Protein complexes in base excision repair:
Biochemical and kinetic analysis of mismatch uracil DNA glycosylase

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Abstract

Mismatch uracil DNA glycosylase (MUG) is an *E. coli* enzyme involved in the repair of ethenocytosine and uracil through the base excision repair pathway. MUG is known to bind the abasic site tightly. This may act to protect the abasic lesion, but the question then is how is the site handed over to the AP Endonuclease? Much has been made of the increase in turnover of some DNA glycosylases by AP endonucleases, but it is not clear whether this occurs via an active displacement mechanism or by passive diffusion. We are addressing these questions by studying the kinetics of MUG interactions with its product and Exonuclease III, the main AP Endonuclease in *E. coli*. We used fluorescence anisotropy and fluorescence resonance energy transfer assays to investigate MUG & DNA interactions. These revealed that MUG binds abasic DNA in a cooperative manner and that binding of two MUG’s is needed for efficient DNA repair. Higher salt concentrations reduced cooperativity leading to a 1:1 binding and reduced MUG’s activity. We also used these assays to investigate whether ExoIII displaces MUG from the DNA through an active or passive mechanism.

MUG’s role in the *in vivo* repair of etheno lesions has been explored by treating *E. coli* cells with urethane, a chemical, known to introduce etheno lesions in the DNA. A eukaryotic DNA glycosylase, hSMUG was shown to repair etheno lesions; hence we investigated hSMUG’s ability to complement *mug* deficiency in *E. coli* cells. hSMUG was found to reduce cell viability, increase mutation rates and provided a boost in cell divisions in *mug* deficient cells whereas it didn’t affect wild type cells. We then asked the question whether hAPE1, the AP endonuclease believed to increase the turnover rate of hSMUG, can reverse the effects of hSMUG expression in *E. coli*. 

# Table of Contents

Abstract ......................................................................................................................... 2
Table of Contents ........................................................................................................... 3
List of Figures ................................................................................................................ 10
List of Tables .................................................................................................................. 16
Abbreviations ................................................................................................................. 17
Dedication ....................................................................................................................... 20
Acknowledgements ........................................................................................................ 21
Declaration ..................................................................................................................... 23

Chapter 1: Introduction ................................................................................................. 25
  DNA: The basis of life .................................................................................................... 25
  Biological Responses to DNA Damage ........................................................................ 28
  The base excision repair (BER) pathway .................................................................... 35
  Uracil DNA Glycosylases .......................................................................................... 43
  Uracil DNA Glycosylase (UDG) ............................................................................... 44
  MUG and TDG Enzymes ......................................................................................... 50
  Single-strand selective monofunctional uracil-DNA glycosylase (SMUG) .............. 56
  Binding stochiometry of DNA glycosylases ............................................................... 59
  AP Endonuclease Family ......................................................................................... 66

Chapter 2: Mismatch DNA Glycosylase: DNA binding and activity assays .......... 72
  Aim ......................................................................................................................... 72
  Background ............................................................................................................. 72
Fluorescence measurements ................................................................. 74
Fluorescence and its properties ............................................................ 74
Intensity ............................................................................................... 75
Lifetime ................................................................................................. 78
Polarization ............................................................................................ 79
Objectives ............................................................................................... 81
MUG Glycosylase binds DNA cooperatively ......................................... 81
Ionic strength of the reaction effects binding cooperativity of MUG .......... 84
Lifetime analysis of the fluorophore in DNA binding by MUG ............... 87
Competition titration using HEX-labeled oligonucleotides ..................... 93
MUG activity and cooperativity .............................................................. 95
Salt dependence of MUG activity .......................................................... 98
Discussion ............................................................................................. 100
MUG glycosylase binds DNA cooperatively .......................................... 100
Time-resolved measurements of the fluorophore upon DNA binding by MUG.... 101
Competition and activity experiments suggest that two MUG’s are required for
DNA binding and cleavage ..................................................................... 102
An increase in ionic strength reduces binding cooperativity and glycosylase activity
of MUG .................................................................................................. 103

Chapter 3: Characterization of MUG & DNA complexes ....................... 107
Aim ........................................................................................................ 107
Background ........................................................................................... 107
Objectives ............................................................................................... 108
Optimization of the electrophoretic mobility shift assay to analyze MUG & DNA complexes ............................................................................................................................. 109
Oligomerizing MUG glycosylase molecules retard the electrophoretic mobility of specific and non-specific DNA ........................................................................................... 111
Competition experiments suggest that the second band (band 2a) is due to specific binding .......................................................................................................................... 113
Longer DNA leads to the formation of additional MUG & DNA complexes .......... 115
Sedimentation equilibrium to analyze self-association of MUG in the absence of DNA .............................................................................................................................. 120
Discussion .............................................................................................................. 123

Chapter 4: ............................................................................................................. 127
FRET to understand enzyme communication in base excision repair ....................... 127
Aim ....................................................................................................................... 127
Background .......................................................................................................... 127
Stopped Flow System ............................................................................................. 136
Objectives ............................................................................................................. 138
Labeling MUG and the DNA ................................................................................. 138
FRET between MUG-Alexa488 and HEX-DNA ...................................................... 142
Determining optimal MUG: DNA concentrations for FRET .................................... 146
Stopped flow fluorescence anisotropy ...................................................................... 148
Kinetics of Exonuclease III binding & cleavage of abasic DNA .............................. 149
Exonuclease III binds to abasic DNA specifically but to uracil containing DNA non-specifically .................................................................................................................. 149
FRET to monitor dissociation of MUG from the DNA ............................................. 153
FRET between MUG-Alexa488 and HEX- U·G -Hairpin ........................................ 164
Exonuclease III does not actively displace MUG glycosylase from abasic DNA ....170
Analysis of the enhancement of MUG’s glycosylase activity by Exonuclease III ....173
Electrophoretic mobility shift assay to visualize MUG and Exonuclease III on the DNA ........................................................................................................................................174
Discussion ........................................................................................................................................176
Exonuclease III and the hairpin oligonucleotides .........................................................178
The effect of Exonuclease III on MUG & DNA complexes ........................................178
The effect of Exonuclease III on MUG bound to AP·A ..............................................179
The effect of Exonuclease III on MUG bound to AP·G ..............................................180

Chapter 5: In vivo DNA repair by MUG and SMUG .....................................................184
Aim ........................................................................................................................................184
Background .........................................................................................................................184
Mutations in E. coli cells ......................................................................................................186
Objectives ............................................................................................................................187
Results ....................................................................................................................................187
Does MUG Glycosylase give an advantage to cells when treated with urethane? .187
Can SMUG glycosylase repair lesions introduced by urethane in E. coli? ............192
Does SMUG glycosylase affect mutation frequencies and cell survival of E. coli cells? ........................................................................................................................................193
Can human AP Endonuclease I balance SMUG’s effect on cell viability and mutation frequencies? .........................................................................................................................195
Discussion ............................................................................................................................200
Does MUG glycosylase confer the cells an advantage upon urethane treatment? .200
Can the eukaryotic glycosylase SMUG complement base excision repair defects in E. coli cells? ........................................................................................................................................201
Does AP endonuclease work together with SMUG?.................................203
Future experiments ..................................................................................204
Background about adaptive mutations in Escherichia coli ...................204
Experiments ............................................................................................208

Chapter 6: General Discussion & Conclusions........................................212
Binding of MUG glycosylase to DNA .....................................................212
Two MUG’s are needed for maximum activity .......................................213
Electrophoretic mobility shift assay provides additional information about MUG binding .......................................................................................214
MUG binding is affected by an increase in ionic strength ....................216
DNA glycosylases and oligomerization ..................................................217
FRET to understand Enzyme Communication in Base Excision Repair ....225
Conclusions ............................................................................................230

Chapter 7: Materials and Methods .........................................................233
DNA Substrates .......................................................................................233
Media and Antibiotics ...........................................................................235
Enzymes .................................................................................................236
Competent Cell Lines ............................................................................237
Making Glycerol Stocks .........................................................................238
Preparation of electroporation competent cells ....................................238
Preparation of chemically competent cells ............................................239
Agarose Gels .........................................................................................240
Polyacrylamide Gels .............................................................................240
Denaturing Urea PAGE .......................................................... 240
Protein Analysis: SDS PAGE .................................................. 240
Electrophoretic mobility shift assay (EMSA) .......................... 241
MUG binding Assays ............................................................. 242
MUG Expression & purification ............................................. 242
Equilibrium DNA Binding assays ......................................... 243
Binding models ................................................................... 244
Time-resolved fluorescence anisotropy analysis .................... 245
Mismatch DNA glycosylase activity assays ......................... 246
Analytical ultracentrifugation measurements ........................ 247
Fluorescence Resonance Energy Transfer ............................. 247
  MUG labeling with Alexa488 .............................................. 247
  Fluorescence polarization Stopped-Flow measurements ....... 249
Exonuclease III activity assays ............................................. 250
In vivo DNA repair by MUG glycosylase ............................... 251
SMUG Cloning strategy .......................................................... 251
Polymerase Chain Reaction .................................................. 251
Ligation in pGEM-T Easy ...................................................... 252
Restriction digest .................................................................. 252
Ligation .................................................................................. 253
Oligonucleotides .................................................................. 253
Experiments to determine the effect of SMUG and urethane ... 254
Addition of urethane .............................................................. 254
Spotting to determine urethane’s effects on cell viability ....... 254
Measuring the mutation frequency ........................................ 254
List of Figures

Chapter 1: Introduction

Figure 1-1 The four bases in DNA.................................................................26
Figure 1-2 The DNA double helix ...............................................................27
Figure 1-3 Major sites of hydrolytic and oxidative damage in DNA .............29
Figure 1-4 Common damages that occur to DNA bases.................................32
Figure 1-5 Uracil DNA Glycosylase recognizes a uracil residue in the DNA.....37
Figure 1-6 Lesions found in DNA and glycosylases repairing them...............38
Figure 1-7 Reaction schemes of monofunctional and bifunctional DNA glycosylases..40
Figure 1-8 An overview of base excision repair pathways: Short and long patch-BER.42
Figure 1-9 Uracil in DNA leads to G: C→A: T/U transition mutations if unrepaired ...43
Figure 1-10 Sequence alignment of human UDG and the five characteristic UDG motifs .................................................................46
Figure 1-11 Initial damage recognition by Ser-Pro region of UDG .................47
Figure 1-12 Structural organization of the active site of human UDG.............48
Figure 1-13 Proposed reaction mechanism for cleavage of the N-C1’ glycosidic bond by UDG..................................................................................49
Figure 1-14 Conserved active site sequences in UDG and MUG Glycosylases .......51
Figure 1-15 Structures of MUG and UDG..........................................................53
Figure 1-16 MUG makes hydrogen bonds with the “widowed” guanine on the complementary strand .................................................................54
Figure 1-17 Structures of etheno lesions..........................................................55
Figure 1-18 SMUG was co-crystallized with an uracil containing 12-mer ............57
Figure 1-19 Structure of MUG glycosylase with DNA ...................................61
Figure 1-20 Structure of the nonspecific MUG-DNA base excision product complex ..62
Figure 1-21 Overview of the structure of TDG: DNA complex. hTDGcat binds the DNA in a 2:1 complex.

Figure 1-22 Two SMUG’s bound to DNA non-specifically.

Figure 1-23 An overview of the structure of APE1: DNA complex.

Figure 1-24 Catalytic sites and cleavage mechanism of AP endonucleases.

Chapter 2: Mismatch DNA Glycosylase: DNA binding and activity assays

Figure 2-1 Structure of the MUG dimer complexed with DNA.

Figure 2-2 Jablonski diagram.

Figure 2-3 Schematic diagram for the measurement of fluorescence anisotropy.

Figure 2-4 MUG binds DNA with positive cooperativity.

Figure 2-5 Higher salt concentrations diminish cooperativity of MUG binding while also reducing MUG’s affinity to DNA.

Figure 2-6 Binding of MUG glycosylase to non-specific DNA is affected by salt.

Figure 2-7 The average fluorescence lifetime change with the addition of MUG.

Figure 2-8 Time resolved anisotropy measurements of HEX labelled AP•G DNA upon DNA binding by MUG.

Figure 2-9 Anisotropy decays for 0, 280, 400 and 1400nM MUG.

Figure 2-10 Competition experiments suggest two MUG molecules bind abasic DNA.

Figure 2-11 A 2:1 ratio of MUG: DNA is required to fully cleave uracil from DNA containing a U•G mismatch.

Figure 2-12 Two MUG’s are required for maximum activity.

Figure 2-13 Higher ionic strength reduces cooperativity and slows down cleavage by MUG.
Chapter 3: Characterization of MUG & DNA complexes

Figure 3-1 MUG & DNA complexes were analyzed via electrophoretic mobility shift assay (EMSA) ................................................................. 109

Figure 3-2 Betaine glycine freezes MUG & DNA complexes ................................. 110

Figure 3-3 MUG glycosylase binds abasic and non-specific DNA with different affinities....................................................................................... 112

Figure 3-4 Non-specific DNA leads to formation of higher MUG & DNA aggregates, the second band is due to MUG binding DNA specifically....................... 114

Figure 3-5 Shorter DNA leads to the formation of fewer MUG & DNA complexes... 115

Figure 3-6 Equilibrium dissociation constant and cooperativity index of MUG binding abasic DNA (H12AP•G) .................................................................................. 117

Figure 3-7 Equilibrium dissociation constant and cooperativity index of MUG binding shorter abasic DNA (H6AP•G) ................................................................. 118

Figure 3-8 Non-specific binding by MUG glycosylase depends on the oligonucleotide size................................................................. 119

Figure 3-9 Sedimentation equilibrium of MUG in free solution ............................ 122
Chapter 4: FRET to understand enzyme communication in base excision repair

Figure 4-1 FRET Process .......................................................................................................................... 134

Figure 4-2 FRET is used to follow MUG binding the DNA and its dissociation from the DNA ................................................................................................................................. 135

Figure 4-3 Stopped flow instrument ....................................................................................................... 137

Figure 4-4 Structures of fluorophores ................................................................................................. 140

Figure 4-5 Emission and excitation spectra of Alexa488 and 6-carboxy-2', 4, 4', 5', 7, 7'–Hexachlorofluoresceine (HEX) ......................................................................................................................... 141

Figure 4-6 Reaction of Alexa Fluorophore 488 succinimidyl ester with primary amine of proteins ................................................................................................................................. 142

Figure 4-7 FRET between H12AP·G and MUG-Alexa488 ................................................................. 144

Figure 4-8 Labeling MUG with Alexa488 does not affect the activity of the enzyme. 145

Figure 4-9 Energy transfer between MUG-Alexa488 and HEX-12AP·G ................................. 146

Figure 4-10 Increase in DNA: MUG ratio leads to a decrease in ∆E .............................................. 147

Figure 4-11 Fluorescence anisotropy was measured using the stopped flow Instrument ................. 148

Figure 4-12 Exonuclease III specifically binds to abasic DNA but non-specifically to uracil containing DNA ................................................................................................................................. 150

Figure 4-13 Reaction profile of Exonuclease III with abasic and uracil-containing hairpin DNA substrates ........................................................................................................................................ 152

Figure 4-14 Addition of abasic competitor in excess to MUG & HEX-AP·A complex leads to a decrease in ∆E and anisotropy ........................................................................................................... 154

Figure 4-15 ∆E is different when competing with abasic, non-specific competitor or Exonuclease III .............................................................................................................................................. 155

Figure 4-16 Change in anisotropy in the presence of competitor DNA or ExoIII....... 157

Figure 4-17 Addition of Exonuclease III & 1mM MgCl₂ to the MUG & HEX-AP·A complex leads to a decrease in ∆E ............................................................................................................... 159

Figure 4-18 Exonuclease III & 10mM MgCl₂ lead to a larger decrease in ∆E........... 160
Figure 4-19 MgCl₂ affects energy transfer between Alexa488 and HEX

Figure 4-20 10mM MgCl₂ has a non-specific effect on FRET

Figure 4-21 Reduction in ∆E by MgCl₂ is not due to MUG’s dissociation

Figure 4-22 Energy transfer between 200nM MUG-Alexa488 and 100nM HEX-U·G-Hairpin DNA

Figure 4-23 Passive diffusion in the presence of the abasic competitor

Figure 4-24 MgCl₂ does not affect the decrease in FRET due to passive diffusion

Figure 4-25 Exonuclease III leads to a small decline in FRET which proceeds faster than passive diffusion

Figure 4-26 FRET decrease with ExoIII & 10mM MgCl₂ is due to 10mM MgCl₂ affecting FRET and not due to Exonuclease III displacing MUG from the AP·G site

Figure 4-27 The effect of ExoIII, ExoIII & 1mM MgCl₂, ExoIII & 10mM MgCl₂ and 10mM MgCl₂ on ∆E is the same over longer time periods

Figure 4-28 ExoIII does not actively displace MUG glycosylase from the abasic site

Figure 4-29 ExoIII does not actively displace MUG glycosylase from the abasic site

Figure 4-30 Electrophoretic mobility shift assay with MUG and ExoIII

Chapter 5: In vivo DNA repair by MUG and SMUG

Figure 5-1 Urethane kills wild type and mug⁻ E. coli cells

Figure 5-2 Cell survival following urethane treatment

Figure 5-3 Urethane introduces mutations in wild type cells but not in mug⁻ cells

Figure 5-4 The effect of urethane treatment on mug⁻-SMUG cells

Figure 5-5 SMUG increases mutation frequencies and is cytotoxic for E. coli cells

Figure 5-6 SMUG expressing mug⁻ cells have a boost in cell division

Figure 5-7 The effect of SMUG and APE1 on the viability of wild type and mug⁻ cells
Figure 5- 8 APE1 does not decrease the high mutation frequencies caused by SMUG 198
Figure 5- 9 SMUG and APE1 strongly enhance cell division in mug deficient cells....199
Figure 5- 10 A model for post-replication repair of damaged DNA.........................205

Chapter 6: General Discussion and Conclusions

Figure 6- 1 Structure of the MUG dimer complexed with DNA ...............................219
Figure 6- 2 Overview of the structure of the TDG:DNA complex ............................221
List of Tables

Table 1- 1 Biological responses to DNA damage ................................................................. 34
Table 2- 1 $K_d$ values and Hill coefficients of MUG binding abasic and non-specific DNA 83
Table 2-2 MUG's binding to non-specific DNA is affected by salt ........................ 104
Table 2-3 MUG's binding to abasic DNA and its activity are affected by salt ........ 104
Table 3- 1: Comparison of $K_d$ and cooperativity values from anisotropy measurements and EMSA 124
Table 7- 1 Sequences and melting temperatures of DNA substrates used in MUG binding, dissociation and activity assays ................................................................. 234
Table 7- 2 Recipe for LB medium used for cell cultures .......................... 235
Table 7- 3 Recipe for M61 Minimal medium used for mutation studies .......... 235
Table 7- 4 summarizes stock and working concentrations of antibiotics used in cellular assays .................................................................................................................. 236
Table 7- 5 summarizes genotypes of cell lines, which were used in cellular assays .... 237
Table 7- 6 Excitation and emission maxima of Alexa488 and HEX ................. 248
Table 7- 7 Summary of the PCR cycle to amplify hSMUG ................................. 251
Table 7- 8 Primer sequences for PCR amplification of hSMUG ......................... 253
Table 7- 9 Primer sequences for sequencing of hSMUG ................................. 253
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>εC</td>
<td>Etheno Cytosine</td>
</tr>
<tr>
<td>εA</td>
<td>Etheno Adenine</td>
</tr>
<tr>
<td>εG</td>
<td>Etheno Guanine</td>
</tr>
<tr>
<td>U</td>
<td>Uracil</td>
</tr>
<tr>
<td>FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>HmU</td>
<td>5-Hydroxymethyl-uracil</td>
</tr>
<tr>
<td>AP</td>
<td>Apurinic / apyrimidin</td>
</tr>
<tr>
<td>BER</td>
<td>Base excision repair</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribose nucleic acid</td>
</tr>
<tr>
<td>E.coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>ExoIII</td>
<td>Exonuclease III</td>
</tr>
<tr>
<td>APE1</td>
<td>AP endonuclease I</td>
</tr>
<tr>
<td>MUG</td>
<td>Mismatch uracil DNA glycosylase</td>
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<tr>
<td>UDG</td>
<td>Uracil DNA Glycosylase</td>
</tr>
<tr>
<td>APE1</td>
<td>Human AP Endonuclease</td>
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<td>SMUG</td>
<td>Single-strand selective monofunctional uracil-DNA glycosylase</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>HEX</td>
<td>Hexachlorofluoresceine</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
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<td>Electrophoretic mobility Shift Assay</td>
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<tr>
<td>TBE</td>
<td>Tris-borate EDTA</td>
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<td>TEMED</td>
<td>$N,N,N',N'$ – tetramethylethylenediamine</td>
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<td>Thymine DNA glycosylase</td>
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<td>OH</td>
<td>Hydroxyl</td>
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<td>amp</td>
<td>Ampicillin</td>
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<td>cam</td>
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### Abbreviations for Amino Acids

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<td>Serine</td>
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<td>Histidine</td>
<td>T</td>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
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<td>Ile</td>
<td>Isoleucine</td>
<td>V</td>
<td>Val</td>
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<tr>
<td>K</td>
<td>Lys</td>
<td>Lysine</td>
<td>W</td>
<td>Trp</td>
<td>Tryptophan</td>
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<tr>
<td>L</td>
<td>Leu</td>
<td>Leucine</td>
<td>Y</td>
<td>Tyr</td>
<td>Tyrosine</td>
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</tbody>
</table>
Dedication

I would like to dedicate my thesis

To my Mom and Dad who have dedicated their lives to us
To Laurent, the love of my life
To little Mallorca who is about to join our family…
Acknowledgements

I would like to firstly thank my PhD supervisor Geoff Baldwin. Thank you for being a great support throughout my PhD, for your patience, criticism and the stimulating discussions.

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and my sister Elif and my brother Ozan for always being there for me…

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I would also like to thank my examiners Dr. Mahendra Deonarain and Professor Malcolm White for their input, criticism and discussions.
Declaration

I declare that the work in this thesis was carried out in accordance with the Regulations of Imperial College London. The work is original except where indicated by specific references in the text and no part of the thesis has been submitted for any other degree.

Any views expressed in this thesis are those of the author and in no way represent those of Imperial College.

This thesis has not been presented to another University for examination either in the United Kingdom or overseas.
Chapter 1

Introduction
Chapter 1: Introduction

DNA: The basis of life

It wasn’t until the 1950’s that Deoxyribonucleic Acid, DNA, was definitively shown to be the carrier of genetic information. Shortly after, its structure was discovered by James Watson and Francis Crick (Watson & Crick 1953) based on the X-ray structure obtained by Rosalind Franklin in 1953 (Franklin & Gosling 1953). Half a century may have passed since the double helix made its debut and we are continuously learning something new about this fascinating molecule made of bases, sugar and phosphates. Today, the notion that DNA is a static double helix is outmoded and we know that it is a dynamic molecule showing structural changes based on its base sequence, environmental conditions, interactions with other molecules and the needs of the cell (Wang et al. 1979; Brown et al. 1999).

The X-ray diffraction photographs of fibers of DNA revealed that it is formed of two chains that wound in a regular helical structure. The four nucleotide monomeric units of DNA each comprise a phosphate group, a pentose sugar (lacking the 2’- hydroxyl group) and a heterocyclic purine or pyrimidine base. The difference between the nucleotides lies in the base units: Guanine (G), Adenine (A), Thymine (T) and Cytosine (C). Adenine pairs with thymine via two hydrogen bonds whereas cytosine makes three hydrogen bonds with guanine. The bases are linked with each other via phosphodiester bonds which gives one strand a 5’ → 3’ orientation while the other strand has a 3’ → 5’ orientation (Figure 1-1).
The four bases in DNA

Four bases that are linked with each other on the same strand via phosphodiester bonds and pair with bases on the complementary strand via hydrogen bonds make up the DNA. Adenine, guanine, cytosine and thymine are bases that make up the DNA. Adenine makes two hydrogen bonds with thymine, whereas cytosine makes 3 hydrogen bonds with guanine. Two polynucleotide strands linked via 5’ → 3’ phosphodiester bonds run in anti-parallel directions coupled via complimentary hydrogen bonding between the bases.

The sugar-phosphate backbones are on the outside and therefore, the purine and pyrimidine bases lie on the inside of the helix. The bases are nearly perpendicular to the
helix axis and adjacent bases are separated by 3.4Å so that there are 10 bases per turn of helix (Figure 1-2).

DNA molecules in eukaryotes and E. coli chromosomes are made from the same building blocks, which are base, sugar and phosphates but the DNA in eukaryotes and E. coli is packaged in different ways. Human chromosomes with their linear form are wrapped around proteins called histones whereas bacterial chromosomes are circular. When thinking about parallels between eukaryotic and prokaryotic organisms’ storage and repair of genetic information, this detail should be considered.
Biological Responses to DNA Damage

When DNA was acknowledged to be the molecule that encodes the genetic information more than half a century ago, the assumption that heredity is a stable process led to a false belief which was: the primary structure of DNA was fundamentally stable and was not subject to chemical alteration. Now we know that DNA, the molecule on which all free living organisms depend on to store genetic information is labile and decomposes. DNA reacts continuously with oxygen and water, resulting in spontaneous lesions. The major sites of such oxidative and hydrolytic damage are summarized in Figure 1-3.
Figure 1-3 Major sites of hydrolytic and oxidative damage in DNA

A segment of one DNA strand is shown with four principal DNA bases. The major sites of hydrolytic depurination are shown by long blue arrows. Short blue arrows show other sites of hydrolytic attack. Major sites of oxidative damage are indicated by the red arrows.
A common change that can occur to DNA bases is deamination. Deamination of amino groups in cytosine and 5-methylcytosine results in generation of uracil and thymine respectively, both mismatched to guanine. Deamination of cytosine is estimated to be 100 - 500 events per cell in a day and will result in C → T transition mutations unless repaired (Lindahl 1974; Frederico et al. 1990; Shen et al. 1994). Adenine and guanine are also prone to deamination; adenine deamination leads to hypoxanthine (Figure 1-4) whereas guanine deamination leads to xanthine.

Oxidation by reactive oxygen species (ROS) also poses a threat to DNA. The rate of oxidative damage is hard to measure but it is clear that oxidation reactions constitute major sources of DNA damage (Klungland et al. 1999; Lindahl & Wood 1999). ROS’s can be formed as by-products from oxidative metabolism or from γ-radiation and are dangerous since they steal hydrogen atoms from DNA bases. Over 80 products of DNA base damage caused by reactive oxygen species are known. Examples are the highly mutagenic 8-hydroxyguanine or ring-opened forms of purines and pyrimidine glycols which are cytotoxic and mutagenic (Figure 1-4) (Ide et al. 1985; Rouet & Essigmann 1985; Lindahl 1993; Klungland et al. 1999; Marnett 2000). The accumulation of oxidative lesions in DNA have been associated with aging (Shigenaga et al. 1994; Souza-Pinto et al. 1999) and are also strongly correlated to cancers resulting from known exposure to oxidative environmental carcinogens (Beckman & Ames 1997; Ichinose et al. 1997).

Loss of bases from DNA occurs continuously. The missing 2’ OH of the ribose which makes the phosphodiester bond resistant leads to a labile N-glycosyl-bond. The instability of the glycosyl bond was initially measured by using DNA with 14C-labelled purine or pyrimidine residues and by determining the rates of release of free bases as a function of temperature, pH and ionic strength. A more sensitive method based on looking at the sensitivity of circular DNA molecules to cleavage by alkali or AP endonucleases, which both cleave abasic sites further helped understand the number of
abasic sites in a cell (Lindahl & Nyberg 1972). Through hydrolysis of the $N$-glycosidic bond of purines, ~10,000 purines are lost per human genome in a day (Lindahl & Nyberg 1972; Nakamura et al. 1998). Guanine and adenine are lost from the DNA at similar rates whereas cytosine and thymine are lost at 5% of the rate of purines. At the site of the base loss, the DNA chain is weakened and undergoes cleavage by a $\beta$-elimination process within a few days (Lindahl & Andersson 1972).

Other metabolic products such as alkylating agents, oestrogens, chlorinating agents, reactive nitrogen species, glycoxidation products, heme precursors and amino acids (Burcham 1999) can damage DNA as well. Figure 1-4 illustrates examples of DNA bases damaged by endogenous or exogenous agents.
Figure 1-4 Common damages that occur to DNA bases.

Chemical groups not found in the normal DNA bases are shown in red. Figure was taken from Krokan et al. (Krokan et al. 1997).
Instability of the DNA caused by the great variety of DNA damaging agents would be an overwhelming problem for cells and organisms if they didn’t have the specific repair mechanisms. The genome of every organism from viruses to humans encodes information to protect its own integrity. DNA repair enzymes continuously monitor chromosomes to correct damaged nucleotide residues degenerated by exposure to carcinogens, cytotoxic compounds or simple metabolites. Given the huge spectrum of damage that the genome can suffer, it is understandable that cells have evolved a multitude of mechanisms by which either damaged DNA is removed from the genome or the potentially lethal effects caused by interference with normal DNA metabolism are mitigated. Table 1-1 summarizes the different biological responses to DNA damage that have been identified so far.
Table 1 - Biological responses to DNA damage

- Reversal of base damage (Excision of damaged, mispaired, incorrect bases)

  Base excision Repair (BER)
  Nucleotide Excision Repair (NER)
  Transcription-coupled nucleotide excision repair (TC-NER)
  Alternative excision repair (AER)
  Mismatch Repair (MMR)

- Strand break repair
  Single-strand break repair (SSBR)
  Double-strand break repair (DSBR)

- Tolerance of base damage
  Translesion DNA synthesis (TLS)
  Post-replicative gap filling
  Replication fork progression

- Cell cycle checkpoint activation

- Apoptosis
A good characterization of steps in DNA repair is important since it is also a prerequisite to rational drug design in developing efficient enzyme inhibitors to down-regulate DNA repair in pathogenic bacteria and viruses. Our lab focuses on base excision repair (BER), which is the pathway that addresses the modifications of the heterocyclic bases in DNA (Lindahl 1993).

**The base excision repair (BER) pathway**

Base damage is the most common form of DNA damage in cells, therefore BER is one of the major pathways for dealing with most DNA damage (Lindahl & Wood 1999). Base excision repair (BER) pathway protects cells from the mutagenic and cytotoxic effects of DNA lesions by excising and replacing defective DNA bases caused by environmental or cellular mutagens. The importance of base excision repair can be seen by the fact that BER deficiency leads to cellular hypersensitivity to certain toxins as well as to genomic instability and mutations (Engelward et al. 1998).

BER and the proteins that initiate the pathway first came to light when uracil DNA glycosylase was discovered in the search for an enzyme that acts on deaminated cytosine residues in DNA (Lindahl 1974). It was exciting to see that the pathway can be reconstituted using purified proteins from bacteria and mammals and is conserved throughout evolution (Dianov & Lindahl 1994; Kubota et al. 1996). BER starts when a DNA glycosylase specific for a lesion recognizes the damaged or mismatched base and binds to it. Most DNA glycosylases remove several structurally different damaged bases, while a few have very narrow substrate specificity. They often contain a conserved motif of helix-hairpin-helix (HhH), which enables them to bind the DNA. Once a glycosylase recognizes a lesion, it flips out the abnormal nucleoside residue to accommodate the altered base in its recognition pocket and cleaves the N-glycosyllic bond that links the base to the deoxyribose backbone of the DNA leaving an apurinic/
apyrimidinic site in the DNA. As an example, figure 1-5 shows how uracil DNA glycosylase flips out uracil followed by uracil binding by the pyrimidine binding pocket and cleavage of the N-glycosidic bond between sugar and base (Slupphaug et al. 1996). It is critical for the cell to ensure proper repair of the abasic site since abasic sites can be mutagenic or lethal to the cell (Kavli et al. 1996; Otterlei et al. 2000; Sobol et al. 2003; Boiteux & Guillet 2004).
Figure 1-5 Uracil DNA glycosylase recognizes a uracil residue in the DNA.

The glycosylase binds and flips out the abnormal nucleoside residue to accommodate the altered base in its recognition packet and cleaves the N-glycosyl bond that links the base to the deoxyribose backbone of DNA. The structure of UDG in complex with a 10 base double stranded DNA bearing a U-G mismatch reveals insertion of a wedge into the DNA and cleavage of the glycosidic bond with free uracil (blue) bound in the specificity pocket. (Slupphaug et al. 1996) (PDB ID code: 1SSP). The figure was generated using PyMOL (DeLano, 2002).

Examples for lesions in DNA, which may occur and DNA glycosylases that repair them are given in Figure 1-6.
Figure 1-6 Lesions found in DNA and glycosylases repairing them

Figure summarizes lesions found in DNA and glycosylases which are responsible for repairing those lesions. Chemical groups not found in the normal DNA bases are shown in red. The reported substrates for the various DNA glycosylases are numbered in accordance with the left panel. For DNA glycosylases recognizing multiple substrates, the number of the proposed preferred substrate is shown in red.

Organisms: S. cerevisiae, Saccharomyces cerevisiae; M. thermoautotrophicum, Methanobacterium thermoautotrophicum; S. pombe, Schizosaccharomyces pombe; A. thaliana, Arabidopsis thaliana; D. melanogaster, Drosophila melanogaster; M. luteus, Micrococcus luteus; N. mucosa, Neisseria mucosa.

Figure taken from: Krokan et al. (Krokan et al. 1997).
DNA glycosylases can be grouped in two classes based on how they remove the inappropriate base and what kind of end they leave after removal. (1) monofunctional DNA glycosylases cleave the glycosidic bond followed by the action of AP endonuclease. The subsequent AP endonuclease activity produces a 3’ OH terminus, which is a substrate for DNA polymerase. (2) bifunctional DNA glycosylases/AP lyases, which form a Schiff’s base that undergoes enzyme catalysed β-elimination cleave the phosphodiester bond 3’ from the abasic site, leaving a 3’deoxy ribose phosphate (dRP) (Dodson et al. 1994) (Figure 1-7). This 3’dRP is not a substrate for DNA polymerase and is thus a block to DNA replication and must therefore be further processed by an AP endonuclease (Figure 1-7). AP endonucleases are thus essential enzymes in both of these sub-pathways.

Examples for monofunctional glycosylases are: Uracil-DNA glycosylase (Lindahl et al. 1977; Olsen et al. 1989; Nilsen et al. 1997; Aylon & Kupiec 2004), Thymine/Uracil mismatch glycosylase (Neddermann et al. 1996), Mismatch specific DNA Glycosylase (MUG) (Barrett et al. 1998), SMUG1 (Haushalter et al. 1999) and MBD4 (Hendrich et al. 1999; Petronzelli et al. 2000) which all show activity against bases that arise through deamination of cytosine and/or 5-methyl cytosine residues (Figure 1-7).

Bifunctional glycosylases include 8-oxoguanine DNA glycosylase (Radicella et al. 1997), which removes oxidized purines and hNTH1 (Aspinwall et al. 1997; Ikeda et al. 1998), which removes oxidized pyrimidines (Figure 1-7).
Figure 1-7A demonstrates the reaction mechanism of monofunctional DNA glycosylases. A water molecule in the active site is deprotonated by a conserved carboxylic acid side chain of the glycosylase and positioned for nucleophilic attack. Substantial positive charge is developed on the ribose sugar ring. The product is an abasic site, which stays bound to most DNA glycosylases as a product inhibitor. Figure 1-7B reveals the reaction mechanism of bifunctional DNA glycosylases. The mechanism used is similar to monofunctional glycosylases except for the fact that the nucleophile is a lysine residue of the enzyme. The product undergoes rearrangement and base catalyzed β-elimination leading to scission of the C3-O bond between the abasic site and the phosphate to the 3’ site. The figure was taken from Scharer and Jiricny (Scharer & Jiricny 2001).

