart, 1977), sonic cavitation (Peterson and Wolk, 1978), or by sonication (Russel et al., 1988 and Razquin et al., 1996). The heterocyst purification in Paper II (Figure 7 in this thesis) was optimized for *Nostoc punctiforme* based on the protocols developed for *Nostoc muscorum* (Almon and Böhme, 1980), *Anabaena* sp. strain CA (Smith et al., 1988), and *Anabaena* sp. PCC 7119 (Razquin et al., 1996). To achieve preparations with high yields of pure heterocysts a few points should be taken into account. First, the effectiveness of the sonication step will be determined by the lysozyme incubation, thus a freshly prepared lysozyme solution should always be used. 1 mg ml⁻¹ is the most common concentration recommended for incubation; higher concentrations will cause in fact more degradation, but it is not necessary since the differences in degradation are only noticeable after several hours of incubation. One hour incubation is enough time to weaken the vegetative cell walls so that there is complete disintegration after the sonication treatment; while half-an-hour is insufficient—at least, for *Nostoc punctiforme*. During sonication, one minute and the full amplitude of a Sonic Vibracell VC-130 ultrasonicator was found to be the optimal for complete disintegration of the vegetative cells; shorter times and lower amplitudes were insufficient. The ten seconds intervals are advisable to avoid overheating of the sample. If after one minute of sonica-

tion there are still filament fragments or single vegetative cells remaining in the extract it is an indication that the lysozyme incubation was ineffective, probable because the enzyme is old or the cell suspension was not well mixed during incubation.

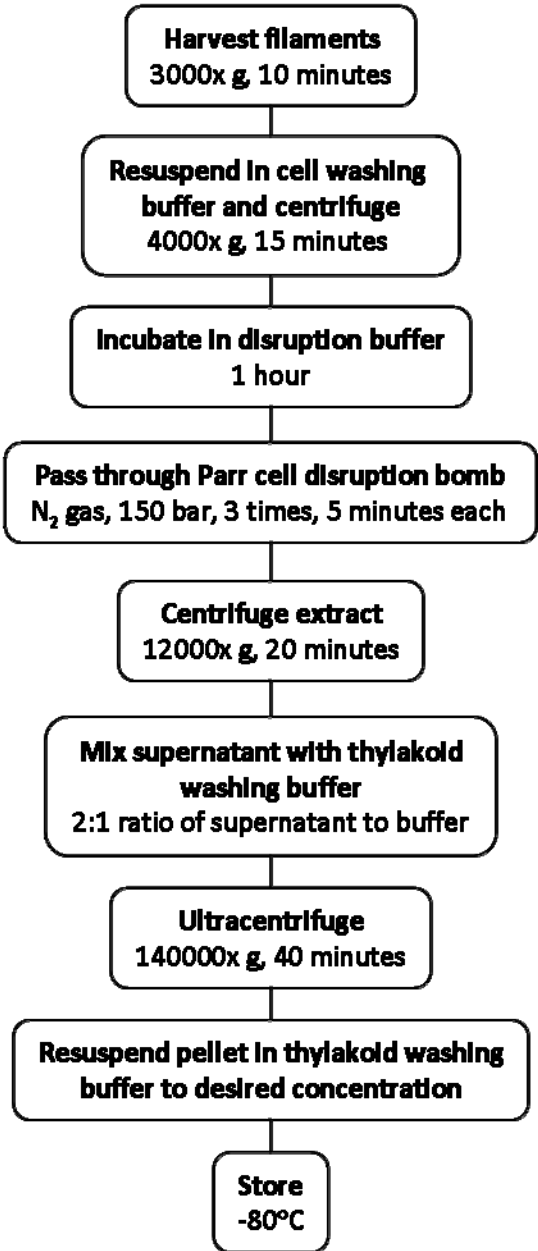


Figure 6. Step-by-step isolation of thylakoids from vegetative cells.

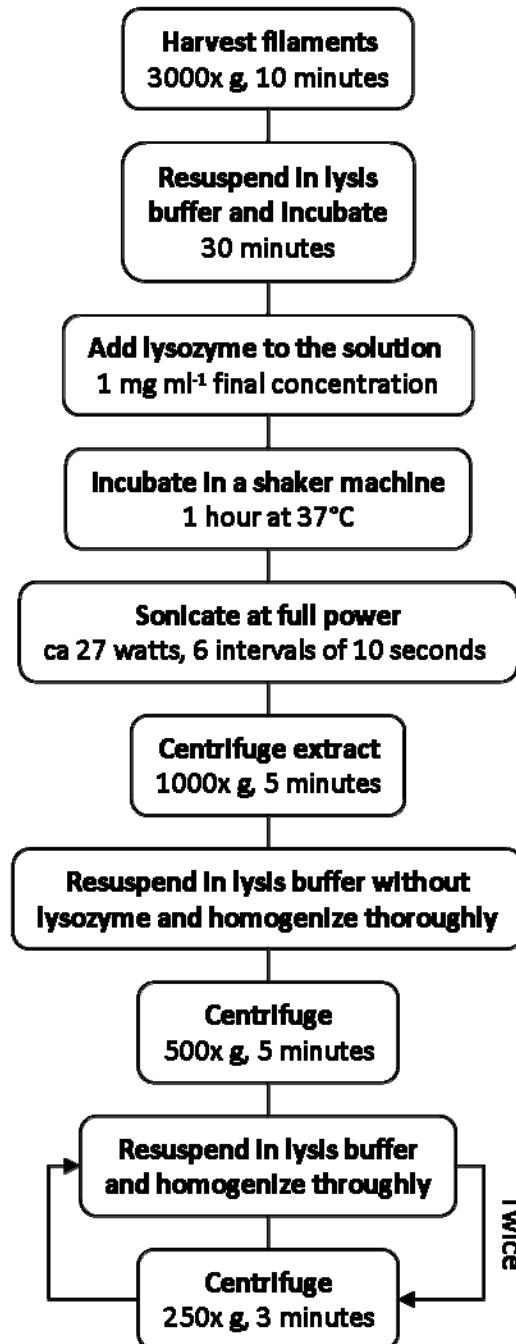


Figure 7. Step-by-step purification of heterocysts.

The last step of the protocol is the differential centrifugation. Several low speed centrifugations are required for cleaning the heterocysts from the debris and the cytoplasmic content of the vegetative cells. After each centrifugation step, the heterocyst precipitate should be thoroughly homogenized with a Potter pestle until there are no remaining aggregates. To ensure maximum purity, the washing and centrifugation steps should be repeated until the supernatant is clear and has lost the reddish color from the PBS released by the vegetative cells. If desired, the interested researcher should continue with the thylakoid preparation from the now pure heterocysts or store them at -80°C .

3.2.1 Assessment of sample purity

Several types of contamination from vegetative cells may be found in the heterocysts sample: (i) the contamination may be caused by unbroken filament fragments, single vegetative cells, or debris; (ii) the heterocysts may be contaminated with cytoplasmic material or abundant proteins like Rubisco and PBS; or (iii) the sample may be contaminated with remaining thylakoid membranes that somehow have wrapped around the heterocyst. Before proceeding with the isolation of thylakoids from the purified heterocysts samples, it is very important to have a control of the possible contaminants: below, I will describe the strategies that we implemented to make sure that our preparations had the required quality for further experimentation.

3.2.1.1 Light microscopy

Light microscopy is a very useful and practical tool for keeping track of each step of the protocol during heterocysts purification and ensuring that the lysozyme treatment, sonication, and final cleaning were carried out effectively. A successful preparation should have negligible amounts of unbroken vegetative cells or debris, as far as what can be detected by direct observation. Light microscopy is also a very convenient way to check the integrity of the heterocysts; they should retain the polar bodies and their typical blue-green color (Smith et al., 1988). To test whether the outer envelope has been preserved after the sonication, the heterocysts can be specifically stained with Alcian blue as described by Liu and Golden (2002); with our protocol the outer envelope remains intact (Figure 8).

3.2.1.2 Laser scanning confocal microscopy

When excited with laser light, vegetative cells show a strong fluorescence emission due to the high content of PBS. On the contrary, in the heterocysts a substantial amount of the antenna is degraded after compound nitrogen depletion (Thomas, 1970; Baier et al., 2004; and Wolf and Schussler, 2005); thus with the confocal microscope it is very easy to spot any contaminating vegetative cells, debris, remaining thylakoids or PBS aggregates; which are

otherwise impossible to see with a light microscope. For details on the exact measurements the reader is suggested to refer to Cardona et al. (2008) or to Paper II; there we determined that on average the fluorescence intensity from a vegetative cell is 20 to 25 times higher to the intensity from a heterocyst when exciting the antenna with 488 nm laser light. Figure 9 shows a confocal microscopy picture of filaments (panels A, B, and C) where it is very clear that a heterocyst has much less fluorescence than a vegetative cell. The confocal microscopy pictures helped us to confirm that a successful preparation should not contain any remaining intact vegetative cells or debris (Figure 9, panels D, E, and F).

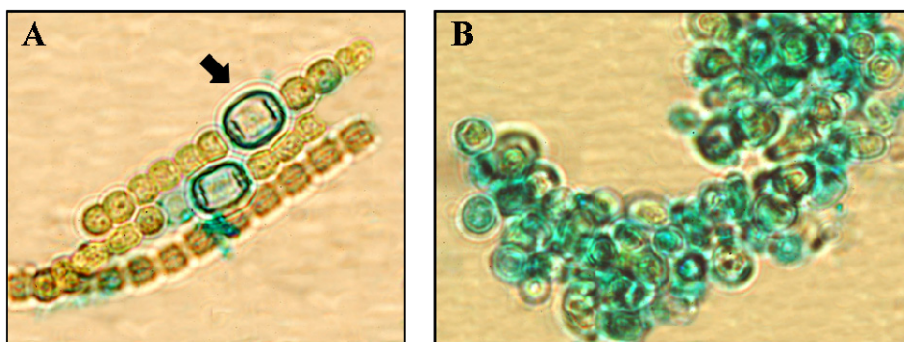


Figure 8. Alcian blue staining. (A) Filaments, the black arrow points towards the blue stained heterocysts. (B) Isolated heterocysts.

3.2.1.3 Rubisco western blots

To have a more quantitative control of the possible contamination from vegetative cells' cytoplasmic material, western blots could be performed on total protein extracts from vegetative cells and the purified heterocysts, using antibodies against the Rubisco large subunit (RbcL). Since heterocysts do not fix CO₂ they should be depleted of the Rubisco enzyme (Wolk et al., 1994), making this a reliable strategy to assess contamination from vegetative cell contents. We calculated then, that our heterocysts preparations were 93% to 97% pure (Paper II).

3.2.1.4 Light induced DCPIP reduction

Another way to prove that the purified heterocysts are not contaminated with thylakoid membranes from vegetative cells is to measure light-driven electron transfer in PSII, from water to an artificial electron acceptor, DCPIP. Since heterocysts do not possess water splitting activity no reduction of DCPIP should be observed, by simulating a hypothetical contamination from thylakoids corresponding to 1%, 5%, 10%, and 20% we were able to estimate that even at contaminations below 10%, electron donation from water to DCPIP should be easily detectable. This experiment was performed on

isolated thylakoids from heterocysts and the results of it will be discussed further on in this thesis.

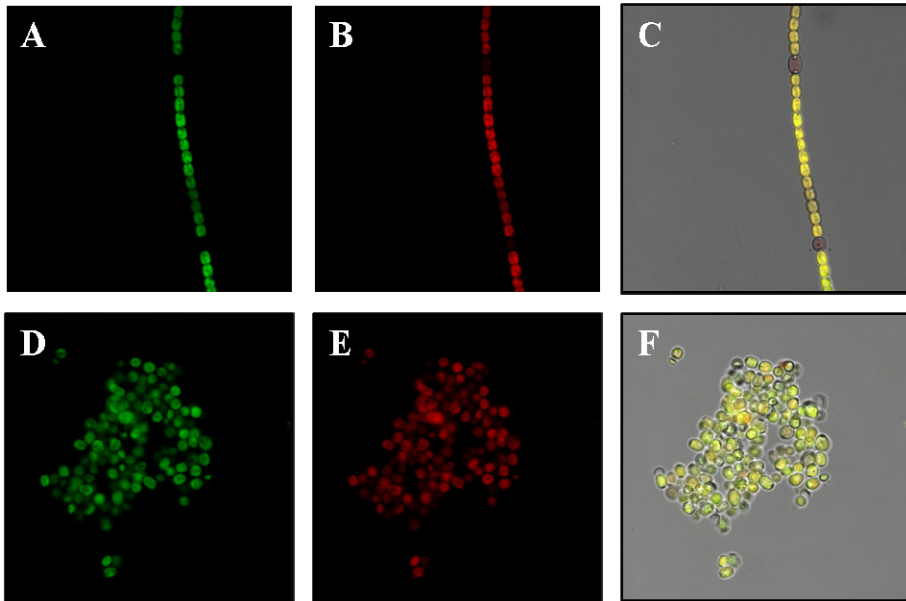


Figure 9. Laser scanning confocal microscopy of filaments and heterocysts. Panels **A**, **B**, and **C** depict a filament; while panels **D**, **E** and, **F** show purified heterocysts. The green color in panels **A** and **D** represents PBS fluorescence emission recorded from 650 to 690 nm. The red color in panels **B** and **E** represents fluorescence emission recorded for Chl-*a* from 700 to 740 nm. Panels **C** and **F** are a combination of the green and red channels with a picture taken in the transmission mode. Note: the sensitivity of the detector has been raised in panels **D**, **E**, and **F** in order for the heterocysts to be visible.

3.3 Isolation of thylakoid membranes from heterocysts

The protocol for the isolation of heterocyst thylakoid membranes is almost identical to that of the vegetative cells: however, a few modifications were necessary to the cell disruption step to maximize heterocyst breakage and thylakoid yield. First, the pressure was raised to 170 bar and second, the heterocysts were passed five times through the Parr vessel (see Figure 10). After the third time the homogenate was centrifuged, the supernatant containing the first release of thylakoids was kept on ice, the heterocyst pellet was resuspended in new disruption buffer, and passed once more through the Parr vessel: this cycle was repeated a second time. At the end, the 3 supernatants were pooled together and centrifuged at high speeds to precipitate the thylakoids.

Because a significant fraction of the membranes still remained attached to the cell walls of the heterocysts after five passages through the pressure vessel; the pellet composed of heterocysts debris and fragments was also stored and used later on for membrane proteomics analysis, as described in Paper II.

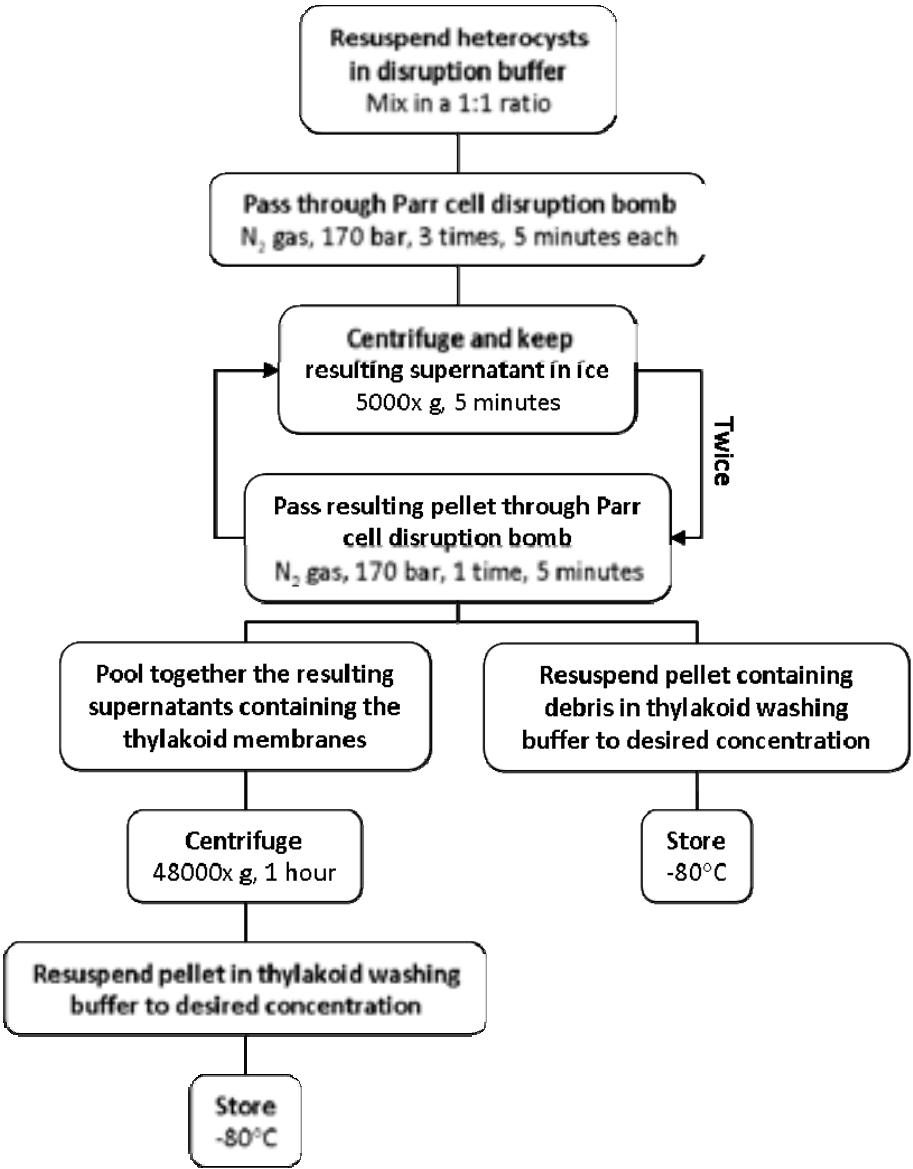


Figure 10. Step-by-step isolation of thylakoids from heterocysts.

4 The thylakoid membrane proteome

Proteomics is the study of the function of all expressed proteins (Tyers and Mann, 2003). It is not limited to the identification and listing of the proteins in a biological sample, it is high-throughput biochemistry aimed towards a direct understanding and description of all cellular processes. In such context, the thylakoid membrane proteome should give us greater insight into the bioenergetic processes that govern oxygenic solar energy conversion for the fixation of CO₂ in vegetative cells and anaerobic solar energy conversion for the fixation of N₂ in heterocysts, within the same organism and at the same time. Needless to say, this is the first time that intact photosynthetic complexes has been separated, identified, and analyzed for both vegetative cells and more remarkably, for the heterocysts of a filamentous cyanobacterium. Some of that insight we have gained into the bioenergetics of *Nostoc punctiforme* will be presented below.

The membrane proteome from the isolated thylakoids was separated by 2D Blue Native/SDS Polyacrylamide Gel Electrophoresis (2D BN/SDS-PAGE) and the proteins were identified by mass spectrometry (Matrix-assisted laser desorption/ionization time-of-flight, MALDI-TOF). BN electrophoresis is a technique for the separation of intact membrane multiprotein complexes conserving their native, quaternary conformation. The thylakoid membranes are first solubilized in a mild non-ionic detergent (n-dodecyl β -D-maltoside) and then with a proper gradient polyacrylamide gel, it is possible to separate complexes that range in molecular weight from 10 MDa down to approximately 10 kDa (Wittig et al., 2006). In our case, we focused in a molecular weight range from 2 MDa down to approximately 80 kDa, which is the size range for most complexes from the photosynthetic machinery. In the second dimension, a denaturing SDS polyacrylamide gel permits the separation of the membrane complexes into their respective subunits. In order to identify the proteins; the spots are excised out from the 2D gels and cleaved into small peptides by site-specific proteases. The size of the resulting peptides is sequence dependent and unique for different proteins: thus, the masses of the peptides can be determined by mass spectrometry and then by comparison with the theoretical mass of a translated gene product fragment from a given genome, the identity of the parent peptide is obtained (Whitelegge, 2003). Our 2D BN/SDS-PAGE, and MALDI-TOF methodology was based on that published by Herranen et al., (2004) and it is described in both Paper I and Paper II.

We identified 28 proteins from the vegetative cell thylakoid proteome (Paper I, Table 3) and 23 proteins from the heterocyst thylakoid membranes (Paper II, Table 1) in different multimeric complexes from all major components of the photosynthetic electron transport chain: they include PSII, PSI, Cyt *b₆f* complex, and ATP synthase; the respiratory complex NDH-1; and other proteins of relevance. I would like to remind the reader, that from heterocysts two separate membranes fractions were isolated and used for proteomics—as mentioned in section 3.3: a first one consisting of the membranes released after the passages through the Parr bomb, which I will be calling ‘thylakoid fraction’; and a second one consisting of the membranes that were still attached to the cell wall debris from the broken heterocysts, which I will refer to as ‘cell-wall fraction’. In the following sections I will present our membrane proteomics studies on the thylakoid membranes from vegetative cells and heterocysts, the differences in thylakoid composition and structures, and the possible implications that this differences have regarding each cell type specific physiological demands. I will correlate such findings with other experiments we have done to characterize the properties of the thylakoid membranes (e.g. EPR, electron transfer measurements, Mn concentration) and with the existing literature.

4.1 Photosystem II

In the membranes from vegetative cells six subunits from PSII were identified. They are the reaction center D1 and D2 proteins, the reaction center antenna proteins CP43 and CP47, the Cyt *b₅₅₉* subunit α , and the extrinsic protein PsbO involved in the stabilization of the water oxidizing complex (Paper I, Figure 4). The presence of PsbO is an indication of the intactness of the donor side of PSII in at least some of the centers. In addition, PSII was found to separate as a dimer and a monomer in agreement with the general accepted view of the oligomerization forms *in vivo* for both cyanobacteria (Zouni et al., 2001; Kamiya and Shen, 2003; Ferreira et al., 2004; Loll et al., 2005; and Guskov et al., 2009) and plants (Danielsson et al., 2006 and Adachi et al., 2009). However, recently this view has been challenged by Takahashi et al., (2009) whose experiments suggest PSII might be only a monomer *in vivo* and the dimerization occurs as an artifact caused by lipid deprivation during solubilization or purification.

Unexpected and very surprisingly, intact PSII complexes were found in the thylakoid fraction from heterocysts. Five subunits were identified: they are the D1, D2, CP43, CP47, and PsbO (see Figure 11, spots 13 to 16; and refer to Table 1 in Paper II). All of them were assembled as a monomer of PSII with a molecular weight of approximately 300-350 kDa, identical in size as the PSII monomer found in the vegetative cells. In contrast, PSII proteins were not detected at all in the cell-wall fraction (Figure 11 B). Indi-

vidual subunits of PSII have been found before in heterocysts (Houchins and Hind, 1984; Braun-Howland and Nierzwicki-Bauer, 1990; Thiel et al., 1990; Baier et al 2004; and Black and Osborne, 2004) but their experiments did not determine whether these proteins were part of a fully assembled PSII, or just remnants from degradation.

It has been hypothesized that the lack of O₂ evolution and the inactivation of PSII in heterocysts could be due to a depletion of Mn within the cell. Tel-Or and Stewart (1977) working with *Anabaena cylindrica* calculated that the concentration of Mn in the heterocysts was ca 9% of that in the vegetative cells. We wanted to test if a similar phenomenon could be found in *Nostoc punctiforme*, so we quantified the Mn concentration by EPR as described in Paper II and calculated that the amount of Mn in the heterocysts was ca 27% of that in the vegetative cells: plenty of Mn to maintain the water oxidizing complex.

To test whether the monomeric PSII present in heterocysts possesses an active electron transport chain of cofactors, light-driven electron transfer was measured from H₂O to an artificial electron acceptor, DCPIP, or from an artificial electron donor, DPC, to DCPIP. It was found that while the heterocyst thylakoid fraction was unable to reduce DCPIP with water as the natural electron donor of PSII; DPC did reduce DCPIP at 13% of the rate found in vegetative cell thylakoids on a Chl-*a* basis. Such an activity was inhibited by the specific PSII herbicide, DCMU (Paper II, Table 2); confirming that the recorded activity can be ascribed to PSII and not to any other redox process.

From the Mn concentration determination and the electron transfer measurement through PSII we can conclude that even though there is enough Mn in the heterocysts and that monomeric PSII is capable of charge separation, the water oxidizing complex is not assembled. We hypothesized that a possible explanation why there is PSII complexes in the heterocysts is to serve as a 'back up' system for electron donation after periods of darkness when the supply of carbohydrates from vegetative cells might be low (see Paper II, section 3.7 for a lengthier discussion). It is reasonable to think that under the right circumstances there could be photoactivation of PSII and water splitting activity below compromising levels for the inhibition of O₂ intolerant enzymes.

4.2 Photosystem I

We identified from the vegetative cell thylakoids the two main reaction center subunits of PSI, PsaA and PsaB. In the heterocyst membranes we identified in addition, the extrinsic protein located at the stromal side of PSI, PsaD (Jordan et al., 2001); the PsaL subunit essential for the formation of trimers (Chitnis and Chitnis, 1993 and Schluchter et al., 1996); and the PsaF, a subunit that might be involved in the stabilization of the reaction center core and

may also be involved in interactions with the PBS (Fromme and Grotjohann, 2006).

In total we separated six different oligomeric forms of PSI in vegetative cells and five in heterocysts, ranging in molecular weight from approximately 350 kDa to 1 MDa. The majority of PSI separated in the monomeric form in vegetative cells and in both heterocyst fractions. A second form of PSI of approximately 600 kDa also stained strongly and was assigned in Paper I and Paper II as trimers. In Herranen et al. (2004) a PSI complex of similar size was also identified and assigned as PSI dimers. Both assignments are probably inappropriate since dimers of PSI have never been reported to exist, and the size of the complexes in the BN gel is not consistent with either dimers or trimers. A clue about the nature of the higher molecular weight complexes of PSI isolated from *Nostoc punctiforme* might come from the heterocyst thylakoid fraction; where the FtsH protease was identified together with the PsaA and PsaB proteins from the highest molecular weight (1 MDa) 'PSI supercomplex' (Figure 11, spot 1; see Table 1 in Paper II also). FtsH is known to exist as large molecular assemblies reaching 1 MDa and it is in charge of the proteolysis of damaged membrane proteins (Sai-kawa et al., 2004 and Ito and Akiyama, 2005). While FtsH proteases are known to be involved in the degradation of PSII after photoinhibition in both plant's chloroplasts and cyanobacteria (Spetea et al., 1999; Yamamoto, 2001; Adam et al., 2004; Nixon et al., 2005; Komenda et al., 2006; also see Kato and Sakamoto 2009 for a recent review), FtsH has never been implicated in the degradation of PSI. To our knowledge, only one report exists to date that relates an FtsH protease with PSI; that of Mann and coworkers (2000) who found that a knock-out mutant of an FtsH protease from *Synechocystis* sp. PCC 6803 caused a 60% deficiency in the abundance of PSI. They concluded that FtsH might be involved in PSI assembly. Whether the FtsH protease is working in the assembly or disassembly of PSI, the most likely explanation for the presence of such an impressive number of oligomeric forms of PSI is that each one of those corresponds to steps in the biogenesis or degradation of this complex.