The abasic site generated by cleavage of the N-glycosidic bond is subsequently addressed by one of two BER pathways, “short-patch” or “long-patch” BER (Figure 1-8). In “short-patch” BER, removal of the aberrant base is followed by cleavage of the strand 5’ to the resulting abasic site by an AP endonuclease which creates a 3’-OH end (Barzilay & Hickson 1995). This step is followed by a DNA polymerase attaching the appropriate base to the generated 3’-OH and displacing the baseless sugar by its inherent AP-lyase activity. The circle is completed with DNA ligase sealing and completing the repair process (Figure 1-8) (Kubota et al. 1996). In mammalian cells, DNA ligase III interacts with DNA polymerase β through the XRCC1 protein (Kubota et al. 1996). The interactions between DNA Ligase III, XRCC1 and Polβ as well as APE1 and Polβ (Bennett et al. 1997) plus increasing evidence for functional interaction between APE1 and DNA Glycosylases (Waters et al. 1999) suggest that short-patch BER is a highly coordinated process (Figure 1-8).
The long-patch pathway differs from the short-patch pathway in that it involves the removal of up to six damaged nucleotides (Zharkov 2008). Short-patch repair pathway predominates and in certain cases appears to be used exclusively for example when base excision is mediated by DNA glycosylases/AP lyases (Fortini et al. 1999). Long-patch BER may serve as a back-up pathway and could be important for the removal of modified abasic sites that are resistant to the AP Lyase activity of Polβ (Klungland & Lindahl 1997). Long-patch repair has also been detected in *E. coli* cell extracts and the decision for long-patch versus short-patch base excision repair is influenced by the ratio of DNA polymerase I to DNA ligase (Sung & Mosbaugh 2003).
In short-patch BER pathway, which is predominant, an abasic site (AP site) is created through excision of the mismatched base (■) by a glycosylase or by spontaneous base loss. If the DNA glycosylase in action is a monofunctional DNA glycosylase, the AP site is processed by an AP endonuclease that cleaves 5’ to the abasic site creating a 3’ OH terminus, which is a substrate for DNA polymerase. In the presence of a bifunctional DNA glycosylase, which has an AP lyase activity, the formed 3’deoxy ribose phosphate (▲) has to be removed by a 3’ Phosphodiesterase first. The lesion is then repaired by DNA polymerase, which attaches the appropriate base and DNA ligase that seals the nick to restore the original sequence. The long-patch pathway differs from the short-patch pathway in that it involves the removal of up to six damaged nucleotides. The flap is removed by a Flap endonuclease FEN1, which is probably loaded on the free 5’end of the flap and tracks along until it encounters the junction with double stranded DNA. (Zharkov 2008). DNA glycosylase, AP endonuclease, DNA polymerase and DNA ligase are thus essential enzymes in both of these sub-pathways. FEN1 is required for the removal of the flap in the long-patch pathway.
Uracil DNA Glycosylases

Uracil arises in DNA either by the misincorporation of dUTP during DNA synthesis which results in a U·A base pair or by spontaneous deamination of cytosine. The conversion of cytosine which pairs with guanine, into uracil, a base-pairing partner for adenine, causes G·C→A·T transition mutations in half of the progeny during replication (Figure 1-9). Therefore deamination of the cytosine to uracil represents one of the major instabilities in the encoding of genetic information in DNA.

Figure 1-9 Uracil in DNA leads to G·C→A·T/U transition mutations if unrepaired.

Hydrolytic deamination of cytosine generates uracil (A) Unrepaired G-U mismatches produce G·C→A·T/U transition mutations in half the daughter duplexes on replication (B).
The first DNA glycosylase to excise uracil from DNA was discovered in *Escherichia coli* (Lindahl 1974). On the basis of sequence similarity, known uracil DNA glycosylases have been classified into 4 families and are found in almost all kingdoms including bacteria, viruses and animals (Pearl 2000). Family-1 enzymes, which include uracil DNA glycosylase UDG, are active against uracil in single and double stranded DNA and recognise uracil in an extrahelical conformation via a combination of protein and bound-water interactions. Family-2 enzymes include thymine DNA glycosylase (TDG) and mismatch specific uracil DNA glycosylase (MUG), which are mismatch specific and recognise the widowed guanine on the complementary strand rather than the extrahelical pyrimidine (Barrett *et al.* 1998; Pearl 2000; Maiti *et al.* 2008). Family-3 enzymes include single-strand selective monofunctional uracil-DNA glycosylases (SMUG), which recognise uracil and 5-hydroxymethyl-uracil but exclude thymine from their binding pocket through a mechanism similar, but different from UDG’s. The fourth family was identified in the genome of thermophilic bacterium *Thermotoga maritime* and has homologues in a range of thermophilic and mesophilic bacteria and in archaea (Pearl 2000).

**Uracil DNA Glycosylase (UDG)**

Uracil DNA glycosylase (UDG) is the main uracil removing enzyme in cells (Slupphaug *et al.* 1991). UDGs are highly conserved in evolution, with the exception of UDG’s from pox viruses; the active site is completely conserved (Pearl 2000). Uracil glycosylases remove uracil from single stranded DNA as well as from U: A pairs resulting from misincorporation of dUMP during replication and from mutagenic U: G mispairs resulting from deamination of cytosine in the preferential order ssU > U: G > U: A (Slupphaug *et al.* 1995). No activity has been detected against any normal DNA base or against uracil in RNA (Lindahl *et al.* 1977; Krokan & Wittwer 1981). *E. coli* and yeast with mutations in UDG have 4-30 fold increases in G: C →A: T transition mutations depending on the context (Duncan & Weiss 1982; Impellizzeri *et al.* 1991).
UDG is highly selective for uracil in DNA but it also removes certain closely related bases at a much slower rate. These include 5-fluorouracil which is formed in DNA after treatment with 5-fluorouracil (Ingraham et al. 1980) as well as isodialuric acid, 5-hydroxyuracil and alloxan, which are all formed from cytosine in DNA after exposure to $\gamma$-irradiation or oxidative stress (Hatahet et al. 1994; Zastawny et al. 1995; Dizdaroglu et al. 1996).

UDG removes uracil at different rates from different double-stranded DNA sequence context and its activity is not dependent on the base partner of uracil (Nilsen et al. 1995; Bellamy & Baldwin 2001). UDG is hypothesized to flip uracil out of the DNA base stack using a “push-pull” mechanism in which a leucine side chain penetrates into the DNA (push) and complementary interactions from the uracil recognition pocket facilitate binding (pull) (Slupphaug et al. 1996). The enzyme uses steric exclusion and specific electrostatic interactions to recognize uracil and to prevent other bases from docking in its active site (Parikh et al. 2000). DNA binds along a positively charged groove in the enzyme, the uracil binding pocket located at the base of the groove is too deep and narrow to allow binding of uracil-DNA unless uracil is flipped-out of the DNA helix (Figure 1-5) (Slupphaug et al. 1996).

There are five characteristic UDG motifs key to at least one aspect of BER initiation: These are the Leu272 loop (268-HPSPLSVYR-276), the 4-Pro loop (165-PPPPS-169), the Gly-Ser-loop (246-GS-247), the uracil specificity region (201-LLLN-204) and the water activating loop (145-DPYH-148) (Figure 1-10).
The five characteristic UDG motifs are indicated by bars below the water activating loop (light blue), the 4-Pro loop (red), the uracil recognition strand (magenta), the Gly-Ser loop (dark blue) and the Leu272 loop (green) sequences (GenBank accession number: 1296803).

The Leu272 loop, the 4-Pro-loop and the Gly-Ser loop interact with the DNA phosphodiester backbone compressing the intra strand phosphate distance of the uracil containing DNA strand and kinking DNA (Parikh et al. 1998). Leucine272 (Leu$^{272}$) located directly above the uracil-binding pocket helps in scanning and pushing of the dUMP residue from the dsDNA but as seen by the flipped out uracil in the L272A mutant, Leu$^{272}$ is not indispensible for nucleotide flipping. Leu272 is critical for the enzymatic activity as can be seen from the L272A mutant which is severely impaired with uracil excision efficiencies < 1% that of the wild type enzyme (Parikh et al. 1998) (Figure 1-11).

In the UDG-DNA complexes, clamping the Ser-Pro pinch loops around the flipped-out nucleotide brings the catalytic residues into an active conformation. Movement of the Leu$^{272}$ loops into the minor groove helps form the uracil recognition pocket by bringing His-268 within hydrogen-distance of uracil O2 (Figures 1-11 and 1-12).
Ser-Pro loops compress the uracil-containing DNA strand at the phosphates 5’ and 3’ of the uracil nucleotide in the directions indicated by the arrows. Leu272 loop (red) penetrates into the helix flipping out the uracil residue. The nucleophilic attack by His 268 (purple) leads to cleavage of the glycosidic bond. Leu272 is critical for the enzymatic activity as can be seen from the L272A mutant which is severely impaired with uracil excision efficiencies < 1% that of the wild type enzyme (PDB ID code: 1EMH) (Parikh et al. 1998). The figure was generated using PyMOL (DeLano, 2002).

The conserved and buried uracil-binding pocket of UDG is characterized by shape and electrostatic complementarity to uracil. Several hydrogen bonds are established from the conserved UDG residues such as histidine of the HPSPLS motif (H187 in Escherichia coli UDG, H268 in human UDG) and asparagine of the GVLLLNN motif (N123 in E. coli UDG and N204 in human UDG) to positions 2, 3 and 4 of uracil (Figure 1-12). Specificity of these contacts avoids cytosine binding in the pocket (Mol et al. 1995; Savva et al. 1995; Kavli et al. 1996). Furthermore, the side chain of tyrosine of the GQDPYH motif (Y66 in E. coli UDG and Y147 in human UDG), which is in van der Waals’ contact with the C5 position of the uracil, excludes thymine with a methyl
group at this position, or the purines with bulky rings (Figure 1-12). This steric shielding was verified by site specific mutagenesis of Tyr^{147} of human UDG to Ala, which results in a mutant that excises thymine in addition to uracil from DNA (Kavli et al. 1996).

![Structural organization of the active site of human UDG](image)

Figure 1-12 Structural organization of the active site of human UDG

Residues in the uracil specificity pocket make hydrogen bonds with uracil. Figure shows key UDG residues in catalysis, lesion binding and specificity. Hydrogen bonds are established from the conserved UDG residues such as histidine of the HPSPLS motif (H187 in *E. coli* UDG, H268 in human UDG) and asparagine of the GVLLLN motif (N123 in *E. coli* UDG, N204 in human UDG) to positions 2, 3 and 4 of uracil. Furthermore, the side chain of tyrosine of the GQDPYH motif (Y66 in *E. coli* UDG and Y147 in human UDG), which is in van der Waals’ contact with the C5 position of uracil excludes thymine that has a methyl group at the C5 position. (PDB ID code: 1SSP). The figure was generated using PyMOL (DeLano, 2002).

Site directed mutagenesis of human UDG has demonstrated that effective catalysis is critically dependent on Gln144, Asp145 and Asn204 whereas His268, Ser169 and Ser-270 are also important (Mol et al. 1995).

Based on the data obtained from mutagenesis and structural studies, two possible reaction mechanisms have been proposed, both involving the imidazole group of His-
In a nucleophilic substitution mechanism, His-268 would directly attack the C1’ atom of the furanose ring and cleave the glycosidic bond. If a general base mechanism persists, His-268 would abstract a proton from a water molecule, creating an OH⁻ nucleophile that would then carry out an attack on C’ of the furanose ring. For both mechanisms, the reaction ends with the addition of a second water molecule, leaving a free uracil and an abasic site in DNA (Figure 1-13).

Figure 1-13 Proposed reaction mechanism for cleavage of the N-C1’ glycosidic bond by UDG

The interactions made by the enzyme with positions 2,3 and 4 of uracil facilitate the nucleophilic attack by the imidazole group of His-268. The intermediate is stabilized by interactions of its O2 with the backbone amides of Gln144 and Asp145. Nucleophilic attack by a water molecule leads to a hydrogen atom being added to N1 of uracil and an OH group added to the C1’ atom of the furanose ring. An alternative mechanism would have histidine abstracting a proton from water. The N-C1’ glycosidic bond would then be attacked by the resulting OH ion on the C1’ atom of the ring. Asn204 is not directly involved in the catalytic mechanism but interacts with N3 and O4 ensuring only a single uracil being bound by the catalytic site (Mol et al. 1995).

Family 1 UDGs are found in many organisms, including bacteria, yeast, mammalian cells, plants and large DNA viruses. Enzymes in this family have not been detected in Archaea and insects, which led to the evolution of different types of UDGs in these organisms, as described below.
Mammalian cells contain at least two other type I family UDG’s: SMUG and TDG but UNG appears to be quantitatively dominating as determined from in vitro assays done with human cell-free extracts (Slupphaug et al. 1995). The ability of human UNG to complement ung\(^{-}\) E.coli led to the assumption that hUNG is the functional ortholog of E. coli UNG fulfilling the same roles of excising uracil arising from both misincorporation and deamination.

**MUG and TDG Enzymes**

Prior to the identification of mismatch uracil DNA glycosylase (MUG), and its human homologue thymine DNA glycosylase (TDG), glycosylases were characterized by their activity against uracil in ssDNA or dsDNA, susceptibility to inhibition by the phage UGI protein (Wang & Mosbaugh 1989) and strong conservation of the key residues implicated in specificity and catalysis (Mol et al. 1995; Savva et al. 1995). A new enzyme with N-glycosylase activity against thymine in G: T mismatches was identified and characterized by Neddermann and Jiricny (Neddermann & Jiricny 1993), which was also active against uracil but only in the context of G:U mispairs (Neddermann & Jiricny 1994). Subsequently, homologues of TDG were identified in bacteria (Gallinari & Jiricny 1996). This new family of thymine DNA glycosylases and mismatch UDG (TDG/MUG) were different from UDG in being insensitive to inhibition by UGI (Gallinari & Jiricny 1996), being inactive on ss-uracil-DNA and lacking the conserved catalytic residues of the family-1 UDG’s (Barrett et al. 1998).

The crystal structure of MUG from E.coli (Barrett et al. 1998) shows remarkable structural homology to the family-1 UDGs, despite only ~10% sequence identity, which led to identification of a second uracil-glycosylase family (family 2). MUG processes uracil mismatched with guanine (Gallinari & Jiricny 1996), mismatches of εC∙C base pairs (Hang et al. 1998; Saparbaev & Laval 1998; O'Neill et al. 2003) and several other
mismatches (see below). As in UDG, MUG has a deep binding pocket specific for uracil. The highly conserved active site sequences in UDG have precise topological equivalents in MUG and have very similar confirmations to their UDG equivalents suggesting that MUG’s pocket may serve a similar function, and that MUG/TDG enzymes, like UDG’s employ a nucleotide-flipping mechanism when they process their substrates. The highly conserved active site sequences in UDGs that contain the catalytic aspartate and histidine have precise topological equivalents in MUG (Figure 1-14).

<table>
<thead>
<tr>
<th>UDG</th>
<th>86-GQDPY-90</th>
<th>210-HPSPLS-215</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUG</td>
<td>16-Gl NPGL-20</td>
<td>140- NPSGLS-145</td>
</tr>
</tbody>
</table>

Figure 1-14 Conserved active site sequences in UDG and MUG glycosylases

The highly conserved active site sequences in UDGs that contain the catalytic aspartate and histidine have precise topological equivalents in MUG. These sequences have very similar confirmations to their UDG counterparts. The catalytic residues are replaced by asparagines.

One of the faces of the putative uracil binding pocket in MUG is provided by Phe-30 corresponding to Phe-101 in the structure of HSV1 UDG (shown in red in Figure 1-15). Although this residue is not phenylalanine in all known MUG/TDG sequences, it is always aromatic and therefore capable of the stacking interactions with uracil, as observed in UDG-base complexes (Mol et al. 1995; Savva et al. 1995). The sequence motif from residues 86-90 which is totally conserved in all UDG’s forms one face of the uracil binding pocket (purple in Figure 1-15). In the structural alignment of E. coli MUG, this corresponds to the sequence GINPG from residues 16-20 (magenta in Figure 1-15), is totally conserved in all known MUG/TDG family sequences and adopts a virtually identical conformation to the homologous sequence in UDG (Figure 1-15).

In UDG, the presence of tyrosine in the sequence two residues downstream the catalytic aspartate (GQDPYH) is responsible for exclusion of thymine from the UDG binding
pocket (orange in Figure 1-15). The side chain of the tyrosine packs against the C5 position of thymine, sterically excluding the 5-methyl group of thymine. In the known MUG/TDG sequences, this residue is a conserved glycine (GINPG). The absence of a “barrier” to a 5-methyl group at this position explains thymine glycosylase activity of TDG and MUG enzymes. Cleavage of thymine by MUG (Neddermann & Jiricny 1994) is by multiple folds lower compared to cleavage of uracil and ethenocytosine (O'Neill et al. 2003). Tight binding of uracil and discrimination against cytosine is due to a specific pattern of hydrogen-bonding interactions (Barrett et al. 1998).

The catalytic aspartate residue of UDG’s in the motif GQDPY is an asparagine GINPG in the MUG/TDG sequences (Figure 1-15). The structures of HSV-1 UDG and human UDG-DNA complex, suggest a role for the asparagine residue in binding a water molecule and its activation by abstraction of a proton which makes the water molecule attack the N-glycosidic bond of the deoxyuridine. In MUG, the water molecule is in equivalent position bound between the main-chain carbonyl and side-chain amide of Asn-18 so the nucleophilic attack on the N-glycosidic is attacked in a similar manner as by Asp-88 in UDG.
A distinct feature of the MUG/TDG family is their interaction with the guanine on the complementary strand (Barrett et al. 1998; Maiti et al. 2008). Flipping-out of the mismatched pyrimidine by MUG is mediated by the push of the wedge formed by Gly-143, Leu-144 and Arg-146 into the duplex DNA. Leucine (Leu-144 in MUG and Leu-272 in human UDG) and glycine (Gly-143) occupies the gap left by the flipped out uracil residue. Interaction of MUG with DNA is unique and different from UDG since it makes contacts with the “widowed” guanine which is absent in UDG (Figure 1-16). The head group of Arg-146 makes a hydrogen bond with the peptide oxygen of Leu-144 on one side and to a water molecule on the other, which is in turn hydrogen-bonded to the deoxyribose ring oxygen of the widowed guanine. Gly-143, Leu-144 and Arg-146 and
the associated water molecule form a “wedge” which penetrates the base stack of the DNA. Once the deoxyuridine is displaced and the wedge is inserted into the DNA duplex, strong hydrogen bonding connections are formed between the N1 imino group of the widowed guanine and the carbonyl oxygen of Gly-143 and between the N2 exocyclic amino group of the widowed guanine and the carboxyl oxygens of Gly-143 and Ser-145. These interactions which involve the Watson-Crick base pairing groups on the guanine are specific for guanine and can only be made when the base partner (uracil) is flipped out (Barrett et al. 1998) (Figure 1-16).

Figure 1-16 MUG makes hydrogen bonds with the “widowed” guanine on the complementary strand

The intercalation wedge which is formed by Gly-143, Leu-144, Arg-146 and the associated water molecule (shown in green) induce “flipping out” of the damaged nucleotide into the active site. Specific hydrogen bonds are made with the “widowed” guanine on the complementary strand (taken from Barrett et al., 1998).

In addition to uracil from U:G mispaired bases, MUG also processes mismatches of εG·C (Saparbaev & Laval 1998) and εC·G base pairs (Lutsenko & Bhagwat 1999; O’Neill et al. 2003). Its ability to process εC from εC·G mismatches is by magnitudes faster than uracil from U·G pairs (O’Neill et al. 2003). Similar to its recognition of uracil, MUG was found to recognize εG only when present in a double-stranded
oligonucleotide (Saparbaev & Laval 1998). Etheno bases (ε) are the most extensively studied exocyclic lesions and arise through the addition of a two carbon unit to DNA bases (Figure 1-17).

Recently, it has been shown that MUG can excise, with very low efficiency, εA, hypoxanthine and 5-hydroxycytosine residues when present in DNA (O'Neil et al. 2003). Although its activity is weaker compared to a U•G mismatch, MUG is also active against U•A lesions (Liu et al. 2002).
Another glycosylase, which removes uracil from DNA has been identified through a genome-wide screening for DNA glycosylases by selecting for their ability to bind synthetic inhibitors that target the active site typical for glycosylases (Haushalter et al. 1999). The glycosylase was named: Single-strand selective monofunctional uracil DNA glycosylase because of its initially discovered activity against single stranded DNA (Haushalter et al. 1999). SMUG is active against uracil (Haushalter et al. 1999; Nilsen et al. 2001; Wibley et al. 2003) and is the primary repair mechanism against oxidation damage product 5-hydroxymethyl-uracil in mammalian cells (Boorstein et al. 2001). Similar to Uracil DNA glycosylase, SMUG does not process G:T mismatches (Wibley et al. 2003), similar to MUG (Gallinari & Jiricny 1996); it is not inhibited by the UDG inhibitor UGI (Haushalter et al. 1999).

SMUG is not found in bacteria and yeast, but is present in higher eukaryotes. It is ~700 fold more active against a double stranded uracil than single stranded DNA substrate. It excises uracil and 5-hydroxymethyl-uracil (HmU) with comparable efficiency but hydrolyses thymine >10^6 times more slowly than G:U or G:HmU (Wibley et al. 2003).

Co-crystals of SMUG with a uracil containing 12-mer revealed that the enzyme had dissociated from the AP site after excising uracil and two SMUG molecules had bound on either side of the oligonucleotide (Wibley et al. 2003). Although the enzyme was found bound to the ends of the oligonucleotide and not to the abasic site, a reasonable model for the interaction of SMUG with DNA could be constructed using DNA-bound structures of other members of the UDG superfamily (Slupphaug et al. 1996) (Barrett et al. 1998; Parikh et al. 1998; Barrett et al. 1999) (Figure 1-18).
SMUG has a pyrimidine binding pocket that is topologically equivalent but chemically different, consisting of catalytic residues found in family-1 UNG and in family-2 MUG/TDG enzymes (Barrett et al. 1998; Pearl 2000). The enzyme is less than 8% identical to UDG but has similar motifs to both: UDG and MUG. Motif I of MUG has the sequence GINPG-N9-F and the crystal structure suggests that asparagine in the third position might be responsible for the nucleophilic activation of a water molecule that attacks the N-glycosidic bond like in UDG. The terminal phenylalanine stacks up against uracil. In HSV-1 UDG, this motif has the sequence GQDPY-N10-F, aspartic acid rather than asparagine is used to activate the water molecule (Figure 1-14).

Figure 1-18 SMUG was co-crystallized with an uracil containing 12-mer

The enzyme had dissociated from the AP site after excising uracil and two SMUG molecules had bound on either side of the oligonucleotide. Residues, which form the wedge (Pro-Ser-Pro-Arg-Asn) inserted into the DNA are in purple, residues labeled red form the short helix (Pro-Gln-Ala-asn-Lys), which is also part of the wedge inserted into the DNA. (PDB ID code: 1OE4). The figure was generated using PyMOL (DeLano, 2002).
As in family 1 and 2 enzymes of the UDG superfamily, SMUG flips the mismatch nucleotide through the major groove and inserts a “wedge” into the DNA duplex. The wedge is formed by a loop (251-Pro-Ser-Pro-Arg-Asn-255) (in purple in Figure 1-18) and a short α-Helix (256-Pro-Gln-Ala-Asn-Lys-260) (labeled red in Figure 1-18). This short α-Helix is specific to SMUG with no equivalent in UDG and MUG1 structures. The combination of the loop and helix generates a much bigger wedge than in other UDG family members. When compared to UNG and MUG, interaction of SMUG with DNA leads to a substantially bigger disruption of the DNA duplex (Wibley et al. 2003).

In UDG, the presence of tyrosine hinders thymine from entering the active site. MUG has a glycine in this site which does not block thymine from entering the active site. SMUG’s motif I has the sequence GMNPG-N<sub>10-F</sub>. Interestingly SMUG is also able to discriminate against thymine although it has a glycine at position 5 just like MUG. This suggests SMUG using a different mechanism than UDG to exclude thymine from its catalytic site.

Pyrimidine specificity of UNG; MUG and SMUG is provided through different mechanisms. Family 1- UNG’s are exquisitely selective for excision of uracil only and have no activity against thymine or cytosine. Discrimination against cytosine is mediated by an asparagine in the pyrimidine binding pocket, whose side chain and associated water molecules provide bonding points for uracil but are completely incompatible with cytosine (Pearl 2000). Thymine’s hydrogen binding pattern is identical to uracil but is rejected from entering the binding pocket via steric constriction. The uracil-C5-hydrogen is small enough to be accommodated but the larger C5-methyl of thymine cannot.

The mechanism of thymine rejection in SMUG is different from the mechanism used by UDG since there is no aromatic side chain barrier, which would block thymine from
entering the binding pocket. Instead, in SMUG the equivalent position to the barrier side chain is occupied by a water molecule, which makes three hydrogen bonds when DNA is bound across the pyrimidine pocket (Gly98, Met102, Glu146). When uracil is bound in the pocket, this water molecule is not displaced but retained in van der Waals contact with C5 of the pyrimidine ring and has a hydrogen bond with the O4-carbonyl. Thymine could only occupy the SMUG pyrimidine pocket by displacing this water molecule at the expense of three hydrogen bonds which would not be compensated by the SMUG-thymine complex. So a water molecule cannot provide a barrier like the aromatic ring in UDG does but its displacement imposes a sufficient large penalty so that there is \( >10^6 \) fold discrimination against thymine excision compared to uracil.

Determining the structure of SMUG bound to 5-hydroxymethyl-uracil (HmU), a base generated \textit{in vivo} by oxidation damage of thymine or oxidation and deamination of 5-methylcytosine, showed that HmU displaces the water molecule making the same hydrogen bonds with SMUG that uracil does. HmU is excised by SMUG at a similar rate to uracil (Wibley \textit{et al.} 2003).

**Binding stochiometry of DNA glycosylases**

As described above, uracil DNA glycosylase, MUG and SMUG glycosylases have similar active site architecture, however the key difference in how they bind the abasic reaction product relates to the loop that intercalates into the DNA double helix. In all three enzymes this is likely to stabilize the extra-helical conformation of the abasic site. It is thought that these interactions are responsible for the stability of the enzyme-DNA complex (Pearl 2000). The highly conserved active site sequences in UDG have precise topological equivalents in MUG and SMUG and have very similar confirmations to their UDG equivalents suggesting that MUG and SMUG’s pocket may serve a similar function, and that MUG/TDG enzymes, like UDG’s employ a nucleotide-flipping mechanism when they process their substrates. A distinct feature of the MUG/TDG
family is their interaction with the guanine on the complementary strand (Barrett et al. 1998; Maiti et al. 2008).

Studies providing information about the binding stoichiometry of glycosylases have been mostly structural studies of ligand complexes with family-1 UDGs from Herpex simplex virus type 1 (Savva et al. 1995), humans (Mol et al. 1995; Slupphaug et al. 1996; Parikh et al. 1998) and E.coli (Xiao et al. 1999). The crystal structure of MUG glycosylase, TDG and SMUG have also been analyzed using oligonucleotides bearing a lesion (Barrett et al. 1998; Barrett et al. 1998; Barrett et al. 1999; Wibley et al. 2003; Maiti et al. 2008).

Structural analyses as well as investigation of UDG binding to DNA using fluorescence anisotropy suggest a 1:1 binding of UDG to its substrate (Mol et al. 1995; Slupphaug et al. 1996; Parikh et al. 1998; Bellamy & Baldwin 2001). When the crystal structure of MUG glycosylase was obtained by Barrett et al. (Barrett et al. 1998), the authors observed that two MUG molecules had bound to the oligonucleotide of interest. Interestingly, although the two DNA strands were complementary to each other, the DNA in the cocrystal did not exist as a self-complementary blunt-ended duplex but, instead, had formed a continuous “nicked” double stranded DNA molecule. The base pairing in the DNA duplex was offset by six nucleotides positioning uracil on one strand opposite a guanine on the other strand, generating a G∙U mismatch. Because both MUG’s had excised one uracil each, the authors assumed the dimer observed in the cocrystals was due to interactions of each MUG glycosylase with the crystal lattice as well as each MUG independently binding to a U∙G mismatch but not due to a functional necessity. Therefore the structure was published as a monomer (Barrett et al. 1998)(Figure 1-19).
MUG glycosylase was published as a monomer bound to uracil containing DNA (PDB ID code: 1MWJ). The figure was generated using PyMOL (DeLano, 2002).

Another study by Barrett et al. where MUG was crystallized with a self-complementary oligonucleotide containing two G·T mismatches has revealed the enzyme had processed the G·T mismatch and detached from the abasic site. Two MUG molecules had bound at the end of the oligonucleotide non-specifically, making contacts with the exposed G·C base pairs. Apart from some disordering of the loop around residues 74-78 in one of the protein chains, the structure of the protein was similar to MUG in the absence of DNA and was therefore not considered significant (Barrett et al. 1998) (Figure 1-20).
Figure 1- 20 Structure of the nonspecific MUG-DNA base excision product complex

The two MUG molecules that had excised thymine from the T-G mismatch had dissociated from the DNA and rebound at the end of the oligonucleotide non-specifically, making contacts with the exposed G-C base pairs (PDB ID code: 1MTL). The figure was generated using PyMOL (DeLano, 2002).

Interestingly, crystallization of hTDG catalytic domain (hTDG\textsuperscript{cat}, residues 111-308) bound to a 22-bp DNA containing a tetrahydrofuran nucleotide (a chemically stable mimic of the natural abasic site) revealed a 2:1 stochiometry in binding, one subunit bound at the abasic site (product complex) and the other at an undamaged (nonspecific complex) (Figure 1-21). While the specific subunit was making contacts with the target strand and complementary strand, the nonspecific subunit was found to interact predominantly with the complementary strand and the contacts were less extensive than the product complex. Although the nucleotide was not flipped into the active site, the phosphate contacts 3’ on the target site in the product complex were present in the nonspecific complex, as were the long-range contacts with Lys-246 and Lys-248 that are made with the phosphate backbone on the complementary strand at positions 8 and 9 on
the 5' side of the target site (Maiti et al. 2008). The product complex made interactions with the widowed guanine similar to MUG (Figure 1-16).

In order to evaluate whether 2:1 binding is required for catalytic activity, the authors performed single-turnover kinetics using two G·U containing substrates, a 28-bp duplex (G·U28) and a 15-bp duplex (G·U15). Isothermal titration experiments suggested only one hTDG molecule binding to G·U15 whereas two hTDG’s bound to G·U28. Full length hTDG was found to exhibit nearly the same activity for G·U28 as for G·U15, which suggested that 2:1 binding is not required for full catalytic activity of TDG (Maiti et al. 2008).
Another DNA Glycosylase found to bind the DNA at two sites was SMUG Glycosylase. Co-crystals of SMUG with a uracil containing 12-mer revealed that the enzyme had dissociated from the AP site after excising uracil and two SMUG molecules had bound on either site of the oligonucleotide. Although this end-binding was mostly through nonspecific contacts, the complex provided an insight into the binding mechanism of the enzyme. SMUG is believed to bind the DNA as a monomer and the dimer observed was considered to be due to lattice contacts (Wibley et al. 2003) (Figure 1-22).
SMUG was co-crystallized with an uracil containing 12-mer, the enzyme had dissociated from the AP site after excising uracil and two SMUG molecules were found to be bound on either site. Residues, which form the wedge (Pro-Ser-Pro-Arg-Asn) inserted into the DNA are in purple, residues labeled red form the short helix (Pro-Gln-Ala-asn-Lys), which is also part of the wedge inserted into DNA (PDB ID code: 1OE4). The figure was generated using PyMOL (DeLano, 2002).

A requirement for a 2:1 Enzyme: DNA ratio for maximum glycosylase activity has been demonstrated for MutY glycosylase. MutY DNA glycosylase exhibits adenine glycosylase activity toward G·A, C·A and OG·A mismatches (Pope et al. 2002). A study by Wong et al. has demonstrated that maximum substrate cleavage occurs when two MutY molecules per one DNA molecule are available (Wong et al. 2003).
AP Endonuclease Family

The hydrolytic breakage of the N-glycosidic bond to produce apurinic/apyrimidinic (AP) sites is the most significant structural abnormality that arises in cellular DNA and can (1) either be generated by spontaneous hydrolysis of the N-glycosidic bond or (2) as an intermediate in DNA base excision repair or (3) by the action of endogenous factors such as reactive oxygen species produced by cellular metabolism or exogenous damaging agents such as ionizing radiation (for a review see Barzilay & Hickson 1995). It is estimated that between 2000 and 10000 purine bases alone are lost and regenerated in each human cell per day (Lindahl & Nyberg 1972). As AP sites are both cytotoxic and mutagenic due to lack of coding information, the repair of AP sites is essential for cell viability, therefore all organisms have developed repair mechanisms to eliminate AP sites.

*E. coli* cells have two main types of AP Endonucleases; Exonuclease III (ExoIII) and Endonuclease IV (EndoIV), among these ExoIII is constitutively expressed and is the main AP Endonuclease in *E. coli* (Saporito *et al.* 1988). In humans, the overwhelming majority of 5’ AP endonuclease activity is provided by the ExoIII homolog designated APE1 (Demple *et al.* 1991). APE1 is also known by the names HAP1, REF1, and APEX (Mol *et al.* 2000) and is 27% identical to ExoIII. Expression of human APE1 in *E.coli* cells deficient in Exonuclease III and Endonuclease IV (Cunningham *et al.* 1986) restores resistance of these cells to methyl methanesulfonate (MMS) indicating a conserved mechanism among species (Demple *et al.* 1991). The proteins of the APE1/Exonuclease III family exhibit a range of enzymatic activities on duplex DNA including 3’ →5’ exonuclease activity, 3’ phosphatase activity, 3’ repair phosphodiesterase activity, and RNase H activity (Richardson & Kornberg 1964; Richardson *et al.* 1964; Demple *et al.* 1986; Bernelot-Moens & Demple 1989).
The structure of ExoIII (Mol et al. 1995) revealed the characteristic four-layered α, β-sandwich fold, which is also conserved in the human homolog APE1 (Gorman et al. 1997). The structure of APE1 shows that the enzyme encompasses both DNA strands, mainly making connections with the AP-DNA strand and inserts loops into the DNA to flip out the abasic site similar to the mechanism used by DNA glycosylases. Across from the flipped-out AP site, a methionine residue (Met-270) inserts through the minor groove to pack against the orphan base partner of the abasic site and occupies the space (Mol et al. 2000) (Figure 1-23).

Figure 1-23 An overview of the structure of APE1: DNA complex
APE1 makes contacts with the DNA 5’ and 3’ to the abasic site using 5 DNA-binding regions (red). Red marked residues penetrate the DNA major groove, Met-270 (blue) inserts into the DNA to pack against the orphan base partner to the abasic site (PDB ID code: 1DEW). The figure was generated using PyMOL (DeLano, 2002).
The proposed catalytic mechanism based on the crystal structure of both enzymes involves a single active site. Both models involve amino acid residues that are conserved in the APE1/Exonuclease III family (Sander et al. 1991; Mol et al. 1995). APE1 is slightly different from ExoIII as it can be separated into two functionally distinct regions, the N-terminal domain, which is found to activate the DNA binding of some oxidized transcription factors in vitro and the C-terminal domain, which contains the AP endonuclease activity (Masuda et al. 1998).

Figure 1-24 shows the catalytic mechanism used by both AP Endonucleases to cleave the phosphodiester bond. Critical residues of the catalytic sites of APE1 and ExoIII are shown. Asn (blue); Asp (red) and His (violet) are common to both catalytic sites. Asn forms hydrogen bonds with the target phosphate. Asp-229 forms a hydrogen bond with His-259 and stabilizes the positive charge that develops when histidine abstracts a proton from a water molecule. The resulting nucleophilic hydroxide ion then attacks the phosphate group which proceeds to a penta-covalent transition state. Glu-34 binds the metal ion, which interacts with the negatively charged phosphate group and aids the nucleophilic attack of the hydroxyl group (Figure 1-24B) (Mol et al. 1995).
Figure 1-24 Catalytic sites and cleavage mechanism of AP endonucleases

Critical residues of the catalytic sites of (A) Exonuclease III (ExoIII) and human AP Endonuclease (APE1) are shown. Asn (blue); Asp (red); His (violet) are common to both catalytic sites. Asn forms hydrogen bonds with the target phosphate whereas Asp and His are responsible for the hydrolytic cleavage of the phosphodiester bond. (B) Proposed mechanism for the hydrolytic cleavage of the phosphodiester bond 5' to the abasic site based on ExoIII active site residues. Asp-229 forms a hydrogen bond with His-259. The resulting nucleophilic hydroxide ion then attacks the phosphate group. Glu-34 binds the metal ion which interacts with the negatively charged phosphate group and aids the nucleophilic attack of the hydroxyl group. Figure B was taken from Mol et al.; 1995 (Mol et al. 1995). Figure A were generated using PyMOL (DeLano, 2002).
ExoIII and APE1 are both strongly stimulated by metals such as MgCl₂ (Richardson & Kornberg 1964; Kane & Linn 1981; Mol et al. 1995). MgCl₂ does not affect binding of ExoIII or APE1 to abasic site containing DNA but was found to have different effects on abasic site cleavage and on product dissociation by APE1 (Masuda et al. 1998). In the absence of MgCl₂, APE1 exhibits relatively slow dissociation from AP-DNA (Masuda et al. 1998). 1mM MgCl₂ leads to more product formation but decreases APE1’s dissociation from the DNA as determined by Electrophoretic Mobility Shift Assays. 10mM MgCl₂ increases APE1’s dissociation from the DNA (Masuda et al. 1998).
Chapter 2

Mismatch DNA Glycosylase: DNA binding and activity assays
Chapter 2: Mismatch DNA Glycosylase: DNA binding and activity assays

Aim

Preliminary studies in our lab (O'Neill 2004) suggested a positive cooperativity in the binding of mismatch DNA uracil glycosylase (MUG) to abasic DNA. This finding was unexpected since the general assumption was that the enzyme binds and cleaves DNA as a monomer. To gain more insight, we aimed to further examine cooperative binding by MUG and explore if cooperativity is important for its glycosylase activity. Another objective was to determine the optimal MUG: DNA stoichiometry for maximum glycosylase activity. The effect of an increase in ionic strength on the binding characteristics of MUG as well as on its activity has been investigated.