Remarkably, we noticed contrasting variation in the distribution of the PSI oligomeric complexes between the thylakoid fraction and the cell-wall fraction from heterocysts. In the former we observed significantly more stained spots for five of the complexes, in the latter the spots are stained rather weakly and the largest complex is missing completely. This suggests that each fraction might represent a discrete and separate region of the heterocyst thylakoid membranes. I will discuss in section 4.8 the meaning of these findings.

4.3 Photosystem I to Photosystem II ratio

A direct quantification of the PSI:PSII ratio can be acquired with EPR by measuring the proportion of $P700^+$ from PSI to the $Y_D^\bullet$ radical from PSII formed on a given thylakoid sample. Because the EPR absorption is directly proportional to the concentration of the molecular species giving the signals; the EPR absorption of $P700^+$ and $Y_D^\bullet$ can be used to determine the concentration of each reaction center (see Paper I for details of calculation). On vegetative cell thylakoids we calculated the ratio of PSI to PSII to be 4:1, if we assume that PSI contains 96 Chl-*a* molecules (Jordan et al., 2001) and PSII 35 Chl-*a* molecules (Guskov et al., 2009), then we can estimate that approximately 90% of the total Chl-*a* in *Nostoc punctiforme* belongs to PSI and the other 10% to PSII.

A similar EPR quantification of the amount of PSII and PSI in heterocysts has been difficult due to sample limitations. Nevertheless, an indirect estimate of the PSI to PSII ratio can be deduced from the reduction of DCPIP rates reported in (Paper II, Table 2). If we start from the assumptions that the 10% of the Chl-*a* in PSII from the vegetative cell thylakoid sample gave an activity of 34 $\mu\text{mol DCPIP reduced mg}^{-1} \text{ Chl-}a \text{ h}^{-1}$ and that all the PSII centers in heterocyst thylakoids are capable of electron transfer; then an activity of 3.9 $\text{mg}^{-1} \text{ Chl-}a \text{ h}^{-1}$ would correspond to ca 1% Chl-*a* belonging to PSII in the heterocyst thylakoids. In other words, a vegetative cell would have about 10 times more PSII than a heterocyst.

4.4 Cytochrome *b₆f* complex

From the eight subunits from the Cyt *b₆f* complex we identified in the vegetative cell thylakoids the four major proteins: the c-type Cyt *f* (PetA), the Cyt *b₆* (PetB), the Rieske iron-sulfur protein (PetC), and the subunit IV (PetD). In the heterocyst fractions we identified only PetA by mass spectroscopy; PetB and PetD were assigned by comparison with the vegetative cell staining pattern in the 2D gels. Unfortunately, PetC was not clearly separated for identification.

The Cyt *b₆f* complex functional form *in vivo* is the dimer (Kurusu et al., 2003), with the monomeric form considered as an artificial product of solubilization (Breyton et al., 1997). In our thylakoid preparations from both vegetative cells and heterocysts, the Cyt *b₆f* complex is separated in its native dimeric form. In the heterocysts the Cyt *b₆f* complex appears to be located almost exclusively on the cell-wall fraction.

4.5 ATP synthase

Seven subunits that made part of the ATP synthase in cyanobacteria were identified for the vegetative cell thylakoids and eight for the heterocyst thylakoids. These include the α , β , γ , δ , ϵ , b and b' subunits that form the F_1 soluble domain and the membrane spanning subunit c from the H^+ pumping rotary domain, F_o . The subunit c was not identified in vegetative cell thylakoids.

The ATP synthase separated as a monomer of approximately 500 kDa. Evidence of a dimeric form has been given for ATP synthase in mitochondria from very diverse organisms and in chloroplast from algae and higher plants (Schwassmann et al., 2007); we did not observe any ATP synthase dimers at all, at the current resolution of our gels. The complex was evenly distributed in both membrane fractions from heterocysts; however, they stained heavier than the vegetative cell thylakoid gels, suggesting that the abundance of this complex might be elevated in heterocysts.

4.6 NADH:quinone oxidoreductase

From the NDH-1 complex we identified a total of 3 subunits all belonging to the extrinsic hydrophilic domain, these are: NdhH and NdhK in both vegetative cells and heterocysts, plus NdhI only identified in the heterocysts. The complex separated in the BN gels from vegetative cell thylakoids in two forms corresponding in size to the NDH-1M and NDH-1L forms in Zhang et al. (2004) and Herranen et al. (2004). In both heterocyst membrane fractions we could observe the corresponding NDH-1M form, more abundantly present in the cell-wall fraction than in the thylakoids fraction. The NDH-1L appeared extremely faint and just barely visible.

The NDH-1M possibly has a dual function mediating inorganic carbon uptake and cyclic photophosphorylation in cyanobacteria (Ohkawa et al., 2000 and Zhang et al., 2004). Because heterocysts do not fix CO_2 , mechanisms for the concentration of inorganic carbon would be unnecessary, it is then likely that the NDH-1M in heterocysts works only in cyclic electron flow by returning the electrons in NADH or NADPH back to the PQ pool. On the other hand, the NDH-1L complex has been proposed to be vital for photoheterotrophic growth and respiration (Ohkawa et al., 2000 and Zhang et al., 2004). Since heterocysts depend on respiration and a heterotrophic-like metabolism for energy supply (Wolk et al., 1994), it is logical to think that they would contain higher amounts of NDH-1L, yet in both membrane fractions we observed only trace amounts of this complex.

4.7 Ferredoxin:NADP⁺ oxidoreductase

Two protein spots corresponding to FNR were identified only in the membranes from heterocysts: the first one separated to an apparent molecular weight of approximately 25 kDa (Figure 11, spot 20), it migrated in the vicinity of the Cyt *b*₆*f* complex, almost overlapping with the Cyt *f* subunit (Figure 11, spot 21), and it was present in both membranes. The second spot assigned to FNR separated to an apparent molecular weight of approximately 30 kDa, and it was present only in the cell-wall membrane fraction (unpublished results; Figure 11 B).

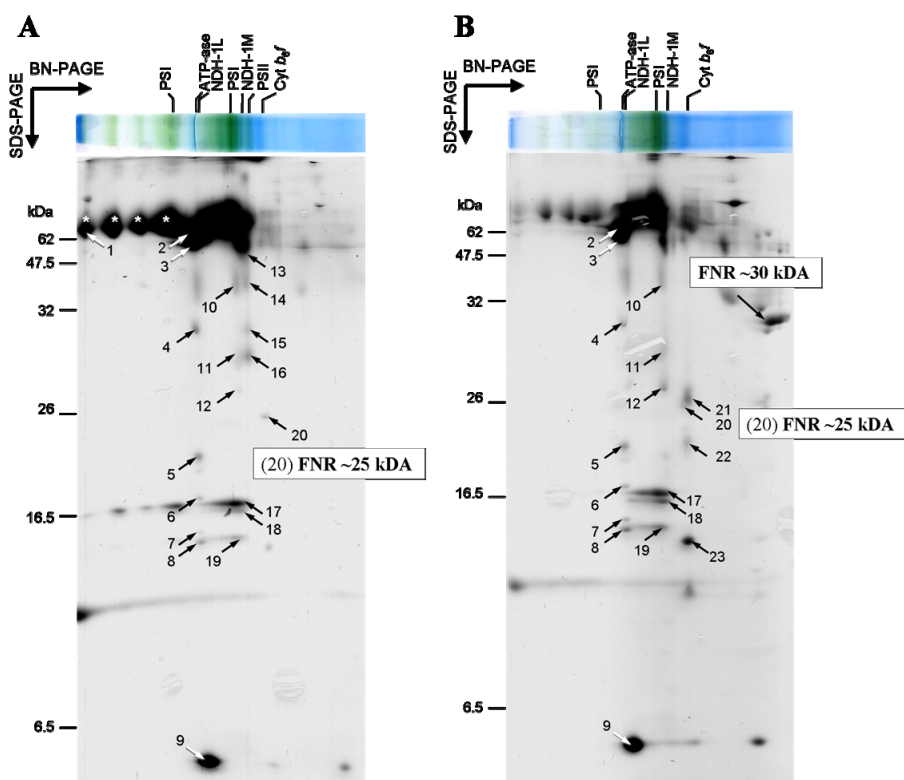


Figure 11. Two-dimensional gel electrophoresis of membranes from heterocysts. (A) Purified thylakoid membranes and (B) membranes belonging to the cell-wall fraction. The upper gel lane is a BN gel where membrane protein complexes have been separated in their native form. For the identity of the spots the reader is suggested to see Table 1 in Paper II.

The presence of two FNR forms has been demonstrated for cyanobacteria capable of heterotrophic growth (Thomas et al., 2006); a large FNR of molecular weight close to 46 kDa was suggested to be essential for photoauto-

trophic growth and to function as a NADP⁺ reductase, while the smaller FNR with a molecular weight close to 35 kDa was observed to accumulate during heterotrophic growth and thus suggested to function as a NADPH oxidase. Genetic studies on *Anabaena* sp. PCC 7119 and *Nostoc* sp. PCC 7120, showed the existence of two promoters and two transcription start points for the FNR gene generating respectively two different mRNAs; one of the promoters was active constitutively under all conditions, the second one was active under nitrogen deficient conditions and seemed to be exclusively used in the heterocysts (Valladares et al., 1999). It is hard to tell whether our observations of two FNR in the heterocysts membranes correspond to the isoforms reported in Thomas et al. (2006), especially because of the variation in size. Nevertheless, it is interesting that the small FNR in both heterocysts fractions separated on the BN gels to a molecular weight of approximately 200-250 kDa; while the bigger separated to a molecular weight of approximately 100 kDa in the cell-wall fraction only, suggesting that each form may be associated to the membrane in distinct regions and through different complexes, possibly fulfilling different roles in each case.

4.8 Different domains in the heterocyst thylakoids

As the reader might have noticed in the previous sections of this chapter, the two heterocyst fractions are somewhat different from each other in protein composition. The differences are listed and compared qualitatively in Table 2: briefly, the thylakoid fraction is made mostly of PSI, ATP synthase, and it also harbors all the monomeric PSII found in heterocysts. On the other hand, the cell-wall fraction appears to contain most of the NDH-1 complexes, the Cyt *b₆f* complex, and also have significant amounts of ATP synthase. The distinction between both membrane fractions is also reflected by the 38% higher Chl-*a* to protein ratio in the thylakoid fraction compared to the cell-wall fraction, which means that the former is composed mainly of the photosynthetic apparatus while the latter is richer in respiratory complexes. It may be that each fraction represents a discrete region or domain of the membrane system with a specialized function. In fact, it has been shown by hyperspectral confocal microscopy that the antenna proteins, PSII, and PSI are localized in different regions of the thylakoid membranes from the cyanobacterium *Synechocystis* sp. PCC 6803; PBS and PSII fluorescence seemed to be originating preferentially from the periphery of the thylakoids while PSI fluorescence more toward the inner layers of the membranes (Vermaas et al., 2008). They suggest that linear electron flow might occur at the periphery and cyclic photosynthesis through PSI at the inner layers.

Table 2. *Distribution of protein complexes in the heterocyst membrane fractions.*

	Thylakoid fraction	Cell-wall fraction
PSII	++	-
PSI	+++	+
Cyt <i>b₆f</i> complex	+	+++
ATP synthase	+++	+++
NDH-1	+	+++
FNR small	++	++
FNR large	-	+++

5 Quantitative shotgun proteomics

In the previous section I dealt with membrane proteins in their intact native form. In this chapter I will focus my attention on the entire proteome of the heterocysts as analyzed by quantitative shotgun proteomics. This work was performed in two different species of heterocystous cyanobacteria: first in *Nostoc* sp. PCC 7120 (Paper III) which was published as a continuation of a previous proteomic studies limited only to whole filaments in N₂ fixing and non-N₂ fixing conditions (Stensjö et al., 2007); and in *Nostoc punctiforme* (Paper IV) which would serve as foundation for future proteomic studies in mutant strains of this species with enhanced H₂ evolution.

One of the most important aspects of shotgun proteomics is that it not only identifies a vast number of proteins but it allows the quantification of identified proteins. The quantification is made possible thanks to the application of isobaric tags for relative and absolute quantitation (iTRAQ) commercialized by Applied Biosystems. It uses four (Paper IV) or eight (Paper III) amine specific tagging reagents to label the primary amines of peptides from four or eight different biological samples, respectively (Aggarwal et al., 2006; Chong et al., 2006).

In our experiments, 100 µg of total protein extracts from filaments and isolated heterocysts from both species are digested into peptides separately and labeled with a specific tag. Then the labeled peptides from each sample are mixed together and fractionated via strong-cation exchange liquid chromatography (SCX-LC). A second peptide separation via reverse-phase liquid chromatography (RP-LC) is done before identification and quantification of the peptides by tandem mass spectrometry (MS/MS), see Figure 1 in both Paper III and Paper IV. The protein quantification is achieved by comparing the peak area of the reporter ions derived from the tags in the mass spectrum. In our experiments, for *Nostoc* sp. PCC 7120 we compared the proteome of purified heterocysts relative to the proteome of the residual vegetative cell extracts from where they were isolated (heterocyst study) and we also analyzed the proteome of N₂ fixing filaments relative to that of the ammonia grown non-N₂ fixing filaments in a single 8-plex experiment (filament study); 8-plex meaning that eight different tags were used to label the samples. For *Nostoc punctiforme* in Paper IV, the proteome of isolated heterocysts was compared to the proteome of the parent N₂-fixing filaments in one 4-plex experiment; plus the comparison of both type of filaments in a second 4-plex experiment. Similarly, 4-plex indicates that only four tags were used

to label the samples. In all the experiments, for each type of sample two biological replicates were used and each one of them received its own tag.

Once the mass spectrometry is completed thousands of peptide spectra are obtained and then filtered for a 99% confidence protein identification and quantification using a stringent statistical analysis (see Paper III and IV for details). The quantification values reported here are presented as the ratio of the protein abundances from two chosen samples (e.g. heterocysts vs. N_2 fixing filaments) averaged for the two biological replicates. Based on the variation between replicates, the changes in abundances higher than 1.6-fold or lower than 0.6-fold in the protein ratio were considered to be significantly different. Finally in Paper III a total of 402 proteins were quantified and about 23% of those showed significant variation in the entire 8-plex experiment for both heterocyst study and filament study. In Paper IV a total of 497 proteins were quantified for the filament 4-plex study; from those approximately 15% of the proteome showed significant variation. In the heterocyst 4-plex study 377 proteins were quantified and about 60% of the proteome showed variation.

Due to the very large amount of data generated from these experiments I shall only devote my attention to nitrogen assimilation, the carbohydrate metabolism, and proteins from the photosynthetic apparatus; comparing the changes between the isolated heterocysts and the N_2 fixing filaments in *Nostoc punctiforme*. Nevertheless, throughout the following sections when judged necessary I will also compare similarities and differences to the *Nostoc* sp. PCC 7120 proteome.

5.1 Nitrogen assimilation

Because heterocysts develop for the purpose of N_2 fixation it is expected that the proteins directly involved in this process should be more abundant than in the vegetative cells. This is indeed the case for both organisms: in *Nostoc punctiforme* the components of the nitrogenase enzyme NifH (dinitrogenase reductase subunit) and NifK (dinitrogenase β chain) were found to be 1.89 ± 0.06 and 2.25 ± 0.11 times more abundant in the heterocysts than in the vegetative cell respectively. In *Nostoc* sp. PCC 7120, NifH and NifK were found 2.22 ± 0.05 and 4.54 ± 0.03 times higher in the heterocysts. In addition, the NifD (dinitrogenase α chain), a protein involved in the nitrogenase cofactor biosynthesis NifZ, and the heterocyst specific ferredoxin FdxH, the direct electron donor to nitrogenase, were also quantified with a 3.12 ± 0.10 -, 2.32 ± 0.05 -, and 8.33 ± 1.8 -fold increase in abundances, respectively.

Heterocysts use glutamine synthase (GlnA) as the main protein required for the assimilation of the ammonia produced by nitrogenase. It catalyses the binding of ammonia into glutamate generating glutamine; which in turn will be exported to the vegetative cells (Wolk et al., 1976 and Thomas et al.,

1977). We would then expect it to be highly abundant in the heterocysts; this was the case for *Nostoc* sp. PCC 7120 which showed a 1.96 ± 0.09 in contrast to *Nostoc punctiforme* that was below the cut-off limit at 1.42 ± 0.05 .

5.2 Carbohydrate metabolism

Although the proteome identified consist of less than 10% of the total number of proteins predicted in the genome of these organisms, almost all enzymes involved in glycolysis, pentose phosphate pathway, and citric acid pathway were quantified, allowing us to draw a detailed uninterrupted map of reactions that clearly portraits the differences in metabolism between heterocysts and the vegetative cell and the strategies used to fit each cell type energy requirements.

5.2.1 Glycolysis

Let's start with glycolysis (Figure 12), the metabolic route for the conversion of glucose into pyruvate. A first glance Figure 12 reveals that the process is a bit more complex than expected: the first step is the phosphorylation of glucose to yield glucose-6-phosphate (G6P). This step is ATP dependent and the enzyme is in lower abundance in the heterocysts compared to the parent filaments, this is in agreement with previous studies suggesting that glycolytic enzymatic activities are not very high in heterocysts (reviewed in detail by Wolk et al., 1994). The next step in glycolysis is the isomerization of G6P to fructose-6-phosphate (F6P) catalyzed by G6P isomerase and no significant variation between the type of cells was found.

The next step in the pathway showed a novel result, the enzyme that catalyzes the phosphorylation of F6P to fructose-1,6-bisphosphate (FBP) was not found: on the contrary, two isoforms of fructose-1,6-bisphosphatase (FBPase) were quantified, the enzyme that catalyzes the irreversible dephosphorylation of FBP into F6P in gluconeogenesis. One of them encoded by the gene NpF4023 was 2.62 ± 0.24 times higher in the heterocysts, while the second enzyme encoded by NpF3917 was found in much lower abundance at 0.14 ± 0.13 . NpF4023 is located in an operon with other pentose phosphate pathway proteins and therefore belongs to the class I FBPase; while the second one was annotated in the genome as GlpX and probably is a class II FBPase. Class I enzymes are present in both prokaryotes and eukaryotes while class II is almost exclusively present in prokaryotes (Jules et al., 2009). In plants, FBPase occurs also as two isoenzymes: one involved in gluconeogenesis and sucrose synthesis located in the cytosol and the other one is present in the stroma of chloroplasts and is a key component of photosynthetic carbon fixation, however both are derived from a class I FBPase (Tamoi et al., 1998). The observation that class I FBPase is more abundant

in heterocysts while the class II FBPase is mostly found in the vegetative cell is new and deserves more investigation. It is logical to think that in heterocysts, the class I enzyme works in cooperation with the pentose phosphate pathway to regenerate G6P; whether the class II FBPase in the vegetative cell is performing the same role or is working in the carbon fixation pathway is an open question.

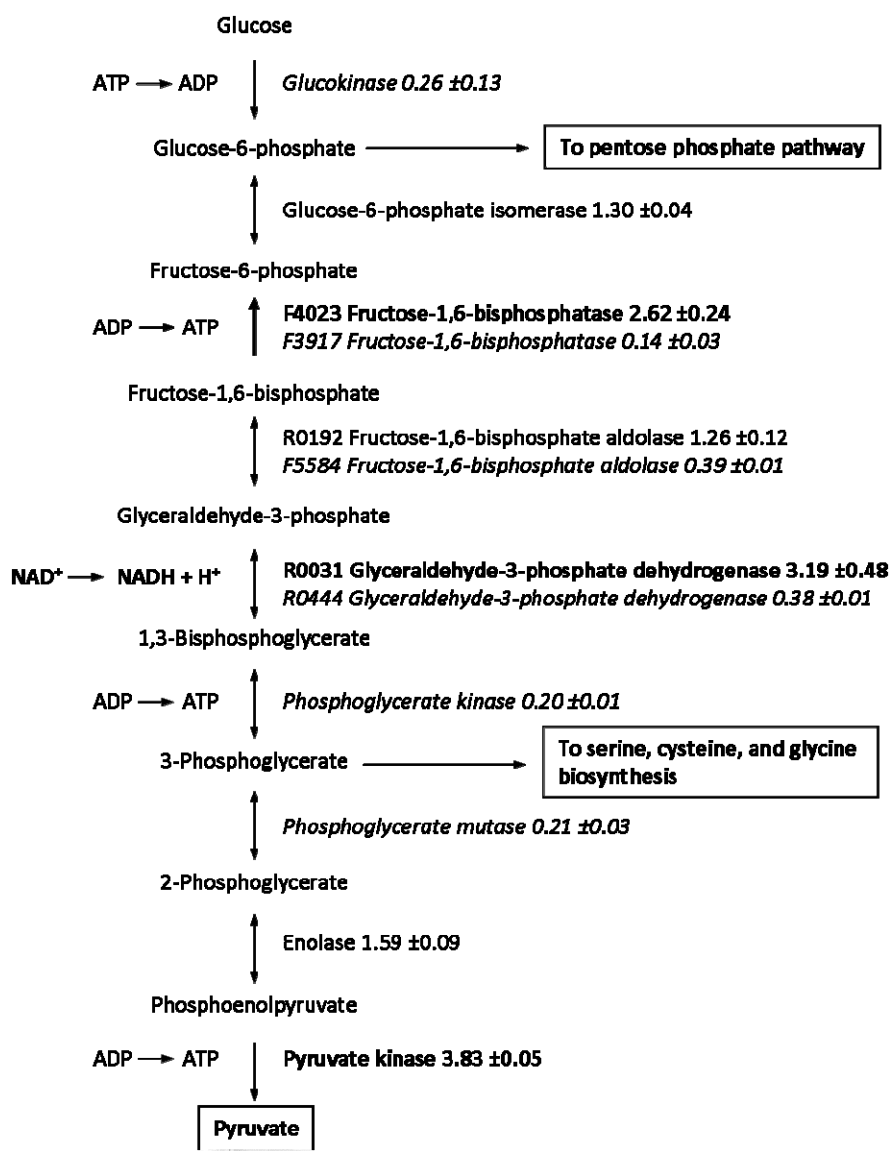


Figure 12. Glycolysis. Enzymes in bold represent those that were quantified with higher ratios for the heterocysts, while in italics those with lower ratios.

The next reaction is the cleavage of the six carbons FBP resulting in the three carbon sugars glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate catalyzed by FBP aldolase. Similar to the step before, there are two non-homologous classes of aldolases: class I is present mostly in eukaryotes, especially in mammals and plants, and class II is present mostly in prokaryotes (Nakahara et al., 2003) and requires divalent ions for catalysis (e.g. Zn^{2+} or Fe^{2+}). Both classes are present in cyanobacteria; interestingly the eukaryotic class I aldolase seemed to have gotten into cyanobacteria by an ancient horizontal gene transfer event from an eukaryotic red alga, a very rare and intriguing case indeed (Rogers et al., 2007). The proteome from *Nostoc punctiforme* revealed that both classes of aldolases are present to different extents in heterocysts compared to the filaments. The gene NpR0192 encoding the eukaryotic class I aldolase showed no variation between the cell types (1.26 ± 0.12); on the other hand, the class II aldolase encoded by the gene NpF5584 seems to be more abundant in the vegetative cells (0.39 ± 0.01). As for the case with FBPases such differential expression of aldolase isoenzymes has not yet been reported in the literature for heterocystous cyanobacteria.

The following step in glycolysis, the reversible conversion of GAP into 1,3-bisphosphoglycerate (BPG) with the generation of NADH catalyzed by GAP dehydrogenase (GAPDH) shows exactly the same pattern as the two previous steps: the presence of two enzyme forms differentially localized in each cell type. Something similar is seen in plants where there are two nucleus encoded GAPDH, one eukaryotic expressed in the cytosol and one chloroplast-targeted originating from cyanobacteria (Harper and Keeling, 2003). The two GAPDH forms described here have a single origin within the cyanobacteria and are not closely related to the eukaryotic form. In the *Nostoc punctiforme* proteome, we identified a GAPDH encoded by the gene NpR0031, similar to the Gap1 form annotated for other cyanobacteria, highly abundant in the heterocysts (3.19 ± 0.48); and a second one encoded by the gene NpR0444, annotated as Gap2, and predominating in the filaments (0.38 ± 0.01). In the *Nostoc* sp. PCC 7120 proteome we obtained the same result where Gap1 was more abundant in the heterocysts and Gap2 was higher in the filaments. In contrast to *Nostoc punctiforme*, *Nostoc* sp. PCC 7120 possesses an extra GAPDH denominated Gap3; Valverde et al., (2001) working with *Nostoc* sp. PCC 7120, and two other strains of closely related heterocystous cyanobacteria showed that Gap2 dominated in the vegetative cells while Gap3 did so in the heterocysts. In the same study Gap1 was not detected at all. Based on those results they suggested that Gap2 may function in photosynthetic carbon fixation while Gap3 would participate in glycolysis. In our work we suggested that Gap1, highly expressed in heterocysts might function in the reverse path for the regeneration of glucose-6-phosphate to feed the pentose phosphate pathway. Of course, more bio-

chemical evidence for the directionality and activity of the GAPDH is needed to confirm our hypothesis.

The next two enzymes in glycolysis, phosphoglycerate kinase and phosphoglycerate mutase were quantified with considerably lower abundances in the heterocysts (0.20 ± 0.01 and 0.21 ± 0.01); the following protein, enolase was just at the upper edge of the statistical cut-off at 1.59 ± 0.09 ; and very interestingly, the last enzyme in glycolysis catalyzing the formation of pyruvate from phosphoenolpyruvate (PEP) by pyruvate kinase was highly abundant in the heterocysts with a ratio of 3.83 ± 0.05 . Based on our results, if we assume that glycolysis is down-regulated in heterocysts, that aldolase and GAPDH works in the inverse reaction to assist the pentose phosphate pathway; I have to ask myself how PEP is formed in the heterocysts in enough quantities to feed the very abundant pyruvate kinase? The most likely explanation would be that it is synthesized in the vegetative cells and transported to heterocysts bypassing glycolysis, however no evidence for this have been given experimentally.