Background

When the crystal structure of MUG glycosylase was obtained by Barrett et al., the authors observed that two MUG molecules had bound to the oligonucleotide of interest (Barrett et al. 1998). The authors assumed the dimer observed in the cocrystals was due to interactions of each MUG glycosylase with the crystal lattice as well as each MUG independently binding to a U·G mismatch but not due to a functional necessity. Although the two MUG molecules had bound forming a protein-protein interface with each other, the structure was published with one MUG glycosylase bound to DNA with the argument that the dimer was functionally not relevant (Figure 1-19). Based on the structure of the enzyme, MUG glycosylase bound to DNA as a dimer was constructed by our group and can be seen in Figure 2-1.
Anisotropy measurements in our lab suggested a positive cooperativity in MUG binding to abasic DNA (O'Neill 2004). Cooperative binding to DNA is so far not common among DNA glycosylases. The only other DNA glycosylase to which a cooperative binding has been allocated is MutY. MutY exhibits adenine glycosylase activity toward G·A, OG·A and C·A mismatches (Pope et al. 2002). It has been shown that for maximum substrate cleavage, two MutY molecules are required per one DNA molecule (Wong et al. 2003).
In this chapter, binding characteristics of MUG glycosylase were explored by monitoring change in fluorescence anisotropy upon MUG glycosylase binding to DNA. The optimal MUG: DNA stoichiometry for maximum glycosylase activity was also determined by monitoring base excision at different MUG: DNA ratios. The effect of different salt concentrations on the binding characteristics of MUG as well as its activity has been analyzed. The association between cooperativity and activity was also explored.

**Fluorescence measurements**

Fluorescence is the emission of light from a substance and occurs from electronically excited states. Fluorescence measurements are widely applied, particularly in biology and medicine. It is important to extract maximum information from fluorescence signals since they can give very valuable information about the local molecular environment or interactions of molecules with each other. With the appropriate instrumentation, parameters such as fluorescence intensity, excitation and emission wavelength, decay time and polarisation can be measured.

**Fluorescence and its properties**

When a fluorophore absorbs a photon it can be excited to either the $S_1$ or to higher singlet electronic states (Figure 2-2). It will lose energy to the surrounding environment as its population returns to the lowest vibrational level of $S_1$. This is called thermal relaxation by internal conversion. The fluorophore will then return to the ground state ($S_0$) either radiatively with the emission of a photon, known as fluorescence, or non-radiatively. Internal conversion generally occurs in $10^{-12}$s or less whereas fluorescence lifetimes are typically near $10^{-8}$s so internal conversion is generally complete prior to
emission. At room temperature, thermal energy is not adequate to significantly populate the excited vibrational states. Absorption typically occurs from molecules with the lowest vibrational energy. When a molecule is excited, another process called phosphorescence can also occur by intersystem crossing from the excited singlet state $S_1$ level to the first excited triplet state $T_1$ followed by decay to the ground state by emission of a photon. The lifetime of phosphorescence is longer than the fluorescence lifetime.

Figure 2- 2 Jablonski diagram

$S_0$ and $S_1$ represent the singlet ground and first electronic states respectively. 0, 1, 2 and 3 are the vibrational states that a fluorophore can exist in. $T_1$ is the first triplet state.

**Intensity**

There are several properties of fluorescence that can be used to study a fluorophore and its environment. The most commonly used property is the fluorescence intensity, which is related to the number of photons emitted from a fluorophore. The quantum efficiency,
$F$, is the ratio of emitted to absorbed photons and can be expressed by the equation 2.1 (Eq 2.1) (Lakowicz 1999).

$$F = \frac{k_r}{k_r + k_{nr}}$$  
(Eq 2.1)

where $k_r$ is the radiative decay rate coefficient and $k_{nr}$ is the non-radiative rate coefficient. The quantum efficiency is therefore the fraction of photons that are emitted radiatively. Different fluorophores have different quantum efficiencies and therefore have different intensities. This can be used to identify different fluorophores when imaging a sample. Moreover, $k_{nr}$ is highly dependent on the environment, which means the fluorescence intensity can be used as an environmental sensor.

**Wavelength**

Every fluorophore has a unique emission spectrum due to the discrete energy differences between the electronic states. This provides additional contrast in an image that might not be obtainable with intensity imaging alone. It should be noted that fluorescence always occurs from the lowest vibrational level of $S_1$ to the highest vibrational level of $S_0$, therefore the emission wavelength is longer than the excitation wavelength. According to equation 2.2 (Eq 2.2), where $\lambda_{em}$ is the emission wavelength, $h$ is Planck’s constant, $c$ is the speed of light and $\Delta E$ is the energy gap, the emitted photon always has a lower energy than the excitation photon. This is known as the Stokes shift, due to the first observation made by Sir G.G. Stokes in 1852 (Lakowicz 1999).

$$\lambda_{em} = \frac{hc}{\Delta E}$$  
(Eq 2.2)
The spectrum of a fluorophore can be dependent on the pH, ionic strength and presence of other fluorophores so it can be used to provide information about its chemical environment.
**Lifetime**

The average time an electron spends in the excited state before returning to the ground state is known as the fluorescence lifetime. Its value, $\tau$, is therefore the inverse of the total decay rate as shown in equation 2.3 (Eq 2.3).

$$\tau = \frac{1}{k_r + k_{nr}}$$  
(Eq 2.3)

The radiative decay rate in the equation is dependent on the fluorophore as previously discussed, allowing it to be used to identify different fluorophores. While the non-radiative decay rate is dependent on the local environment so the lifetime can be used to report on temperature (Benninger et al. 2006), viscosity (Benninger et al. 2005) and ionic concentration (Agronskaia et al. 2004).

Unlike intensity based measurements where the sample is continuously illuminated, lifetime measurements require the sample to be excited with a laser pulse with a width that is typically shorter than lifetime value itself. After excitation the intensity of the sample will decay exponentially according to the following equation,

$$I(t) = I_0 \times e^{-t/\tau}$$  
(Eq 2.4)

where $I(t)$ is the intensity at time $t$ and $I_0$ is the intensity at time zero (Lakowicz 1999). In this case lifetime is defined as the time it takes for the intensity to decay exponentially to 1/e times its peak value after excitation.
Polarization

Fluorescence anisotropy is a very sensitive method for monitoring binding of a protein to DNA. Upon excitation with polarized light, emission from samples is also polarized. The extent of polarization of the emission is described in terms of anisotropy ($r$). Depolarization of the emission can be caused by a number of phenomena; rotational diffusion of fluorophores is a common cause for depolarization. Polarization or anisotropy measurements reveal an average angular displacement of the fluorophore that occurs between absorption and subsequent emission of a photon. This angular displacement is dependent on the rate and extent of rotational diffusion (tumbling) in solution during the lifetime of the excited state. The rate and extent of rotational diffusion can be influenced by the size and shape of the diffusing molecule. Like the measurement of the lifetime, the measurement of the anisotropy is also ratiometric and concentration independent. It can be used to probe the fluorophore’s rotational mobility, which provides information about its molecular size, whether it is bound or unbound and the viscosity of the environment (Lakowicz 1999).

The measurement of fluorescence anisotropy is illustrated in Figure 2-3. The sample is excited with vertically polarized light. The electric vector of the excitation light is oriented parallel to the vertical or $z$-axis. The intensity of the emission is then measured via a polarizer. When the emission polarizer is oriented parallel ($I_{\parallel}$) to the direction of the excitation, the observed intensity is called $I_{\parallel}$. Likewise, when the polarizer is perpendicular $I_{\perp}$ to the excitation, the intensity is called $I_{\perp}$. These values are used to calculate anisotropy ($r$). The difference in both intensities ($I_{\parallel} - I_{\perp}$) is normalized by the total intensity, which is $I_T = (I_{\parallel} + 2I_{\perp})$ (Eq 2.5).

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad \text{(Eq 2.5)}$$
Figure 2-3 Schematic diagram for the measurement of fluorescence anisotropy

The sample is excited with vertically polarized light. The electric vector of the excitation light is oriented parallel to the vertical or z-axis. When the emission polarizer is oriented parallel to the direction of the excitation (II), the observed intensity is called \( I_{II} \). Likewise, when the polarizer is perpendicular to the excitation (\( \perp \)), the intensity is called \( I_{\perp} \). These values are used to calculate anisotropy \( r \).

To calculate the actual intensities an additional factor, G factor, must be measured. G factor determines the efficiency of the detection system to measure the ratios of vertically and horizontally polarized light. For example, a G factor of 2 indicates that the emission polarizer passes vertically polarized light twice as efficiently as horizontally polarized light.

Binding of a protein to DNA leads to an increase in rotational correlation time due to the increased mass of the enzyme-DNA complex relative to free DNA. This leads to an increase in anisotropy, which can be measured by using the equation (Eq 2.5). The extent of fluorescence polarization of a molecule also depends on the relative values of the fluorescence lifetime and the rotational correlation time, which is proportional to the
molecular volume of the molecule when the temperature and viscosity of the medium are kept constant (Lakowicz 1999).

**Objectives**

- Work out the binding mode of MUG glycosylase to DNA
- Study the MUG: DNA stoichiometry required for maximum glycosylase activity
- Investigate if cooperativity in MUG binding is important for MUG’s activity

**MUG Glycosylase binds DNA cooperatively**

Using fluorescence anisotropy, solution-based DNA binding experiments were performed to measure the affinity of MUG glycosylase to DNA containing abasic furanose analogue (AP), paired with guanine and labeled with HEX on its 5’ terminus (H12AP∙G). Binding experiments were also carried out with non-specific DNA simply composed of normal DNA bases. The non-specific DNA has the same sequence as H12AP∙G except it has a cytosine instead of the abasic site (H12C∙G). Increasing concentrations of MUG glycosylase were added to a sample containing 100nM H12AP∙G or H12C∙G. When the protein binds the fluorescently labeled DNA, it leads to a change in the rotational diffusion of the fluorophore (leading to increase in rotational correlation time) due to the change in mass of the protein-DNA complex relative to free DNA. This in turn leads to an increase in fluorescence anisotropy. The advantage of this technique is that the DNA is free to move in solution. DNA binding experiments were initially performed in standard MUG reaction buffer containing 50mM NaCl at 25°C. In order to elucidate the role of the size of the oligonucleotide, binding was also examined.
with two oligonucleotides H6AP-G and H6C-G, which are shorter than H12AP-G and H12C-G. These oligonucleotides have 6 basepairs 5’ to the site of interest whereas H12AP-G and H12C-G have 12 basepairs 5’ to the site of interest.

When MUG was titrated into H6C-G and H12C-G a large increase in anisotropy was observed, indicating that MUG binds well to non-specific DNA (Figure 2-4A). When fitted to a hyperbolic binding curve, a systematic deviation in the data was observed indicating that a single site binding did not describe the equilibrium (not shown). Instead, the data is shown with the best fit to the Hill equation, which describes cooperative binding of multiple ligands. Hill equation provides an equilibrium dissociation constant ($K_d$) and a Hill coefficient ($n$). $K_d$ and $n$ of non-specific binding to H6C-G ($K_d = 53.5 \pm 2$ nM; $n: 1.7 \pm 0.1$) and H12C-G ($K_d = 29.6 \pm 0.9$ nM; $n: 1.6 \pm 0.0$) were found to be similar. The Hill coefficient is a measure of the cooperativity of the system, it provides a measure of the minimum number of interacting ligands, but is not a direct measure of stoichiometry (Holt & Ackers 2009).

When MUG was titrated against abasic product DNA, an even more pronounced sigmoidal response, characteristic of positive cooperativity, was observed (Figure 2-4B). This data is also shown with the best fit to the Hill equation, $K_d$ and $n$ of MUG binding to H6AP-G ($K_d = 125.1 \pm 1.1$ nM; $n: 3.6 \pm 0.1$) and H12AP-G ($K_d = 186.5 \pm 3.0$ nM; $n: 3.4 \pm 0.2$) were the same. The significant increase of the Hill coefficient to 3.4 indicates a higher degree of cooperativity within the system. Although $K_d$ for abasic DNA is apparently weaker than $K_d$ for the non-specific DNA, it is an average value of all bound protomers and thus does not represent a discrete value for binding to the abasic product site. Table 2-1 summarizes $K_d$ and cooperativity values obtained with H6C-G, H12C-G, H6AP-G and H12AP-G.
Figure 2-4 MUG binds DNA with positive cooperativity

Binding of MUG glycosylase to HEX labeled non-specific oligonucleotides H6C∙G (17bp) and H12C∙G (25bp) was analyzed by monitoring the increase in fluorescence anisotropy as described for binding reactions (Materials & Methods) (A). Fitting the data to the Hill equation gave a $K_d = 53.5 \pm 2$ nM and a Hill coefficient of $n = 1.7 \pm 0.1$ for H6C∙G. $K_d$ for H12C∙G was found to be $29.6 \pm 0.9$ nM and the Hill coefficient was $1.6 \pm 0.0$. The length of non-specific oligonucleotide did not affect the affinity or cooperativity of MUG binding.

Binding of MUG glycosylase to the abasic oligonucleotides H6AP∙G (17bp) and H12AP∙G (25bp) was analyzed by monitoring the increase in fluorescence anisotropy (B). Fitting the data to the Hill equation gave a $K_d = 125.1 \pm 1.1$ nM and a Hill coefficient of $3.6 \pm 0.1$ for H6AP∙G. $K_d$ for H12AP∙G was found to be $186.5 \pm 3.0$ nM and the Hill coefficient was $3.4 \pm 0.2$. These values demonstrate that the length of the oligonucleotide does not change the affinity or cooperativity of MUG binding to abasic DNA. Table 2-1 summarizes $K_d$ values that were obtained for the different abasic and non-specific oligonucleotides.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>$K_d$ (nM)</th>
<th>Cooperativity</th>
</tr>
</thead>
<tbody>
<tr>
<td>H6AP∙G</td>
<td>125.1 ± 1.1</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td>H12AP∙G</td>
<td>186.5 ± 3.0</td>
<td>3.4 ± 0.2</td>
</tr>
<tr>
<td>H6C∙G</td>
<td>53.5 ± 2</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>H12C∙G</td>
<td>29.6 ± 0.9</td>
<td>1.6 ± 0.0</td>
</tr>
</tbody>
</table>

Table 2-1 $K_d$ values and Hill coefficients of MUG binding abasic and non-specific DNA were determined by fitting the fluorescence anisotropy data to the Hill equation.
Ionic strength of the reaction effects binding cooperativity of MUG

It has been suggested that macromolecular association, as for example in the formation of protein-DNA complexes, can proceed rapidly by molecules first binding non-specifically and then diffusing along each other. Non-specific electrostatic forces have been proposed as the most likely source for the non-specific interactions between proteins. In the first phase, two proteins form a low affinity non-specific complex, which is held together by long-range electrostatic interactions. The second phase is the docking of the two proteins, to give the final high affinity complex (Schreiber & Fersht 1996; Halford 2009). Increasing salt concentration has been shown to have a negative effect on protein - protein association (Schreiber & Fersht 1996). Increase in ionic strength has a negative effect on the non-specific interactions between proteins and DNA as well (Record et al. 1976). The finding that MUG binds DNA non-specifically and cooperatively led us to investigate the effects of an increase in ionic strength on MUG binding the DNA.

In order to investigate the effects of salt, MUG’s binding to H12AP-G was evaluated at different salt concentrations by monitoring the change in anisotropy. The standard MUG reaction buffer contains 50mM NaCl. Binding at 150mM and 300mM NaCl was investigated in comparison to binding at 50mM NaCl. 150mM NaCl was found to reduce cooperativity of MUG from 3.1 ±0.2 to 1.9 ± 01. Equilibrium dissociation constant of MUG for DNA at 150mM NaCl ($K_d = 143.3 \pm 5nM$) was the same as in standard MUG reaction buffer (50mm NaCl) ($K_d = 122.1 \pm 2.6nM$). At 300mM NaCl, MUG binding fit best to a one-site binding model. The affinity of the enzyme to DNA was significantly reduced at 300mM NaCl ($K_d = 1384.5 \pm 80.5nM$) (Figure 2-5).
MUG was added to 100nM H12AP: G in standard binding buffer with 50mM, 150mM or 300mM NaCl. Change in anisotropy was monitored. Cooperativity decreased with increasing NaCl concentrations from 3.1 (50mM NaCl) to 1.9 (150mM NaCl) and to 1 (300mM NaCl). With increasing the salt concentration to 300mM, affinity of MUG to DNA with the abasic site decreased dramatically. $K_d$’s observed were: $K_d = 122.1 \pm 2.6\,\text{nM (50mM NaCl)}$, $K_d = 143.3 \pm 5\,\text{nM (150mM NaCl)}$; $K_d = 1384.5 \pm 80.5\,\text{nM (300mM NaCl)}$.

The effect of an increase in ionic strength on binding to non-specific DNA was also evaluated by monitoring the change in fluorescent anisotropy upon MUG binding to H12C-G at the salt concentrations above. Binding at 50mM NaCl showed a cooperativity index of $n=1.7 \pm 0.1$ whereas cooperativity was reduced to $0.99 \pm 0.1$ at 150mM NaCl. A reduction of MUG’s affinity to non-specific DNA was also observed at higher salt concentrations with a $K_d$ increase from $32.5 \pm 0.1\,\text{nM (50mM NaCl)}$ to $94.2 \pm 4.4\,\text{nM}$.
Non-specific binding at 300mM NaCl was linear and did not saturate (Figure 2-6).

Figure 2-6 Binding of MUG glycosylase to non-specific DNA is affected by salt

MUG was added to 100nM H12C-G in a standard binding buffer at 25°C (A) containing 50mM NaCl (B) 150mM NaCl (C) 300mM NaCl. Change in anisotropy of H12C-G was monitored. The binding data with 50mM and 150mM NaCl were fitted to the Hill equation. Cooperativity as well as affinity of MUG to DNA was reduced at higher NaCl concentrations. Cooperativity at 50mM NaCl was 1.7 ±0.1 and was reduced to 0.99±0.1 at 150mM NaCl. Reduction of the affinity of MUG to non-specific DNA was recognized by the increase of \( K_d \) from 32.5 ±0.1 nM (50mM NaCl) to 94.2 ±4.4 nM (150mM NaCl). Non-specific binding at 300mM NaCl was linear.

An increase in ionic strength has a bigger effect on the affinity of MUG glycosylase to non-specific than to abasic oligonucleotide. Increasing the salt concentration from 50mM to 150mM NaCl did not lead to an increase in the \( K_d \) of MUG binding to abasic DNA.
whereas it led to a threefold increase in the $K_d$ of non-specific binding. Increase in ionic strength is known to weaken the binding to non-specific DNA much more than specific DNA (Powell et al. 1998). At 300mM NaCl, a linear increase in anisotropy signal was observed with non-specific DNA, indicating that electrostatically mediated non-specific binding was eliminated at this salt concentration.

**Lifetime analysis of the fluorophore in DNA binding by MUG**

The DNA binding assays described above are dependent on a change in the steady state anisotropy of the fluorophore, which changes as the rotational correlation time of the DNA is altered due to the increased mass when bound by MUG. However, associated changes in fluorescence intensity and lifetime with MUG binding can interfere with steady state anisotropy measurements. We therefore wished to determine the rotational correlation time directly from time-resolved studies. This provides an independent measure of the rotational freedom of the DNA and will thus determine whether the measured anisotropy is subject to significant deviations due to intensity or lifetime changes, and whether it provides a suitable method for monitoring free and bound DNA species.

Interpretation of the steady-state anisotropies usually depends on an assumed form of the anisotropy decay, which is not directly observed in the experiment. Additional information can be obtained by measuring the time-dependent anisotropy, which is the values of $r(t)$ following pulse excitation. The form of the anisotropy decay depends on the size, shape and flexibility of the fluorescently labeled molecule. For instance, for a spherical molecule, the anisotropy is expected to decay with a single rotational correlation time ($\theta$). Anisotropy decays of fluorescent molecules are frequently more complex than a single exponential. The shape of the anisotropy decay can provide information about the shape of the macromolecule and its flexibility. This shape
information is lost during averaging of the anisotropy over the decay time while doing steady-state anisotropy measurements.

Fluorescence intensity decays were collected using a custom-built time-resolved spectrofluorometer, previously described in Manning et al. (Manning et al. 2008). This spectrofluorometer permits the fluorescence intensity or anisotropy to be measured in a nanosecond timescale (Manning et al. 2008). The measurements were conducted together with Professor French’s group and the analysis was performed by Tom Robinson in Professor French’s group. Analysis of lifetime and anisotropy decays was performed using TRFA data processor (Scientific Software Technologies Center, Minsk, Belarus).

For the analysis of fluorescence intensity decays, MUG was added to 100nM HEX H12AP: G in standard MUG binding buffer at 25°C. The data was best fitted using two fluorescence lifetime components and two rotational correlation times. The change in the average fluorescence lifetime values with addition of MUG are shown in Figure 2-7. We hypothesize that the average lifetime value changes because the binding of MUG causes a change in the vibrational freedom of the fluorophore and therefore a change in the non-radiative decay rate. The change in the average fluorescence lifetime is small therefore any significant deviations in steady-state anisotropy are not of great significance.
Fluorescence anisotropy decays were fitted with a double exponential model resulting in a short rotational correlation time (0.28 ± 0.08 ns) which did not change significantly with the addition of MUG. This can be attributed to the fast rotational diffusion of the HEX about its flexible linker which is not affected by the binding of MUG (Powell et al. 1998). The second rotational correlation time increased from approximately 3 ns to 18 ns due to the overall rotational diffusion of the DNA-enzyme complex. Both of the rotational correlation times are shown in Figure 2-8A. Figure 2-8B shows how the corresponding steady-state anisotropy calculated from the same data set varied with MUG binding and it clearly reproduces the same trend as the long rotational correlation time (Figure 2-8A). This suggests that there is therefore not a significant change in fluorescence lifetime of the HEX upon MUG binding. This is also confirmed by figure
2-7, which shows the average lifetime variation is relatively small (~5%) and so should have only a minor affect on steady-state anisotropy measurements.

The change in rotational correlation time was monitored as MUG was added to 100nM H12AP:G in standard MUG binding buffer at 25°C. Fluorescence anisotropy decays were fitted with a double exponential model resulting in a short rotational correlation time ($\theta_1 = 0.28 \pm 0.08$ ns) which did change significantly with the addition of MUG. The second rotational correlation time ($\theta_2$) increased from approximately 3 ns to 18 ns due to the overall rotational diffusion of the DNA-enzyme complex (A). The variation in the corresponding steady-state anisotropy has also been calculated from the same acquired data set (B).

Figure 2-8 Time resolved anisotropy measurements of HEX labelled AP•G DNA upon DNA binding by MUG
Figure 2-9 shows four anisotropy decays to illustrate how the addition of MUG changes the rotational correlation times. The fast rotational correlation time remains present with and without MUG reflecting the fast rotational diffusion of the fluorophore about its flexible linker. However when MUG is added in increasing amounts the longer anisotropy component increases reflecting an increase in the overall rotational diffusion of the complex due to the mass increase.
Figure 2-9 Anisotropy decays for 0, 280, 400 and 1400nM MUG

The figure shows four anisotropy decays to illustrate how the addition of MUG changes the rotational correlation times (A). When MUG is added in increasing amounts, the longer component increases reflecting an increase in the overall rotational diffusion of the complex due to the rise in mass (A). Figures 2-9 B-E show residuals (color coded to concentrations) for 0, 280, 400 and 1400nM MUG.
Competition titration using HEX-labeled oligonucleotides

In order to further investigate the MUG: DNA binding stoichiometry, competitive titrations have been used. Initially, complexes were formed between MUG and H12AP-G. Unlabeled 12AP-G (competitor) was titrated into the mixture, and the decrease in anisotropy, as H12AP-G became displaced, was measured (Figure 2-10). The initial concentration of H12AP-G was at 500nM to be above the $K_d$ for the abasic product, the concentration of MUG in the initial complex was increased in stoichiometric equivalents from a 1:1 ratio with the DNA up to 4:1 (Figure 2-10A).

With an initial 1:1 stoichiometry of MUG: H12AP-G, the starting anisotropy was very low, and this was readily competed off with unlabeled competitor 12AP-G DNA. At a 2:1 ratio, the initial anisotropy was much higher, and this was competed off directly with unlabeled 12AP-G as well. At higher stoichiometric equivalents of 3:1 and 4:1, the starting anisotropy was slightly higher than with 2:1, but there was a lag before the unlabeled DNA was able to compete off the labeled MUG:DNA complex indicating the presence of unbound MUG in the sample.

At the low MUG concentration (1:1), the anisotropy was very low indicating that the DNA was not in a fully bound state and that a 1:1 stoichiometry of MUG: DNA is insufficient to fully bind the abasic H12AP-G. At 2:1, the anisotropy was much higher, consistent with a near saturated complex. The relatively small increase in anisotropy observed with higher MUG concentrations may be due to complete saturation and/or further binding of MUG in a non-specific manner. The lag observed with higher enzyme concentrations is consistent with the unlabeled competitor first binding excess free MUG or MUG bound in a weak, non-specific manner. Only once this excess MUG has been
bound, does the competitor begin to compete for MUG bound in a tighter, specific complex.

The stoichiometry can be further examined by plotting the anisotropy versus the stoichiometric balance, calculated from the stoichiometry of MUG: [H12AP-G] minus the stoichiometric equivalents of competitor DNA added ([12AP-G] / [H12AP-G]) (Figure 2-10B). Plotting the data in this way clearly demonstrates that the specific complex is only competed off once there is a stoichiometry of 2 x MUG: 1 x H12AP-G remaining. The slight shift to the right of the higher concentration curves can be accounted for by additional non-specific binding of MUG to H12AP-G. The observed competition data are thus consistent with cooperative binding of MUG to the abasic DNA product with a 2:1 stoichiometry.

**Figure 2- 10 Competition experiments suggest two MUG molecules bind abasic DNA**

500nM HEX labeled abasic DNA (H12AP-G) was incubated with 500nM, 1000nM, 1500nM and 2000nM MUG in standard MUG binding buffer at 25°C for 15 minutes to reach equilibrium. Increasing amounts of unlabeled abasic DNA (12AP-G) was titrated in and anisotropy was measured. The anisotropy data are shown plotted (A) vs. concentration of competitor DNA and (B) vs. the stoichiometric balance, calculated as the stoichiometry of MUG:H12AP-G ([MUG]/[H12AP-G]), minus the stoichiometric equivalents of unlabeled DNA added ([12AP-G]/[H12AP-G]).
**MUG activity and cooperativity**

Solution-based DNA binding experiments illustrated that MUG glycosylase binds abasic DNA as well as DNA without a lesion cooperatively. Competition titration experiments suggested a MUG: DNA binding stoichiometry of 2:1. In order to investigate the optimal MUG: DNA stoichiometry required for maximum glycosylase activity, product formation was monitored at various MUG: DNA ratios.

The turnover of MUG glycosylase is limited by the rate of product dissociation \( (k_{\text{off}}) \) shown in equation 2.6. The kinetic parameter \( k_{cl} \) reflects the maximal rate of product formation and is governed by the reaction steps after DNA binding and before product release. The kinetic parameter \( k_{\text{cat}} \) reflects the maximal steady-state turnover of MUG and reflects the same steps as \( k_{cl} \) plus product release. Under single turnover conditions (DNA \(<\ll\) MUG), when the substrate is saturated with enzyme, the observed rate will be equal to \( k_{cl} \), the chemical rate of hydrolysis. Therefore \( k_{cl} \) can be determined by quantifying product formation under single-turnover conditions. \( k_{\text{cat}} \) is difficult to obtain from conventional steady-state kinetics due to the exceedingly slow product release by MUG.

\[
E + S \leftrightarrow ES \rightarrow EP \leftrightarrow E + P
\]

\( k_{cl} \quad k_{\text{off}} \)

\( k_{\text{cat}} \)

Eq. 2.6

Product formation by MUG glycosylase has been analyzed by O’Neill *et al.* (*O’Neill et al.* 2003) and it was shown that under single turn-over conditions MUG cleaves its substrate with a rate of \( k_{cl} = 0.037 \pm 0.002 \ s^{-1} \).
The binding data presented before clearly demonstrates higher order binding complexes of MUG with its abasic product DNA. However it does not provide any indication of whether this has an impact on the catalytic function of the enzyme. We therefore performed activity assays with MUG and a HEX labeled oligonucleotide containing a U·G mismatch (H6G·U). In this fixed-time quench assay, 400nM HEX labeled H6G·U was incubated with increasing amounts of MUG for 15 minutes. Based on results of O’Neill et al. (O’Neill et al. 2003) and our own results, 15 minutes is an adequate time to allow complete substrate cleavage by MUG. Since our group has also established that MUG has very poor turnover kinetics (O’Neill et al. 2003), there will be no multiple reactions of the enzyme within this time phase.

Fixed-time quench assays were quenched with NaOH, which stops the reaction and cleaves abasic sites after heating to 90°C for 20 minutes. Substrate and product were then separated by denaturing PAGE and quantified using the Phoretix 1D software. The gel and its quantitation is shown in Figure 2-11. For complete cleavage of uracil from 400nM H6G·U, a minimum of 800nM MUG is required (Figure 2-11). This indicates a necessity for a 2:1 MUG: DNA ratio for complete activity.
A 2:1 ratio of MUG: DNA is required to fully cleave uracil from DNA containing a U-G mismatch. 400nM HEX labeled DNA with a U-G mismatch (H6G∙U) was incubated with increasing amounts of MUG in reaction buffer at 25°C. The reaction was allowed to proceed for 15 minutes before quenching with NaOH and analyzing by denaturing PAGE (A). The bands corresponding to the product (P: blue) and substrate (S: red) were quantified by using the Phoretix 1D software (B). Formation of product and depletion of substrate were plotted to determine the minimum MUG concentration required for complete cleavage. 800nM MUG is required for complete cleavage of uracil from 400nM H6G-U.

To validate the requirement for a twofold excess of MUG to process the DNA, the activity test was also performed with 200nM and 600nM H6G-U. The oligonucleotides were incubated with increasing amounts of MUG. Substrate cleavage and product formation were analyzed as before and the percentage of substrate (%) was plotted against [MUG]/[DNA]. In each case, complete cleavage coincided with an enzyme concentration that is double the DNA concentration (Figure 2-12). This is consistent with the 2:1 binding stoichiometry observed in competition experiments (Figure 2-10).
and also indicates that multiple protein binding is important for catalytic activity (Figure 2-12).

**Figure 2-12 Two MUG’s are required for maximum activity**

200nM, 400nM and 600nM 6HG-U were incubated with different amounts of MUG in reaction buffer at 25°C. The reaction was allowed to proceed for 15 minutes before quenching with NaOH and analyzing by denaturing PAGE. Substrate cleavage was quantified using the Phoretix 1D software. Depletion of the substrate was plotted for each DNA concentration; product formation is omitted for clarity. In each case, complete cleavage coincides with an enzyme concentration that is double the DNA concentration.

**Salt dependence of MUG activity**

We observed that the interaction of MUG with DNA is significantly affected by the ionic strength of the sample: with increasing NaCl, binding cooperativity decreased, accompanied by an increase in $K_d$. There was no observed cooperativity with non-specific DNA at 150 mM NaCl, and with abasic DNA the cooperativity was abolished at the higher NaCl concentration of 300 mM (Figures 2-5 and 2-6). Having found that an increase in ionic strength reduces MUG’s binding cooperativity, we investigated substrate cleavage at a higher ionic strength. Cleavage of H6G-U by MUG was analyzed...
in MUG reaction buffers containing 50mM, 150mM and 300mM NaCl. Since MUG is product inhibited, the reaction was monitored under single turnover conditions (MUG>>DNA) to ensure substrate saturation, even at the higher ionic strength conditions, so that product formation can be monitored directly. 5μM MUG processed 100nM H6G·U with a rate of $k_{cl}: 0.062 \pm 0.003 \text{ s}^{-1}$ at 50mM NaCl, which is the standard ionic strength for the MUG reaction. This rate is consistent with that previously observed under saturating conditions (O’Neill et al. 2003). MUG’s activity was reduced at 150mM and preceded with a rate of $k_{cl}: 0.016 \pm 0.002 \text{ s}^{-1}$. At 300mM NaCl, the reaction rate was significantly reduced ($k_{cl}: 0.0004 \pm 0.0001 \text{ s}^{-1}$) (Figure 2-13).

Figure 2-13 Higher ionic strength reduces cooperativity and slows down cleavage by MUG

100nM H6G·U was incubated with 5μM MUG in reaction buffer containing 50mM NaCl (*), 150mM NaCl (•) and 300mM NaCl (•). The reaction was allowed to proceed for 15 minutes before quenching with NaOH and analyzing by denaturing PAGE. Product formation was analyzed by quantifying the product band using the Phoretix 1D software. Data for H6G·U is shown with the best fit to a single exponential with rates of 0.062 s$^{-1}$ (±0.003), 0.016 s$^{-1}$ (± 0.002) and 0.0004 s$^{-1}$ (0.0001) for 50mM, 150mM and 300mM NaCl, respectively.
Discussion

Our study furthers the knowledge about the binding and cleavage mechanism of MUG glycosylase. MUG glycosylase and its human homologue TDG are assumed to bind DNA as a monomer, on the other hand a MUG dimer had been observed in co-crystals but dimerization was not taken into account since it was assumed to be due to crystal lattice attachments (Barrett et al. 1998b). An independent study found that TDG, the human homologue of MUG, bound to DNA in a 2:1 stoichiometry (Maiti et al. 2008). Fluorescence anisotropy measurements in our lab had suggested a positive cooperativity in the binding of MUG glycosylase to DNA. We therefore conducted further experiments to characterize binding of MUG to DNA.

MUG glycosylase binds DNA cooperatively

In order to investigate how MUG binds the DNA, we monitored the increase in fluorescence anisotropy upon binding. The binding data fit well to the Hill equation, which describes cooperative binding of multiple ligands. Hill equation provides us with a Hill coefficient (n). It also gives one general equilibrium dissociation constant without measuring the individual binding constants. The Hill coefficient gives a measure of the minimum number of interacting ligands, but is not a direct measure of stoichiometry (Holt & Ackers 2009).

When we first titrated MUG into non-specific DNA, we observed a large increase in anisotropy, which indicates that MUG binds well to the non-specific DNA. Binding to non-specific DNA could not be fit to a one-site binding model since the curve was sigmoidal. When the enzyme was titrated against abasic DNA, an even more pronounced sigmoidal response, characteristic of positive cooperativity, was detected.
Although $K_d$ is apparently higher with abasic than non-specific DNA, it is an average value of all bound protomers and thus does not represent a discrete value for binding to the abasic product site. The Hill coefficient or $K_d$ of MUG binding to non-specific as well as abasic DNA did not change with an increase in the number of basepairs 5’ to the site of interest.

**Time-resolved measurements of the fluorophore upon DNA binding by MUG**

Steady-state fluorescence anisotropy measurements have frequently been used as a convenient means for measuring DNA binding in free solution (Powell *et al.* 1998; Krusong *et al.* 2006). Unfortunately, while the steady-state anisotropy varies with the rotational correlation time, it is also a function of the fluorescence lifetime, and changes in fluorescence lifetime upon binding can introduce significant ambiguity into anisotropy measurements (Lakowicz 1999). We have utilized a multidimensional fluorimeter (Manning *et al.* 2008) to determine the variation in rotational correlation time and fluorescence lifetime as a function of MUG binding. Analysis of the fluorescence decay profiles suggested complex anisotropy decays and the data were fitted to a double exponential model resulting in short and long rotational correlation times. The short rotational correlation time (0.28 ± 0.08 ns) did not change significantly with the addition of MUG and can be attributed to the fast rotational diffusion of the HEX about its flexible linker, which would not be affected by the binding of the enzyme (Powell *et al.* 1998). The second rotational correlation time increased from approximately 3 ns to 18 ns and was attributed to the overall rotational diffusion of the enzyme-DNA complex, which varied as expected with increased MUG binding. While this is a useful and robust readout of MUG binding, the data acquisition and analysis is time consuming and steady-state anisotropy measurements would be much more convenient. The corresponding steady-state anisotropy calculated from the same data set varied with MUG binding and clearly reproduced the same trend as the long rotational correlation time. This suggests that there is therefore not a significant change
in fluorescence lifetime of the HEX upon MUG binding. This was also confirmed by measurement of the average lifetime variation, which is relatively small (~5%) and so should have only a minor affect on steady-state anisotropy measurements.

**Competition and activity experiments suggest that two MUG’s are required for DNA binding and cleavage**

Competition titration experiments were performed in order to further investigate the stoichiometry of MUG: DNA binding. Starting with a pre-bound mixture of MUG and HEX labeled AP·G DNA, the enzyme was competed off with increasing concentrations of unlabeled AP·G. The stoichiometry was examined by plotting the anisotropy versus the stoichiometric balance, calculated from the stoichiometry of MUG:([HAP·G]) minus the stoichiometric equivalents of competitor DNA added ([AP·G]/[HAP·G]). Plotting the data in this way clearly demonstrated that the specific complex is only competed off once a MUG:DNA stoichiometry of 2:1 was obtained. The observed competition data are thus consistent with cooperative binding of MUG to the abasic DNA product with a 2:1 stoichiometry.

In order to investigate whether the higher order binding complexes of MUG with its abasic product DNA have an impact on the catalytic function of the enzyme, activity assays were performed. For this, different concentrations of DNA containing a U·G mismatch were incubated with increasing concentrations of MUG under standard reaction conditions. As the concentration of MUG increased, the concentration of substrate decreased in a linear fashion until complete cleavage was observed. Complete cleavage coincided with an enzyme concentration that is double the DNA concentration, which is consistent with the 2:1 binding stoichiometry observed and indicates that multiple protein binding is important for catalytic activity as well as abasic product binding.
An increase in ionic strength reduces binding cooperativity and glycosylase activity of MUG

We then investigated the effect of an increase in ionic strength in the binding cooperativity of MUG and its activity. An increase in salt concentration has been shown to have a negative effect on protein - protein association (Schreiber & Fersht 1996) and on the non-specific interactions between proteins and DNA (Record et al. 1976). MUG’s binding to abasic and non-specific DNA was evaluated at three different salt concentrations by looking at the change in anisotropy upon MUG binding. Increasing salt concentrations reduced cooperativity of MUG binding to non-specific DNA and abasic DNA and also reduced MUG’s affinity to both oligonucleotides. Tables 2-2 and 2-3 summarize cooperativity and equilibrium dissociation constants of MUG binding to both oligonucleotides.

Binding of MUG to nonspecific DNA was affected more dramatically than its binding to abasic DNA by an increase in ionic strength. 150mM NaCl increased equilibrium binding constants for non-specific DNA three fold whereas an increase to 150mM did not affect binding to abasic DNA significantly. The fact that MUG’s $K_d$ for abasic DNA stays the same but its cooperativity changes as ionic strength of the buffer increases from 50mM to 150mM suggests that salt mainly affects protein-protein interactions.
### Table 2

<table>
<thead>
<tr>
<th>NaCl Concentration</th>
<th>Cooperativity</th>
<th>$K_d$ (nM)</th>
<th>$k_{cl}$ (s$^{-1}$)</th>
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<td>50mM</td>
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<tr>
<td>150mM</td>
<td>0.99 ± 0.1</td>
<td>94 (±4.4)</td>
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<tr>
<td>300mM</td>
<td>N.D.</td>
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Table 2- MUG’s binding to non-specific DNA is affected by salt

The table summarizes equilibrium dissociation constants and cooperativity of MUG binding to non-specific DNA at different salt concentrations.

<table>
<thead>
<tr>
<th>NaCl Concentration</th>
<th>Cooperativity</th>
<th>$K_d$ (nM)</th>
<th>$k_{cl}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50mM</td>
<td>3.1 ± 0.2</td>
<td>122 ± 3</td>
<td>0.062 ± 0.003</td>
</tr>
<tr>
<td>150mM</td>
<td>1.9 ± 0.1</td>
<td>143 ± 5</td>
<td>0.016 ± 0.002</td>
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<tr>
<td>300mM</td>
<td>1</td>
<td>1385 ± 81</td>
<td>0.0004±0.0001</td>
</tr>
</tbody>
</table>

Table 2- MUG’s binding to abasic DNA and its activity are affected by salt

The table summarizes equilibrium dissociation constants and cooperativity of MUG binding to abasic DNA at different salt concentrations. Rates for cleavage of the N-glycosidic bond at each salt concentration are also shown. Reduction in binding cooperativity is associated with reduced rate constants, which indicates a diminished glycosylase activity.
When MUG’s glycosylase activity was tested at these salt concentrations, the reduction of cooperativity correlated with a significant decrease in MUG’s activity. MUG binding at standard MUG binding buffer proceeded with a cooperativity index: 3.1 and a rate constant $k_{cl}: 0.062 \pm 0.003$ s$^{-1}$. An increase of the ionic strength to 150mM NaCl did not change MUG’s $K_d$ for DNA but only reduced its binding cooperativity to 1.9, which led to an almost four fold reduction in activity (Table 2-3). An increase of salt concentration to 300mM led to reduction of cooperativity to 1 coinciding with a 155 fold reduction in MUG’s cleavage rate. At this salt concentration, although MUG binding was saturated, only 20% glycosylase activity was detected (Figure 2-13; Table 2-3).

Even though we cannot rule out the possibility that salt may be affecting MUG’s activity due for a different reason, the fact that saturation of binding does not correspond with the enzyme’s glycosylase activity suggests a requirement for cooperativity in the cleavage of the N-glycosidic bond.
Chapter 3

Characterization of MUG & DNA complexes
Chapter 3: Characterization of MUG & DNA complexes

Aim

We aimed to further investigate MUG & DNA complexes using an electrophoretic mobility shift assay. MUG glycosylase was also analyzed via analytical ultracentrifugation to gain insight into whether the protein is present as a monomer or an oligomer in the absence of DNA.

Background

As described in the previous chapter, crystal structures of MUG glycosylase revealed that MUG bound DNA as a dimer (Barrett et al. 1999). Although the authors assumed that the dimer is not required for the functionality of the enzyme, it was interesting that TDG, the human homologue of MUG, was also found to bind DNA in a 2:1 stoichiometry (Maiti et al. 2008). Fluorescence anisotropy measurements described in the previous chapter revealed that MUG glycosylase binds abasic as well as non-specific DNA with positive cooperativity. In addition, competition titration experiments suggested a 2:1 binding stoichiometry for the MUG: DNA complex. In support of the binding stoichiometry, a minimum MUG: DNA ratio of 2:1 is required for maximum glycosylase activity. Increase in ionic strength led to a reduction in cooperativity, which resulted in diminished activity. This suggests a requirement for cooperative binding for maximum glycosylase activity. In this chapter, MUG & DNA complexes were further investigated using an electrophoretic mobility shift assay. MUG glycosylase was also analyzed via analytical ultracentrifugation to gain insight into whether the protein is present as a monomer or as an oligomer in the absence of DNA.
The electrophoretic mobility shift assay (EMSA) is a common affinity electrophoresis technique used to study protein & DNA interactions. A particular strength of this technique is its ability to resolve protein-DNA complexes of differing stoichiometries. It is based on the differential migration velocities of protein & DNA complexes relative to the more rapidly migrating unbound DNA during electrophoresis in non-denaturing polyacrylamide gel. EMSA has been used to measure the dissociation constants \( (K_d) \) for proteins to an oligonucleotide of interest (Winston et al. 1999; Pope & David 2005); to determine effects of inhibitors of protein & DNA complexes (Park et al. 2004) and also to analyze affinities and determine dissociation constants \( (K_d) \) of glycosylases such as 3-methyladenine DNA glycosylase (AAG) (Lee et al. 2009), mMYH; the murine MutY homologue (Pope & David 2005) and MutY (Chepanoske et al. 1999).

In this chapter, optimal conditions for EMSA were established and EMSA was employed to further investigate MUG & DNA complexes.

**Objectives**

- Work out the optimal conditions of the electrophoretic mobility shift assay to investigate the mode of MUG binding to DNA
- Investigate the oligomeric complexes MUG forms with DNA
Optimization of the electrophoretic mobility shift assay to analyze MUG & DNA complexes

The electrophoretic mobility shift assay (EMSA) was performed by incubating H12AP∙G with increasing concentrations of MUG glycosylase (0 →2000nM). The complexes were incubated at 25°C for 30 minutes for the binding to reach equilibrium. MUG & DNA complexes were loaded and ran on a non-denaturing 8% polyacrylamide gel. A slight band shift was observed (Figure 3-1).

![Figure 3-1](image)

Figure 3-1 MUG & DNA complexes were analyzed via electrophoretic mobility shift assay (EMSA)

Increasing concentrations of MUG were incubated with 100nM H12AP-G in standard MUG binding buffer at 25°C for 30 minutes. MUG&DNA complexes were analyzed by running a native polyacrylamide gel followed by visualization of the bands using a phosphoimager. The fastest running band is free DNA (*). A band shift was observed with increasing MUG concentrations. The smeary nature of the bands indicates that MUG is dissociating from the DNA as it migrates.

The smeary nature of the bands indicates dissociation of MUG as it migrates with the DNA on the gel. In order to preserve the complexes, we took advantage of the method that uses osmotic stress to ‘freeze’ mixtures of protein & DNA complexes. The method is based on the observation that neutral osmolytes such as betaine glycine can strongly slow
down the rate of protein & DNA complex dissociation (Sidorova et al. 2005). To determine the optimal betaine glycine concentration to preserve MUG & DNA complexes, the osmolyte was added to the MUG & DNA complex up to different final concentrations (0.1M →5M) before analyzing the complexes via native polyacrylamide electrophoresis. For this assay, 100nM H12AP-G was incubated with 400nM MUG. The first lane in each subset shows free DNA (*), the second lane of each subset has 400nM MUG (M). Free DNA band has higher faint bands which coincide with bands in the protein lanes. This may be due to carry over of samples in the wells or alternative DNA structures from annealing or due to contaminations in the oligonucleotide. As they are faint, the major free DNA band can be recognized easily. 1M betaine glycine was chosen as the optimal concentration since it gave a good separation of unbound DNA from higher bands (Figure 3-2).

Figure 3-2 Betaine glycine freezes MUG & DNA complexes

100nM H12AP-G and 400nM MUG were incubated at 25°C for 30 minutes. Increasing concentrations of betaine glycine was added to MUG & DNA complexes (0.1M →5M). Samples were loaded on an 8% non-denaturing polyacrylamide gel and run for 210 minutes at 40V. The first lane in each subset shows free DNA (*), the second lane of each subset has 100nM DNA with 400nM MUG (M).
Oligomerizing MUG glycosylase molecules retard the electrophoretic mobility of specific and non-specific DNA

Once the optimal betaine glycine concentration was determined as described above, binding of MUG glycosylase to its product as well as to non-specific DNA was investigated by EMSA in order to further characterize individual MUG & DNA complexes.

HEX labeled DNA with an abasic site (H12AP∙G) (Figure 3-3A) and H12C∙G (Figure 3-3B) were incubated for 30 minutes with increasing concentrations of MUG (100nM →2000nM) in 25°C in standard MUG reaction buffer, 1M glycine betaine was added to each and the samples were then subjected to electrophoresis on a 8% non-denaturing polyacrylamide gel. The fastest migrating bands with both oligonucleotides, H12AP-G (Figure 3-3A) and H12C-G (Figure 3-3B), which are marked with “1a” and “1b” represent free DNA. When MUG was added to H12AP-G, band 2a was observed. Starting at a MUG: DNA ratio of 4:1 a higher band (3a) became predominant. At each MUG concentration, additional weaker bands that are likely to be due to non-specific binding were visible (*) (Figure 3-3A).

When MUG was bound to H12C-G, multiple non-specific bands were visible (Figure 3-3B). Both complexes, MUG & H12AP-G and MUG & H12C-G were run at the same voltage for the same amount of time, but as can be seen from the unbound DNA band in both EMSA experiments (bands 1a and 1b), abasic DNA and non-specific DNA ran with different speeds, therefore it was not possible to compare the higher bands between gels based on their migration distance. Comparison of MUG binding to abasic and non-specific DNA revealed that the affinity of the enzyme to abasic DNA is significantly higher as can be seen from the disappearance of the free DNA with abasic DNA.
Figure 3- 3 MUG glycosylase binds abasic and non-specific DNA with different affinities

Increasing concentrations of MUG were incubated with 100nM H12AP∙G (A) and with H12G∙C (B) in standard MUG binding buffer at 25°C for 30 minutes. MUG & DNA complexes were analyzed by running an 8 % native polyacrylamide gel followed by visualization of the bands using a phosphoimager. The fastest running bands, which were free DNA in both cases (bands 1a and 1b), were quantified using the Phoretix 1D software and the data was fit to a Hill equation, which is used to determine the degree of cooperativity in binding. MUG has higher binding affinity to DNA with an abasic site ($K_d= 95 \pm 2$ nM) (C) compared to non-specific DNA ($K_d=624 \pm 132.8$ nM) (D) and binds both DNA’s cooperatively. Cooperativity index for abasic DNA was 2.2 (±0.1) whereas cooperativity index for non-specific binding was 1.5 (±0.3).
In order to analyze MUG’s affinity for abasic and non-specific DNA, the unbound DNA bands (Band 1a and 1b) were quantified using the Phoretix 1D software. When fitted to a hyperbolic binding curve, a systematic deviation in the H12AP·G and H12C·G binding data was observed indicating that a single site binding did not describe the equilibrium (not shown). Instead, the data is shown with the best fit to the Hill equation, which describes cooperative binding of multiple ligands (Materials and Methods). Our EMSA results indicate that MUG has higher binding affinity to DNA with an abasic site ($K_d = 95 \pm 2nM$) (Figure 3-3C) compared to non-specific DNA ($K_d = 624 \pm 132.8nM$) (Figure 3-3D) and that the enzyme binds both oligonucleotides cooperatively. Cooperativity index for abasic DNA was 2.2 (±0.1) whereas cooperativity index for non-specific binding was 1.5 (±0.3).

EMSA experiments were also performed to investigate binding of MUG glycosylase to abasic and non-specific DNA was also performed using 200nM and 400nM H12AP·G and H12C·G and the same bands were observed as with 100nM DNA (data not shown).

**Competition experiments suggest that the second band (band 2a) is due to specific binding**

Competition experiments provided us with more information about the binding characteristics of MUG glycosylase. For this, complexes of 100nM 12HAP·G DNA were formed with increasing concentrations of MUG (100nM →2000nM). After incubation for 30 minutes at 25°C for equilibrium binding, 1M Betaine glycine was added and MUG & DNA complexes were run on an 8% non-denaturing polyacrylamide gel (Figure 3-4A). Bands 1a, 2a and 3a were observed in addition to weaker bands (*), which are likely to be due to non-specific binding. In order to gain more insight into the characteristics of MUG & DNA complexes, 5μM unlabeled non-specific DNA (12C·G) (Figure 3-4B) or 5μM unlabeled abasic DNA (12AP·G) (Figure 3-4C) were added.
before analyzing the complexes on a native polyacrylamide gel. Addition of excess unlabeled non-specific DNA led to formation of higher MUG-DNA complexes (*), which migrated even higher than the original complexes. These are likely to be non-specific aggregates of MUG & DNA (Figure 3-4B). Competition with 5μM unlabeled abasic DNA (12AP·G) led to elimination of most bands except band 2a, which suggests that this is the most stable MUG & DNA complex and is formed due to specific binding of MUG to abasic DNA (Figure 3-4C).

Figure 3-4 Non-specific DNA leads to formation of higher MUG & DNA aggregates, the second band is due to MUG binding DNA specifically.

Increasing concentrations of MUG were incubated with 100nM H12AP·G (A) 5μM unlabeled, 12C·G (B) or 5μM unlabeled, 12AP·G were added (C) to the complex before analyzing the complexes on a native polyacrylamide gel.
Longer DNA leads to the formation of additional MUG & DNA complexes

The knowledge that MUG binds DNA non-specifically led us to question if a different binding profile would be observed with a shorter oligonucleotide. We therefore compared binding of MUG to a HEX labeled oligonucleotide that has 6 base pairs 5’ to the abasic site (H6AP·G) (Figure 3-5B) along with H12AP·G, which has 12 base pairs 5’ to the basic site (Figure 3-5A). MUG formed two major complexes with 6AP·G and 12AP·G (1→3). With 12AP·G, additional non-specific, weaker complexes (*) were observed (Figure 3-5A).

Figure 3- 5 Shorter DNA leads to the formation of fewer MUG & DNA complexes

In order to determine the influence of the oligonucleotide size on the MUG & DNA complexes formed, MUG was complexed with H6AP·G (B) and H12AP·G (A) and the complexes were studied by running a 8% non-denaturing polyacrylamide gel. In both cases, bands 1, 2 and 3 were observed. MUG formed additional non-specific, weaker complexes (*) with H12AP·G.
In order to calculate $K_d$ and cooperativity from the EMSA experiment results more accurately, EMSA was performed by mixing lower concentrations of MUG with the H12AP·G oligonucleotide (Figure 3-6A). Binding of MUG to H12AP·G leads to formation of two predominant complexes (bands 2 and 3) that run slower than free DNA (band 1). The amount of free DNA as a function of increasing concentration of MUG glycosylase was analyzed using Grafit 5.0.1 software. The data did not fit well to a one site binding model and is shown with the best fit to a Hill equation describing cooperative binding (Figure 3-6B). Fitting the data to a Hill equation gave a $K_d$ value = 96.5 ± 2.9nM and a cooperativity index: 1.6 ±0.1. The weaker bands which are indicated with (*) are likely to be due to non-specific binding since they are not observed with the shorter oligonucleotide (H6AP·G) (Figure 3-5B and 3-7) and are easily eliminated in the competition experiment (Figure 3-4C).
In order to calculate $K_d$ and cooperativity for binding to abasic DNA from the EMSA experiment results more accurately, EMSA was performed by mixing lower concentrations of MUG with 100nM H12AP∙G oligonucleotide. The amount of free DNA as a function of increasing concentration of MUG glycosylase was analyzed using Grafit 5.0.1 software. Fitting the data to a Hill equation gave a $K_d$ value = $96.5 \pm 2.9$ nM and a cooperativity index $n = 1.6 \pm 0.1$.

In order to calculate the $K_d$ and cooperativity of MUG binding to the H6AP∙G oligonucleotide, EMSA was performed with a wide range of MUG concentrations (Figure 3-7A). Binding of MUG to H6AP∙G leads to formation of two predominant complexes (bands 2 and 3) that run slower than free DNA (band 1). Free DNA was quantified using Phoretix 1D software and plotted against MUG concentration. The data
did not fit a one-site binding model and is shown with the best fit to a Hill equation (Figure 3-7B). $K_d$ was determined as $81.2 \pm 2nM$ and cooperativity index was $2.0 \pm 0.1$.

![Figure 3-7 Equilibrium dissociation constant and cooperativity index of MUG binding shorter abasic DNA (H6AP•G)](image)

In order to calculate $K_d$ and cooperativity for binding to the shorter abasic DNA (H6AP•G), EMSA was performed by mixing lower concentrations of MUG with 100nM H6AP•G. The amount of free DNA as a function of increasing concentration of MUG glycosylase was analyzed using Grafit 5.0.1 software. Fitting the data to a Hill equation gave a $K_d$ value = $81.2 \pm 2nM$ and a cooperativity index: $2.0 \pm 0.1$. 
In order to analyze the effect of oligonucleotide size on the number of non-specific complexes MUG forms with DNA, MUG binding to H6C·G was analyzed via EMSA as well (Figure 3-8). MUG was found to form three complexes (→), which were significantly weaker in intensity compared to complexes formed with H12C·G. MUG was found to form four complexes that were stronger in intensity with H12C·G (Figure 3-3B), which demonstrates the number of non-specific MUG & DNA complexes is dependent on the oligonucleotide size (Figure 3-8).

![Figure 3- 8 Non-specific binding by MUG glycosylase depends on the oligonucleotide size](image)

In order to investigate the influence of the oligonucleotide size on the non-specific binding by MUG, the enzyme was complexed with H6C·G and complexes were studied by running on an 8% non-denaturing polyacrylamide gel. Three MUG & DNA complexes were observed when increasing amounts of MUG were added to 100nM H6C·G. Fewer bands were observed with H6C·G compared to H12C·G (Figure 3-3B). The intensity of these bands were significantly weaker compared to complexes formed with H12C·G, which demonstrates that non-specific binding by MUG is dependent on the oligonucleotide size.
Sedimentation equilibrium to analyze self-association of MUG in the absence of DNA

Analytical ultracentrifuge sedimentation equilibrium measurements were used to determine the apparent $M_r$ of the MUG species in free solution. This is a powerful technique for characterizing the solution-state behavior of macromolecules and has been successfully used to study oligomerization of proteins (Weinberg et al. 2004; Rajakulendran et al. 2009). The analytical ultracentrifuge is used to determine the equilibrium distribution of solutes, which enables measurement of self-association strengths and stoichiometries. The transport of the sample down the cell is balanced by the desire of the sample to diffuse against the concentration gradient created by the gravitational field. After a certain time, these two forces will balance out and an equilibrium distribution will be obtained. These measurements and analysis were conducted by Dr. Jacqui Marshall in Professor Halford’s group at University of Bristol.

To determine the molecular weight and self-association of MUG in solution, sedimentation equilibrium assay was performed using a Beckman XL-A instrument. The analysis was performed in MUG reaction buffer containing three different NaCl concentrations. The reaction buffers contained 50 mM Tris-HCl, pH 8.0, 1 mM EDTA and 50mM, 150mM or 300mM NaCl concentrations with three different MUG concentrations (33, 17, 8 μM). Optical measurements, or scans, of the radial distribution of the protein were recorded at 280nm. The scans were taken at different time increments and increasing rotor speeds:

- Scan 1 at 3000 rpm for 30 minutes;
- Scan 2 - 3 at 15 000 rpm at 20 and 24 hrs, respectively;
- Scan 4 - 5 at 20 000 rpm at 20 and 24 hrs, respectively;
- Scan 6 - 7 at 25 000 rpm at 20 and 24 hrs, respectively;
- Scan 8 - 9 at 40 000 rpm at 6 and 17 hrs, respectively.
The data was fit globally with using 9 datasets: 3 protein concentrations (33, 17, and 8 μM) at 3 speeds (15 000, 20 000 and 25 000 rpm) all at the 24 hour collection point. Figure 3-9 shows a representative example for a MUG dataset (16.7 μM) at (A) 50mM (B) 150mM and (C) 300mM NaCl. The lower panel (radius versus absorbance) shows blue dots which are the data at 20,000 rpm after 24 hours. The red line represents the fit to the whole group of data (all 3 concentrations of MUG at three speeds= 9 datasets). The upper panel shows the residuals, i.e. The difference between the data points obtained at 16.7 μM, 20 000 rpm and the fit to the larger group of datasets. At all concentrations, 9 datasets at each salt concentration (50mM, 150mM and 300mM NaCl) were globally fitted by non-linear least squares to a single value for molecular weight (M_r). M_r obtained was 18.7 kDa, which corresponds to MUG being present in a monomeric form.
Figure 3-9 Sedimentation equilibrium of MUG in free solution

A dataset from the global fit using SEDNTERP. The main panels A, B and C show the absorbance at 280nm as a function of the centrifugal radius, following centrifugation of MUG at 20°C of a sample of MUG (16.7μM) in MUG reaction buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA and (A) 50mM NaCl (B) 150mM NaCl and (C) 300mM NaCl. The sample was centrifuged for 24 hours at 20000rpm. The best fit to the model for a single species is shown as a red line through the experimental data points. The upper panels show the deviation, or residuals, between the data and the fit.
Discussion

In this chapter, electrophoretic mobility shift assay (EMSA) was used to investigate MUG & DNA complexes. In the initial EMSA experiments, smeary bands had been observed, which indicated that MUG dissociates from the DNA during electrophoresis. We therefore took advantage of a method that uses osmotic stress to ‘freeze’ mixtures of protein & DNA complexes (Sidorova et al. 2005). The method is based on the observation that neutral osmolytes such as betaine glycine can strongly slow down the rate of protein & DNA dissociation. Addition of betaine glycine significantly improved EMSA by allowing us to differentiate higher bands from unbound DNA. The ideal concentration for the analysis of MUG & DNA complexes was determined to be 1M betaine glycine and this concentration was used in all EMSA experiments.

Although EMSA is very valuable for the analysis of protein & DNA complexes, there is speculation concerning the “equilibrium nature” of the method. When interpreting band shifts with EMSA, one should take into consideration that protein & DNA complexes of interest may be experiencing dissociation. Fluorescence anisotropy allows solution based analysis of proteins binding to DNA and therefore monitors equilibrium conditions. The EMSA analysis reported here reveals $K_d$ values for MUG binding abasic DNA similar to the $K_d$ values observed with fluorescence anisotropy measurements (Table 3-1). Equilibrium dissociation constants obtained by EMSA are 1.5- 2 fold tighter than values acquired through anisotropy measurements whereas cooperativity indexes obtained by EMSA are 1.5- 2 fold lower than values acquired through anisotropy measurements. The fact that the difference is consistent for both oligonucleotides (H6AP·G and H12AP·G) suggests that the slight discrepancy may be due to experimental differences. Gel mobility shift assays are known to be sensitive to buffer-dependent artifacts. The fact that EMSA gels were run in TBE buffer (pH 8.3), which has a pH close to MUG reaction buffer (pH 8.0) also provides favorable conditions for MUG & DNA complexes.
<table>
<thead>
<tr>
<th>Method</th>
<th>Oligonucleotide</th>
<th>$K_d$(nM)</th>
<th>Cooperativity</th>
</tr>
</thead>
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<tr>
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<td>125.1 ± 1.1</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td>EMSA</td>
<td>6AP·G</td>
<td>81.2 ± 2</td>
<td>2.0 ±0.1</td>
</tr>
<tr>
<td>Fluorescence Anisotropy</td>
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<td>186.5 ± 3.0</td>
<td>3.4 ± 0.2</td>
</tr>
<tr>
<td>EMSA</td>
<td>12AP·G</td>
<td>96.5 ± 2.9</td>
<td>1.6 ±0.1</td>
</tr>
</tbody>
</table>

Table 3-1: Comparison of $K_d$ and cooperativity values from anisotropy measurements and EMSA

The advantage of fluorescence anisotropy measurements is that they are solution based but they cannot differentiate between multiple MUG & DNA complexes. Analysis of MUG & DNA complexes through EMSA has been beneficial in providing further information about the complexes formed by MUG glycosylase. MUG glycosylase formed two prominent bands with H6AP·G and H12AP·G, which are oligonucleotides bearing an abasic site opposite a guanine residue. Their only difference is the number of basepairs 5’ to the abasic site; H12AP·G has 12 basepairs 5’ to the abasic site hence is slightly longer than H6AP·G, which has 6 basepairs 5’ to the abasic site. In addition to the two strong bands, MUG forms additional weaker complexes with H12AP·G. The fact that these bands were not observed with H6AP·G and are significantly fainter than the two major bands suggests that they are non-specific as the only difference between these two oligonucleotides is the number of non-specific basepairs. Analysis of MUG binding to H6C·G and H12C·G also revealed that the enzyme forms more complexes with the longer oligonucleotide. Binding to H6C·G was significantly weaker than to H12C·G, which can be seen through the fact, that most of H6C·G was free when run on the native gel. These results support the notion that non-specific binding is affected by the number of oligonucleotides available. H12AP·G and H12C·G have been used in fluorescence anisotropy experiments unless depicted otherwise.
In competition experiments, MUG & DNA complexes were formed and MUG was competed off with an unlabeled abasic or with a non-specific oligonucleotide. When unlabeled non-specific DNA was added in excess, higher complexes were formed, which are likely to be due to non-specific protein & DNA aggregates. Addition of unlabeled abasic DNA in excess led to elimination of most bands except the second one, which suggests that the second band is the most stable MUG & DNA complex and is formed due to specific binding of MUG to abasic DNA.

In order to gain further insight into whether MUG is monomeric or oligomeric in solution in the absence of DNA, analytical centrifugation sedimentation equilibrium analysis method was utilized. This is a powerful technique for characterizing the solution-state behavior of macromolecules. The analysis was performed at 50mM, 150mM and 300mM NaCl concentrations with three different MUG concentrations (33, 17, 8 μM). The datasets for each salt concentration were globally fitted by non-linear least squares to a single value for molecular weight ($M_r$) separately. At 50mM, 150mM and 300mM a molecular weight of 18.7 kDa was obtained, which corresponds to MUG being present in a monomeric form in solution in the absence of DNA.
Chapter 4

FRET to understand enzyme communication in base excision repair
Chapter 4:

FRET to understand enzyme communication in base excision repair

Aim

To develop a fluorescence resonance energy transfer (FRET) assay for monitoring interactions of enzymes in base excision repair. FRET will be used to monitor association of MUG glycosylase with DNA and its dissociation from the DNA. MUG will be labeled with a donor fluorophore, and DNA containing a lesion of interest will be labeled with an acceptor fluorophore. Change in energy transfer (ΔE) will be monitored as MUG binds DNA containing a lesion. The question of whether Exonuclease III affects MUG’s dissociation and if it is through an active or a passive mechanism will be addressed by mixing Exonuclease III with the MUG & DNA complex in equilibrium. If Exonuclease III leads to MUG dissociating from the DNA, a decrease in ΔE will be observed and its rate can be quantified. The dissociation rate due to Exonuclease III will be compared with the passive diffusion rate in order to address whether Exonuclease III displaces MUG through an active or a passive mechanism.

Background

Base excision repair involves several proteins acting on the site of damage and it is important that the order of their activity is precisely coordinated. Once a mismatched base is removed and an abasic site is created, it is important for the next enzyme in the pathway to step in and cleave the abasic site so that the correct base is incorporated by DNA polymerase, followed by DNA ligase sealing the nick. If the resulting AP site is
improperly repaired, removal of a base by DNA glycosylases is potentially mutagenic or lethal for the cell (Kavli et al. 1996; Otterlei et al. 2000; Sobol et al. 2003; Boiteux & Guillet 2004). In order to protect the cell from the potentially cytotoxic and mutagenic effects of BER, proteins in BER are thought to interact with each other to temporally order and guide the parade of proteins that ‘trade places’ on the DNA, a model known as ‘protein hand-off’.

Interactions between various enzymes in base excision repair have been observed before. The yeast-two-hybrid system has revealed an interaction between the human Polβ and APE1, which was also confirmed by an electrophoretic mobility shift assay (Bennett et al. 1997). Another eukaryotic BER protein, which was found to interact with Polβ was DNA ligase. In a study where crude nuclear extracts from bovine testis were applied to affinity columns with antibody against Polβ, a complex containing DNA ligase I co-eluded with Polβ. The interaction was also confirmed when DNA ligase I and Polβ were co-immunoprecipitated from the testis nuclear extracts (Prasad et al. 1996). Protein-protein interactions between AP endonucleases and DNA glycosylases have been shown by some studies. Direct association between recombinant TDG and Human AP Endonuclease (APE1) was demonstrated using a GST interaction assay (Tini et al. 2002). APE1 was found to co-immunopurify with hMYH, the human homologue of MutY from HeLa nuclear extracts (Parker et al. 2001). MutY, the E. coli homologue of hMYH, was also found to interact with ExoIII as observed by an electrophoretic mobility shift assay (Pope et al. 2002).

Product inhibition of DNA glycosylases also brings up the question whether a protein hand-off mechanism exists in base excision repair (O'Neill et al. 2003; Fitzgerald & Drohat 2008). To find out if AP endonucleases stimulate dissociation of glycosylases from the abasic site, a number of studies have investigated the effect of AP endonucleases on the turnover rate of DNA glycosylases. APE1 has been demonstrated to affect the turnover of SMUG (Kavli et al. 2002), TDG (Waters et al. 1999; Fitzgerald
& Drohat 2008) and hOGG1 (Hill et al. 2001). Endonuclease IV and Exonuclease III, the two main AP endonucleases in *E. coli*, were found to enhance the activity of MUG and MutY glycosylases (Hang *et al.* 2002; Pope *et al.* 2002; Guliaev *et al.* 2004).

AP Endonucleases were found to affect product release rather than the chemical step of the glycosylase reaction. The mechanism of coordination between glycosylases and AP endonuclease is largely unknown. Two basic ideas for the mechanism have emerged: a passive or an active mechanism. For the passive enhancement mechanism, the AP endonuclease stimulates glycosylase turnover by depleting the concentration of abasic DNA by cleaving it to a 5’ nicked AP-DNA, thereby preventing glycosylase from re-associating with the product and increasing the effective concentration of free glycosylase. If an active displacement mechanism exists, AP endonuclease would interact with the glycosylase and/or DNA adjacent to the AP-site to disrupt the complex.

If AP endonuclease stimulates glycosylase turnover by depleting the concentration of abasic DNA, thereby preventing glycosylase from re-associating with the product and increasing the effective concentration of free glycosylase, we would expect both AP endonucleases in *E. coli*; Exonuclease III and Endonuclease IV to equally stimulate DNA glycosylases of interest. A study by Pope *et al.* demonstrates that Endonuclease IV stimulates product formation by MutY to a higher extent than Exonuclease III on a G·A substrate (Pope *et al.* 2002). But the study also shows that neither AP endonuclease has an effect on product formation by MutY on a A·OG substrate which is the substrate to which MutY has a higher affinity for and is thought to be the particularly important biological substrate of this glycosylase (Li *et al.* 2000). Considering this, the question is whether the effect of these Endonucleases is biologically relevant.

The study by Pope *et al.* also suggests that EndoIV and ExoIII interact with MutY in different ways (Pope *et al.* 2002). ExoIII binds to the MutY & DNA complex to give a
super-shifted band in the gel retardation assay. In contrast, EndoIV results in
displacement of MutY from the DNA duplex and formation of an EndoIV & DNA
complex. This finding suggests a product & glycosylase specific interaction with the AP
endonucleases. The fact that APEI which is the human homolog of ExoIII does not
stimulate the turnover rate of MutY with a G•A substrate although ExoIII does also
supports that recognition of the AP site alone is not responsible for the enhanced
turnover of the glycosylase.

The finding that ExoIII and EndoIV affect the activity of an E. coli glycosylase to
different extents also came from a study with MUG glycosylase (Guliaev et al. 2004).
ExoIII was found to stimulate the removal of 3, N⁴-ethano-Cytosine by MUG to a bigger
extent compared to EndoIV.

Another finding that supports substrate dependent interaction between glycosylases and
AP endonucleases is the fact that the effect of APE1 on the turn-over rate of
glycosylases is dependent on the type of mismatch. For instance in the case of TDG;
enhancement of product formation by APE1 is seen with substrates to which the
enzymes have weaker affinity (Waters et al. 1999; Fitzgerald & Drohat 2008). In the
study by Waters et al., APE1 increased the rate of turnover of TDG with C•U DNA by
more than 150-fold so that the reaction was complete in 15 minutes. The same
APE1:TDG ratio led to a less prominent increase on Thymine removal from a G•T
mismatch (Waters et al. 1999). Since TDG was found to dissociate more rapidly from a
C•AP site than a G•AP site, this suggests that APE1’s effect on the glycosylase is
dependent on the affinity of the glycosylase to the mismatch (Waters et al. 1999).
Another study by Fitzgerald et al. found that $k_{cat}$ of TDG for 5-fluorouracil paired with
guanine (G•FU) is much faster than for G•U substrates (indicating faster $k_{off}$ for the
G•FU reaction) and the concentration of APE1 required for maximum enhancement is
lower for G•FU versus G•U (Fitzgerald & Drohat 2008).
Analysis of turn-over rates of truncated glycosylases supports the presence of protein-protein interactions between glycosylases and AP endonuclease. A MutY mutant, in which the C-terminus has been removed, shows the same biphasic behavior as full-length MutY in product formation under steady-state conditions, which suggests a similar mode of product inhibition like full-length MutY. When the activity of this truncated protein was tested in the presence of EndoIV, its activity was not enhanced by EndoIV, although EndoIV increased activity of full length MutY (Pope et al. 2002). Another experiment was performed where the effect of APE1 on the turn-over rates of TDG and truncated TDG, which lacks the N- and C-terminal domains but retains the catalytic domain (TDG\textsuperscript{cat}) was measured. TDG\textsuperscript{cat} has nearly the same catalytic activity as hTDG. APE1 was found to increase $k_{\text{cat}}$ of TDG\textsuperscript{cat} to a higher extent compared to $k_{\text{cat}}$ of TDG, which suggests an active displacement mechanism involving interaction between these enzymes. This result cannot be due to TDG\textsuperscript{cat} falling off its product easier compared to TDG as TDG\textsuperscript{cat} has a turnover rate similar to TDG (Fitzgerald & Drohat 2008).