5.2.2 Citric acid pathway and branches

In cyanobacteria the citric acid cycle is incomplete because the enzyme required for the conversion of 2-oxoglutarate into succinyl-CoA is missing: in consequence, the “cycle” is mostly used for biosynthetic purposes (Stainer and Cohen-Bazire, 1977). In our case pyruvate will enter the citric acid pathway and it will be metabolized to 2-oxoglutarate with the generation of NADH.

2-Oxoglutarate is essential to heterocysts because it is the precursor of glutamate, which binds ammonia to form glutamine, thus connecting the nitrogen with the carbon metabolism. It is expected that the citric acid pathway will play an important role in the heterocysts (Laurent et al., 2005 and Zhang et al., 2006) and indeed all the enzymes required for metabolizing pyruvate into 2-oxoglutarate were quantified in the heterocysts (see Figure 13). Starting with the four components of the pyruvate dehydrogenase multienzyme complex that in a series of reactions decarboxylates pyruvate into Acetyl-CoA generating NADH and CO_2 , three of them showed higher abundance in heterocysts: namely, the pyruvate dehydrogenase β subunit (2.20 ± 0.02), the dihydrolipoamide acyltransferase component (1.76 ± 0.02), and the dihydrolipoamide dehydrogenase component (1.81 ± 0.05). The pyruvate dehydrogenase α subunit did not show any changes in abundance compared to the vegetative cell suggesting that the stoichiometry of this complex may be altered in heterocysts (1.17 ± 0.07).

The next step in the route is catalyzed by citrate synthase to generate citrate out of Acetyl-CoA, this enzyme showed also a very high ratio for the heterocysts (3.82 ± 0.02). Besides, isocitrate dehydrogenase, the enzyme that

catalyzes the formation of 2-oxoglutarate with the concomitant production of NADH and CO₂ was found to be also more abundant (1.68 ± 0.07).

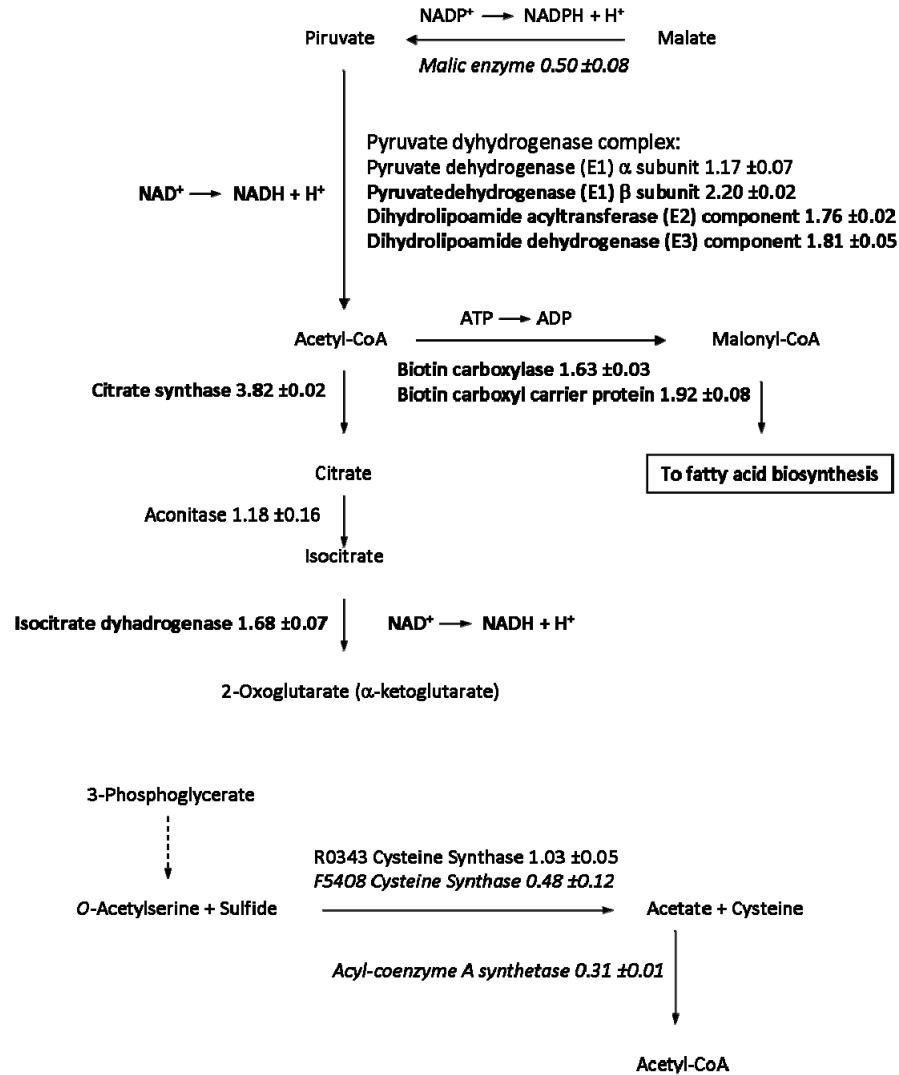


Figure 13. Citric acid pathway and branches. Enzymes in bold represent those ones that were quantified with higher ratios for the heterocysts, while in italic those that were found with lower ratios. The dashed arrow indicates that there is more than one enzymatic step in between the two products for which the respective enzymes were not identified.

We also found enzymes that branch out from or share intermediates with the citric acid pathway. For example, we quantified higher ratios two enzymes that catalyze the first committed step towards fatty acid biosynthesis. These

ones were biotin carboxylase (1.63 ± 0.03) and biotin carboxyl carrier protein (1.92 ± 0.02) involved in the ATP dependent formation of malonyl-CoA from Acetyl-CoA.

Acetyl-CoA can be formed from acetate by the enzyme Acyl-CoA synthase, and acetate can be formed from the synthesis of cysteine by cysteine synthase (although these two reactions are not necessarily connected). Acyl-CoA synthase was 0.31 ± 0.01 -fold lower in the heterocysts; while again, as for the cases in glycolysis, two isoforms of cysteine synthase were found. The first one showed no significant variation among the cell types (NpR0343: 1.03 ± 0.05) and the second one had a lower abundance in the heterocysts (NpF5408: 0.48 ± 0.12).

We also quantified the malic enzyme which catalyzes the reversible formation of pyruvate to malate with generation of NADPH. In *Synechocystis* sp. PCC 6803, this enzyme has also been shown to connect the citric acid pathway with glycolysis during heterotrophic growth in glucose-containing media (Yang et al., 2002); malate can also be formed from the oxidation of fatty acids. In heterocysts the malic enzyme seems to be about half the amount present in the filaments (0.5 ± 0.08).

5.2.3 Pentose phosphate pathway

It is generally accepted that due to the inability of heterocysts to split water, the reduction of O_2 and N_2 in heterocysts is supported by carbohydrates from the vegetative cell and metabolized in the pentose phosphate pathway (Wolk et al., 1994). This metabolic route will oxidize G6P in several steps producing ribulose-5-phosphate (Ru5P), an intermediate in the Calvin cycle and generating NADPH (see Figure 14). Then, in a series of isomerization reactions, Ru5P will be transformed into F6P and GAP which are intermediates in glycolysis and could be used to replenish G6P. The NADPH produced in the heterocysts might donate electrons to the respiratory chain through NDH-1 or to PSI through the Cyt *b₆f* complex, in both cases via FNR (Schmetterer, 1994 and Goldbeck, 1994). Our proteomic results quantified all the enzymes necessary to metabolize G6P into the glycolytic intermediates, the two enzymes in the pathway responsible for the production of NADPH: G6P dehydrogenase and 6-phosphogluconate dehydrogenase, were 4.29 ± 0.10 and 2.41 ± 0.08 times more abundant in the heterocysts, consistent with the activity measurements performed in purified heterocysts homogenates (Winkenbach and Wolk, 1973; Schrautemeier et al., 1984; Böhme and Schrautemeier, 1987; and Summers et al., 1995).

6-Phosphogluconate will be decarboxylated by 6-phosphogluconate dehydrogenase to produce Ru5P and NADPH; the next enzyme that should transform Ru5P into ribose-5-phosphate (R5P), R5P isomerase seems to be lower in abundance in the heterocysts (0.60 ± 0.01), while the last two enzymes transaldolase and transketolase seem to remain unchanged. Although

it is very risky to draw any conclusions, the low ratio of R5P isomerase might instead reflect a higher abundance in the filaments. This could be because this protein participates in the Calvin cycle and it has been shown that in isolated heterocysts from *Anabaena variabilis*, R5P supported nitrogenase activity via the pentose phosphate pathway (Böhme and Schrautemeier, 1987).

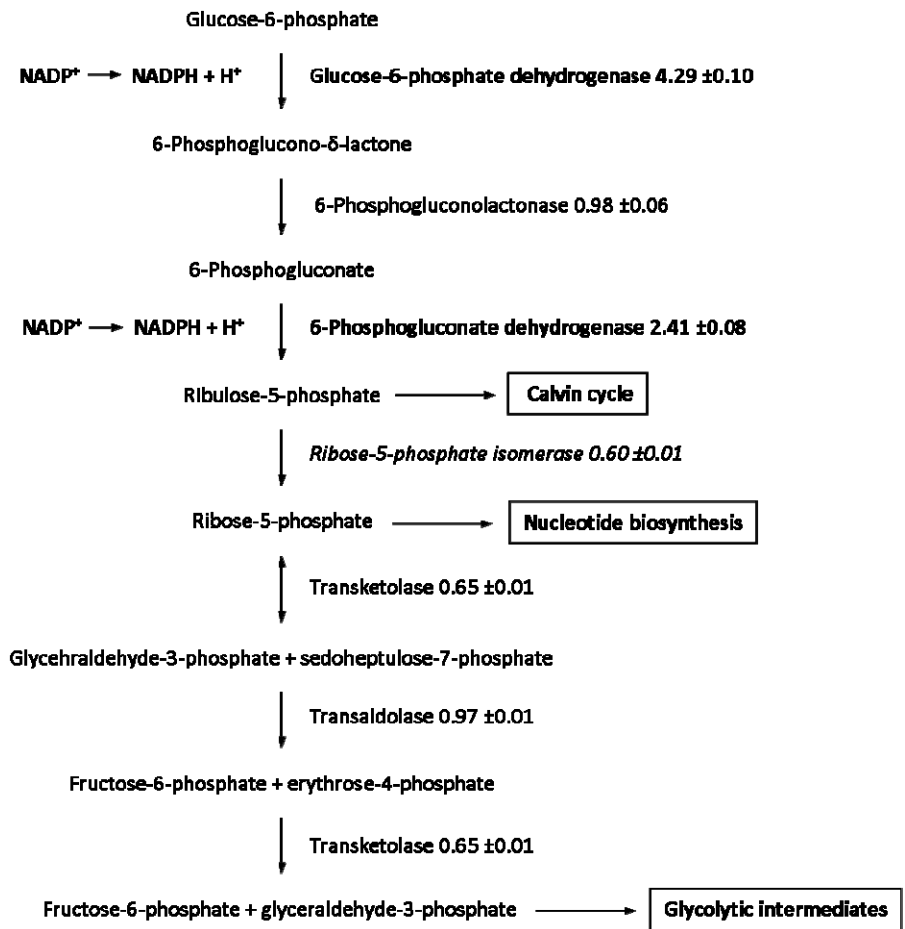


Figure 14. The pentose phosphate pathway. Enzymes in bold represent those that were quantified with higher ratios for the heterocysts, while that in italic was found with lower ratio.

5.2.4 Heme and Chlorophyll synthesis

Other proteins that appeared in the proteome of *Nostoc punctiforme* were from the biosynthetic pathway of tetrapyrroles and their derivatives, such as hemes, Chl-*a*, and vitamin B12 (Figure 15). Tetrapyrroles are synthesized

from small precursor molecules by two distinct mechanisms depending on the organism: the first one starts with glycine and succinyl-CoA, and it is utilized by some phototrophic bacteria, fungi and animals; the second one that is the mechanism used by cyanobacteria and plants starts with glutamate as a precursor (Beale and Weinstein, 1991). It is important to remember here that glutamate is essential for ammonia assimilation.