Although stimulation of the activity of glycosylases by AP endonuclease has been observed and is aligned with the protein hand-off theory in base excision repair, the high molar ratios of AP endonucleases needed to see an affect on glycosylase turnover in some studies is thought provoking. In order to see a twofold effect in thymine removal by TDG, hundred fold access of APE1 was needed (Waters et al. 1999). While the study by Waters et al. did not show a significant increase in thymine removal when APE1 and TDG were at equimolar amounts, a TDG: APE1 ratio of 1:1 was enough to induce a 26 fold enhancement in removal of uracil from G·U mismatches as determined by a coupled-enzyme fluorescence assay by Fitzgerald et al. (Fitzgerald & Drohat 2008). Other glycosylases, which were found to be stimulated by APE1 are SMUG and OGG. A molar ratio of 10:1 was needed to see an affect of APE1 on SMUG (Hill et al. 2001; Kavli et al. 2002; Pettersen et al. 2007). A molar ratio of 15:1 was needed to see a two fold increase in product formation by OGG1 (Hill et al. 2001).
A reason for the high APE1 concentrations needed in the in vitro experiments to increase the turnover rate of glycosylases might be the absence of in vivo modifications, which may be required for product release. Interaction of TDG with APE1 was shown to be inhibited by acetylation (Tini et al. 2002). Product bound hTDG was found to be sumoylated, which increases the dissociation rate of the enzyme from the abasic site (Hardeland et al. 2002). Interestingly, an equimolar amount of APE1 increased product formation of sumoylated TDG but did not affect non-sumoylated TDG (Hardeland et al. 2002). Lack of these modifications in vitro might make high ratios of AP endonucleases for some substrates necessary. Although the importance of these modifications for the turnover of TDG has only been demonstrated in eukaryotes, the presence and importance of modifications in bacterial BER enzymes cannot be ruled out.

The high AP endonuclease: glycosylase ratios required and the discrepancies between experiments call for a more direct and quantitative approach to look at AP endonuclease induced glycosylase turnover. We are interested in dissociation of mismatch DNA glycosylase (MUG) from the abasic site and whether Exonuclease III (ExoIII), the E. coli homologue of the human AP endonuclease has an effect on MUG dissociating from abasic DNA. In order to answer if Exonuclease III, which is the main AP endonuclease in E. coli, actively displaces MUG from the DNA, we developed and used a fluorescence resonance energy transfer (FRET) assay. FRET has previously been widely used to investigate nucleic acid hybridization (Cardullo et al. 1988), polymerase-substrate interactions (Allen & Benkovic 1989) and to monitor real time kinetics of restriction endonuclease cleavage (Ghosh et al. 1994).

FRET is a powerful tool for looking at real time dynamics and interactions of biomolecules. It is based on the transfer of electronic excitation energy from a fluorescent donor (D) chromophore to an unexcited acceptor chromophore (A) (Figure 4-1). Thus, there is a concerted quenching of donor and activation of acceptor fluorescence. Energy transfer occurs without the appearance of a photon and is the result
of long-range dipole-dipole interactions between the donor and acceptor. The term resonance energy transfer is used because the process does not involve the appearance of a photon.

The rate of energy transfer is highly dependent on many factors, such as the extent of spectral overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor, the quantum yield of the donor, the relative orientation of the donor and acceptor transition dipoles, and, most importantly, the distance between the donor and acceptor molecules. The emission spectra of donor molecule and the absorbance spectra of the acceptor molecule have to overlap and these molecules have to be within angstrom (Å) distances to each other (10-100 Å). FRET is highly efficient if donor and acceptor are positioned within Förster radius (the distance at which half the excitation energy of the donor is transferred to the acceptor, typically 3-6nm) (Sekar & Periasamy 2003) (Figure 4-1).
Figure 4-1 FRET Process

Upon excitation, the excited-state donor molecule transfers energy non-radiatively to a proximal acceptor molecule located at distance \( r \) from the donor. The acceptor releases the energy either through fluorescence or through non-radiative channels. The spectrum shows the absorption (Abs) and emission (Em) profiles of one of the most commonly used FRET pairs: Fluorescein (donor) and rhodamine (acceptor). Fluorescein can be efficiently excited at 480 nm and emits at around 520 nm. The spectral overlap between fluorescein emission and rhodamine absorption, as defined by \( J(\lambda) \), is observed at 500–600 nm. The Förster distance \( R_0 \) for this pair is 55 Å. Thus, in an optimal configuration (\( r < 55 \) Å), excitation of fluorescein at under 500 nm can result in significant FRET emission of the rhodamine at above 600 nm. \( A= \)normalized absorption, \( I_F= \)normalized fluorescence. (Figure taken from Kim E. Sapsford et al. (Sapsford et al. 2006)).

In order to monitor FRET, MUG (donor) and DNA containing a mismatch of interest (acceptor) will be labeled with two different fluorophores that have overlapping emission and absorbance spectra. When MUG binds the lesion, donor and acceptor come in close proximity. Fluorescence energy is transferred from the donor fluorophore to the acceptor fluorophore upon excitation with light at the appropriate wavelength. Using FRET will also enable us to monitor dissociation of MUG from the DNA since a decrease in energy transfer (\( \Delta E \)) will be observed when MUG dissociates from the DNA (Figure 4-2).
FRET is used to follow MUG binding the DNA and its dissociation from the DNA

DNA containing a lesion, e.g., an abasic site (AP) opposite guanine, is labeled with a fluorophore ■ (HEX) (A). MUG glycosylase is labeled with a fluorescence molecule. When MUG binds the abasic site (AP), MUG’s fluorophore comes in close proximity with the fluorophore on the DNA. Energy is transferred from donor fluorophore to acceptor fluorophore (B). AP endonuclease is the next enzyme in base excision repair. When AP endonuclease binds the DNA, there are two scenarios: AP endonuclease and MUG bind DNA simultaneously, MUG dissociates after a delay time (passive mechanism) (D). Or AP endonuclease displaces MUG from the DNA (active mechanism) (E). If they bind simultaneously, we would continue to see fluorescence resonance energy transfer after adding AP endonuclease. If MUG dissociates when AP endonuclease binds, FRET will cease since donor (MUG’s fluorophore) and acceptor (DNA’s fluorophore) are not in close proximity.
Stopped Flow System

The stopped flow instrument is a rapid mixing device used to study the kinetics of a reaction in solution. Syringes are filled with solutions to be mixed; reactant A in syringe A is rapidly mixed with reactant B in syringe B and enters a spectrophotometer cell. Once the flow is stopped, change in fluorescence is monitored (Figure 4-3). The double push mode is optional and enables mixing of further reactants (reactant C and D) with reactant A and B right away or after a delay time. FRET and anisotropy measurements in the following experiments were performed using a stopped flow instrument from TGK Scientific (formerly Hi-Tech Ltd) equipped with a fluorescence polarization accessory. Stopped-flow anisotropy measurements were performed using a T-format with excitation and emission polarizers.
The stopped-flow instrument can mix solutions in a couple of milliseconds and measure the dynamics of the occurring reaction. The instrument can be utilized in a single push mode to mix reactant A and B using the A and B drive syringes. Alternatively the double push mode can be used to mix reactants C and D with reactants A and B.
Objectives

- Optimize labeling of MUG with a donor fluorophore.

- Optimize FRET conditions between MUG and the DNA to analyze association and dissociation rates.

- Use FRET to answer the biological question whether Exonuclease III actively displaces MUG from the DNA.

Labeling MUG and the DNA

Initially, Fluoresceine-5-maleimide (FAM) was picked as the FRET donor because of the overlap of its emission spectrum with the absorption spectrum of HEX. Absorption spectrum of HEX has a maximum at 535nm and emission spectrum of fluorescein-5-maleimide (FAM) has a maximum at 518nm. Exciting the MUG & DNA complex with light at 495nm leads to energy transfer from FAM to HEX when both fluorophores are in close proximity.

Maleimide labeling reagents such as Fluoresceine-5-maleimide are effective for labeling free sulfhydryl-containing molecules. Maleimide groups react predominantly with sulfhydys at pH: 6.5 – 7.5 forming a stable thioether bond. MUG has two cysteine residues (cysteine<sub>15</sub> and cysteine<sub>64</sub>), which are potential targets for FAM. Neither of the cysteine residues is close to the DNA interface of MUG. During repair of a mismatch, a “wedge” is formed by residues 141 – 146 which penetrates the body of the double helix and the space left by the flipped-out nucleotide becomes occupied by residues Ser-142,
Gly-143, Leu-144 and Ser-145 (Barrett et al. 1998). Neither cysteine15 nor cysteine64 is known to contribute to MUG’s interaction with the DNA (Barrett et al. 1998; Barrett et al. 1999). A disadvantage in having internal cysteines is the possibility of labeling interfering with the protein’s activity. Another disadvantage might arise if both cysteines are not labeled at the same efficiency or if labeling efficiency is inconsistent between experiments. In order to obtain homogenous labeling of MUG and to ensure that cysteine residues are labeled specifically, single cysteine → serine mutants of MUG (MUGC15S and MUGC64S) as well as the cysteine → serine double mutant (MUGC15SC64S) were constructed. MUGC15S and MUGC64S could be used in case FAM labeling of wild type MUG interferes with MUG’s activity. Alternatively, specific cysteine residues could be introduced into MUGC15SC64S for labeling. Each construct was expressed in BL21-DE3 E. coli cells and purified as described previously (O’Neill et al. 2003). The activity of the MUG cysteine mutants was significantly reduced compared to wild type MUG, therefore a protocol for labeling MUGWT with Fluoresceine-5-maleimide was optimized (data not shown). Even though the protocol to label MUG with FAM and FRET experiment were developed successfully, following experiments revealed inconsistencies between the efficiency of labeling. We also found the fluoresceine-5-maleimide signal to fade quickly when FRET was monitored over extended periods of time (data not shown). The inconsistency of fluoresceine-5-maleimide is likely to be due to the pH sensitivity of the fluorophore. Fluoresceine-5-maleimide is known to be less stable at pH values > 7.5. In order to choose a more stable fluorophore with a more consistent labeling, Alexa488 which is a more stable fluorophore used for amino-terminal labeling of proteins was tested (Figure 4-4).
Emission spectrum of Alexa488 has a maximum at 518nm and absorption spectrum of HEX has a maximum at 535nm as shown in Figure 4-5. Exciting the MUG & DNA complex with light at 495nm leads to energy transfer from Alexa488 to HEX when both fluorophores are in close proximity. HEX excitation can be measured by monitoring emitted fluorescence signal at 556nm (Figure 4-5).
Emission spectrum of Alexa488 overlaps with the excitation spectrum of HEX. When the complex is excited with light around 495nm, Alexa488 is excited and emits light that has a maximum around 518nm. HEX has an absorption maximum of 535nm, hence is excited with light emitted from Alexa488. HEX emits light with a maximum around 556nm. Detection of emitted light at 556nm enables us to monitor energy transfer from Alexa488 to HEX.

**Absorbance and fluorescence emission spectra of Alexa488 labeled protein**

**Absorbance and fluorescence emission spectra of HEX**

--- Excitation

--- Emission

* Taken from Invitrogen Spectra Viewer

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Figure 4-5 shows the structure of Alexa Fluorophore 488 succinimidyl ester (A) and its reaction scheme with primary amines on proteins (B). Succinimidyl esters are ideal for the conjugation of proteins because they form a very stable amide bond with primary amines (R-NH₂) in proteins. Amine reactive reagents react with non-protonated aliphatic amine groups, including the amino terminus of proteins and the ε-amino group of lysines. The ε-amino group has a pKₐ of around 10.5; in order to maintain this amine group in non-protonated form, the conjugation must take place at slightly basic pH. More specific labeling of the amino terminus can be achieved using a buffer closer to natural pH as the pKa of the terminal amine is lower than that of the lysine ε-amino group.
group (between pH 7.5 – 10). Alexa fluorophores are also known for their good photostability and pH insensitivity (active at pH 4–10).

![Succinimidyl Ester to Carboxylamide](image)

Succinimidyl Ester \[ \rightarrow \] Carboxylamide  

Figure 4-6 Reaction of Alexa Fluorophore 488 succinimidyl ester with primary amine of proteins  

AlexaFluorophore488 succinimidyl ester reacts with primary amine of proteins to give a carboxylamide.

**FRET between MUG-Alexa488 and HEX-DNA**

In order to monitor the binding and dissociation of MUG to and from DNA, MUG labeled with Alexa488 was used as the donor and H12AP-G was used as the acceptor. FRET upon addition of H12AP-G to MUG-Alexa488 was monitored by exciting the complex at 494nm and measuring emission at 518nm using the Fluoromax-3 spectrophotometer. Increasing amounts of H12AP-G were added to 200nM MUG-Alexa488 in MUG reaction buffer at 25°C and the complex was excited at 494nm. Alexa488 emission was monitored at 518nm. As energy transfer took place between Alexa488 and HEX, Alexa488 emission signal decreased. Decrease of Alexa488 emission was partially due to emission being quenched by the buffer added to the medium. As a control, the decrease in Alexa488 emission was also monitored upon addition of buffer to MUG- Alexa488 and emission signal was plotted against % dilution by added buffer (Figure 4-7B). Decrease in Alexa488 signal due to dilution best fits to a linear equation and was corrected for by subtracting from the Alexa488 signal in the
FRET experiment. Data was best fit to a single ligand binding equation using Grafit 5.0 (Erithacus software) and $K_d$ was determined as $148.8 \pm 12.7\text{nM}$ (Figure 4-7A). The observed $K_d$ value is consistent with values previously observed in our lab and with the $K_d$ value observed in the anisotropy experiments described in Chapter 2 and shown in Figure 2-4.

The change in energy transfer ($\Delta E$) was measured using the relative fluorescence intensity of the donor, by calculating the ratio of fluorescence at the end of the reaction (in the presence of the acceptor ($F_{DA}$)) and fluorescence in the beginning of the reaction (in the absence of acceptor ($F_D$)) (Eq. 4.1).

$$\Delta E = 1 - \frac{F_{DA}}{F_D} \quad \text{Eq. 4-1}$$

Change in energy transfer ($\Delta E$) was calculated and plotted against the DNA concentration, data was fitted to a single ligand binding equation (Materials and Methods Eq. 7.1) using Grafit 5.0 (Erithacus software) and $K_d$ was determined as $157 \pm 10.7\text{nM}$. $K_d$ determined from the Alexa488 emission curve ($148.8 \pm 12.7\text{nM}$) was similar to the $K_d$ determined from the $\Delta E$ curve ($157\pm10.7\text{nM}$) (Figure 4-7C).
Figure 4- 7 FRET between H12AP·G and MUG-Alexa488

(A) In order to monitor FRET between MUG-Alexa488 and H12AP·G upon binding, H12AP·G was added to 200nM MUG-Alexa488 at 25°C. The complex was excited with light at 494nm and MUG-Alexa488 emission signal (518nm) was measured using Fluoromax-3 and plotted against the DNA concentration (A). Background, which is the decrease in emission signal due to dilution, was determined by adding buffer to the reaction mix containing MUG only (B). In order to normalize for dilution, background was subtracted for each data point. Data is shown with the best fit to a single ligand binding equation using Grafit 5.0 (Erithacus software) and $K_d$ was determined as 148.8 +/- 12.7nM. (A) Change in energy transfer ($\Delta E$) was plotted against the DNA concentration, data was fitted to a single ligand binding equation using Grafit 5.0 (Erithacus software) and $K_d$ was determined as 157 +/- 10.7nM (C).

After observing FRET between Alexa488 and HEX upon MUG binding the abasic site, the activity of MUG-Alexa488 was tested in comparison with native MUG to ensure that labeling did not interfere with the enzyme activity. For this, 100nM H12U·G was reacted separately with 1µM MUG and with 1µM MUG-Alexa488. Product formation was followed over time by taking aliquots, quenching with NaOH and analyzing on
denaturing polyacrylamide gel. Labeling with Alexa488 did not interfere with the activity of MUG. Data is shown with the best fit to a single exponential with rate constants of MUG $k_{cl} = 0.06 \pm 0.003 \text{ s}^{-1}$; MUG-Alexa488 $k_{cl}=0.07 \pm 0.003 \text{ s}^{-1}$ (Figure 4-8).

![Graph showing product formation over time for MUG and MUG-Alexa488](image)

**Figure 4- 8 Labeling MUG with Alexa488 does not affect the activity of the enzyme**

Activity of MUG labeled with Alexa488 (MUG-Alexa488) was tested side by side with unlabeled MUG. 100nM H12U∙G was reacted with 1µM MUG and with 1µM MUG-Alexa488. Product formation was followed over time. Data is shown with the best fit to a single exponential with rate constants for MUG $k_{cl}= 0.06 \pm 0.003 \text{ s}^{-1}$ and for MUG-Alexa488 $k_{cl}= 0.07 \pm 0.003 \text{ s}^{-1}$.
Determining optimal MUG: DNA concentrations for FRET

In order to monitor the kinetics of FRET, energy transfer was analyzed between 200nM MUG-Alexa488 and 100nM H12AP-G using the stopped flow instrument. DNA and MUG were mixed followed by simultaneous measurement of the HEX and Alexa488 fluorescence. 21.2% change in HEX signal and 19.4% change in Alexa signal was observed upon mixing 100nM H12AP-G and 200nM MUG-Alexa488 (Figure 4-9). To monitor FRET, the complex was excited at 495nm; the emitted fluorescence was collected through a 520nm filter with 20nm band pass to follow donor emission and a 550nm cut-off filter to monitor acceptor emission.

Change in energy transfer (ΔE) between 100nM H12AP•G and 200nm MUG-Alexa488 was calculated using Equation 4-1 and plotted. The rate in ΔE was measured by fitting the data to a single exponential. ΔE proceeded with 275 ±1.5 *10^{-6} M^{-1}s^{-1} (Figure 4-
9B). 100nM H12AP-G DNA was used in following experiments as it gave sufficient signal to monitor FRET.

The $\Delta E$ between 200nM, 400nM and 800nM MUG-Alexa488 and 100nM H12AP-G was also evaluated. The $\Delta E$ between 100nm H12AP•G and 100nM MUG-Alexa488 was found to be the same as between 100nm H12AP•G and 200nM MUG-Alexa488. $\Delta E$ decreased when higher concentrations of MUG-Alexa488 were used. Data is shown with the best fit to a single exponential with rates of $259 \pm 5 \times 10^{-6}$; $145 \pm 3 \times 10^{-6}$; $72 \pm 1 \times 10^{-6}$ and $224 \pm 13 \times 10^{-6}$ M$^{-1}$s$^{-1}$ for 100nM, 200nM, 400nM and 800 nM MUG-Alexa488 respectively (Figure 4-10). The amplitude of $\Delta E$ between 800nM MUG-Alexa488 and DNA was significantly lower than $\Delta E$ with 100, 200 and 400nM MUG-Alexa488. This could be due to increased background fluorescence signal from the donor which does not necessarily participate in the reaction. In the following experiments a 100nM DNA and 200nM MUG was used.

![Figure 4-10 Increase in DNA: MUG ratio leads to a decrease in $\Delta E$](image)

$\Delta E$ was measured between 100nM H12AP•G and 100nM (■), 200nM (■), 400nM (■) and 800nM (■) MUG-Alexa488. Data is shown with the best fit to a single exponential with rates of $259 \pm 5 \times 10^{-6}$; $145 \pm 3 \times 10^{-6}$; $72 \pm 1 \times 10^{-6}$ and $224 \pm 13 \times 10^{-6}$ M$^{-1}$s$^{-1}$ for 100, 200, 400 and 800 nM MUG-Alexa488 respectively.
**Stopped flow fluorescence anisotropy**

Fluorescence anisotropy measurements with H12AP•G were made using the stopped flow instrument in T-format with polarizers. 1000nM MUG and 100nM H12AP•G were rapidly mixed and fluorescence from HEX was obtained by exciting at 546 nm and collecting the emission on both channels through a WG540 cut-off filter (Comar) with emission polarisers at horizontal and vertical. The data from each channel was collected and mathematically converted to anisotropy by the KinetAssyst 3 software (TGK Scientific) (Figure 4-11). Anisotropy was plotted against time. The data is shown with the best fit to a single exponential with a rate \( k = 370 \pm 4.7 \times 10^6 \text{ M}^{-1}\text{s}^{-1} \).

**Figure 4-11** Fluorescence anisotropy was measured using the stopped flow instrument.

Binding of MUG to DNA was monitored by mixing 100nM H12AP•G with 1000nM MUG using the stopped flow instrument. Anisotropy was measured by exciting at 546nm and observing emitted light in perpendicular and parallel axis simultaneously. The data from each channel was collected and mathematically converted by the KinetAssyst 3 software (TGK Scientific) to anisotropy. Anisotropy was plotted against time, the data is shown with the best fit to a single exponential with a rate \( k = 370 \pm 4.7 \times 10^6 \text{ M}^{-1}\text{s}^{-1} \).
Kinetics of Exonuclease III binding & cleavage of abasic DNA

Once the parameters of the fluorescence resonance energy transfer assay were established, FRET was used to monitor dissociation of MUG from the DNA. In order to analyze whether Exonuclease III actively displaces MUG from the DNA, a MUG & DNA complex needs to be formed and ∆E monitored upon addition of Exonuclease III (ExoIII) to the complex. Due to the 3'→5' exonuclease activity of ExoIII, a hairpin DNA with a cap structure at its 3’ terminus (a single strand overhang with methyl phosphonate residues) which makes it resistant to digestion with ExoIII was used in these experiments. When annealed the DNA forms a double stranded hairpin structure. Oligonucleotides with a U·G or a U·A mismatch (HEX-Hairpin-U·G or HEX-Hairpin-U·A) and oligonucleotides with an abasic site (HEX-Hairpin-AP·A) were designed to be used in assays with ExoIII. FRET between MUG-Alexa488 and HEX-AP·A-Hairpin, HEX-U·A-Hairpin, HEX-U·G-Hairpin and H12AP•G proceeded with similar rates showing that the hairpin structure did not interfere with the energy transfer (data not shown).

Exonuclease III binds to abasic DNA specifically but to uracil containing DNA non-specifically

Binding of Exonuclease III to DNA with an abasic site (HEX-Hairpin-AP·A) and to DNA with a uracil lesion (HEX-Hairpin-U·A) was analyzed by titrating in Exonuclease III to 100nM DNA and monitoring the change in anisotropy. Exonuclease III binds the DNA in the absence of MgCl₂ but requires this divalent cation to cleave the abasic site and dissociate from it. No MgCl₂ was used in anisotropy experiments, which allowed us to follow binding of ExoIII only. Anisotropy values were plotted against Exonuclease III concentration (Figure 4-12). Binding of Exonuclease III to the abasic DNA follows a hyperbolic binding curve. The data was fitted to a one site binding model using Grafit.
5.0 (Erithacus software). $K_d$ was determined as $23.1 \pm 3.6\text{nM}$ (Figure 4-12A). Upon addition of 2.5mM MgCl$_2$, as expected anisotropy went down to background levels due to cleavage of the abasic site (data not shown). Binding of Exonuclease III to DNA with a uracil lesion was non-specific as can be seen by the linear progression of the curve. The data of Exonuclease III binding to uracil containing DNA was fitted to a linear equation (Figure 4-12B).

![Figure 4-12](image)

Figure 4-12 Exonuclease III specifically binds to abasic DNA but non-specifically to uracil containing DNA.

Exonuclease III was added to 100nM (A) HEX labeled DNA with an abasic site (HEX-Hairpin-AP·A) and to (B) DNA containing a uracil (HEX-Hairpin-U·A) lesion. Anisotropy was measured using Fluoromax 5.0. Binding of Exonuclease III to the abasic DNA follows a hyperbolic curve whereas binding to DNA with a uracil lesion is linear and does not saturate, which is indicative of non-specific binding (B). Data for binding to abasic DNA is shown with the best fit to a one-site binding model using Grafit 5.0 (Erithacus software). $K_d$ for ExoIII binding to abasic DNA was determined as $23.1 \pm 3.6\text{nM}$.

An activity assay was performed to monitor the activity of ExoIII towards DNA with an abasic site and towards DNA containing a uracil residue. This assay also provided a control for the resistance of the DNA against the 3’→5’ Exonuclease activity of ExoIII. Processing of HEX-Hairpin-AP·A and HEX-Hairpin-U·A by Exonuclease III was tested by incubating 100nM of each oligonucleotide at 37°C in Exonuclease III reaction buffer, which contains 10mM MgCl$_2$. Aliquots were taken at 10s, 20s, 40s, and 60s, 2min,
5min, 10min, and 30min. The cleaved products were separated on a 20% denaturing polyacrylamide gel. In the standard reaction buffer 1nM Exonuclease III was enough to cleave 100nM abasic DNA within 10 seconds. Cleavage of the abasic site created the “specific” product (¥) which was separated from uncleaved substrate (*) by denaturing gel electrophoresis. 100nM Exonuclease III created a shorter band (§) when incubated with HEX-Hairpin-AP∙A DNA. The short band is likely to be arising from the 3’→5’ exonuclease III activity once the abasic site is cleaved and an unprotected 3’ end is created for Exonuclease III. ExoIII was not active against the U∙A substrate as can be seen by the lack of change in substrate concentration (Φ). When 1nM ExoIII was incubated with 100nM HEX-Hairpin-U∙A DNA, faint non-specific bands were seen after 5 minutes. After 5 minute incubation time, 100nM ExoIII cleaved these non-specific bands to the shorter band also seen with HEX-Hairpin-U∙A DNA (§), which again is likely to be due to the 3’→5’ exonuclease III activity of the enzyme (Figure 4-13).
Figure 4- 13 Reaction profile of Exonuclease III with abasic and uracil-containing hairpin DNA substrates

Processing of HEX-Hairpin-AP·A and HEX-Hairpin-U·A by Exonuclease III was tested by incubating 100nM of each oligonucleotide at 37°C in Exonuclease III reaction buffer. Aliquots were taken at indicated time points and cleaved products were separated on a 20% denaturing polyacrylamide gel. Aliquots were taken at: 10s, 20s, 40s, and 60s, 2min, 5min, 10min, and 30min. 1nM Exonuclease III was enough to cleave 100nM abasic DNA within 10 seconds. Cleavage created the “specific” product (¥), which was separated from the uncleaved substrate (*) easily. At higher concentrations, Exonuclease III created a shorter band (§). The short band is likely to be arising from the 3’→5’ exonuclease III activity once the abasic site is cleaved and an unprotected 3’ end is created for Exonuclease III. Exonuclease III did not show activity towards the U·A substrate except creating some non-specific products after 5 minutes (■). These non-specific bands were converted into the shorter product by 100nM ExoIII (§).
**FRET to monitor dissociation of MUG from the DNA**

Anisotropy measurements described in the second chapter had shown that MUG dissociates from the DNA upon addition of unlabeled, abasic (competitor) DNA (12AP•G) (passive diffusion) (Figure 2-10). Monitoring FRET upon addition of competitor DNA to the MUG & DNA complex should allow us to determine the kinetics of passive diffusion. In order to monitor the change in energy transfer (ΔE) upon competition with abasic competitor, we first made a MUG & DNA complex by incubating 100nM HEX-AP∙A-Hairpin with 200nM MUG-Alexa488 for 15 minutes at 25°C. 1000nM unlabeled abasic oligonucleotide was added rapidly using the stopped flow instrument. ΔE was fitted to a single exponential, finding a rate constant \( k = 1.32 \pm 0.01 \text{ s}^{-1} \) (Figure 4-14A). Anisotropy was also measured in a separate experiment by mixing 1000nM unlabeled AP-DNA with the MUG & HEX-AP∙A-Hairpin complex. Decrease in anisotropy was fitted to a single exponential using Grafit 5.0 (Erithacus software) and the rate was determined as 3.31 ± 0.08 s\(^{-1}\). The rates for decrease in ΔE and anisotropy were close to each other, which is in alignment with the process of simple diffusion (Figure 4-14).
The effect of an unlabeled non-specific competitor (12C·G) containing the same sequence as the abasic competitor (12AP·G) on MUG’s dissociation was tested by FRET analysis. For this, 100nM HEX-AP-A-Hairpin DNA was incubated with 200nM MUG-Alexa488 for 15 minutes at 25°C. 1000nM non-specific competitor was added rapidly using the stopped flow instrument. ΔE was fitted to a single exponential finding a rate constant $k = 0.51 \pm 0.02 \text{ s}^{-1}$ (Figure 4-15B). The rate of ΔE when competing with 1000nM abasic competitor is also shown for comparison (Figure 4-15A). Comparison of decrease in ΔFRET with two different oligonucleotides shows that competition with abasic DNA leads to a higher degree of MUG dissociation compared to the non-specific competitor.
Figure 4- 15 ΔE is different when competing with abasic, non-specific competitor or Exonuclease III

200nM MUG-Alexa488 was incubated with 100nM HEX-AP-A-Hairpin for 15 minutes at 25°C. 1000nM abasic competitor (A), 1000nM non-specific competitor (B) or 200nM Exonuclease III (C) were rapidly mixed with the MUG & DNA complex using the stopped flow instrument. HEX and Alexa488 fluorescence signals were measured simultaneously. A decrease in ΔE was seen in all three cases. Upon mixing abasic competitor (12AP•G), a negative ΔE with a rate constant $k = 1.32 \pm 0.01 \text{ s}^{-1}$ was observed (A). Change in ΔFRET was measured in a separate experiment by mixing in 1000nM non-specific competitor (12C•G) with the MUG & AP-A complex. Rate in ΔE decrease was found to be $0.51 \pm 0.02 \text{ s}^{-1}$. In order to understand if Exonuclease III actively displaces MUG from its product, 200nM Exonuclease III was rapidly mixed with the MUG & DNA complex in the absence of MgCl$_2$. A negative ΔE was observed with a rate $k = 1.33 \pm 0.03 \text{ s}^{-1}$ (C).
Once the rate of passive diffusion of MUG from AP·A DNA was determined, we analyzed the effect of Exonuclease III on the MUG & AP·A complex. If Exonuclease III actively displaces MUG from the abasic DNA, a significant increase in the dissociation rate would be expected. In order to understand if Exonuclease III actively displaces MUG from its product, 200nM MUG-Alexa488 was incubated with 100nM HEX-AP·A-Hairpin for 15 minutes at 25°C for binding to reach equilibrium. 200nM Exonuclease III was rapidly mixed with the MUG & DNA complex in the absence of MgCl₂ using the stopped flow instrument. A decrease in ∆E was observed, fitting the data to a single exponential provided us with a rate for decrease in ∆E; \( k = 1.33 \pm 0.03 \text{ s}^{-1} \) (Figure 4-15C).

Change in anisotropy upon competition with abasic (12AP·G), non-specific (12C·G) competitor and Exonuclease III was also monitored using the same solutions as above, but changing the optical setup of the instrument. Data was again collected by mixing a MUG & HEX-Hairpin-AP·A against specific and non-specific competitor and ExoIII. The data in all three cases was fitted to a single exponential. Upon competition with abasic DNA, decrease in anisotropy proceeded with a rate of \( k=3.31 \pm 0.08 \text{ s}^{-1} \) (Figure 4-16A). Competing off MUG from the MUG & HEX-AP·A-Hairpin complex with non-specific competitor led to a very small anisotropy change that also proceeded very slowly \( (k= 0.19 \pm 0.01 \text{ s}^{-1}) \) (Figure 4-16B). When Exonuclease III was added to the MUG & HEX-AP·A-Hairpin complex, a rapid increase in anisotropy was seen with a rate \( k= 224.71 \pm 11.47 \text{ s}^{-1} \) (Figure 4-16C).
Figure 4: Change in anisotropy in the presence of competitor DNA or ExoIII

1000nM abasic competitor was mixed with the MUG & DNA complex and change in anisotropy was measured. A decrease in anisotropy was observed as MUG dissociated from the DNA in the presence of the abasic competitor. The data was fitted to a single exponential with the rate constant \( k = 3.31 \pm 0.08 \text{ s}^{-1} \) (A). Competing off MUG from the MUG & HEX-AP-A-Hairpin complex with non-specific competitor led to a small anisotropy change which was also very slow \( (k = 0.19 \pm 0.01 \text{ s}^{-1}) \) (B). When ExoIII was added to the MUG & HEX-AP-A-Hairpin complex, a rapid increase in FRET was observed with a rate \( k = 224.71 \pm 11.47 \text{ s}^{-1} \) (C).
Exonuclease requires MgCl$_2$ to cleave abasic DNA and to dissociate from its product. 1mM MgCl$_2$ is enough for Exonuclease III to cleave abasic DNA. In order to analyze if cleavage of the abasic site leads to a change in FRET, $\Delta E$ upon mixing Exonuclease III & 1mM MgCl$_2$ with the MUG & DNA complex was monitored in comparison to Exonuclease III only. A decrease in $\Delta E$ was seen upon mixing Exonuclease III & 1mM MgCl$_2$ with the MUG & DNA complex. The rate of decrease in $\Delta E$ was found to be $k=0.55 \pm 0.01$ s$^{-1}$. The amplitude and the rate of $\Delta$FRET were similar in the presence ($k=0.55 \pm 0.01$ s$^{-1}$) and absence of MgCl$_2$ ($k=0.98 \pm 0.01$ s$^{-1}$) (Figure 4-17).
Addition of Exonuclease III & 1mM MgCl₂ to the MUG & HEX-AP·A complex leads to a decrease in ∆E

200nM Exonuclease III & 1 mM MgCl₂ was rapidly mixed with the MUG & DNA complex. A decrease in ∆E was seen. The rate of decrease in ∆E was \( k = 0.55 \pm 0.01 \text{ s}^{-1} \), which is similar to the decrease in energy transfer in the presence of ExoIII only (\( k = 0.98 \pm 0.01 \text{ s}^{-1} \)).

We also wanted to evaluate if higher MgCl₂ concentrations, which increase dissociation of APE1 (Masuda et al. 1998), the human homologue of Exonuclease III, from the abasic site, affect FRET between MUG-Alexa488 and HEX-DNA to a different extent. Decrease in ∆E was measured upon mixing ExoIII & 10mM MgCl₂ with the MUG & DNA complex. Exonuclease III & 10mM MgCl₂ led to a bigger ∆FRET decline with a rate \( k = 6.4 \pm 0.1 \text{ s}^{-1} \). This rate is higher than the rates observed upon mixing ExoIII in the absence of MgCl₂ and in the presence of 1mM MgCl₂ (0.98 ± 0.01 s⁻¹ and 0.55 ± 0.01s⁻¹ respectively) (Figure 4-18).
Comparison of ExoIII’ s effect on ΔFRET with and without 1mM MgCl₂ revealed that the presence of 1mM MgCl₂ does not affect MUG’s dissociation from the abasic site. When Exonuclease III & 10mM MgCl₂ was mixed with the MUG & DNA complex, a larger reduction in ΔE was observed compared to ExoIII only or ExoIII & 1mM MgCl₂. In order to eliminate the possibility of an artifact caused by higher MgCl₂ concentrations, different MgCl₂ concentrations (without ExoIII) were mixed with the MUG & DNA complex. When buffer or 1mM MgCl₂ were rapidly mixed with the MUG & DNA complex, a minor decrease in energy transfer was seen, which occurred with similar rates ($k_{\text{Buffer}}=11.3 \pm 1.2 \text{s}^{-1}$ and $k_{\text{MgCl}_2}=8.8 \pm 0.9 \text{s}^{-1}$). The low amplitude of ΔE upon mixing buffer and 1mM MgCl₂ suggests that the change is most likely a dilution effect. The effect of 1mM MgCl₂ on the MUG & DNA complex was the same as buffer, which indicates that 1mM MgCl₂ on its own does not have any
effect on FRET between MUG and the DNA. At higher MgCl₂ concentrations, a concentration dependent ∆FRET decline was seen (Figure 4-19A and B).

![Graph showing energy transfer changes with different MgCl₂ concentrations](image)

**Figure 4-19** MgCl₂ affects energy transfer between Alexa488 and HEX

In order to see if higher MgCl₂ concentrations lead to a larger decline in ∆E, 200nM MUG-Alexa488 was incubated with 100nM HEX-AP-A-Hairpin for 15 minutes at 25°C. A range of MgCl₂ concentrations were rapidly mixed with the MUG & DNA complex using the stopped flow instrument. A concentration dependent reduction in ∆E was observed with MgCl₂ (A). When buffer (■) or 1mM MgCl₂ (■) were rapidly mixed with the MUG & DNA complex, a minor decrease in energy transfer was seen which occurred with the same rate ($k_{\text{Buffer}}=11.3 \pm 1.2 \text{ s}^{-1}$ and $k_{\text{MgCl₂}}=8.8 \pm 0.9 \text{ s}^{-1}$). The effect of 1mM MgCl₂ on the MUG & DNA complex was the same as buffer, which indicates that 1mM MgCl₂ on its own does not have any effect on FRET between MUG and the DNA.

The rate of ∆FRET decline due to 10mM MgCl₂ was fitted to a single exponential and was determined as $k= 7.8 \pm 0.2 \text{ s}^{-1}$. This rate is the same as observed upon mixing ExoIII & 10mM MgCl₂, $k= 6.4 \pm 0.1 \text{ s}^{-1}$ (Figure 4-20). FRET change with 10mM MgCl₂ and with ExoIII & 10mM MgCl₂ are very similar in amplitude and rate suggesting the FRET change observed with upon mixing ExoIII & 10mM MgCl₂ is due to 10mM MgCl₂.
Figure 4- 20 10mM MgCl$_2$ has a non-specific effect on FRET

The decline in $\Delta$FRET was compared upon mixing 10mM MgCl$_2$ (---) or Exonuclease III & 10mM MgCl$_2$ (---) with MUG & HEX-AP::A-Hairpin complex. Both data were fit to a single exponential. The rate of FRET decrease due to 10mM MgCl$_2$ was determined as $k = 7.8 \pm 0.2$ s$^{-1}$. This rate is the same as with ExoIII & 10mM MgCl$_2$ $k = 6.4 \pm 0.1$ s$^{-1}$.

In order to further understand if MgCl$_2$ dependent reduction in $\Delta$E is due to MUG dissociating from the DNA due to MgCl$_2$, the effect of MgCl$_2$ was investigated by monitoring the change in fluorescence anisotropy. As a control, buffer and 1mM MgCl$_2$ were also mixed with the complex. No decrease in anisotropy was seen when 10mM MgCl$_2$, 1mM MgCl$_2$ or buffer were mixed with MUG & HEX-AP::A-Hairpin (Figure 4-21). The fact that there is no decrease in anisotropy but decrease in $\Delta$E suggests that MgCl$_2$ concentrations higher than 1mM have an effect on FRET from Alexa488 to HEX independent of MUG’s dissociation. MgCl$_2$ may also be leading to a molecular rearrangement that causes the reduction in FRET.
Figure 4- 21 Reduction in ΔE by MgCl₂ is not due to MUG’s dissociation

Decrease in ΔE upon addition of MgCl₂ could be due to dissociation of MUG from the abasic DNA caused by MgCl₂ or alternatively, MgCl₂ may be affecting energy transfer. In order to differentiate between these possibilities, the effect of MgCl₂ on fluorescence anisotropy was analyzed by mixing buffer (A), 1mM MgCl₂ (B) and 10mM MgCl₂ (C) with the MUG & HEX-AP-A-Hairpin complex. Upon mixing buffer, 1mM or 10mM MgCl₂ with MUG & HEX-AP-A-Hairpin, no change in fluorescence anisotropy was observed.
**FRET between MUG-Alexa488 and HEX- U•G -Hairpin**

To compare the effect of Exonuclease III on MUG’s dissociation from AP-G DNA, DNA with the same sequence, hairpin, and 3 ’cap structure and a 5’ HEX label that has a U•G site instead of a U•A was used (HEX-U•G-Hairpin).

We first monitored change in energy transfer (ΔE) upon rapid mixing of 200nM MUG-Alexa488 with 100nM HEX-U•G-Hairpin. As expected, an increase in ΔE was seen upon mixing MUG-Alexa488 with HEX-U•G-Hairpin. When MUG binds DNA with the U•G mismatch, uracil is cleaved and MUG stays bound to the abasic site. Change in FRET and its rate constants were the same upon binding of MUG to U•A and to U•G DNA, suggesting that cleavage of the N-glycosidic bond does not affect FRET. Change in FRET upon MUG-Alexa488 binding to HEX-U•G-Hairpin was fitted to a single exponential with a rate constant: \( k = 400 \pm 4.5 \times 10^6 \text{ M}^{-1}\text{s}^{-1} \) (Figure 4-22). This rate is similar to the rate obtained when MUG binds to the non-hairpin oligonucleotide H12AP-G \( k = 275 \pm 1.5 \times 10^6 \text{ M}^{-1}\text{s}^{-1} \) (Figure 4-9B) and HEX-AP-A-Hairpin \( k = 290 \pm 3 \times 10^6 \text{ M}^{-1}\text{s}^{-1} \) (data not shown).
We first analyzed ∆E when MUG passively diffuses from the AP-G DNA in the presence of excess amount of abasic competitor. To analyze the effect of abasic competitor on the dissociation of MUG from the AP-G site, 100nM HEX-AP-G-Hairpin was mixed with 200nM MUG-Alexa488 and incubated at 25°C for 30 minutes to allow completion of uracil excision and equilibrium binding. After 30 minutes, 1000nM unlabeled, abasic competitor (AP•G) was mixed rapidly using the stopped flow instrument. Change in ∆E was fitted to a single exponential using Grafit 5.0 (Erithacus software). The rate of passive diffusion was determined as $k = 1.86 \pm 0.02$s$^{-1}$ (Figure 4-23). The rate of passive diffusion of MUG from AP-A DNA ($1.32 \pm 0.01$ s$^{-1}$) and AP-G DNA ($1.86 \pm 0.02$s$^{-1}$) were similar. This result is very interesting as it implies that guanine across the abasic site does not have that big of an effect on passive diffusion.
The effect of an abasic competitor on MUG’s dissociation from HEX-AP•G-Hairpin DNA was analyzed by making a MUG & DNA complex after incubating 200nM MUG-Alexa488 with 100nm HEX-U•G-Hairpin for 30 minutes at 25°C. 1000nM abasic competitor (12AP•G) was added to the complex and decrease in ΔE was monitored. The data was fitted to a single exponential. MUG dissociated from HEX-AP•G-Hairpin DNA with a rate constant \(k = 1.86 \pm 0.02 \text{ s}^{-1}\).

In order to monitor if MgCl\(_2\) affects FRET decline due to passive diffusion, abasic competitor was added to the MUG & HEX-U•G-Hairpin complex in the absence and presence of 1mM and 10mM MgCl\(_2\). No difference in the amplitude or rate in FRET reduction was observed in either case (Figure 4-24).
Abasic competitor leads to dissociation of MUG from the DNA through passive diffusion. The effect of MgCl₂ on FRET decrease due to passive diffusion was analyzed by adding 1mM and 10mM MgCl₂ together with 1000nM abasic competitor. No significant difference in the amplitude or rate in FRET reduction was seen in the absence or presence of MgCl₂. Rates were determined by fitting the data to a single exponential and were found to be $k = 1.17 \pm 0.02 \text{ s}^{-1}$; $0.91 \pm 0.01 \text{ s}^{-1}$ and $0.74 \pm 0.02 \text{ s}^{-1}$ for AP; AP & 1mM MgCl₂ and for AP & 10mM MgCl₂, respectively.

To analyze the effect of Exonuclease III on MUG’s dissociation from the AP-G site, 100nM HEX-U·G-Hairpin was mixed with 200nM MUG-Alexa488. After 30 minutes, 200nM Exonuclease III was mixed rapidly using the stopped flow instrument. A decrease in ΔE was seen upon mixing Exonuclease III, a similar result was obtained upon mixing Exonuclease III with 1mM MgCl₂. Decrease in ΔE in the presence of Exonuclease III and Exonuclease III & 1mM MgCl₂ is shown with the best fit to a single exponential with rates $k = 85.5 \pm 32.7 \text{ s}^{-1}$ and $k = 68.2 \pm 5.7 \text{ s}^{-1}$ respectively (Figure 4-25). The amplitude of FRET decrease upon ExoIII addition was smaller than in passive diffusion but the rates in FRET decline were ~54 times faster than in passive diffusion (Figures 4-23 and 4-25).
The degree in FRET change is much smaller when Exonuclease III is mixed with HEX-U·G-Hairpin (Figure 4-25) compared to HEX-AP-A-Hairpin (Figure 4-17).

![Figure 4-25 Exonuclease III leads to a small decline in FRET which proceeds faster than passive diffusion](image)

The effect of Exonuclease III on energy transfer in the absence and presence of 1mM MgCl$_2$ was analyzed by making a MUG & DNA complex after incubating 200nM MUG-Alexa488 with 100nm HEX-U·G-Hairpin. A reduction in $\Delta E$ was seen in the absence of 1mM MgCl$_2$ and presence of 1mM MgCl$_2$ with rates of $k = 85.5 \pm 32.7$ s$^{-1}$ and $k = 68.2 \pm 5.7$ s$^{-1}$ respectively.

Cleavage of the abasic site does not affect dissociation of MUG from the abasic site as can be seen from the fact that the amplitudes and rates of the decrease in $\Delta E$ were the same in the absence and presence of 1mM MgCl$_2$. The effect of 10mM MgCl$_2$ on $\Delta E$ was evaluated by adding ExoIII & 10mm MgCl$_2$ or 10mM MgCl$_2$ to the MUG & HEX-AP-G-Hairpin complex. A larger decrease in energy transfer was seen when ExoIII was mixed with the MUG & DNA complex in the presence of 10mM MgCl$_2$ compared to in the absence of MgCl$_2$ (Figure 4-26).
Figure 4-26 FRET decrease with ExoIII & 10mM MgCl₂ is due to 10mM MgCl₂ affecting FRET and not due to Exonuclease III displacing MUG from the AP·G site.

The effect of Exonuclease III & 10mM MgCl₂ on ΔE was analyzed by forming a MUG & DNA complex. ExoIII & 10mM MgCl₂ or 10mM MgCl₂ alone was added to the complex. A decrease in ΔE was seen in both cases. 10mM MgCl₂ with ExoIII leads to a larger decrease in energy transfer compared to ExoIII with 1mM MgCl₂ or without MgCl₂. The data is shown with the best fit to a single exponential with the rate constant $k = 24 ± 0.9 \text{ s}^{-1}$ for ExoIII & 10mM MgCl₂. A similar decrease in ΔE was seen in the presence of 10mM MgCl₂ with a rate $k = 23.9 ± 0.9 \text{ s}^{-1}$ suggesting the decrease is due to 10mM MgCl₂ and not due to Exonuclease III.

A comparison of the ΔE due to Exonuclease III & 10mM MgCl₂ ($k = 24 ± 0.9 \text{ s}^{-1}$) and 10mM MgCl₂ showed that the energy transfer decrease observed was due to 10mM MgCl₂ ($k = 23.9 ± 0.9 \text{ s}^{-1}$) and not due to Exonuclease III displacing MUG from the AP·G site (Figure 4-26). The effect of ExoIII, ExoIII & 1mM MgCl₂, ExoIII & 10mM MgCl₂ and 10mM MgCl₂ on FRET was also monitored over longer time periods. Data obtained over longer times showed that there was no further decline in FRET over a longer time period (Figure 4-27).
Exonuclease III does not actively displace MUG glycosylase from abasic DNA

We have observed a significant difference in the dissociation rates between passive diffusion ($k=1.86 \pm 0.02 \text{s}^{-1}$; Figure 4-24) and ExoIII induced diffusion ($k=85.5 \pm 3.3 \text{s}^{-1}$; Figure 4-25) of MUG glycosylase from the DNA containing a AP•G site. The considerably big difference between the rates of each process might indicate an active mechanism induced by Exonuclease III. On the other hand, there is also a considerable discrepancy between the changes in energy transfer between these two processes. Exonuclease III only leads to a 20% decrease in energy transfer compared to passive diffusion. As a control, we investigated the effect of mixing only buffer with the MUG HEX-AP-G-Hairpin complex. Figure 4-28 shows the changes in energy transfer upon mixing buffer (A) and ExoIII (B) with the MUG & DNA complex. Upon mixing buffer
or ExoIII, a small decrease in energy transfer is observed which proceeds with similar rates ($k_{Buffer} = 104.9 \pm 12.6 \text{ s}^{-1}$; $k_{ExoIII} = 85.5 \pm 3.35 \text{ s}^{-1}$).

Figure 4-28 ExoIII does not actively displace MUG glycosylase from the abasic site

200nM MUG-Alexa488 was mixed with 100nM HEX-U∙G-Hairpin. The complex was incubated for 30 minutes at 25°C and then mixed with (A) Buffer or with (B) 200nM ExoIII, the decrease in ΔE was monitored. The data was fitted to a single exponential. When mixed with buffer, a small change in ΔE was observed, which proceeded with the rate $k= 104.9 \pm 12.6 \text{ s}^{-1}$ (A). Change in ΔE upon mixing ExoIII was similar to change with buffer and proceeded with $k= 85.5 \pm 3.3 \text{ s}^{-1}$ (B).

If MUG was still bound to the abasic site upon the addition of ExoIII, mixing in additional unlabeled competitor should lead to a decrease in energy transfer due to passive diffusion of the still bound MUG. In order to address this, 200nM MUG was mixed with 100nM HEX-U∙G-Hairpin and the complex was incubated at 25°C for 30 minutes in order to allow complete cleavage of uracil and equilibrium binding. 200nM Exonuclease III was added to the complex and incubated for 5 minutes at 25°C. After 5 minutes, 1000nM unlabeled DNA containing a G∙U mismatch was added. If MUG was present on the DNA after the addition of Exonuclease III, a further change in FRET would be expected upon addition of the unlabeled competitor. As can be seen from Figure 4-29, a further decrease in ΔE was observed upon addition of unlabeled competitor DNA. ΔE was fitted to a single exponential giving a rate $k=0.17 \pm 0.002 \text{ s}^{-1}$. 
Figure 4- 29 ExoIII does not actively displace MUG glycosylase from the abasic site

200nM MUG-Alexa488 was mixed with 100nM HEX-U·G·Hairpin. The complex was incubated for 30 minutes at 25°C. For comparison, 200nM ExoIII was mixed with MUG & DNA complex using the stopped flow instrument and the decrease in ΔE was monitored (A). The data was fitted to a single exponential. MUG dissociated from HEX-U·AP·Hairpin DNA with a rate constant \( k = 85.5 \pm 3.3 \text{ s}^{-1} \). (B) Unlabeled competitor leads to dissociation of MUG from the DNA through passive diffusion. Dissociation of MUG was monitored by mixing 1000nM DNA containing a G·U mismatch. The dissociation rate was determined by fitting the data to a single exponential and was found to be \( k = 0.07 \pm 0.0007 \text{ s}^{-1} \). (C) In order to find out if the addition of ExoIII leads to MUG’s dissociation, MUG & DNA complex was formed and ExoIII was added to the complex. Following incubation for 5 minutes at 25°C, 1000nM unlabeled DNA containing a G·U mismatch was added to the complex using the stopped flow instrument. The data was fitted to a single exponential with a rate \( k = 0.17 \pm 0.002 \text{ s}^{-1} \).
To summarize, the fact that ExoIII and buffer have the same effect on the energy transfer between MUG-Alexa288 and the AP•G DNA suggests that ExoIII does not actively displace MUG from the AP•G site. Addition of unlabeled competitor further led to a decrease in FRET with the same rate as passive diffusion, which confirmed that MUG is still bound to the AP•G DNA after ExoIII addition.

**Analysis of the enhancement of MUG’s glycosylase activity by Exonuclease III**

As described above, the role of Exonuclease III in MUG’s displacement from the abasic site was investigated via the fluorescence resonance energy transfer assay. We also wanted to explore whether ExoIII stimulates the base excision repair activity of MUG. For this analysis, 200nM HEX-U-G-Hairpin was incubated with 20nM MUG and increasing concentrations of Exonuclease III. The effect of increasing ExoIII concentrations on product formation by MUG was analyzed on a denaturing polyacrylamide gel. Under these conditions, since the DNA >> MUG, MUG has to dissociate from the DNA in order to rebinding and process all the DNA. If ExoIII increases the turnover rate of MUG glycosylase, we would expect an increase in product formation. The glycosylase activity of MUG creates an abasic site, which upon NaOH treatment is cleaved creating a product with a 3’PO₄ end. Exonuclease III cleaves 5’ to the abasic site creating a product with a 3’OH end. Both products can be differentiated on a denaturing gel as the 3’OH containing product runs slower than the 3’PO₄ containing product (data not shown). A striking observation was that increasing ExoIII concentration led to an increase in both 3’OH and 3’PO₄ products which can only come about due to a contaminating glycosylase activity in the commercial ExoIII preparation. Repetition of the experiment with different batches of ExoIII from the same provider led to the same result making us come to the conclusion that the glycosylase activity present in the ExoIII sample is probably due to the purification method used by New England
Biolabs. Further experiments to investigate the effect of ExoIII on MUG’s glycosylase activity will be performed with Exonuclease III cloned and expressed in our lab.

**Electrophoretic mobility shift assay to visualize MUG and Exonuclease III on the DNA**

In order to investigate if Exonuclease III actively displaces MUG from the abasic site, we attempted to use an electrophoretic mobility shift assay (Figure 4-30A). 100nM HEX-U•G-Hairpin was incubated with 200nM and 400nM MUG. MUG & DNA complexes were incubated at 25°C for half an hour to allow the uracil to be cleaved; Exonuclease III was added equimolar to MUG. The samples were run on a native polyacrylamide gel after incubation with ExoIII (Figure 4-30). Band shifts consistent with those previously seen with MUG were observed, however the presence of ExoIII neither appeared to displace MUG from the DNA, nor did it lead to the formation of any higher complexes. The addition of ExoIII alone did not produce a clearly visible DNA complex with abasic DNA, so it is not clear whether the lack of an observable effect with MUG was due to limitations of the assay. Faint bands were observed when DNA was incubated with ExoIII alone (Figure 4-30B). The technique needs optimization in order to be used to answer the question whether ExoIII displaces MUG from the abasic site.
Electrophoretic mobility shift assay with MUG and ExoIII

(A) 100nM HEX-U•G-Hairpin was incubated with 200nM (Lane 2) and 400nM MUG (lane 4). EMSA was performed as before. The first lane shows DNA on its own. After incubation of MUG & DNA complexes at 25°C for half an hour, Exonuclease III was added equimolar to MUG. Lane 3 contains DNA & 200nM MUG with 200nM ExoIII whereas lane 5 contains DNA & 400nM MUG with 400nM ExoIII. As a control, 200nM (Lane 6) and 400nM (Lane 7) ExoIII were mixed with HEX-U•G-Hairpin. 200nM and 400nM MUG bind the HEX-U•G-Hairpin and lead to two slower migrating bands (Bands 1 and 2 in Lanes 2 and 4). Although lane 5 which contains 400nM MUG and 400nM ExoIII with DNA contains a higher migrating band (*) the same band can also be seen in lane 4, which has MUG and DNA. As can be seen from lanes 6 and 7, ExoIII did not lead to a visible band shift with DNA. (B) Increase of the band intensities makes a slight band shift with ExoIII visible. This band cannot be seen in lanes 3 or 5.
Using fluorescence resonance energy transfer (FRET), we aimed to answer the question: “Does Exonuclease III (ExoIII) displace MUG from the DNA through an active or passive mechanism?” To our knowledge, this experimental approach has not been employed before to address this question in base excision repair. Using FRET, we determined the rates for MUG’s dissociation from the DNA by adding ExoIII and compared them to the rate of passive diffusion. This method provides a quantitative measure and can be used to investigate interactions of other enzymes in base excision repair as well.

In order to follow multi-component complexes in DNA repair using FRET, Alexa488 and HEX were used to label MUG and DNA, respectively. These fluorophores are suitable for FRET since their emission and absorption spectra overlap. Under the conditions employed, Alexa488 specifically binds primary amines on N-terminal amine groups of proteins. MUG was successfully labeled with Alexa488 (MUG-Alexa488), activity testing revealed that labeling did not interfere with MUG’s glycosylase activity. Our analysis using the fluorimeter revealed that FRET occurred between MUG-Alexa488 and the HEX labeled oligonucleotide (H12AP-G). Change in energy transfer (ΔE) was calculated by taking the ratios of donor fluorescence in the beginning and the end of the reaction duration. We were able to determine the equilibrium dissociation constant (K_d) for binding by adding H12AP-G in small increments to MUG-Alexa488 and plotting ΔE. Fitting the data to a single ligand binding equation using Grafit 5.0 (Erithacus software) enabled us to determine the K_d as 157 +/- 10.7nM. This K_d is consistent with the K_d determined through anisotropy measurements (K_d = 186.5 ± 3.0nM). This firstly shows that binding of MUG-Alexa488 has the same characteristics as MUG and also confirms that change in ΔE reflects binding of MUG to the abasic site.
Stopped flow instrument is very useful to monitor the kinetics of protein-ligand interactions; hence we used it to determine association and dissociation rates of MUG via FRET measurement. Change in energy transfer was measured upon rapid mixing of MUG-Alexa488 and H12AP-G, the rate was determined by fitting the data to a single exponential. Analysis of ΔE between H12AP-G and different MUG-Alexa488 concentrations revealed that 100nM H12AP-G gave a good signal for monitoring energy transfer between MUG and the DNA.

Investigation of MUG binding to DNA with an AP·G site suggested that the enzyme binds DNA in a 2:1 stoichiometry but non-specific binding by MUG was also observed and was dependent on the size of the oligonucleotide used. Analysis of FRET between MUG-Alexa488 and HEX-AP·G at ratios: 1:1, 2:1, 4:1 and 8:1 revealed that the change in energy transfer is the same when MUG binds DNA in a 1:1 or 2:1 ratio but decreases when four or eight MUG molecules are bound. Reduced ΔE may be an artifact of multiple MUG molecules binding to DNA. MUG molecules might be non-specifically binding to close to the HEX on the 5’end or maybe even partially on it, hence blocking FRET. Another reason for reduction in ΔE may be increased background fluorescence signal from the donor which does not necessarily participate in the reaction. All FRET experiments were conducted at a 2:1 MUG: DNA ratio.

FRET experiments revealed that the association rates of MUG with AP·A, AP·G and U·G bearing oligonucleotides were the same (290 ± 3 *10^6 M⁻¹s⁻¹ ; 275 ±1.5*10^6 M⁻¹s⁻¹ and 400 ±4.5* 10^6 M⁻¹s⁻¹ respectively).
Exonuclease III and the hairpin oligonucleotides

When analyzing the effect of Exonuclease III on MUG & DNA complexes, the 3'→5' exonuclease activity of ExoIII had to be circumvented. We therefore used an oligonucleotide bearing a cap structure at its 3’ terminus, which when annealed forms a double stranded hairpin structure. Oligonucleotides tested contained an abasic site or a uracil across adenine and are also labeled at their 5’ terminus (HEX-AP∙A-Hairpin and HEX-U∙A-Hairpin). Analysis of Exonuclease III binding to AP∙A and U∙A revealed that ExoIII binds the abasic site with nanomolar affinity ($K_d = 23.1 ± 3.6$ nM) but binding to U∙A was non-specific and was fit to a straight line. Exonuclease III requires MgCl$_2$ in order to cleave 5’ to the abasic site. As expected, upon addition of MgCl$_2$, anisotropy went down to background levels (data not shown). Exonuclease III processed AP∙A very efficiently and as expected did not process U∙A, except non-specific products created at high concentrations over an extended period of time.

The effect of Exonuclease III on MUG & DNA complexes

When MUG and DNA are in equilibrium, bound MUG can be competed off from the DNA by adding competitor oligonucleotide in excess. The rate of dissociation will be dependent on the diffusion rate of MUG glycosylase. The rate of passive diffusion was thus determined by competing off MUG with unlabeled abasic or non-specific DNA in excess. The effect of Exonuclease III on MUG & DNA complexes was determined; the rates and extent of change in energy transfer through passive diffusion and ExoIII were compared. Change in FRET has been assessed when MUG was bound to AP∙A and AP∙G containing oligonucleotides.
The effect of Exonuclease III on MUG bound to AP·A

Passive diffusion of MUG from the AP·A site due to the unlabeled abasic competitor or non-specific competitor proceeded with similar rates; $k = 1.32 \pm 0.01 \text{ s}^{-1}$ and $k = 0.51 \pm 0.02 \text{s}^{-1}$, respectively.

Addition of Exonuclease III to the MUG & AP·A complex led to a decrease in $\Delta E$ with a rate similar to passive diffusion ($1.33 \pm 0.03 \text{ s}^{-1}$). The striking observation was that non-specific competitor and ExoIII led to a much smaller $\Delta E$ (-0.02 and -0.06, respectively) compared to abasic competitor (-0.11). Comparison of rates and amplitude of $\Delta E$ suggest ExoIII does not actively replace MUG glycosylase from the AP·A site. The small change in energy transfer upon mixing ExoIII that occurs with a rate similar to passive diffusion may be due to dissociation of MUG molecules that were bound non-specifically to the DNA.

As expected, mixing unlabeled abasic, non-specific competitor and ExoIII led to changes in anisotropy. A minute decrease in anisotropy was observed upon addition of non-specific competitor whereas abasic competitor led to a larger decrease in anisotropy. The rates of passive diffusion as determined by $\Delta E$ and anisotropy change were similar for abasic and non-specific competitor. Addition of ExoIII led to a rapid increase in anisotropy with a rate 70 fold faster than passive diffusion ($k = 224.71 \pm 11.47 \text{ s}^{-1}$). The fact that ExoIII binds rapidly but $\Delta E$ upon addition of ExoIII proceeds with the speed of passive diffusion confirms the presence of a non-active mechanism.

Then we asked the question: Is there any change in MUG’s diffusion when Exonuclease III cleaves 5’ to the abasic site? Dissociation of MUG from the abasic site was monitored by adding ExoIII and 1mM MgCl₂ simultaneously to the MUG & AP·A complex. $\Delta E$ and the rates were similar in the presence and absence of 1mM MgCl₂.
This suggests that cleavage of the abasic site does not lead to MUG’s dissociation. At MgCl₂ concentrations higher than 1mM, an MgCl₂ dependent decrease in ΔE was observed. This is likely to be due to ionic effects by MgCl₂. MgCl₂ may be leading to a molecular rearrangement that causes the reduction in FRET. No anisotropy change was observed upon mixing 1mM or 10mM MgCl₂ with the MUG & DNA complex, which indicates that MgCl₂ on its own does not lead to dissociation of MUG from the DNA.

We cannot draw a conclusion about the effect of Exonuclease III in the presence of concentrations higher than 1mM MgCl₂ due to the concentration dependent effect of MgCl₂ on FRET. MgCl₂ concentrations higher than 1mM may be causing structural changes in the oligonucleotide or in MUG. Mg²⁺ was found to affect the stabilities of RNA tertiary structures (Grilley et al. 2009). MgCl₂ ions were found to promote the structural rigidity of proteins as evidenced in the higher resistance to the heat-induced unfolding of the nuclear inclusion protein A in the presence of the MgCl₂ ions (Kim et al. 2000). Fluorescence resonance energy transfer (FRET)-based sensors have previously been developed to monitor Zn²⁺ levels, hence the binding of Mg²⁺ could be interfering with FRET between Alexa488 and HEX (Vinkenborg et al. 2009).

The effect of Exonuclease III on MUG bound to AP·G

MUG is known to make hydrogen bonds with the guanine on the complementary strand (Barrett et al. 1998), thus the consequence of Exonuclease III on the MUG & DNA complexes was also investigated with an oligonucleotide containing a AP·G mismatch. For this, MUG was mixed with HEX-U·G-Hairpin, which is processed to a HEX-AP·G-Hairpin upon cleavage of the glycosidic bond by MUG. Change in energy transfer upon mixing MUG with HEX-U·G-Hairpin occurred with a similar rate to HEX-AP·A-Hairpin and HEX-12AP·G oligonucleotides. Passive diffusion of MUG from the AP·G site (k= 1.86 ± 0.02 s⁻¹) upon mixing unlabeled abasic competitor progressed with the same rate as from the AP·A site (k= 1.32 ± 0.01 s⁻¹). In the case of MUG & AP·G complexes, Exonuclease III led to a rapid change in ΔE with a rate 44 times faster than
passive diffusion (85.5 ± 3.3s⁻¹). The amplitude of ΔE (-0.02) was again much smaller than in passive diffusion (-0.1). Addition of 1mM MgCl₂, which leads to ExoIII cleaving 5’ to the abasic site, did not change the rate or amplitude of ΔE. Comparison of ΔE upon mixing ExoIII versus buffer revealed that the change in amplitude and the rate was the same with buffer and ExoIII indicating ExoIII does not actively displace MUG from the AP•G site.

In order to confirm this, unlabeled competitor was added to the MUG & AP•G complexes after an incubation time with ExoIII to validate that MUG is still bound to DNA after the addition of ExoIII. The effect of ExoIII and passive diffusion on ΔE was investigated as a control. Addition of ExoIII led to a small, rapid decrease in ΔE (k = 85.5 ± 3.3s⁻¹) compared to a larger, slower change in ΔE through passive diffusion (k = 0.07 ± 0.0007 s⁻¹). Addition of unlabeled competitor after ExoIII led to a further and large decrease in ΔE (k = 0.17 ± 0.0002 s⁻¹). The finding that unlabeled competitor leads to a further decrease in ΔE after the addition of ExoIII supports our theory that ExoIII does not actively displace MUG from the AP•G site.

As with the AP•A oligonucleotide, 10mM MgCl₂ mixed with ExoIII led to a larger change in ΔE, which was due to the presence of 10mM MgCl₂ and not due to cleavage of the abasic site by ExoIII. Interestingly, when higher concentrations of MgCl₂ were mixed with unlabeled abasic competitor to induce passive diffusion, high MgCl₂ concentrations did not affect FRET. This suggests that MUG may be binding Mg²⁺ ions leading to reduction in FRET. When MUG diffuses from the abasic site due to competitor DNA, a large decrease in ΔE is observed and the effect of Mg²⁺ ions is not seen.

The rate of passive diffusion of MUG from AP•A DNA (1.32 ± 0.01 s⁻¹) and AP•G DNA (1.86 ± 0.02s⁻¹) were similar. This result is very interesting as it implies that having a
guanine as the complementary base does not affect passive diffusion of MUG from the abasic site.
Chapter 5

In vivo DNA repair by

MUG and SMUG
Chapter 5: *In vivo* DNA repair by MUG and SMUG

**Aim**

To investigate the role of MUG glycosylase in the *in vivo* repair of lesions introduced in bacterial DNA by the mutagen urethane. The potential of SMUG glycosylase compensating for the *mug* deficiency in *E. coli* cells was also explored and the effect of APE1, which is the next enzyme in BER, in SMUG bearing cells was assessed.

**Background**

Exocyclic DNA adducts are a unique class of ring-extended modifications formed by a range of chemicals most of which have been classified as animal and/or human carcinogens (Hang 2004). Etheno bases (ε) are the most extensively studied exocyclic lesions and arise through the addition of a two carbon unit to DNA bases (Figure 1-17). These lesions have attracted much attention as critical candidates in the etiology of human cancers because they lead to misincorporation upon replication or transcription (Moriya *et al.* 1994; Simha *et al.* 1994; Shibutani *et al.* 1996). Etheno adducts in DNA can also be formed by products of lipid peroxidation generated during the cellular metabolism (Chen & Chung 1996; Nair 1999). Increased levels of ε-adducts are observed in abnormalities with persistent oxidative stress such as Wilson’s disease, hepatitis and familial adenomatous polyposis (Bartsch & Nair 2002). Decreased repair of ε-adducts was also discovered in lung adenocarcinoma (Speina *et al.* 2003).
These lesions are primarily repaired by the base-excision repair pathway; nucleotide-excision and mismatch repair pathways have been implicated in the repair of some etheno lesions as well (Hang 2004). MUG and its human homologue TDG have been found to be involved in the repair of etheno lesions (Saparbaev & Laval 1998; Kavli et al. 2002; Saparbaev et al. 2002; O'Neill et al. 2003; Jurado et al. 2004). The ability of MUG glycosylase to process εC from εC-G pairs is 5 fold faster than uracil from U-G pairs (O'Neill et al. 2003). MUG can with very low efficiency also excise εA in DNA (O'Neill et al. 2003). Methyl-CpG binding domain protein (MBD4/MED1) (Petronzelli et al. 2000) and alkyl-N-purine DNA glycosylase (ANPG) (Saparbaev et al. 2002) have also been found to be involved in the repair of etheno lesions.

While no experimental structure of MUG or TDG complexed with DNA containing an εC is available, molecular modelling based on the structure of MUG complexed with the non-hyrolysable 2’-fluoro-deoxyuridine mispaired with deoxyguanidine (βFU·G) demonstrated that εC can be accommodated in the space of MUG’s pyrimidine binding pocket (Barrett et al. 1999). Cell extracts from mug+ cells were found to excise εC whereas this activity was missing in mug deficient cells (Lutsenko & Bhagwat 1999; O'Neill 2004). This suggested that MUG may be the only enzyme in E. coli responsible for removing these mutagenic products. Recent in vivo studies in our lab also showed that εC and εA accumulates in mug deficient E. coli cells suggesting an in vivo role for MUG in the repair of these lesions. It is not clear why E.coli would need an enzyme that repairs ε-adducts. Etheno lesions had not been found in E.coli and lipid peroxidation processes that are known to give rise to these lesions in eukaryotes are not known to exist in E.coli (Bartsch & Nair 2000). We aimed to further explore MUG’s role in the repair of these lesions in E. coli cells.

Urethane, which is a carcinogen associated with tumours of the liver, lung and skin in rodents (Zimmerli & Schlatter 1991) reacts with DNA bases, yielding etheno adducts (Ribovich et al. 1982; Sotomayor & Washington 1996; Barbin 2000). Etheno lesions
have been found to be associated with increased misincorporation of bases by DNA polymerases (Singer et al. 1984; Pandya & Moriya 1996; Shibutani et al. 1996). In this study, the role of MUG glycosylase in the repair of lesions introduced by urethane in the *E. coli* genome was explored. For this, *mug* deficient *E. coli* cells have been exposed to urethane followed by the measurement of cell viability and mutation frequencies. We also further looked in order to see whether the eukaryotic DNA glycosylase SMUG can repair urethane induced lesions in *E. coli* and if APE1 cooperates with SMUG in the repair these lesions.

**Mutations in *E. coli* cells**

When *E. coli* cells are inoculated in minimal media over a period of time they accumulate mutations. *E. coli* cells that are deficient in genes involved in BER have been found to accumulate more mutations compared to their wild type counterparts (Mokkapati et al. 2001). To assess the mutation frequency in *E. coli* cells, reverse or forward mutation assays can be used. Reverse mutation assays score the mutation frequency of kanamycin sensitive *E. coli* cells that accumulate mutations and become resistant to kanamycin. Forward mutation assays measure the mutation frequency of rifampicin sensitive *E. coli* cells that accumulate mutations and become resistant to rifampicin. The rifampicin resistance assay was found as a more sensitive tool for analyzing effects of mutations in DNA repair genes (Mokkapati et al. 2001).

Rifampicin inhibits the function of RNA Polymerase (RNAP) by blocking the transition from transcription initiation to transcription elongation (McClure & Cech 1978). In the presence of rifampicin, RNAP can only synthesize short RNA oligomers, and it was proposed that rifampicin exerts a steric hindrance of RNAP translocation along the nascent RNA path. Mutations in *E. coli* RNAP conferring rifampicin resistance (RifΔ) have been located exclusively on the second largest subunit of RNAP, the β-subunit,
encoded by the *rpoB* gene. Except for one located around the 5’ end, most of the Rif\(^r\) mutations in *E. coli* are found in three clusters near the middle of the *rpoB* gene and affect a limited amino acid segment of the \(\beta\)-subunit (Ovchinnikov *et al.* 1983; Lisitsyn *et al.* 1984; Jin & Gross 1988; Severinov *et al.* 1993). Genetic evidence indicates that amino acid residues in cluster I and cluster III interact, forming the rif region (Singer *et al.* 1993). These mutations define the rifampicin-binding sites in RNAP genetically.

**Objectives**

- Determine if MUG glycosylase is required to protect *E. coli* cells from the effects of urethane
- Analyze if the eukaryotic glycosylase SMUG can enhance the response of *E. coli* cells to urethane
- Monitor if the eukaryotic AP endonuclease (APE1) can improve the high mutation rates and cytotoxicity caused by SMUG

**Results**

**Does MUG glycosylase give an advantage to cells when treated with urethane?**

In order to further investigate the role of MUG glycosylase in the repair of etheno lesions, wild type and *mug*\(^r\) cells were treated with urethane, which is a mutagen that introduces etheno lesions in the DNA. After urethane treatment, the effect of the urethane on cell viability and mutation frequency in cells was measured.
MUG levels in *E. coli* cells increase during the exponential phase of growth and reach a plateau as the cells stop dividing (Mokkapati *et al.* 2001). In order to ensure a maximum level of MUG expression, *E. coli* cells were grown for 24 hours until they reached stationary phase and were then challenged with urethane. Another regulator of MUG activity is RpoS, which is a sigma factor for transcription of stationary phase-specific genes. MUG activity was found to be higher and was sustained up to 96 hours after the cells reach stationary phase in *rpoS*⁺ strains but not in *rpoS*⁻ strains (Mokkapati *et al.* 2001). Since RpoS level increases in response to environmental stresses such as nutrient deprivation, acidic pH, high temperature, oxidative stress and osmotic stress (Lombardo *et al.* 2004) minimal media (M63) was used to ensure maximum RpoS levels.