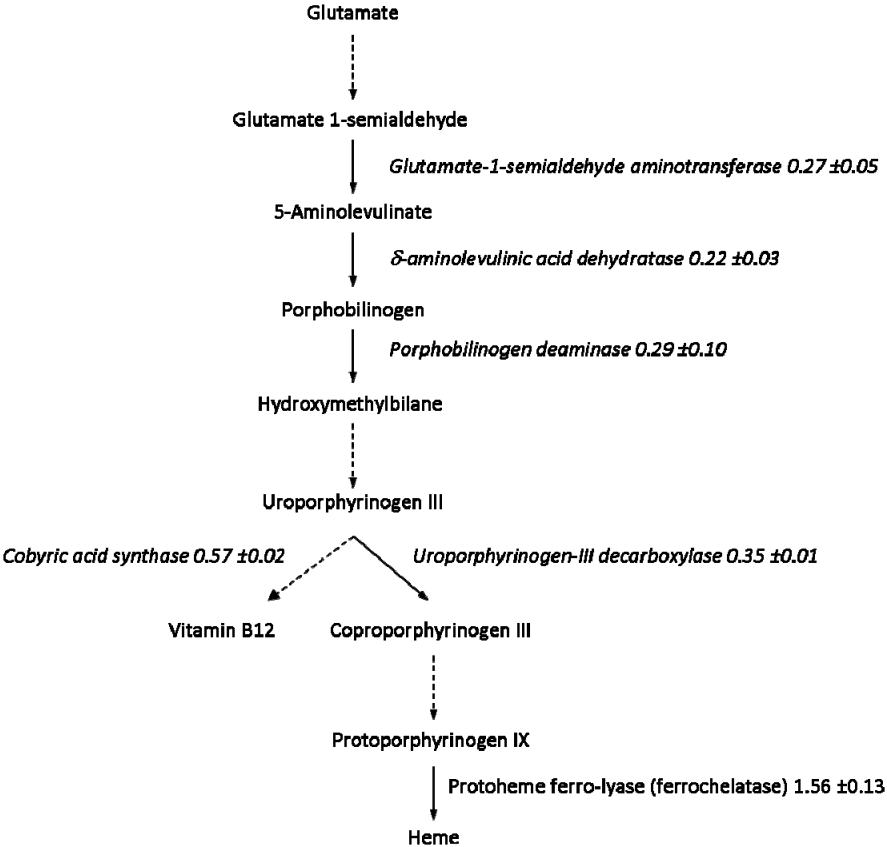


Figure 15. Heme and Chl-*a* biosynthesis. Enzymes in italics represent those that were quantified with lower ratios for the heterocysts. The dashed arrows indicate that there is more than one enzymatic step in between the two products for which not all the respective enzymes were identified.

In our analysis we quantified five proteins which showed consistently low ratios in heterocysts. These ones were, HemL or glutamate-1-semialdehyde aminotransferase, the enzyme that catalyzes the first committed step in tetrapyrrole synthesis (0.27 ± 0.05), HemB or δ -aminolevulinic acid dehydratase (0.22 ± 0.03), HemC or porphobilinogen deaminase (0.29 ± 0.10), and HemE or uroporphyrinogen-III decarboxylase (0.35 ± 0.01). We also quanti-

fied with a low ratio a protein from the branching point that leads to vitamin B12 synthesis, CobQ or cobyrinic acid synthase (0.57 ± 0.02) and with no significant change a protein from the branching point that leads to heme synthesis, HemH or protoheme ferro-lyase (1.56 ± 0.13).

The observation that heme/Chl-*a* synthesis proteins are in lower abundance in heterocysts to my knowledge has never before been reported in the literature. I find it of particular interest because it may suggest that heterocysts are not capable of synthesizing their own electron transport cofactors. This may be because the use of glutamate as precursor conflicts with N₂ fixation and may be a reason to why heterocysts have a short functional lifetime, estimated to be less than a week, based on kinetics studies of nitrogenase activity and turnover (Neilson et al., 1971).

5.3 The photosynthetic apparatus

A total of 52 proteins from the photosynthetic apparatus were quantified in the *Nostoc punctiforme* proteome (see Table 3 and Paper IV); and although it is considered that shotgun proteomic analysis is biased against hydrophobic membrane proteins (Stensjö et al., 2007), this study quantified several membrane spanning subunits from some of the photosynthetic complexes.

5.3.1 Photosystem II

Ten proteins from PSII were quantified for heterocysts of *Nostoc punctiforme*, unfortunately two of them did not appear in Paper IV due to difficulties with the annotation of the genome at that time. This is a confirmation and an extension of the membrane proteomic results from Paper II where we identified intact monomeric PSII in the heterocysts membranes. We observed all proteins that make the reaction center core, D1, D2, CP43, CP47, and the α chain of Cyt *b*₅₅₉; the three extrinsic proteins involved in the stabilization of the Mn₄Ca cluster; one of the small subunits (PsbH) and an additional protein associated to PSII, denominated PsbW, also known in the literature as Psb28, Psb13, or Ycf79 (Kashino et al., 2002). Surprisingly, the quantification showed no significant variation for the ratio of these proteins, with the exception of PsbW that showed a 9-fold decrease in abundance for the heterocysts. In the proteome from *Nostoc* sp. PCC 7120 we also identified four PSII subunits with no significant variation, those were: D1, CP47, PsbU, and also PsbW (see Paper III Table 1).

The PsbW subunit from cyanobacteria differs significantly from the PsbW from higher plants which is reflected in low sequence homology (only 16% identity compared to *Arabidopsis thaliana*). While the PsbW from plants is one of the small subunits of PSII, possessing a transmembrane helix; the PsbW from cyanobacteria is a larger soluble peptide (13 kDa) with

no transmembrane helix (Kashino et al., 2002) and was not detected in the crystal structures from *Synechococcus elongatus* (Loll et al., 2005 and Guskov et al, 2009). The PsbW protein was first identified in a proteomic study of highly pure PSII preparations from *Synechocystis* sp. PCC 6803 (Kashino et al., 2002) and all of the investigations on the cyanobacteria version of PsbW has been in the same organism. More recently, it was demonstrated that PsbW has been detected as a component of PSII depleted of phosphatidylglycerol (Sakurai et al., 2007), suggesting that the protein may be of importance for the assembly of PSII and it is not a true constitutive subunit. Moreover, Dobáková et al., (2009) showed that the majority of PsbW is not bound to PSII, but a fraction of it does interact with the CP47 protein during assembly of the complex. They also showed that deletion mutants grew slower autotrophically than wild type and had accelerated turnover of the D1 protein, faster PSII repair, and a decrease in the amounts of PSI. The deletion mutant also contained high levels of intermediate molecules on the synthesis of Chl-*a* (e.g. protoporphyrin IX) indicating a malfunction in the last steps of Chl-*a* biosynthesis pathway. Dobáková et al., (2009) hypothesized that PsbW assisted in the last step of Chl-*a* formation which would be immediately bound to the CP47 apoprotein.

The presence of PSII complexes in the heterocysts is very puzzling and whether the low abundance of PsbW in *Nostoc punctiforme* is somehow related to the inactivation of PSII remains to be proven.

Table 3. *Quantification of proteins from the photosynthetic apparatus.*

Accession	Name	Other name	Ratio	Comment
Photosystem II				
NpR5188	PsbA	D1	0.98 ±0.01	Core
NpR2471	PsbB	CP47	1.19 ±0.03	Core antenna
NpR3636	PsbC	CP43	1.29 ±0.01	Core antenna
NpF4553	PsbD	D2	1.29 ±0.05	Core
NpF5551	PsbE	Cyt <i>b</i> ₅₅₉	1.41 ±0.05	Core
NpF4315	PsbH		0.93 ±0.03	Stability
NpF4810	PsbO		1.42 ±0.03	Lumen
NpF4509	PsbU		1.34 ±0.03	Lumen
NpR2790	PsbV	Cyt <i>c</i> ₅₅₀	0.98 ±0.01	Lumen
NpR3943	PsbW, Psb28		0.11 ±0.01	Assembly (?)
Photosystem I				
NpF3818	PsaA		4.22 ±0.09	Core/antenna
NpF3819	PsaB		2.41 ±0.12	Core/antenna
NpF5213	PsaC		1.83 ±0.14	Stroma
NpR5148	PsaD		2.91 ±0.09	Stroma
NpR3937	PsaE		3.35 ±0.29	Stroma
NpF3864	PsaL		3.83 ±0.10	Trimerization

Cytochrome <i>b₆f</i> complex				
NpR0131	PetA	Cyt <i>f</i>	2.11 ±0.01	
NpF0310	PetB	Cyt <i>b₆</i>	1.33 ±0.01	
NpR0132	PetC	Rieske FeS	1.84 ±0.07	
NpF0311	PetD	Subunit IV	2.23 ±0.09	
Electron carriers				
NpR2789	PetE	Plastocyanin	0.26 ±0.01	Soluble
NpR2751	PetH	FNR	2.18 ±0.01	Soluble
ATP synthase				
NpF4863	AtpA	α	3.45 ±0.10	F ₁
NpF4861	AtpB	b	3.61 ±0.15	F _o , stalk
NpF4864	AtpC	γ	2.98 ±0.03	F ₁
NpR4417	AtpD	β	3.61 ±0.13	F ₁
NpF4862	AtpD	δ	3.36 ±0.14	F ₁
NpF4860	AtpF	b'	3.03 ±0.14	F _o , stalk
NADH:quinone oxidoreductase				
NpR5626	NdhA		2.36 ±0.02	Membrane
NpR5623	NdhG		2.97 ±0.10	Membrane
NpF4970	NdhH		2.67 ±0.04	Extrinsic arm
—	NdhI		3.99 ±0.04	Extrinsic arm
NpR5546	NdhJ		2.50 ±0.13	Extrinsic arm
NpR5547	NdhK		3.40 ±0.04	Extrinsic arm
NpF1518	NdhM		2.98 ±0.14	Extrinsic arm
NpF4391	NdhN		2.06 ±0.05	Extrinsic arm
Allophycocyanin				
NpR4842	ApcA	α	0.14 ±0.03	Phycobiliprotein
NpF5388	ApcB	β	0.30 ±0.01	Phycobiliprotein
NpR4840	ApcC	L _C	0.35 ±0.02	Linker
NpF0878	ApcD	α ^B	0.14 ±0.02	Phycobiliprotein
NpR4843	ApcE	L _{CM}	0.45 ±0.02	Terminal emitter
NpR4841	ApcF	β ¹⁸	0.20 ±0.02	Phycobiliprotein
Phycocyanin				
NpF5290	PcyA	α	0.12 ±0.02	Phycobiliprotein
NpF5289	PcyB	β	0.15 ±0.02	Phycobiliprotein
NpF5292	CpcC		0.28 ±0.01	Linker
NpF5291	CpcC2		0.57 ±0.01	Linker
NpF5293	CpcD		0.34 ±0.04	Linker
NpF5295	CpcF	PCbilin lyase	0.40 ±0.02	Biogenesis
NpF3811	CpcG1		0.25 ±0.01	Linker
Phycocerythrin				
NpR3806	CpeA	α	0.15 ±0.04	Phycobiliprotein
NpR3807	CpeB	β	0.11 ±0.02	Phycobiliprotein

5.3.2 Cyclic photophosphorylation

As a contrasting difference with PSII, every single subunit identified for the other complexes in the thylakoid membrane showed consistently higher ratios for the heterocysts. Six subunits from PSI, including the core proteins, the FeS clusters bearing proteins, and PsaL necessary for trimerization, were found to be on average 3-fold higher in abundance in the heterocysts. The four main components of the Cyt *b₆f* complex were quantified to be on average 1.8-fold higher, six proteins from the ATP synthase were 3.3-fold higher, and eight subunits from the NDH-1 respiratory complexes were 2.8-fold higher as well. In addition, two electron transport proteins were also quantified: plastocyanin that is reduced by the Cyt *b₆f* complex and donates electron to PSI and FNR that accepts electrons from PSI. FNR was 2.18 ± 0.01 times higher in abundance and in sharp contrast, plastocyanin was significantly lower. The alternative electron donor from the Cyt *b₆f* complex to PSI, the Cyt *c₆* was not detected in *Nostoc punctiforme*, but it did showed up in *Nostoc* sp. PCC 7120 with a very high 3.5 ± 1.3 ratio; it may be that heterocysts has a different choice when it comes to these proteins (Paper III).

Undoubtedly, cyclic photophosphorylation driven by PSI is essential to heterocyst metabolism. The quantification of the proteome allowed us to elegantly observe the entire energy processes going on within the heterocysts in cooperation with the vegetative cell; connecting the carbohydrate metabolism to generate abundant reducing power, with the process of ATP generation powered by PSI mediated cyclic electron transport in the thylakoid membranes to finally fix N₂.

5.3.3 Phycobilisomes

Heterocysts are known to partially degrade the PBS on the onset of compound nitrogen deficiency (Wolk and Simon, 1969; Thomas, 1970; 1972; Bradley and Carr, 1976; and Baier et al., 2004). Fifteen proteins from the PBS were quantified, including all components of the APC core, several subunits from the PC and PE rods, and CpcF a phycocyanobilin lyase involved in the binding of the phycobilin pigment into PC (Fairchild et al., 1992). All of the proteins were consistently lower in abundance for the heterocysts; although not all of the subunits appeared to have been degraded to the same extent. The proteins that showed the lowest abundances were: α and β subunits of PE (CpeA and CpeB), the α and β subunits of PC (PcyA and PcyB), and the α and α^B subunits of the APC core (ApcA and ApcD), presenting on average a 7.4-fold decrease in abundance in the heterocysts. All of these proteins are pigmented phycobilin carrying proteins (Adir, 2008). The remaining phycobilin bearing proteins, β and β^{18} subunits (ApcB and ApcF), plus the terminal emitter L_{CM} (ApcE), all from the ALC core showed respectively a 2.8-, 4-, and 2.2-fold decrease in abundance. In addi-

tion, the linker peptides which do not contain pigments and are necessary to keep all the parts of the PBS together, showed on average a 2.7-fold decrease in abundance (see Table 3).

Such contrasting difference in abundances from the different components of the PBS, together with the fluorescence studies that I will describe in the next chapter, have led us to postulate that the PBS in the heterocysts has been modified: possibly made of shorter rods with core cylinders composed mainly of the L_{CM} subunit and the β APC chain, with the purpose to transfer energy preferentially to PSI. I will spend a few extra words about the heterocyst PBS in section 6.2.