Urethane’s effect on cell viability of wild type and *mug*⁻ *E. coli* cells was analyzed by determining the viability of wild type and *mug*⁻ *E. coli* cells in LB media. After inoculation in minimal media for 24 hours, wild type and *mug*⁻ cells were treated with urethane (50, 100, 250, 500 and 1000mM). Samples were taken at 24 hours, 48 hours and 72 hours after urethane treatment and tenfold dilutions of each culture were spotted on LB plates (1→6) (Figure 5-1).

Treatment of wild type or *mug*⁻ cells with 50mM or 100mM urethane did not have any effect whereas treatment with 250mM urethane impaired viability of both cell types. Since an effect was observed 72 hours after treatment with 250mM urethane, only results at that time point are shown. Treatment with 500nM or 1000nM urethane exterminated all cells in 24 hours (data not shown). Interestingly, based on spotting experiments, 250mM urethane inhibited the growth of wild type cells more than *mug*⁻ cells. The viability of wild type cells was inhibited at the 10⁻³ dilution when treated with 250mM urethane whereas *mug*⁻ cells were not affected at the 10⁻³ dilution. The difference is minor and could be due to a difference in dilution between the two cell types. For further experiments, *E. coli* cells were treated with 250mM urethane in order to examine the requirement for MUG glycosylase upon urethane treatment.
Wild type and mug⁻ cells were treated with 50mM, 100mM, 250mM and 500mM urethane after inoculation in minimal media for 24 hours; samples were taken 72 hours after the addition of urethane and spotted on LB media to analyze viability. Cells that were treated with 500mM and 1000mM urethane did not grow at all, hence the data is not shown. 50mM or 100mM urethane did not affect the viability of wild type or mug⁻ cells whereas treatment with 250mM urethane reduced viability. Interestingly, when treated with 250mM urethane, viability of wild type cells was impaired at the 10⁻³ dilution whereas mug⁻ cells were not affected at the 10⁻³ dilution.

The effect of urethane on the viability of wild type and mug⁻ cells was also evaluated by quantifying the number of cells per ml in treated and untreated cultures. For this, wild type and mug⁻ E. coli cells were grown for 24 hours and treated with 250mM urethane. After treatment, cells were plated onto LB medium and the number of cells per ml was determined. % survival was defined as the ratio of the number of cells in treated culture to the number of cells untreated culture. Both wild type and mug⁻ cells were affected by...
treatment with 250mM urethane. Wild type cells were affected 48 hours after urethane treatment whereas *mug* cells were affected 24 hours after treatment (Figure 5-2).

![Figure 5-2 Cell survival following urethane treatment](image)

Wild type and *mug* *E.coli* cells were treated with 250mM urethane. The effect of urethane on cell survival was measured by determining the number of cells per 1 ml culture for each cell line. % survival was defined as the ratio of number of cells in the treated culture to the number of cells in the untreated culture. The time of urethane treatment was taken as time point: 0 Hrs. The experiments were performed in duplicates and SEM was calculated as described in Materials and Methods.

In order to analyze if MUG is required to repair lesions introduced in the DNA by urethane, the mutation frequency in wild type and *mug* cells was determined by using the forward mutation assay as described by Mokkapati *et al.* (Mokkapati *et al.* 2001). This assay is based on determining the number of rifampicin sensitive bacteria that become resistant to the antibiotics. Wild type and *mug* cells are both rifampicin sensitive, cells growing on rifampicin plates mostly acquire resistance through accumulation of mutation(s) in the *rpoB* gene (Ovchinnikov *et al.* 1983; Lisitsyn *et al.* 1984; Jin & Gross 1988; Severinov *et al.* 1993). Mutation frequency is calculated by normalizing the number of resistant bacteria against the number of viable cells (*10^8*).
The ratio of the mutation frequency in treated and untreated cultures was taken and plotted against time in order to quantify the effect of 250mM urethane on mutation frequencies. 250mM urethane induced mutations in wild type *E. coli* cells while the mutation frequency of *mug* *E. coli* cells was not affected by urethane (Figure 5-3).

![Figure 5-3 Urethane introduces mutations in wild type cells but not in *mug* cells](image)

Wild type and *mug* *E.coli* cells were treated with 250mM urethane. The mutation frequency was calculated by dividing the number of colonies on rifampicin plates by the number of viable colonies (10^8) per 1ml. The time of urethane treatment was taken as time point: 0 Hrs. The mutation frequency in wild type and *mug* cells was plotted against time in order to quantify the effect of 250mM urethane on mutation frequencies. The experiments were performed in duplicates and SEM was calculated as described in the Materials and Methods section.
Can SMUG glycosylase repair lesions introduced by urethane in *E. coli*?

SMUG is a DNA glycosylase, which was initially found in vertebrates and insect cells (Nilsen *et al.* 2001); its orthologs have recently been identified in prokaryotes (proteobacteria and planctomycetes) as well (Pettersen *et al.* 2007) but this glycosylase does not exist in *E. coli* or yeast. Prokaryotes encoding SMUG also lack orthologs of other members of the UDG family (MUG or UDG) indicating SMUG may be the only uracil-DNA glycosylase in these species (Pettersen *et al.* 2007). It is mainly active against uracil (Wibley *et al.* 2003) and 5-Hydroxy-uracil (HmU) (Boorstein *et al.* 2001) in double stranded DNA. SMUG glycosylase has been found to process etheno cytosine from G•εC mismatches (Wibley *et al.* 2003). We wondered whether human SMUG can act as a functional homologue for MUG in *E. coli* cells. To answer the question, *mug*⁻-SMUG cells were treated with 250mM urethane after 24 hours incubation in minimal media. The ratio of number of viable cells in treated and untreated cultures was taken and plotted against time in order to quantify the effect of 250mM urethane on cell survival. The time of urethane treatment was taken as time point: 0 Hrs. As can be seen from the ratio of treated and untreated cells, SMUG did not give *mug* deficient cells an advantage when treated with urethane (Figure 5-4).
Does SMUG glycosylase affect mutation frequencies and cell survival of *E. coli* cells?

In *E. coli* cells transformed with engineered CDG and TDG, the mutation frequency was increased 10 and 100 fold compared to wild type cells. TDG and CDG also reduced survival of *E. coli* host cells (Kavli *et al.* 1996). This information led us to ask the question whether SMUG glycosylase had an impact on mutation frequencies and survival of *E. coli* cells.

Comparison of the number of viable cells with and without SMUG revealed that SMUG reduced the number of viable *mug* and wild type cells. Cells with SMUG had reduced number of cells per ml culture compared to their counterparts without SMUG. Analysis
of mutation frequencies also revealed that SMUG increased the mutation frequency in wild type and mug− cells (Figure 5-5).

Figure 5- 5 SMUG increases mutation frequencies and is cytotoxic for E. coli cells

The number of viable cells per ml was measured by determining the number of wild type-SMUG (A) and mug-SMUG (B) cells per 1 ml culture. Mutation frequency of wild type-SMUG (C) and mug-SMUG (D) bacteria was assessed by calculating the number of rifampicin resistant bacteria and normalizing against the number of viable cells (∗10^8). Experiments were performed in duplicates and SEM was calculated as described in the Materials and Methods section.

mug deficient cells expressing SMUG had a lower number of viable cells per ml compared to their counterparts without SMUG. To our surprise, mug− -SMUG cells showed increased cell division over time compared to their mug− counterparts. Cell
division (%) was determined by taking the number of cells at time 0 Hrs as 100%. Percentage increase in the number of cells was calculated as a ratio to cells at further time points to the number of cells at time 0 Hrs (Figure 5-6B). Interestingly, SMUG did not provide a boost in cell divisions to wild type cells (Figure 5-6A).

![Graph showing cell division over time for WT and WT-SMUG](image)

Figure 5-6 SMUG expressing mug cells have a boost in cell division

mug cells (B) that express SMUG (■) have an advantage in cell division and divide 9 fold after being plated on LB medium following inoculation in minimal liquid medium, while mug cells divide only 2 fold in 24 hours. WT cells expressing SMUG (A) do not have an advantage in cell division. Experiments were performed in duplicates and SEM was calculated as described in the Materials and Methods section.

Can human AP Endonuclease I balance SMUG’s effect on cell viability and mutation frequencies?

Previous experiments revealed that SMUG glycosylase decreases the number of cells per ml and increases mutation frequencies in wild type and mug cells. SMUG was also found to give mug cells a significant boost in cell division. The activity of SMUG against uracil containing DNA was found to be enhanced by AP endonuclease (APE1) (Kavli et al. 2002; Pettersen et al. 2007). APE1 has also been demonstrated to affect the catalytic rate of several other DNA glycosylases (Hill et al. 2001; Fitzgerald & Drohat 2008). We
speculated that SMUG’s impact on the reduction in cell numbers and increase in mutation frequencies might be due to the glycosylase staying tightly bound to its product leading to cell death or mutations. If that is the case and if AP endonuclease increases the turnover of SMUG \textit{in vivo}, the presence of APE1 could possibly enable SMUG to dissociate from the DNA. To test if APE1 has an effect on cell numbers and high mutation frequencies caused by SMUG, the endonuclease was transformed into SMUG expressing wild type and \textit{mug} cells. The APE1 bearing construct has previously been used to solve the structure of APE1. Although it misses the 35 N-terminal aminoacids, this truncated version of APE1 (APE1$_{36-318}$), has been shown to retain full ‘redox and endonuclease repair activity (Gorman \textit{et al.} 1997). The % survival and mutation frequencies of wild type and \textit{mug} cells bearing SMUG and APE1 was measured. APE1 did not have an effect on the viability of wild type or \textit{mug} cells on its own but also did not improve viability of cells bearing SMUG (Figure 5-7).
Figure 5-7 The effect of SMUG and APE1 on the viability of wild type and mug<sup>-</sup> cells

In order to analyze if the presence of APE1, which has been shown to stimulate SMUG’s activity in vitro, increases the number of wild type and mug<sup>-</sup> cells expressing SMUG, APE1 was transformed into WT-SMUG and mug<sup>-</sup>-SMUG cells. APE1 did not affect viability of wild type (C) or mug<sup>-</sup> (D) cells on its own but also did not improve the decrease in cell numbers caused by SMUG (A and B).

The presence of APE1 on its own increased the mutation frequency in mug<sup>-</sup> cells and did not reduce the high mutation frequencies of mug<sup>-</sup>-SMUG cells (Figure 5-8). APE1 did not increase mutation frequency of wild type cells on its own and increased the mutation frequency of wild type cells at 24 hours which might be an experimental error since it only occurred at one time point (Figure 5-8).
Figure 5-8 APE1 does not decrease the high mutation frequencies caused by SMUG

In order to evaluate if APE1 reduces the mutation frequencies induced by SMUG in wild type and mug cells, the endonuclease was transformed into SMUG bearing cells. APE1 did not induce mutations in wild type cells on its own but increased the mutation frequencies in mug deficient cells. When combined with SMUG, the endonuclease did not affect the mutation frequency in wild type cells except at 24 hours. In mug deficient cells bearing SMUG, APE1 did not reduce the mutation frequency.

We had found that SMUG boosts cell divisions of mug deficient cells but doesn’t have any effect on the cell division of wild type cells. Further analysis on APE1’s influence on cell division of cells bearing SMUG was conducted. Cell division (%) was determined by taking the number of cells at time 0 Hrs as 100%. Percentage increase in the number of cells was calculated as a ratio of cells at further time points to the number of cells at time 0 Hrs. APE1 did not have any effect on cell divisions of wild type cells with or without
SMUG. *mug*<sup>−</sup> cells with APE1 showed the same cell division pattern as *mug*<sup>−</sup> cells whereas APE1 significantly boosted cell divisions in *mug*<sup>−</sup> - SMUG cells (Figure 5-9).

![Figure 5-9 SMUG and APE1 strongly enhance cell division in *mug* deficient cells](image)

We have found that the presence of SMUG boosts cell divisions and increases mutation frequency in *mug* deficient cells. APE1 is believed to stimulate turnover of SMUG, hence the effect of APE1 on cell divisions in SMUG bearing cells was examined. APE1 did not affect cell division in wild type cells. In *mug* deficient cells, the presence of APE1 on its own did not affect cell division whereas the presence of SMUG and APE1 together led to a 100 fold boost in cell divisions compared to *mug*<sup>−</sup> cells once the cells were plated on LB medium after being inoculated in liquid minimal medium.
Discussion

Does MUG glycosylase confer the cells an advantage upon urethane treatment?

MUG glycosylase has been found to repair etheno (ε) lesions *in vitro* and *in vivo* (Saparbaev & Laval 1998; Lutsenko & Bhagwat 1999; O'Neill *et al.* 2003). Urethane, which is a carcinogen associated with tumours of the liver, lung and skin in rodents (Zimmerli & Schlatter 1991) reacts with DNA bases, yielding etheno adducts (Ribovich *et al.* 1982; Sotomayor & Washington 1996; Barbin 2000). It is intriguing for *E. coli* cells to have an enzyme that repairs these lesions since lipid peroxidation processes that are known to give rise to etheno lesions are not known to exist in *E. coli* (Bartsch & Nair 2000).

We aimed to further analyze the role of MUG glycosylase in the repair of ε-lesions *in vivo* and monitored the effect of urethane on wild type and *mug* deficient cells. Both wild type and *mug* deficient cells were exterminated by 500mM and 1000mM urethane whereas 50 or 100mM urethane did not affect cell survival. Treatment of all *E. coli* cells with 250mM urethane reduced survival of both cell types to a measurable level; hence 250mM urethane was selected for experiments to further analyze the effect of urethane on survival and mutation frequencies.

Because wild type cells have the intact repair machinery, which is needed to deal with the lesions caused by urethane, we expected them to be less susceptible to urethane and be able to grow better than *mug* deficient cells. In our study, the presence of MUG glycosylase did not provide any advantage to wild type cells when treated with urethane. It is possible that in *mug* cells, etheno lesions induced by urethane are being repaired by another enzyme. For instance AlkA is another *E. coli* DNA glycosylase found to remove etheno lesions (Saparbaev *et al.* 1995). Since the possibility that urethane might be
causing a variety of lesions in addition to etheno bases can also not be ruled out, we cannot draw a conclusion about MUG’s in vivo role in the repair of etheno lesions induced by urethane.

Analysis of rifampicin revertants revealed that in wild type cells, urethane enhanced mutation frequencies whereas mutation frequencies in mug deficient cells were not affected by the chemical. In addition to base excision repair, a role for nucleotide-excision and mismatch repair pathways have been implicated in the repair of etheno lesions (Hang 2004). Therefore, our results might be due to involvement of alternative mechanisms such as nucleotide excision repair or mismatch repair in mug deficient cells in the repair of urethane induced lesions.

**Can the eukaryotic glycosylase SMUG complement base excision repair defects in E. coli cells?**

Several DNA repair pathways are evolutionarily conserved between prokaryotes and eukaryotes therefore it is not surprising that eukaryotic DNA repair enzymes are able to complement DNA repair deficient E. coli strains (O'Connor & Laval 1990; Tano et al. 1990; Olsen et al. 1991; Otterlei et al. 2000). Eukaryotic uracil DNA glycosylase has previously been found to remove uracil from U: A and U: G base pairs in E. coli (Olsen et al. 1991). Human UNG was found to fully complement an E. coli ung’ strain by lowering spontaneous mutations frequencies to wild type levels (Otterlei et al. 2000). The mammalian DNA repair enzymes 3-methyladenine-DNA glycosylase and O6-methylguanine-DNA methyltransferase have been cloned by phenotypic rescue of DNA repair deficient E. coli mutants (O'Connor & Laval 1990; Tano et al. 1990). We were curious to see if SMUG glycosylase, which has recently been found to repair etheno lesions (Wibley et al. 2003) can compensate for the mug deficiency in E. coli cells, which were treated with urethane.
In our study, SMUG did not provide any advantage to \textit{mug} cells in survival upon urethane treatment. Interestingly, SMUG reduced the survival and increased the mutation frequency of wild type and \textit{mug} cells. These results are in alignment with other studies looking at the effect of over-expression of DNA glycosylases and introduction of excessive abasic sites in \textit{E. coli} cells (Schaaper \textit{et al.} 1982; Otterlei \textit{et al.} 2000). Kavli \textit{et al.} have engineered variants of human uracil-DNA glycosylase (UDG) to remove thymine (TDG: Thymine DNA glycosylase) or cytosine (CDG: Cytosine DNA glycosylase) by replacing active site Asn204 with Asp to obtain a CDG and by replacing Tyr147 with Ala to obtain a TDG (Kavli \textit{et al.} 1996). Expression of these variant glycosylases from an inducible vector was found to lead to accumulation of abasic sites in DNA which resulted in reduced cell survival. Like in our study, \textit{E. coli} cells over-expressing CDG and TDG showed a 10 and 100 fold increase in mutation frequency compared to their wild type counterparts (Otterlei \textit{et al.} 2000). Berdal \textit{et al.} found that over-expression of another DNA glycosylase, AlkA resulted in the removal of normal bases from the DNA and induced mutations (Berdal \textit{et al.} 1998). The increase in mutation frequency was proportional to the efficiency of glycosylases to remove bases from the DNA (Kavli \textit{et al.} 1996) and to the number of apurinic sites introduced (Schaaper \textit{et al.} 1982).

A striking finding of our study was that the presence of SMUG gives a significant boost in cell divisions to \textit{mug} deficient cells but not to wild type cells once the cells are plated on LB medium after being incubated in minimal medium. \textit{mug}^{-} SMUG cells had a tenfold increase in cell divisions compared to their \textit{mug}^{-} counterparts. Possible reasons for this finding are discussed below.
Does AP endonuclease work together with SMUG?

Deficiencies in Exonuclease III or in Endonuclease IV increase cytotoxicity and lead to higher mutation frequencies supporting the role of abasic sites in cytotoxicity and mutagenesis (Otterlei et al. 2000). AP endonuclease has been demonstrated to affect the catalytic rate of several DNA glycosylases in vitro (Hill et al. 2001; Fitzgerald & Drohat 2008) including SMUG (Kavli et al. 2002; Pettersen et al. 2007), which is believed to be due to SMUG being displaced from abasic sites by APE1.

In our study, expression of SMUG glycosylase was found to reduce survival and increase mutation frequencies in wild type and mug deficient E. coli cells. We wanted to explore if AP endonuclease (APE1) would reverse SMUG’s effects on cells by enhancing cell survival and decreasing mutation frequencies. To investigate this; human AP endonuclease was expressed in SMUG bearing wild type and mug cells followed by measurement of mutation frequencies and cell survival. The presence of APE1 in SMUG bearing cells did not improve cell survival or mutation frequencies. In mug deficient cells, APE1 on its own actually led to an increase in mutation frequencies due to an unknown mechanism. It is striking that APE1 on its own increases mutation frequencies of mug deficient cells but not of wild type cells. Although the experiment looking at APE1’s effect on mug cells needs to be repeated since it was performed only once, the result suggests different DNA repair pathways being active in wild type and mug cells. Analysis of the activity of genes responsible for mutagenesis in stationary phase cells will shed light into the difference between responses of wild type and mug cells.

Interestingly, in our study SMUG was found to significantly increase cell divisions in mug deficient cells but did not have an effect on wild type cells. This impact of overexpression of DNA glycosylases has not been explored by other labs. Expression of APE1 together with SMUG gave mug cells over 100 fold boost in cell divisions when
they were plated on LB medium after being inoculated in liquid minimal medium. On its own APE1 did not have any effect on cell divisions. This suggests the presence of a different pathway in these cells, which allows mug⁻ cells to repair abasic sites through a different mechanism than the one used by wild type cells. This mechanism may be allowing mug⁻ cells to divide faster once plated on LB medium.

**Future experiments**

**Background about adaptive mutations in *Escherichia coli***

Stress-induced mutations in bacteria that confer a growth advantage, relieving the stress, are called adaptive mutations (Hersh et al. 2004). Adaptive mutations have been extensively explored in *E. coli* cells that were deleted for the chromosomal lac operon and carried an F’ episome with a mutant lac allele. This allele derives from a fusion of lacI and lacZ and has a +1 base-pair frameshift in the lacI sequence (Calos & Miller 1981). Cells are plated onto solid medium with lactose as the sole carbon source and Lac⁺ revertant colonies accumulate. About half of the colonies observed on the second day of incubation result from mutations that occurred during growth of the cultures prior to plating; the remaining day2 colonies and the ones that appear on later days are stress-induced adaptive mutants, formed after exposure to the lactose medium (Cairns & Foster 1991). Adaptive mutations are distinguished from normal growth-dependent mutations. Adaptive reversion of a lac frameshift mutation requires the RecA-RecBCD pathway for homologous recombination whereas no such association was seen during non-selective growth (Harris et al. 1994). The characteristics of adaptive mutations are also different from growth dependent mutations. Adaptive mutations in the lac operon were found to be single base deletions and nearly all occur in mononucleotide repeats whereas a different and much more diverse spectrum of mutations is observed in the same operon in growing cells. While only one sort of adaptive mutation is observed, the growth dependent
mutations include duplications, insertions, and deletions of 1 from 112 nucleotides while the small deletions are not confined to mononucleotide repeats (Rosenberg et al. 1994).

RecA-dependent adaptive reversion was also found to be dependent on RuvAB and RuvC whereas RecG mutations led to an increase in mutations (Rosenberg et al. 1994). During recombination initiated by RecA-mediated strand exchange, four-way (Holliday) junctions are translocated by junction-specific helicases, which in *E. coli* are RuvAB and RecG. RuvC, which was found to be involved in adaptive mutations (Rosenberg et al. 1994) appears to act in concert with RuvAB (Mandal et al. 1993). Figure 5-10 provides a brief overview of the model for DNA repair through recombination and resolution of Holiday junctions.

Figure 5-10 A model for post-replication repair of damaged DNA

A DNA replication fork encounters a lesion in the template strand (closed triangle) (i) and resumes DNA synthesis downstream, leaving a gap in the daughter strand (ii). RecA protein polymerizes at the gap and initiates strand exchange with the sister duplex (iii). Branch migration by RuvAB or RecG extends the heteroduplex joint beyond the lesion and leads to a Holliday junction (iv). Excision of the dimer (v) followed by further processing of the junction by RuvC cleavage (vi) or by reverse branch migration with RecG or RuvAB (vii) separates the sister duplexes and allows repair to be completed by filling in the remaining gaps. (Adapted from Mandal et al. (Mandal et al. 1993)).
The mechanism of adaptive mutations has been explored with lac operon genes but the lac\textsuperscript{+} adaptive reversions were not only directed to lac genes. Hypermutations were also observed in the tetracycline gene inactivated through a frameshift mutation and in multiple other genes. Hypermutation of unselected genes was seen amongst adaptive lac\textsuperscript{+} revertants but not amongst lac\textsuperscript{-} cells from the starved cultures which suggested that a subpopulation of cells exposed to starvation on Lactose experiences a genome-wide hypermutable state (Torkelson et al. 1997).

In E. coli, the damage repair response which is activated upon DNA damage due to UV light or the mutagen ethyl methanosulphonate is called an SOS response (reviewed in (Schlacher & Goodman 2007)). E. coli responds to DNA damage by calling upon a number of genes contained in the SOS regulon. More than 40 genes are induced as part of the SOS response including error-prone DNA polymerases that lead to adaptive mutations and allow cell growth. SOS response genes are transcriptionally up-regulated following cleavage of the LexA repressor protein mediated by a RecA nucleoprotein filament. In response to DNA damage, RecA becomes activated by loading on ssDNA. Mutations in lexA, recA and umuC led to non-mutability upon UV light and ethylmethanesulphonate (Schlacher & Goodman 2007). Mutations in the lac assay system seem to be due the activation of the SOS response since several SOS-controlled genes are required for the process including dinB, recA, recBC, ruvA and ruvB (Galhardo et al. 2009). Expression of the error prone DNA polymerase DinB is controlled by the stress-response sigma factor, RpoS (Layton & Foster 2003), which also controls the expression of MUG glycosylase in E. coli cells (Mokkapati et al. 2001).

In E. coli, there are two polymerases that are identified with error-prone DNA replication, also called translesion DNA synthesis (TLS). These are the SOS-induced UmuC protein in complex with activated UmuD (UmuD\textsubscript{2}C or Pol V) and the UmuC homologue encoded by dinB dene (pol IV) (Godoy et al. 2007). The expression of both error-prone E. coli DNA polymerases Pol V (UmuD\textsuperscript{’}2C complex) and Pol IV (DinB) increases in response to
DNA damage and is induced through cleavage of LexA through RecA::ssDNA nucleoprotein filaments (Wagner et al. 1999). The umuC and umuD gene products combine to form a potent mutator DNA polymerase, E. coli DNA pol UmuD\textsubscript{2}C, which requires activation by the RecA protein and is responsible for the vast majority of UV-damage-induced chromosomal mutations (Schlacher & Goodman 2007).

In the study by Otterlei et al. engineered variants of UDG that remove thymine (TDG) and cytosine (CDG) from the DNA led to an increase in mutation frequencies in E. coli cells. The study looked at rifampicin revertants similar to our assay. When mutation frequencies were monitored in an umuC deficient background, 2-3 fold reduction in mutation frequencies was observed with CDG and TDG, respectively. The fact that mutagenesis is not completely abolished in the umuC mutant suggests that UmuC is important but not essential for mutagenesis (Otterlei et al. 2000). DinB was found to be responsible for SOS-induced mutagenesis in E. coli (Galhardo et al. 2009). It will be of interest to evaluate whether DinB or the UmuD’\textsubscript{2}C complex is responsible for the increased mutation rates observed in SMUG and APE1 expression cells.

It has been shown that multiple damaged sites consisting of two or more damages within 20 base pairs can lead to double strand break in DNA (Harrison et al. 2006). These findings suggest a signaling pathway, in which excessive abasic sites may lead to double strand breaks resulting in activation of recombination and error-prone DNA replication.

Mutagenesis upon growth limiting conditions and stress is complex. Further examination of the significance of various genes involved in recombination, translesion lesion synthesis and SOS response is necessary to comprehend the players responsible for mutagenesis resulting from abasic sites. It will be highly interesting to decipher the genes involved in mutagenesis, cytotoxicity and boost in cell division in mug deficient cells expressing SMUG and APE1.
Experiments

The mechanisms and regulation of mutations in *E. coli* are of considerable interest because they represent model systems for understanding interactions between the environment and the genome, particularly if and how stressful conditions influence genetic change. Pathways leading to mutation-prone cellular replication mechanisms in bacteria are especially of interest as the knowledge may assist us in developing efficient bacterial therapies and possibly in avoiding antibiotic resistance in bacteria.

It will be intriguing to decipher common signaling pathways underlying the high cytotoxicity and mutation frequencies as well as to dissect the genes that are responsible for the increased cell divisions of *mug* cells.

In order to keep the integrity of the genome; base excision repair, nucleotide excision repair, recombination, mismatch repair work together. For instance, it has been shown that nucleotide excision repair and homologous recombination both repair DNA-protein crosslinks (DPC), but differently. NER repairs DPC’s that are smaller than 12–14 kDa whereas larger DPC’s are processed by RecBCD dependent homologous recombination (Nakano *et al.* 2007). The higher mutation frequencies in *mug* - SMUG cells compared to their wild type-SMUG counterparts might be due to accumulation of more, bigger lesions which are repaired through homologous recombination via the RecBCD pathway (Harris *et al.* 1994); leading to higher mutation frequencies.

Questions important to be answered to delineate the players underlying the high cytotoxicity, mutation frequencies and the increased cell divisions of *mug* cells are:
- Is SOS response being activated in wild type and mug' cells, if yes- is it to the same extent? Upon activation of the SOS response RecA accumulates and activates transcription genes under the recA promoter. A calorimetric assay for β-galactosidase production or luciferase expression under the recA promoter may be used to validate activation of the SOS response.

- RecBCD dependent homologous recombination is responsible for repairing larger DNA-protein cross links. Is it being activated in wild type and mug' cells? In order to dissect the role of recombination in mutagenesis due to SMUG, the effect of mutations in recB, recC and recD on mutagenesis, cell survival and the cell division can be analyzed.

- It will be highly interesting to evaluate what kinds of lesions are predominantly present and which polymerase is responsible for the mutations introduced by SMUG glycosylase in wild type and mug deficient cells. In order to answer these questions, the second largest subunit of RNA polymerase, the β-subunit, encoded by the rpoB gene from rifR colonies should be sequenced. Mutagenesis in our assays has been evaluated by analyzing the number of rifampicin revertants. To explore which error-prone DNA polymerase is responsible for the lesions, mutation frequencies induced by SMUG glycosylase in a dinB', umuC' and dinB' umuC' background may be analyzed.

- Is SOS response activated in response to urethane in wild type and mug' cells? Since wild type cells have a higher mutation frequency than mug' cells when treated with urethane, it will be of interest to evaluate if the SOS response is responsible for the difference between these cell types.
We should note that SMUG and APE1 expression levels will also need to be monitored in order to draw solid conclusions between protein levels and mutagenesis, cell survival and cell division. A significant effect was seen by introducing these eukaryotic enzymes into *E. coli* cells but drawing solid conclusions will require analyzing expression levels of both proteins.
Chapter 6

General Discussion & Conclusion
Chapter 6: General Discussion & Conclusions

Binding of MUG glycosylase to DNA

In this project, binding characteristics of MUG glycosylase to DNA were investigated and the requirement for multiple MUG molecules to process a lesion was explored. We found that MUG binds non-specific and abasic DNA with cooperativity whereas binding to abasic DNA exhibits higher cooperativity than non-specific binding. Although the Hill coefficient is not a direct measure of stoichiometry, it is widely used as a measure of the minimum number of interacting ligands (Lee et al. 2004; Weinberg et al. 2004). Binding affinity and cooperativity was not dependent on the size of the oligonucleotide as it did not change for the two oligonucleotides tested.

In order to determine whether the measured anisotropy is subject to significant deviations due to intensity or lifetime changes, we determined the rotational correlation time directly from time-resolved studies. Monitoring change in rotational correlation times confirmed cooperativity in binding and also demonstrated that lifetime measurements are more sensitive than steady state anisotropy measurements. These results also establish that fluorescence anisotropy is a suitable method for monitoring free and bound DNA species.

Competition binding experiments further supported the claim that two MUG molecules bind one DNA. At a 1:1 MUG:DNA ratio, the anisotropy was lower than observed in 2:1, indicating that the DNA is not in a fully bound state, and that a 1:1 stoichiometry of MUG:DNA is insufficient to fully bind the abasic DNA. At a MUG : DNA ratio of 2:1, the anisotropy was much higher and did not increase much at 3:1 and 4:1 ratios, consistent with a near saturated complex. Plotting the anisotropy versus the
stoichiometric balance of competitor and abasic DNA clearly showed that MUG binds DNA in a 2:1 stochiometry.

**Two MUG’s are needed for maximum activity**

Since our binding experiments suggested a 2:1 binding stochiometry for MUG, we also explored whether the presence of multiple MUG’s is necessary for maximum glycosylase activity. As established by O’Neill *et al.* (O’Neill *et al.* 2003), the turnover of MUG glycosylase is limited by the rate of product dissociation. Analysis of substrate cleavage with different MUG : DNA ratios clearly demonstrated that a minimum MUG : DNA ratio of 2:1 is required for optimal glycosylase activity. A similar result was also obtained with MutY, a DNA glycosylase involved in the removal of adenines from G·A and OG·A mismatches (Pope *et al.* 2002). Pre-steady state active titrations demonstrated that a MutY: DNA ratio of 2:1 is required for maximum glycosylase activity (Wong *et al.* 2003).

The activity assay is sensitive to the presence of inactive protein in the preparation. The possibility that the requirement for a 2:1 ratio of MUG : DNA for maximum glycosylase activity may be due to inactive MUG was considered. Hence the activity assays were repeated with MUG from different enzyme preparations. The fact that the need for the 2:1 MUG : DNA ratio was consistently observed as well as the binding being consistent between different enzyme preparations demonstrates that inactive protein is unlikely to be a problem.
Electrophoretic mobility shift assay provides additional information about MUG binding

In order to gain further insight into the individual MUG & DNA complexes, electrophoretic mobility shift assay (EMSA) was performed with two different oligonucleotides (H6AP-G and H12AP-G) which had also been used in fluorescence anisotropy measurements. MUG glycosylase formed two prominent bands with both oligonucleotides. In addition to these two strong bands, additional weaker complexes were observed with the longer oligonucleotide (H12AP-G). The fact that the extra bands were not observed with H6AP-G and are significantly fainter than the two major bands suggests that they are non-specific as the only difference between these two oligonucleotides is the number of non-specific base pairs. Non-specific binding by MUG was also analyzed by EMSA. Binding to H6C·G was significantly weaker than to H12C·G and MUG also produced additional complexes with the longer non-specific oligonucleotide (H12C·G). These results support the notion that non-specific binding is affected by the length of oligonucleotides, consistent with multiple proteins binding non-specifically to a single DNA molecule.

Non-specific binding is common among DNA binding enzymes. Binding is thought to initially occur at a random, non-specific site and the protein transfers to the specific site either through sliding (one dimensional) or through multiple dissociation/re-association events where the protein hops along the DNA until it reaches the specific site (Halford & Marko 2004; Halford 2009). MUG binding to DNA non-specifically is aligned with other DNA binding enzymes that bind DNA non-specifically (Fried et al. 1996; Pray et al. 1998; Weinberg et al. 2004).

Competition experiments with EMSA analysis provided an interesting insight into the nature of the MUG & DNA complexes. The first MUG & DNA complex formed was
stronger and couldn’t be competed off even in the presence of excess unlabeled abasic DNA. MUG formed aggregates when additional non-specific DNA was added.

Although electrophoretic mobility shift assay (EMSA) is very valuable for the analysis of protein & DNA complexes, there is speculation concerning the “equilibrium nature” of EMSA. Fluorescence anisotropy allows solution based analysis of proteins binding to DNA and is therefore a method that monitors equilibrium conditions. The EMSA analysis reported here revealed $K_d$ values for MUG binding abasic DNA similar to the $K_d$ values observed with fluorescence anisotropy measurements indicating fine qualitative agreement between the two different experiments. Equilibrium dissociation constants and cooperativity indexes obtained by EMSA are 1.5-2 fold lower than values acquired through anisotropy measurements, this isn’t a big difference for $K_d$, but it is a big difference for n. Since fluorescence anisotropy measurement is a solution based technique, it is likely to give measurements closer to true equilibrium conditions. The fact that the difference is consistent for both oligonucleotides (6AP∙G and 12AP∙G) suggests that the slight discrepancy may be due to experimental differences.

Obtaining similar equilibrium dissociation constants in both experiments suggests that bands observed in EMSA are close to equilibrium conditions and can be used to analyze the individual complexes formed by MUG glycosylase. Gel mobility shift assays are known to be sensitive to buffer-dependent artifacts. The fact that EMSA gels were run in TBE buffer (pH 8.3), which has a pH close to MUG reaction buffer (pH 8.0) also provides favorable conditions for MUG & DNA complexes.

Protein – DNA stoichiometries cannot be reliably estimated from the extent of gel shift in EMSA experiments. Therefore, expanding our understanding of the stoichiometry of individual complexes seen in EMSA will require further analysis as discussed below.
MUG binding is affected by an increase in ionic strength

An increase in salt concentration has been shown to have a negative effect on protein-protein association (Schreiber & Fersht 1996) and on the non-specific interactions between proteins and DNA (Record et al. 1976). Increasing salt concentrations reduced cooperativity of MUG binding to non-specific and abasic DNA and also reduced the equilibrium dissociation constant of MUG to both oligonucleotides. Non-specific binding by MUG glycosylase was found to be highly sensitive to salt concentration, suggesting that MUG primarily interacts with the DNA backbone via ionic contacts. An increase of the ionic strength from 50mM to 150mM NaCl led to a threefold reduction of the equilibrium binding constants for non-specific DNA whereas 150mM NaCl did not affect binding to abasic DNA. At 150mM NaCl, MUG’s affinity for abasic DNA stayed the same but its binding cooperativity decreased from 4.2 to 1.6. Increasing the ionic strength to 300mM NaCl reduced binding cooperativity to 1 and led to a 155 fold reduction in cleavage of the abasic site by MUG. Although at 300mM NaCl, an anisotropy value of 0.26 was observed with 5µM MUG, which corresponds to ~ 81% binding, only 20% cleavage of the abasic site by MUG was observed at this salt concentration.