6 Energy transfer

Light fluctuations are common in nature, a cloudy day, the forest canopy and oceanic waters filtering effects, the turbidity of lakes and rivers, are just some examples of them (Schubert et al., 1995). Cyanobacteria as well as eukaryotic algae and plants have evolved the capacity to redistribute the amount of excitation energy that is transferred to each photosystem at any given time if light becomes a limiting factor (Grossman et al., 1993 and McConnell et al., 2002). This redistribution of energy is generally accomplished by the preferential association of the light harvesting antenna to one photosystem or the other: LHCII in green algae and plants, or PBS in cyanobacteria and red algae. This phenomenon is called state transition and was described for the first time by Murata (1969) in the red alga *Porphyridium cruentum* and by Bonaventura and Myers (1969) in the green alga *Chlorella pyrenoidosa*. LHCII and PBS are associated primarily to PSII and in normal conditions most of the excitation energy they harvest will be transferred to it. However, when cells are illuminated with light that excites PSII to the point that it is excessive, so that the PQ pool gets reduced in excess; then, some of the PBS or LHCII will be redistributed to PSI. The process is denominated: transition to State 2. On the other hand, when the cells are illuminated by light that excites PSI (e.g. far-red light) to the point that it is limiting, so that the PQ pool gets over oxidized; then there will be a movement of antenna proteins towards PSII, redistributing and balancing the excitation energy. This process is called transition to State 1 (Mullineaux and Allen, 1990; McConnell et al., 2002). In cyanobacteria besides the movement of PBS (Mullineaux et al., 1997; Aspinwall et al., 2004; Yang et al., 2007), another component has been identified to be playing an important role in the control of excitation energy transfer, the monomerization and trimerization of PSI. It has been suggested that the oligomerization might cause energy spillover from PSII Chl-*a* to PSI Chl-*a* (Rouag and Dominy 1994 and Li et al., 2006) aiding in the dissipation of excess energy.

In chapter 4 and 5, I described all the differences in the photosynthetic apparatus between vegetative cells and the heterocysts. We have learnt that heterocysts rely on cyclic electron flow through PSI for generation of ATP to power N₂ fixation, that they possess an inactive PSII, a partially degraded PBS, and an altogether completely different metabolism. It made me wonder whether heterocysts were capable of regulating the distribution of excitation energy transferred to PSI with changing light quality. Because N₂ in hetero-

cysts is a light dependent process (Wolk et al., 1994), I hypothesized that the heterocysts could have a mechanism to control the amount of excitation energy transferred to PSI in order to optimize N_2 fixation in a fluctuating environment.

In Paper V we attempted to test this hypothesis by using fluorescence spectroscopy and state transition-like illumination regimes on entire filaments and purified heterocysts. I will briefly describe in the next sections the methodology, the most important results, and a few insights we have got on the matter.

6.1 Energy transfer from the phycobilisome to Photosystem I

To induce changes in the distribution of energy to the photosystems, dark adapted filaments and purified heterocysts were illuminated for five minutes with either green or red light followed by rapid freezing in liquid nitrogen (77K) to trap the different states formed. Green light was obtained using an interference filter with maxima at 560 nm while red light illumination was obtained using a 708 nm maxima interference filter (see Paper V for exact details). Green light should be absorbed mainly by the PBS and in the vegetative cell it might be sent preferentially to PSII; red light on the contrary, will be more efficiently absorbed by PSI. We then recorded and compared the 77K fluorescence emission and excitation spectra to see the induced changes.

The fluorescence emission from filaments when the PBS is excited with 570 nm light at 77K is characterized by a strong fluorescence from PC and APC around 640-660 nm (see Figure 16 A) (Adir, 2008). We also observed a peak at 680 nm, corresponding to emission from PSII Chl-*a* and also from the terminal emitters of the ALC core, L_{CM} . A broad fluorescence band with maximum around 730 nm is associated with emission from PSI Chl-*a* (Fork and Mohanty, 1986). Figure 16 B depicts the emission spectrum from heterocysts: similar to the filaments although with lower intensity, we observed emission from PC and APC around 640-660 nm and an intense peak around 680 nm originating from the terminal emitter L_{CM} . Because in the heterocysts the amount of Chl-*a* belonging to PSII is very low (see section 4.3 of this thesis) almost all the emission at 680 nm can be assigned to L_{CM} . Finally, like in the vegetative cells, most of the emission above 700 nm belongs to PSI.

When we compared the 77K emissions spectra from the green and red illuminated cells in filaments and heterocysts (Figure 16), the samples that were illuminated with green light showed a marked quenching of fluorescence intensity from the terminal emitter at the 680 nm peak. Simultane-

ously, we observed the appearance of a peak at 750 nm as opposed to the red light illuminated samples. The shoulder at 750 nm belongs to the longest wavelength emitting Chl-*a* molecules from PSI, and such fluorescence is only seen in trimeric PSI where P700 is reduced (see Karapetyan et al., 2006 for a comprehensive review). This was the first indication that like vegetative cells, the photosynthetic apparatus from a heterocyst was responsive to changes in light conditions; and specifically for this experiment, that for both heterocysts and vegetative cells the green light illumination caused the coupling of the PBS to trimeric PSI.

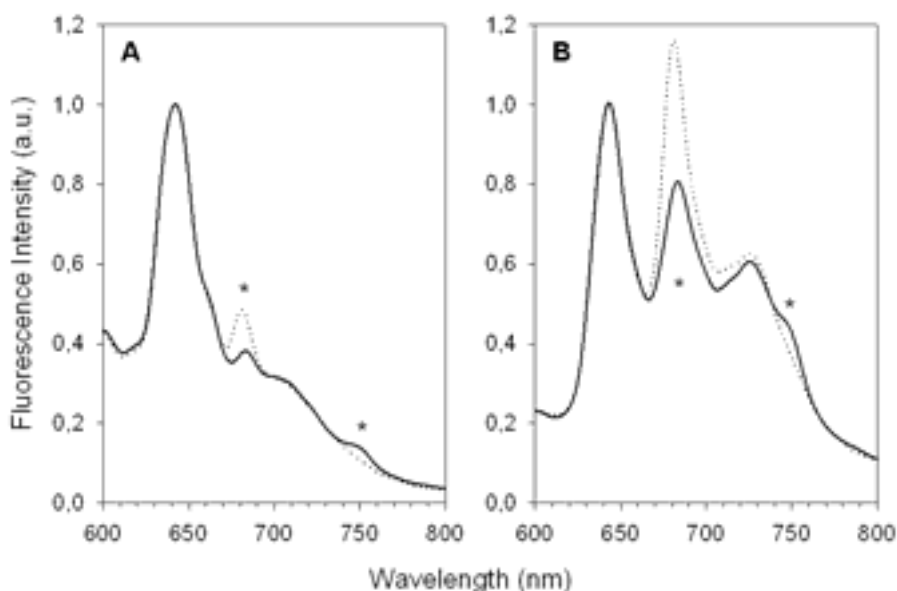


Figure 16. Fluorescence emission spectra recorded at 77K exciting the PBS at 570 nm, from whole filaments (**A**) and heterocysts (**B**). Spectra were recorded after samples had been illuminated at room temperature with either green light (solid) or red light (dotted) and normalized to the emission peak at 645 nm.

To confirm that the quenching at 680 nm was due to energy transfer from the PBS to PSI, we also recorded the excitation spectra after green and red light illumination of the samples (Figure 17). The spectra were taken both at 728 nm, the emission maximum for PSI, and at the 750 nm shoulder where trimeric PSI fluoresced. In the samples that were illuminated by red light there was no difference between 728 nm, 750 nm, and the dark adapted sample (Figure 17, solid line). After green illumination, the region between 550 and 650 nm that correspond to PBS excitation, showed a marked raise in intensity. Suggesting that energy transfer from the PBS to PSI was more

efficient in these samples (Figure 17 dotted and dashed lines). Surprisingly, the spectra recorded at 750 nm (Figure 17 dashed lines), showed an even higher increase in the PBS excitation region than the spectra at 728 nm. We could conclude with more confidence that the quenching of the terminal emitter fluorescence after green illumination was due to the coupling of the PBS to PSI trimers directly through the APC core. Thus, the green illumination led to an increase in energy transfer to PSI, similar to a transition to State 2, but not red illumination. Furthermore, this occurred in filaments and purified heterocysts.

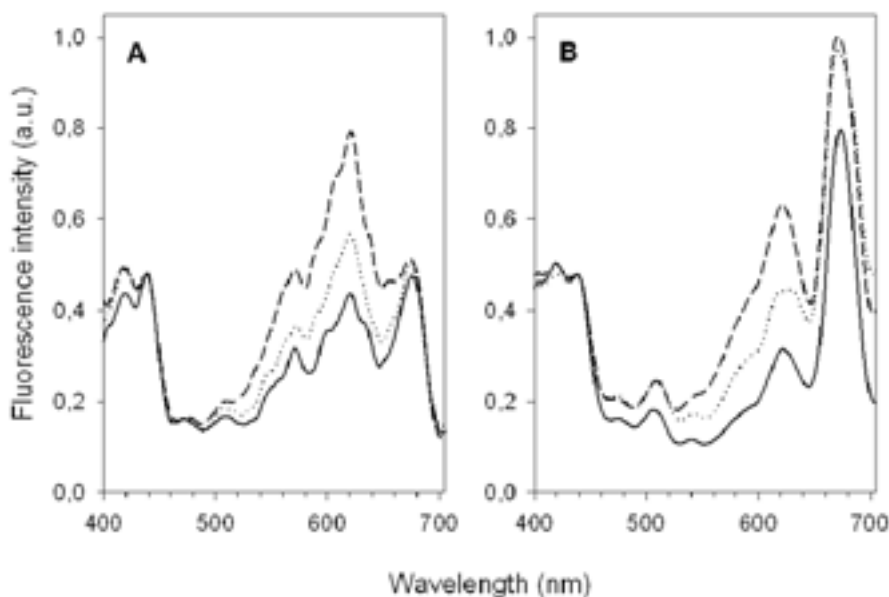


Figure 17. Excitation spectra from whole filaments (**A**) and heterocysts (**B**). Solid lines depict emission in samples that were illuminated with red light, collected at 728 nm. Dotted and dashed lines show emission from samples that were pre-illuminated with green light and collected at 728 nm (dotted) and 750 nm (dashed) respectively. The spectra were normalized at the Chl-*a* excitation peak at 440 nm.

It is noteworthy to mention that because heterocysts do not possess water oxidation: the state transitions are not really possible as a redistribution of the energy transfer to PSII or to PSI. Nevertheless, heterocyst must sustain a highly active cyclic photophosphorylation to generate enough ATP for the nitrogenase (as we have seen in Paper II through IV). In a paper by Ernst and Böhme (1984) it was reported that isolated heterocysts from *Anabaena variabilis* were capable of keeping stable ATP concentrations under varying light intensities and other factors affecting the redox state of the PQ pool,

suggesting that a mechanism to regulate the amount of excitation energy transferred to PSI may exist. Our results demonstrated that heterocysts too are capable of responding to the quality of light by coupling or decoupling the PBS from PSI, and by controlling PSI oligomericization state. Most importantly this takes place completely in the absence of active PSII.

6.2 The heterocyst phycobilisome

On the onset of compound nitrogen deprivation, the first response is a general degradation of the PBS in the entire filament (Bradley and Carr, 1976 and Baier et al., 2004). However, once heterocysts have matured and the nitrogen levels are reestablished the PBS is resynthesized within the vegetative cell (Neilson et al., 1971) but it remains low in heterocysts (Thomas, 1970; 1972; Peterson et al., 1981; Baier et al., 2004; and Paper II). To my knowledge it has never been discussed before why after nitrogen replenishment the heterocysts should not rebuild the PBS. In Paper II and Paper V, based on our fluorescence spectroscopy we demonstrated that what remains of PBS in heterocysts is, as a matter of fact, functional and efficient in energy transfer to PSI. Moreover, the fluorescence spectroscopy also showed that the most affected proteins by degradation were PE and PC, while the terminal emitter seemed not so much affected. This observation found stronger support with the proteomic quantification discussed in section 5.3.3. It has led us to propose that in heterocysts the PBS antenna might be modified: made of very small rods, and a core that has an altered stoichiometry in the subunit composition, with a higher amount of the terminal emitter, L_{CM} . Such modification would optimize light absorption at longer wavelengths that would be more favorable for PSI.

The L_{CM} is a large protein that might reach up to 125 kDa in size (1130 residues and a theoretical 126 kDa molecular weight in *Nostoc punctiforme*) and it is a multifunctional protein made of a phycobiliprotein domain bearing a single phycobilin pigment and several repeated linker domains (see Bald et al., 1996). In the PBS two subunits of the L_{CM} are involved in the organization and general architecture of the entire APC core, they will provide physical anchor to the photosystem, and as the terminal emitters they will funnel the energy from hundreds of pigments into the reaction center antennas of PSII and PSI (Zhao et al., 2005). If our proposal of an APC core composed of a larger number of L_{CM} subunits holds correct, the structure of the heterocyst PBS will then be drastically different to anything we have seen before.

Finally and quite a bit more speculative, I like to think that shorter rods might be also a modification that permits more efficient cyclic photophosphorylation by bringing FNR closer to the acceptor side of PSI. Cyanobacterial FNR is characterized for possessing a non-pigmented phycobiliprotein domain: it has been proven before that FNR can be a subunit of the PBS rods

and that it usually has one or two subunits per PBS (Gomez-Lojero et al., 2003; Thomas et al., 2006; Morsy et al., 2008). The proteomic results have also confirmed that heterocysts contain twice more FNR than the vegetative cells see Paper IV and Table 3. If we imagine a PBS, with rods that bind larger amounts of FNR connected to an APC core specialized in red light utilization; we could say that it would be the perfect antenna for the heterocysts.

6.3 A model for the binding of the phycobilisome to Photosystem I

Our data suggest that energy transfer from the PBS rods to PSI trimers is possible through the APC core. Rakhimberdieva and coworkers (2001) showed that a large fraction of the total PBS of *Spirulina platensis* is functionally connected to PSI; additionally, using mutants in *Synechocystis* sp. PCC 6803 it has been shown that a modified antenna exists, which interacts preferentially with PSI. Such modified PBS would be constituted of a PC rod linked to PSI by a pigment-free linker polypeptide (Kondo et al., 2005 and 2007). Using the available PSI crystal structure at 2.5 Å (Jordan et al., 2001) and the APC structure at 3.5 Å (Murray et al., 2007) I built a crude model of a single APC cylinder binding to a PSI trimer to visually explore the possibilities of a PBS core cylinder interacting directly with PSI (Figure 18).

In PSI a large part of the stromal surface is occupied by the extrinsic proteins, PsaC, PsaD, and PsaE; therefore, the positioning of the APC core cylinders on top of PSI must be somewhat different to that of PSII. We noticed that the three stromal proteins are arranged in such a way that they form an equilateral triangle with flat sides of approximately 15 Å in length, in comparison an APC cylinder is about 120 Å. We also noticed that there is plenty of space left for an APC rod to dock over the surface of PSI, tangentially to the extrinsic proteins across two of the monomers (Figure 18 A). The phycobilin pigments from the APC core would be closer to Chl-*a* and carotenoid molecules at the periphery of PSI which are not covered by the extrinsic proteins (Figure 18 B).

Interestingly, strong excitonically coupled Chl-*a* molecules which might be responsible for the longest wavelength emission fluorescence have been located in the region which is not covered by the extrinsic proteins (Karapetyan et al., 2006). Such docking would also allow some of the PBS rods to be located right above the stromal proteins bringing, hypothetically, any bound FNR closer to the acceptor side of PSI.

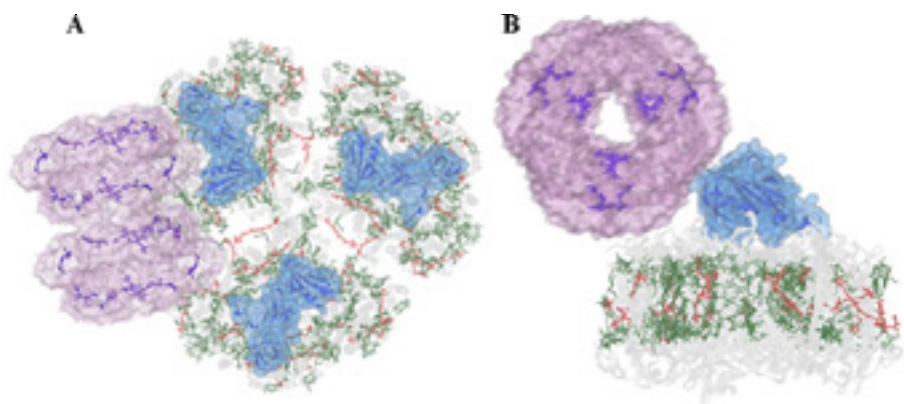


Figure 18. A model for the docking of an APC core cylinder over a PSI trimer. **A** shows a top view and **B** shows a lateral view. The protein matrix of PSI was drawn in light gray, Chl-*a* molecules are green and carotenoids red. The extrinsic proteins of PSI: PsaC, PsaD, and PsaE were drawn in blue. The PBS surface was drawn in translucent pink so the phycobilin pigments are easily observable. The molecular visualization was done with the Accelrys DS Visualizer v2.0 software.

7 Epilogue

During these years as a PhD student, I have focused on the development of reproducible protocols to study heterocyst specific processes. I was able to purify the thylakoid membranes of vegetative cells and heterocysts, separate all the large protein complexes from the photosynthetic apparatus, and I was able to characterize them with a variety of techniques. One of the most surprising results was to find intact PSII complexes within the thylakoid membrane of the heterocysts, causing a lot of debate, interesting discussions and great speculation. We still do not have enough experimental evidence to explain why heterocysts keep an assembled PSII, even though there are specialized enzymatic machineries to quickly process unnecessary proteins; or to arrive to a definitive conclusion about its possible function if it is not water oxidation. Today, I am happy to see that members in our laboratory, new students and postdocs are learning and using routinely those protocols to study hydrogen metabolism in wild type and hydrogen producing mutant strains of *Nostoc punctiforme*. Whether it is to study the localization of the hydrogenase enzyme associated to the thylakoid membrane of heterocysts or for any other reason; it is a gratifying feeling to see already that some of my work has not been in vain.

The proteomic studies on heterocysts provided such an overwhelmingly vast amount of data, even when less than 10% of the total amount of proteins was quantified. Here I described the results that focus on carbohydrate metabolism, nitrogen assimilation, and the photosynthetic machinery, but a lot of information still remains to be dug out from the proteomes: for example differential expression of the amino acid synthesis pathway, nucleotide metabolism, or of the protein translation machinery, to mention just a few examples. Needless to say, the great numbers of differentially expressed proteins which still are hypothetical or unknown.

In conclusion, the characterization of the membranes and quantification of hundreds of proteins have permitted a better understanding of the interplay between the distinct metabolic processes that are carried out on each cell type simultaneously. I truly hope that such understanding would bring inspiration and would serve as foundations and tools for new researchers to develop strategies for efficient and better fuel production. Just as an example of how this could be possible... let's take the case of the last step in glycolysis for the heterocysts that we discussed in section 5.2.1. We have now learnt that the heterocysts of *Nostoc punctiforme* possess a highly abundant pyru-

vate kinase enzyme, which—as we have seen in Figure 12, catalyzes the conversion of PEP into pyruvate coupled with the synthesis of ATP. Higher amounts of the enzyme quite possibly translate into higher amounts of the reactants. Because heterocysts keep an O₂-free environment for the activity of nitrogenase, we can imagine that by expressing only two foreign enzymes involved in the alcoholic fermentation of pyruvate within the heterocysts (a process that is anaerobic), it could be possible to generate abundant ethanol coupled with ATP synthesis! And because all of the energy that arrives to the heterocysts comes from PSII water oxidation in the vegetative cells, we could generate ethanol powered by photosynthesis. It may be crazy and unfeasible: in any case, I believe that such kind of ideas, the ideas that go beyond what we think is possible, will be the driving force we need to continuously move towards that world of our dreams.

8 Acknowledgements

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I should not forget all the people that I have met during these five years in Uppsala, all the friends I made, some for a lifetime, and some for a day. I thank you immensely for expanding my life way beyond the science realms, for all the great experience, for all the adventures, for all the romance, and for all the intimacy, for being a family. Mikeal and Linda from my old corridor, Evalotta, Ale (for making my dreadlocks), Betta, Callis, Ola, Jess, Kasia, Angie, Brinkster, Tobe, Caro, Manda, Sahar, Daniel O, Anna K, Anna E, Aninha... everyone.

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9 Svensk sammanfattning

Cyanobakterier är fotosyntetiserande organismer med hög teknologisk potential tack vare deras kapacitet att producera förnyelsebar vätgas med solljus som energikälla. Produktionen av vätgas är en biprodukt till fixerandet av atmosfäriskt kväve. Denna reaktion katalyseras av enzymet nitrogenas som inaktiveras av syre, till exempel det som produceras av Fotosystem II (FSII) under fotosyntesen. En modellorganism för utveckling av fotobiologisk vätgasproduktion är *Nostoc punctiforme* ATCC 29133, en filamentös, flercellig cyanobakterie. När kvävet i tillväxtmediet tagit slut differentierar denna organism 5 till 10 % av sina vegetativa celler i ett filament till heterocyster, en typ av celler speciellt anpassade för kvävefixering. Differentieringsprocessen innebär dramatiska förändringar hos cellens struktur och fysiologi för att den ska kunna härbärgera nitrogenasmaskineriet och samtidigt skydda och isolera det från syre. Dessa förändringar inbegriper inaktiveringen av FSII och koldioxidfixeringen, en delvis degradering av fotosystemens ljusantennkomplex, bildandet av extra tjocka gastäta cellväggar, med mera. Men även om det finns en grov bild av vilka processer som pågår saknas det kunskap om bioenergetiken som driver kvävefixeringen i heterocysterna, det saknas observationer av processerna samt återstår många andra frågor att besvara. Under mina doktorandstudier har jag försökt karaktärisera de förändringar som sker i det fotosyntetiska maskineriet inom en heterocyst i detalj genom att isolera och karaktärisera fotosyntetiska membraner (tylakoider) med en mängd olika biokemiska och biofysikaliska tekniker, för att få en bättre bild av samspelet mellan fotosyntesen och kvävemetabolismen.

Det första steget i min forskning var att isolera fotosyntetiska membran från vegetativa celler hos *Nostoc punctiforme*. Med detta följde optimeringen av en metod för att rena fram intakta tylakoidmembran som hade kvar sin höga vattenspjälkande FSII aktivitet. En sådan metod skulle vara mycket användbar för isoleringen av tylakoider från heterocyster. Tylakoidmembranens uppbyggnad studerades med hjälp av tvådimensionell elektrofores (nativ/SDS-PAGE) och identifieringen av proteinsubenheter utfördes med masspektrometri (MALDI-TOF). Ytterligare insikt i funktionen hos de fotosyntetiska komplexen uppnåddes genom tekniken paramagnetisk elektronresonans (EPR), mätningar av FSII elektrontransport samt fluorescensspektroskopi (artikel I).

Det andra steget var att optimera metoder för att isolera heterocyster av hög renhetsgrad från kvävefixerande filament. Detta slutfördes framgångs-

rikt vilket kunde bekräftas med hjälp av ljus och konfokalmikroskopi, och ytterligare konfirmeras genom western blots (immunblots) med specifika antikroppar. När heterocysterna var framrenade optimerade jag en metod för att isolera och karaktärisera deras tylakoidmembran. Två membranfraktioner separerades från heterocysterna och studerades med 2D elektrofores och masspektrometri. Heterocysternas membranproteom visade sig jämfört med de vegetativa cellernas vara dominerat av fotosystem I (FSI) och ATP-syntaskomplex, vilka är essentiella för hög nitrogenasaktivitet. I motsats till vad vi väntade oss kunde vi identifiera en signifikant andel av kompletta monomeriska FSII-komplex från heterocysternas tylakoider i en specifik membranfraktion. Vi kunde också mäta ljusdriven elektronöverföring från FSII i heterocysternas tylakoidmembran *in vitro* genom att använda en artificiell elektrondonator, vilket tyder på att heterocysterna kan aktivera FSII under vissa betingelser (artikel II).

Genom så kallad kvantitativ shotgunproteomik kunde vi undersöka de totala proteomen hos två cyanobakterier med heterocyster: *Nostoc* sp. PCC 7120 (artikel III) och *Nostoc punctiforme* (artikel IV). Totalt identifierades 402 respektive 551 proteiner med hög konfidensgrad från *Nostoc* sp. PCC 7120 och *Nostoc punctiforme*, vilket möjliggjorde en detaljerad kartläggning av de metaboliska förändringarna i en heterocyst. Till exempel fann vi en uppreglering av den oxidativa pentosfosfatreaktionsvägen för kolassimilering samt av citronsyrcykeln som genererar de organiska molekyler som krävs för assimileringen av ammoniak. Dessutom fann vi att heterocyster har ett stort överskott av membranproteiner som deltar i det cykliska elektronflödet runt FSI som krävs för att driva nitrogenasaktivitet och mycket annat. Därutöver bekräftade och utvidgade vi identifieringen av FSII-proteiner från studien av membranproteomet.

Slutligen studerade jag hur heterocyster som genomgår förändringar i den fotosyntetiska apparaten svarar på variationer i ljus kvaliteten jämfört med vegetativa celler (artikel V). Genom att använda 77K fluorescensspektroskopi på heterocyster och vegetativa celler som tidigare belysts med ljus av specifika våglängder kunde vi också demonstrera att heterocyster fortfarande besitter ett, möjligen modifierat men funktionellt och mycket dynamiskt, ljusantennsystem, kapabelt att fånga ljus och föra över energin huvudsakligen till FSI. Detta kan vara av vikt för att kontrollera den fotosyntetiska aktiviteten och för att optimera kvävefixeringen under olika förhållanden i miljön.

Sammanfattningsvis fokuserade jag först mina studier på utvecklingen, optimeringen och etablerandet av reproducerbara metoder som möjliggör studier av heterocystspecifika processer som annars skulle vara svåra, eller rent av omöjliga, att genomföra. I nuläget använder vårt laboratorium rutinemässigt dessa metoder för att studera vätgasmetabolismen i vildtypsstammar och vätgasproducerande mutantstammar. Karaktäriseringen av membranproteomet och det totala proteomet innebar möjligheten att teckna en mycket

mer djupgående och integrerad bild av samspelet mellan de distinkta metaboliska processerna som pågår i varje celltyp samtidigt, från oxygenisk fotosyntes och koldioxidfixering i de vegetativa cellerna till den anoxygeniska cykliska fotofosforyleringen som krävs för att driva kvävefixeringen i heterocysterna.

Översatt av Daniel Camsund.

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