Even though we cannot rule out the possibility that salt may be affecting MUG’s activity due to a different reason in addition to reducing cooperativity, the fact that 81% binding does not correspond with the enzyme’s glycosylase activity strongly suggests a requirement for cooperativity in the cleavage of the N-glycosidic bond.
DNA glycosylases and oligomerization

To determine the molecular weight and self-association of MUG in solution, sedimentation equilibrium assay was performed in collaboration with Professor Halford’s group at University of Bristol. Sedimentation equilibrium analytical ultracentrifugation is a powerful technique for characterizing the solution-state behavior of macromolecules. This technique has been widely used to study oligomerization of proteins in solution such as p53 (Weinberg et al. 2004); STAT1 (Wenta et al. 2008) and RAF (Rajakulendran et al. 2009) or to study Uracil-DNA glycosylase complexed with its inhibitor Ugi (Sanderson & Mosbaugh 1996). Molecular weight of MUG obtained at different salt concentrations was 18.7 kDa, which corresponds to MUG being present in a monomeric form. Sedimentation equilibrium assay could not be used to characterize MUG & DNA complexes as EMSA reveals that there are more than one species present.

Here we demonstrate for the first time a 2:1 stochiometry for MUG binding the DNA. A requirement for a 2:1 MUG : DNA ratio for maximum glycosylase activity has also not been demonstrated before. So far MUG glycosylase and its human homologue TDG, both glycosylases responsible for repairing uracil, etheno lesions and thymine mismatches in DNA (Barrett et al. 1998; Barrett et al. 1999) (Neddermann & Jiricny 1993) (Bennett et al. 2006) were assumed to bind DNA as a monomer.

Even though two MUG monomers have previously been observed in co-crystals, dimerization was not taken into account (Barrett et al. 1999). In the study by Barrett et al. where two MUG monomers were observed, both MUG’s had excised one uracil each, therefore the authors assumed the two monomers were due to each MUG independently binding to a U·G mismatch as well as MUG’s interactions with the crystal lattice but not due to a functional necessity. Although the two MUG molecules had bound forming a protein-protein interface with each other, the structure was published with one MUG
glycosylase bound to DNA (Barrett et al. 1998). We have reconstructed the dimer based on the PDB file:1MWI. Figure 6-1 shows the structure of the MUG dimer complexed with DNA. In the protein-protein interface of the MUG dimer, two asparagine residues (78N) which are facing each other are likely to form hydrogen bonds with each other. We therefore aimed to mutate them and analyze the binding characteristics and activity of the mutant (data not shown).
Figure 6-1 Structure of the MUG dimer complexed with DNA

Two MUG molecules had bound to the continuous “nicked” double stranded DNA molecule and excised uracil from the G·U mismatch (PDB ID code:1MWI). The figure was generated using PyMOL (DeLano, 2002). The two asparagine residues (78N) in the protein-protein interface which are potential candidates for making hydrogen bonds with each other are indicated in blue (■). A Close-up of the protein-protein interface showing 78N residues can be seen in (B).

78N was mutated to an aspartic acid (78D) to analyze whether the mutation interferes with MUG’s binding cooperativity to DNA. The mutation did not interfere with the glycosylase activity as determined by comparison of single turnover reaction rates with a G·U mismatch.
containing substrate of the wild type enzyme and MUG78D (data not shown). Monitoring the change in fluorescence anisotropy with MUG78D revealed that the mutation eliminated binding cooperativity. Binding of MUG78D was fitted to a tight binding equation but did not fit a Hill equation like wild type MUG. Further experiments are under way in order to evaluate whether the mutation interferes with the requirement for two MUG molecules per DNA to obtain maximum glycosylase activity as was seen with the wild type enzyme.

It is interesting that TDG\textsuperscript{cat}, a truncated form (residues 111-308) of the human homologue of MUG, was found to bind DNA in a 2:1 stoichiometry (Maiti \textit{et al.} 2008). The crystal structure revealed that one TDG\textsuperscript{cat} subunit had bound at the abasic site (product complex) and the other one was bound to an undamaged site (non-specific complex) (Figure 6-2). While the specific subunit was making contacts with the target strand and complementary strand, the non-specific subunit was found to interact predominantly with the complementary strand and the contacts were less extensive than the product complex. Although the nucleotide was not flipped into the active site in the non-specific complex, the phosphate contacts 3’ on the target site in the product complex were present, as were the long-range contacts with Lys-246 and Lys-248 that are made with the phosphate backbone on the complementary strand at positions 8 and 9; 5’ of the target site (Maiti \textit{et al.} 2008). These residues (Lys-246 and Lys-248) are strictly conserved in vertebrate TDG’s but not in MUG. These long range phosphate contacts are also not seen in complexes with MUG (Barrett \textit{et al.} 1998). Like with MUG, an insertion loop which provides specific contacts with the Watson-Crick regions of the opposing guanine can be seen in the TDG\textsuperscript{cat}. These contacts can only be formed when the target nucleotide is flipped in the active site.
hTDG binds the DNA in a 2:1 complex: one subunit at the abasic site (product complex) and the other at an undamaged site (non-specific complex) (PDB ID code: 2RBA). The figure was generated using PyMOL (DeLano, 2002).

In the TDG\textsuperscript{cat}: DNA complex, the protein subunits had formed a dimer interface with no apparent hydrogen bonds or salt bridges. Sedimentation velocity analytical ultracentrifugation experiments done by the group found TDG\textsuperscript{cat} to be monomeric in the absence of DNA, similar to our results with MUG. EMSA and isothermal titration calorimetry experiments suggested that TDG like TDG\textsuperscript{cat} forms a dimer with the oligonucleotide (Maiti \textit{et al.} 2008).
Residues contributing to the dimer interface, determined by the buried surface area are conserved for vertebrate TDG’s but not with TDG from Drosophila or fission yeast or with MUG. Comparison of the TDG dimer with the relative positions of the two MUG enzymes in the MUG & DNA complex suggests that there is a different mode of interaction. The observed position of the two enzymes is mutually exclusive so that both complexes could not form simultaneously. These structures could provide a starting point for further studies through site-directed mutagenesis and DNA binding & activity experiments.

Dimerization has been observed by several other DNA repair enzymes as well. MutY is thought to function as a dimer (Wong et al. 2003). An assay looking at how many MutY molecules are necessary to process an oligonucleotide bearing an adenine·8-oxoguanine mismatch revealed that two MutY molecules are required to process one mismatch containing oligonucleotide (Wong et al. 2003). Electrophoretic mobility shift analysis demonstrated that MutY and its murine homologue hMYH form multiple bands with DNA (Pope & David 2005).

Human O\textsuperscript{6}-Alkylguanine-DNA Alkyltransferase (hAGT) is another DNA repair enzyme involved in minimizing the mutagenic effects of alkylating agents. hAGT was found to be a monomer in the absence of DNA as determined by analytical ultracentrifugation (Fried et al. 1996). In the presence of DNA, it binds cooperatively and forms dimer→tetramer→octamer on the DNA depending on the size of the oligonucleotide as investigated by sedimentation equilibrium analytical ultracentrifugation and confirmed by EMSA (Rasimas et al. 2003; Rasimas et al. 2007). Electrophoretic mobility shift experiments revealed that the binding of hAGT produced a single mobility-shifted complex at a range of protein and DNA concentrations (Rasimas et al. 2003). This 23kDa DNA repair protein was found to bind the DNA in a 4nt per protein manner. Binding by hAGT seems different than MUG as MUG forms multiple complexes with DNA depending on the size of the oligonucleotide. hAGT was only found to form a single
complex with DNA even when tested with oligonucleotides up to 78 basepairs (Rasimas et al. 2007). We investigated the binding of MUG by EMSA using a 17-mer (6AP•G) and a 25-mer (12AP•G). The 18.6kDa protein bound to the 17-mer creating two higher migrating protein & DNA complexes whereas binding to the 25-mer resulted in two main complexes and minor additional complexes. MUG’s binding to shorter oligonucleotides could be investigated in order to find the minimum nucleotide requirement for binding. Finding a shorter oligonucleotide which results in a single MUG & DNA complex may also allow the use of analytical ultracentrifugation to investigate the stoichiometry of the complex (see below for further discussion).

We speculate that MUG’s dimerization is necessary for its stability and DNA binding. To further investigate the importance of oligomerization for MUG’s binding and glycosylase activity, construction of MUG variants that bear mutations or truncations in the potential protein – protein interface is necessary. As described above, studies are under way in our lab to evaluate the effect of mutations in the potential protein-protein interface of MUG. Analysis of these mutants will help us understand how oligomerization assists DNA binding by MUG.

Further analysis of MUG & DNA complexes using analytical ultracentrifugation sedimentation equilibrium (AUC-SE) may help delineate quantitatively how many MUG molecules are bound to DNA. AUC-SE could not be performed with MUG and DNA since EMSA experiments have shown that MUG forms multiple bands with the DNA. Getting a mixture of the MUG & DNA complexes is difficult to analyze since AUC-SE gives us a global average molecular weight for all the individual complexes. Performing EMSA experiments with a series of shorter oligonucleotides to find an oligonucleotide where only a single MUG & DNA complex exists will enable us to analyze the complex using AUC-SE.
An alternative method which would be useful in the analysis of DNA & MUG complexes is multi-angle light scattering (MALS) which is a sensitive method to measure the molar mass of proteins in solution. MALS measurements are similar to gel filtration but work by calculating the amount of light scattered at each angle as the sample goes through the column. The amount of light scattered is related to the molar mass of the molecule, therefore the molecular weight of the molecule can be determined without reference standards. One disadvantage of MALS is that when the sample is applied to the column, it is diluted therefore equilibrium of the complex will be shifted. In AUC-SE, the complex is in equilibrium. Attempts in the lab are currently on the way to analyze MUG & DNA complexes using a gel filtration column.

Another method which will advance our understanding of the MUG & DNA complexes is DNA footprinting analysis. Crystal structure of MUG suggests the enzyme may cover 5-6 nucleotides (Barrett et al. 1998). MUG’s human homologue, hTDG has twice the molecular weight (46.0 kDa) as MUG (18.7 kDa) and was found by DNA foot printing to cover 12 nucleotides, as suggested by its crystal structure (Maiti et al. 2008). DNA footprint analysis will help us understand whether MUG molecules bind on top of each other or adjacent to each other at ratios higher than 2:1 MUG : DNA. Footprint analysis at MUG : DNA ratios 1:1, 2:1, 3:1 and 4:1 will complement the anisotropy and EMSA experiments. It will also be interesting to monitor whether MUG binds DNA in an overlapping or non-overlapping pattern in the presence of higher MUG : DNA ratios. Asking the same questions with non-specific DNA will also further our understanding about the mode of non-specific binding by MUG glycosylase.
FRET to understand Enzyme Communication in Base Excision Repair

Our results provide the first reported comparison of the effect of Exonuclease III on dissociation of MUG glycosylase from AP·A and AP·G bearing substrates. To our knowledge, fluorescence resonance energy transfer (FRET) assay has so far not been used to investigate protein hand-off in base excision repair. Almost all assays looking at the turnover of glycosylases have either analyzed the degree of base removal or product formation in the presence of AP endonucleases. Here we successfully show that an increase in FRET can be used to monitor the association of MUG glycosylase with DNA and a decrease in FRET can be utilized to analyze its dissociation from the DNA.

Our results suggest that Exonuclease III does not actively displace MUG from the abasic site. This was tested with substrates bearing an abasic site across adenine or guanine as MUG has been shown to bind and process U·A as well as U·G mismatches (Liu et al. 2008). Cleavage of the abasic site by Exonuclease III also did not lead to dissociation of MUG.

In order to further investigate the effect of ExoIII, activity assays are planned to test if Exonuclease III increases the turnover rate of MUG with AP·G complexes. The activity tests performed in our lab were not conclusive since commercially acquired ExoIII was found to be contaminated by an enzyme, likely to be a DNA glycosylase. Experiments monitoring product formation with and without ExoIII revealed that the commercially available ExoIII sample has some glycosylase activity. In addition to that, analysis of the ExoIII by running an SDS-PAGE gel revealed extra protein bands (data not shown). For further analysis, Exonuclease III will be cloned and expressed in our lab in order to investigate its influence on the turnover rate of MUG.
Another study being planned is the optimization of a FRET assay between MUG and ExoIII. Labeling of these proteins with fluorophores that will serve as an acceptor and as a donor will enable us to visualize energy transfer between them upon binding of Exonuclease III to the MUG & DNA complex. Investigation of FRET between MUG and ExoIII using AP·A and AP·G oligonucleotides will allow us to further grasp the differences in the effect of ExoIII on MUG’s dissociation.

Our results do not support the hypothesis that the activity of MUG glycosylase is enhanced by Exonuclease III but are aligned with the presumption that protein-protein interactions might exist between MUG glycosylase and Exonuclease III. The fact that ExoIII affects the decrease in energy transfer between MUG and DNA differently depending on the base opposite the abasic site strongly suggests that ExoIII interacts with MUG and the interaction is dependent on the bonds between MUG and the complementary base.

Substrate specific effect of AP endonucleases was previously seen with various AP endonuclease & glycosylase pairs. In the study by Fitzgerald et el., APE1 increased the turnover rate of TDG with G·U substrates but did not have any effect on the turnover rate with a G·FU substrate, which TDG actually cleaves with a much faster rate (Fitzgerald & Drohat 2008). A substrate specific effect of APE1 on the turnover rate of TDG was also observed by Waters et al. (Waters et al. 1999). In their study, APE1 enhanced the turnover of hTDG more efficiently with a C·U mismatch than a G·T mismatch. The observation was argued to correspond to the weaker affinity of hTDG for the C·AP product than the G·AP product (Waters et al. 1999).

Another example for substrate specificity came from an E. coli DNA glycosylase: MutY, which removes misincorporated residues from OG·A mispairs as well as from G·A and C·A mispairs. Product formation by MutY was found to be enhanced by Endonuclease IV
and Exonuclease III when the DNA had a G·A mismatch but not a G·OA mismatch. MutY also has a weaker affinity for the G·A mismatch than the G·OA mismatch (Pope et al. 2002).

Protein-protein interactions were observed between AP endonucleases and MutY, hMYH, the human homologue of MutY and with TDG. EMSA studies with DNA containing a G·A mismatch demonstrated that EndoIV displaces MutY from the DNA whereas ExoIII leads to a super-shifted band suggesting it forms a complex with MutY on the DNA. Direct interaction between the human homologue of MutY, hMYH and APE1 in vivo has been demonstrated by immunoprecipitation and Western blotting (Parker et al. 2001). Activity of MutY with a truncated C-terminus was not affected by Endonuclease IV, which suggests activation of product formation involves protein-protein interactions between MutY and Endonuclease IV (Pope et al. 2002). Optimization of the electrophoretic mobility shift assay with MUG and ExoIII will also be another useful tool to confirm that they are bound together on the DNA.

The effect of E. coli AP endonucleases on base removal by MUG has been explored before. Hang et al. have studied the effect of Endonuclease IV on substrate cleavage by MUG on DNA containing an 8-HM-εC•G mismatch (Hang et al. 2002). EndoIV was found to stimulate removal of 8-HM-εC•G by MUG by a 1.5 fold, but a MUG: EndoIV ratio of 1:4 was required to see the 1.5 fold effect.

The effect of EndoIV and ExoIII on the removal of 3, N^4-ethano-dC (EC) by MUG was monitored and showed that ExoIII had an almost 2.5 bigger effect on base removal compared to EndoIV. To see an effect, a ExoIII: MUG ratio of 5:1 was required (Guliaev et al. 2004). Besides the high ExoIII: MUG ratio used in the study, the biological significance of EC used in the study compared to εC is also questionable. The same study demonstrates that MUG cleaves εC ~20 times more efficiently compared to EC. It would
be of interest to compare how both AP endonucleases effect removal of εC from the DNA.

εC was found to be a better substrate for MUG glycosylase than U by our group (O’Neill et al. 2003), therefore it would be of interest to use the FRET assay to compare how ExoIII influences MUG’s dissociation from a εC-G mismatch compared to a U-G mismatch.

A study by Sung et al. suggested that EndoIV may lead to dissociation of MUG from DNA containing a U-G mismatch (Sung & Mosbaugh 2000). It would be relevant to use the FRET assay to monitor the effect of EndoIV on dissociation of MUG from the abasic site.

In our experiments equimolar ExoIII to MUG was used, it will be worth to monitor change in energy transfer with a higher ExoIII: MUG ratio as it was used in other studies. But then the question is whether a high ExoIII: MUG ratio is biologically significant or not.

As presented here, there are various previous studies looking at interactions between proteins in base excision repair pathway, the FRET assay described here provides a unique and quantitative approach to address the role of AP endonucleases in the dissociation of glycosylases. Since the FRET assay directly looks at protein-protein interactions, it could be adapted to investigate interactions of various other enzymes in base excision repair as well. Hence further interactions between other members of base excision repair have also been demonstrated. A yeast two hybrid assay has found that AP endonuclease and Polymerase β interact with each other (Bennett et al. 1997). Eukaryotic BER enzymes DNA polymerase β and DNA Ligase III were both found to interact with
XRCC1, which does not have a known BER activity but seems to be a scaffold protein that also suppresses strand displacement by DNA polymerase β. A larger protein complex containing polymerase β was eluted from crude nuclear extract from bovine testis and was able to conduct the complete uracil-initiated BER reaction (Prasad et al. 1996). Interactions between other players of BER could also be tested using FRET.
Conclusions

We have shown that MUG glycosylase binds DNA cooperatively. Two MUG molecules seem to bind the abasic site and are required in order to fully process an abasic site. Lifetime measurements confirmed cooperativity in binding and also indicated that MUG doesn’t bind the DNA until there is a 2:1 ratio of MUG : DNA. MUG also binds non-specific DNA cooperatively. An increase in ionic strength affected cooperativity in binding to abasic and non-specific DNA. A reduction in cooperativity also correlated with a reduction in glycosylase activity of MUG. Equilibrium binding analytical ultracentrifugation experiments showed that MUG exists as a monomer in the absence of DNA. Further analysis of MUG binding stochiometry in the presence of DNA is required.

We also investigated if Exonuclease III, the next enzyme in base excision repair pathway stimulates dissociation of MUG from the abasic site. Our results do not support the hypothesis that ExoIII binding to DNA leads to MUG’s dissociation from the abasic site. The different effect of Exonuclease III on decrease in energy transfer based on the base opposite the abasic site suggests that ExoIII interacts with MUG depending on the mismatch. Cleavage of the abasic site did not affect dissociation of MUG independent of the base across the abasic site.

We were not able to show via electrophoretic mobility shift assay (EMSA) whether MUG and ExoIII are bound together on the DNA. Further optimization of the EMSA conditions will be necessary to show both enzymes bound together to DNA. In house expression of Exonuclease III will also enable us to address the effect of Exonuclease III on the glycosylase activity of MUG. A FRET assay which will enable energy transfer
between MUG and ExoIII will further demonstrate these enzymes binding together to the abasic site.

MUG’s role in the *in vivo* repair of etheno lesions has also been explored by treating *E. coli* cells with urethane, a chemical known to introduce etheno lesions in the DNA. The presence of MUG glycosylase did not provide *E. coli* cells with an advantage when treated with urethane. This might be due to urethane causing multiple and various lesions in the DNA of these cells. A eukaryotic DNA glycosylase, hSMUG was shown to repair etheno lesions; we investigated hSMUG’s ability to complement MUG mutations when treated with urethane. hSMUG did not provide an advantage to cells treated with urethane. hSMUG was found to reduce cell viability and increase mutation rates in *mug* deficient cells whereas it didn’t affect wild type cells. Strikingly, hSMUG gave *mug* deficient cells a significant boost in cell divisions. We then asked the question whether hAPE1, the AP endonuclease believed to increase the turnover rate of hSMUG, can reverse the negative effects of hSMUG expression *in vivo*. The presence of APE1 further increased cell divisions in *mug* deficient cells expressing hSMUG. Increase in mutation rates and cell divisions suggest that an alternative pathway might be activated in *mug* deficient cells expressing these eukaryotic genes. The overall cell viability was reduced in hSMUG & hAPE1 expressing cells suggesting the alternative pathway, which causes cell survival is not activated in the majority of the cells.
Chapter 7

Materials & Methods
Chapter 7: Materials and Methods

All commercial enzymes were from New England Biolabs (Hitchin, Herts). QIAprep® Spin Miniprep Kit was from QIAGEN, Crawley, UK. All remaining chemicals and materials were from Sigma-Aldrich (Dorset, UK) unless stated otherwise.

DNA Substrates

Oligonucleotides were obtained from EUROGENTEC S.A. (Seraing, Belgium). Double stranded substrates were prepared by annealing the HEX labeled strand containing the modified nucleotide to an equal amount of the unlabeled complementary strand. The strands were annealed by heating up to 90°C and slowly cooling down to room temperature. All oligonucleotides used in experiments are listed in Table 7-1. Instead of DNA with an abasic site, DNA with a tetrahydrofuron nucleotide, a chemically stable mimic of the natural abasic product (AP) was used. Oligonucleotides starting with “H” were labeled with 6-carboxy-2’, 4, 4’, 5’, 7, 7’ – hexachlorofluorescein, succinimidylo ester (HEX) on their 5’ terminus. Table 7.1 summarizes oligonucleotides used in experiments in this thesis. H12AP and 12AP were annealed with 12compG to make the double stranded substrates H12AP•G and 12AP•G respectively. The non-specific double stranded substrate (H12C•G) was made by annealing H12C with 12compG. H6AP and H6U were annealed with 6C to make H6AP•G and H6U•G respectively. The hairpin substrates are self-complementary and anneal giving double stranded substrates HEX-AP•A-Hairpin, HEX-U•A-Hairpin and HEX-U•G-Hairpin.
<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence 5’ →3’</th>
<th>TM (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12AP</td>
<td>GCT ATG GAC TAA APAA TGA CTG GGT G</td>
<td>50.9</td>
</tr>
<tr>
<td>H12AP</td>
<td>HEX GCT ATG GAC TAA APAA TGA GCGTG</td>
<td>50.9</td>
</tr>
<tr>
<td>H12C</td>
<td>GCT ATG GAC TAA CAA TGA CTG CGT G</td>
<td>52.6</td>
</tr>
<tr>
<td>12compG</td>
<td>CAC GCA GTC ATT GTT AGT CCA TAG C</td>
<td>52.6</td>
</tr>
<tr>
<td>H6U</td>
<td>GAC TAA UAA TGA CTG CG</td>
<td>37.1</td>
</tr>
<tr>
<td>6G</td>
<td>CGC AGT CAT TGT TAG TC</td>
<td>39.5</td>
</tr>
<tr>
<td>H6AP</td>
<td>GAC TAA APAA TGA CTG CG</td>
<td></td>
</tr>
<tr>
<td>HEXUracil. Hairpin.52</td>
<td>GAC TAA UAA GGA CTC TGA CGT GCG GTT ACG CAC GTC AGA GTC CTT ATT AGT 799 T</td>
<td>65.5</td>
</tr>
<tr>
<td>HEXAbasic.hairpin.52</td>
<td>GAC TAA APAA GGA CTC TGA CGT GCG GTT GTT ACG CAC GTC AGA GTC CTT ATT AGT 799 T</td>
<td>65.5</td>
</tr>
<tr>
<td>HEXUracil/Ghairpin substrate</td>
<td>GAC TAA UAA GGA CTC TGA CGT GCG GTT ACG CAC GTC AGA GTC CTT GTT AGTC<em>T</em>T*T</td>
<td>67</td>
</tr>
</tbody>
</table>

Table 7-1 Sequences and melting temperatures of DNA substrates used in MUG binding, dissociation and activity assays. Bases of interest (uracil and non-specific), their complementary bases and abasic sites (AP) are shown in bold. 7 stands for C-methyl phosphonate and 9 stands for T-methyl phosphonate. C*T*T*T is the same as 799 T.
# Media and Antibiotics

## LB Medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>2 mg/ml</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>1 mg/ml</td>
</tr>
<tr>
<td>Trypton</td>
<td>2 mg/ml</td>
</tr>
<tr>
<td>Agar</td>
<td>6 mg/ml</td>
</tr>
</tbody>
</table>

Table 7-2 Recipe for LB medium used for cell cultures

## 10X M63 Minimal Medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO₄</td>
<td>1 mM</td>
</tr>
<tr>
<td>Thiamine</td>
<td>0.1 mg/ml</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.2%</td>
</tr>
<tr>
<td>Casamino Acids</td>
<td>2 mg/ ml</td>
</tr>
</tbody>
</table>

Table 7-3 Recipe for M63 Minimal medium used for mutation studies
<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Stock concentration (mg/ml)</th>
<th>Working concentration (µg/ml)</th>
<th>dissolved in</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol</td>
<td>34</td>
<td>25</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Tetracyclin</td>
<td>5</td>
<td>25</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>50</td>
<td>50</td>
<td>deionised water</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>10</td>
<td>50</td>
<td>deionised water</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>10</td>
<td>100</td>
<td>DMSO</td>
</tr>
</tbody>
</table>

Table 7-4 summarizes stock and working concentrations of antibiotics used in cellular assays.

### Enzymes

The pTrc99A vector containing the mismatch uracil glycosylase (MUG) was gift from Dr. R. Savva (Birkbeck, London). The pACYC-SMUG construct was made in our lab. APE1 construct was kindly provided by P.S. Freemont and his research group (Centre for structural biology, Imperial College London, UK). The construct is the N-terminal truncated version of APE1 cDNA (APE1_{36-318}), cloned into the expression vector pT7–7 (Novagen), placing the coding region under T7 polymerase control (Gorman et al. 1997). Exonuclease III was purchased from New England Biolabs (M0206S). The expression and purification of MUG is detailed below. Stocks of enzymes were stored in 20% glycerol and 20mM Tris, pH 8.0 at -20°C. Enzyme dilutions were made with the appropriate reaction buffer and discarded after use.
### Competent Cell Lines

*E. coli* K12 strain JM109 (*F'[traD36 proAB+ lacIqΔ(lacZ)M15I] Δ(lac-proAB)gyrA96recA1 relA1 thi1 hsdR17 mcrA supE44*) was from New England BioLabs. The BL21 Rosetta (DE3) strain (*ompT- Ion- dcm-*) was from Novagen (Nottingham, UK). GM31, and BH157 were kindly provided by A.S. Bhagwat. Table 7.5 summarizes cell lines used in *in vivo* DNA repair assays.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Table 7.5 summarizes genotypes of cell lines which were used in cellular assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM31</td>
<td><code>dcm-6 thr-1 hisG4 leuB6 rpsL ara-14 supE44 lacY1 tonA31 tsx-78</code></td>
<td></td>
</tr>
<tr>
<td>GM31 with SMUG</td>
<td><code>dcm-6 thr-1 hisG4 leuB6 rpsL ara-14 supE44 lacY1 tonA31 tsx-78</code></td>
<td></td>
</tr>
<tr>
<td>GM31 with APE1</td>
<td><code>dcm-6 thr-1 hisG4 leuB6 rpsL ara-14 supE44 lacY1 tonA31 tsx-78</code></td>
<td></td>
</tr>
<tr>
<td>GM31 with SMUG and APE1</td>
<td><code>dcm-6 thr-1 hisG4 leuB6 rpsL ara-14 supE44 lacY1 tonA31 tsx-78</code></td>
<td></td>
</tr>
<tr>
<td>BH157</td>
<td><code>dcm-6 thr-1 hisG4 leuB6 rpsL ara-14 supE44 lacY1 tonA31 tsx-78</code></td>
<td></td>
</tr>
<tr>
<td>BH157 with SMUG</td>
<td><code>dcm-6 thr-1 hisG4 leuB6 rpsL ara-14 supE44 lacY1 tonA31 tsx-78</code></td>
<td></td>
</tr>
<tr>
<td>BH157 with APE1</td>
<td><code>dcm-6 thr-1 hisG4 leuB6 rpsL ara-14 supE44 lacY1 tonA31 tsx-78</code></td>
<td></td>
</tr>
<tr>
<td>BH157 with SMUG and APE1</td>
<td><code>dcm-6 thr-1 hisG4 leuB6 rpsL ara-14 supE44 lacY1 tonA31 tsx-78</code></td>
<td></td>
</tr>
</tbody>
</table>
Making Glycerol Stocks

Aliquots of *E. coli* cells were snap frozen in 20% glycerol using dry ice/ethanol. Glycerol stocks were stored at –80°C.

Preparation of electroporation competent cells

Cultures were prepared by inoculating 5mL of an overnight culture into 500mL of LB with the appropriate antibiotic if necessary. Cells were grown at 37°C until an A$_{600}$ of 0.6-0.8 was reached and were stored on ice for 15 minutes. Cells were harvested by centrifuging at 4,000 rpm for 10 minutes at 4°C. Each pellet was resuspended in 250ml chilled sterile water and centrifuged again at 4,000rpm. The water wash was repeated after removing the supernatant. After the second wash, each pellet was resuspended in 10ml of chilled 10% glycerol and centrifuged at 4,000rpm for 20 minutes at 4°C. Pellets were resuspended in a total volume of 1ml 10% glycerol, 60ul aliquots were snap-frozen using a CO$_2$ - ethanol bath and stored at -80°C.

For electroporation, an aliquot of cells was slowly defrosted on ice. Before electroporation, cells were incubated with the transformant on ice for 5 minutes. Electroporation was conducted using BIO RAD GENE PULSER II at 1.8 kV (1mm cuvette) or at 2, 5 kV (2mm cuvette) (capacitance 25µF, resistance 200Ω). 200 μl liquid LB was added immediately to the cells and the cell suspension was transferred into an Eppendorf tube, incubated in the water bath at 37°C for 60 min, plated on LB plates containing antibiotics and incubated overnight at 37°C.

The cuvettes were reused after washing in deionised water, incubating in 70% ethanol for 15 min, sonicating for 20 min in 0.1% NaOH and finally sonicating for 20 min in 0.1% HCl. Between the NaOH and HCl washes, cuvettes were washed in deionised
water and finally stored in 70% ethanol. Before re-use, they were sonicated for 10 minutes.

For co-transformations, GM31 and BH157 cells were first transformed with pACYC-SMUG and glycerol stocks were made. Cells with SMUG were made electrocompetent in order to transform them with the second plasmid. Electrocompetent GM31-SMUG and BH157-SMUG cells were then transformed with pT7-7-APE1 (Gorman et al. 1997) through electroporation. Glycerol stocks were made and cells were stored at -80°C.

**Preparation of chemically competent cells**

Cultures were prepared by inoculating 2ml of an overnight culture into 100ml of LB with the appropriate antibiotic. Cells were grown at 37°C until reaching an A$_{600}$ of 0.3-0.4. Cells were harvested by centrifugation at 4,000rpm for 10 minutes at 4°C. Each pellet was resuspended in 30ml of chilled sterile solution of 80mM MgCl$_2$ 20mM CaCl$_2$ and incubated on ice for 20 minutes. Centrifugation was repeated and 1ml of 0.1M CaCl$_2$ in 10% glycerol was used to resuspend each pellet. Aliquots of heat-shock chemically competent cells were snap-frozen as before.

To transform chemically competent cells, the plasmid of interest was added to an aliquot of cells and incubated on ice for 30 minutes. After 30 minutes, cells were subjected to a 1 minute heat shock at 42°C and were returned to ice for 5 minutes. 300ul LB was added at the end of the ice incubation and cells were incubated at 37°C for 30 minutes. An appropriate volume of the cell suspension was spread on an LB plate containing the appropriate antibiotic and plates were incubated over night at 37°C. In general, 100ul of cell suspension was sufficient to give ~200 colonies and for ligation reactions, the entire cell suspension was spread on a plate to maximize recovery.
**Agarose Gels**

Electrophoresis grade agarose was used to make 1% gels in 1x TAE buffer (40mM Tris-acetate, 1mM EDTA). Ethidium bromide was used at a final concentration of 0.1ng/ml⁻¹.

**Polyacrylamide Gels**

**Denaturing Urea PAGE**

To analyze DNA, denaturing Urea PAGE was performed. A stock solution of 20% polyacrylamide (19:1 acrylamide: bisacrylamide), 7M UREA in 1x TBE was used to make the gels. Polymerization of large volume gels (100ml) was initiated by adding 800µl of 10% ammonium persulfate solution (APS) and 80 µl of N, N, N’,N’ – tetramethylethylenediamine (TEMED). Small gels (5ml) were polymerized by mixing 50ul of 10% APS and 5ul TEMED. Gels were run at either 60W (large) or 250V (small).

After separation of fluorescently labeled DNA fragments, gels were scanned using an FLA-5000 fluorescent image analyzer (Fujifilm). HEX labeled DNA substrates (λ_ex= 535nm, λ_em=556nm) were identified using an excitation laser at 532nm with a 570nm (±20nm) band pass filter. Band intensity measurements were determined from the captured images using Phoretix 1D Gel Analysis software (Newcastle, UK).

**Protein Analysis: SDS PAGE**

Resolving gels comprised of 12% acrylamide (29:1 acrylamide: bisacrylamide); 0.1% SDS in 375mM Tris pH 8.8 and were polymerized with 60µl of 10% APS and 5µl of TEMED for a 5ml gel. Isopropanol was floated on top of the resolving gel and was
washed thoroughly after polymerization. A 5% acrylamide (29:1) stacking gel with 0.1% SDS in 125mM Tris pH 6.8 polymerized with 10µl 10% APS and 2.5µl TEMED per 1.5ml of gel was layered on top of the resolving gel.

Before loading onto the gel, samples were mixed with an equal volume of SDS loading buffer (20% glycerol, 4%SDS, 100mM Tris pH 6.8, 0.2%bromphenol blue, 200mM DTT) and heated at 90°C for 5 minutes. Gels were run at 80V in Tris-glycine buffer (25mM Tris pH 8.3, 250mM glycine, 0.1%SDS).

Polyacrylamide gels were stained in Coomassie blue (45 % ( v/v) methanol, 10% (v/v) acetic acid, 3mM Coomassie Blue R-250) overnight on a rocking platform. Gels were destained in a solution of 30% methanol, 10% acetic acid (v/v) over 3 hours and were scanned using a HP Scanjet 5400c Scanner.

**Electrophoretic mobility shift assay (EMSA)**

In electrophoretic mobility shift assays (EMSA), 100nM of HEX-labeled oligonucleotide duplexes were reacted with a series of MUG concentrations at 25°C for 30 minutes. 1M Betaine glycine was added into the reaction mixture and incubated at 25°C for 5 minutes. 11.1µl of reaction mixture was mixed with 2.2 µl of 6 x loading buffer (0.042% bromophenol blue) and loaded immediately onto an 8% polyacrylamide gel, which has been pre-run in 1 x TBE buffer for 30 minutes. The PAGE was run at constant 40 V in 1 xTBE buffer for 210 minutes in the dark cold room (4 °C). Bands on the gel were visualized using the FLA-5000 fluorescent image analyzer (Fujifilm) and quantified with the PhoretixTM 1D software. The equilibrium binding constants were determined by fitting the data to Hill equation (Eq 7.2) using GraFit 5 (Erithacus Software).
For competition EMSA experiments, MUG and DNA were mixed and incubated in standard reaction buffer at 25°C for 30 minutes. 5μM unlabeled abasic or non-specific competitor was added and incubated for 5 minutes at 25°C. 1M Betaine glycine was added into the reaction mixture and incubated at 25°C for 5 minutes. The samples were loaded, ran and visualized as described above.

**MUG binding Assays**

**MUG Expression & purification**

Mismatch uracil glycosylase (MUG) was expressed from a pTrc99A vector (supplied by Dr. R. Savva (Birkbeck, London) in *E.coli* strain BL21-DE3. An overnight culture of the strain carrying this construct was diluted 1000x into LB broth supplemented with 100μg/ml Ampicillin and grown over night at 37°C before induction with 1mM isopropyl-1-thio-β-D-galactopyranoside. The cultures were grown a further 5 hours at 37°C before the cells were harvested. Harvested cells were lyzed by sonication in buffer A (20mM Tris-HCl, pH 8.3, 1mM EDTA) with “Complete” protease inhibitors (Roche Molecular Biochemicals). The lysate was incubated with 1/10 volume of 10% streptomycin sulfate for 1 hour on ice before centrifugation at 20,000rpm for 40 minutes to remove the cell debris. The supernatant was loaded onto a DEAE-cellulose column, equilibrated in buffer A, and the flow-through was run directly onto an SP-sepharose column, also equilibrated in buffer A. The connected columns were washed with 400ml buffer A; the DEAE column was then removed and the SP sepharose column washed with further 150ml of buffer A. Protein was eluted from the column using a gradient of Buffer B (20mM Tris-HCl, pH 8.3, 1mM EDTA, 1M NaCl) over 400ml. Fractions of the eluate were analyzed on SDS-PAGE and the ones containing MUG were pooled and the volume reduced by ultrafiltration using Millipore ultrafiltration membranes (Millipore Corporation, Billerica, MA) in a stirred cell. The partially purified MUG protein was loaded onto a Sephadex-75 column, equilibrated in Buffer C (20mM Tris-HCl, pH 8, 1mM EDTA, 200mM NaCl) and eluted in the same buffer. Fractions were assayed as
above and the ones containing MUG were pooled and concentrated by ultrafiltration. The protein concentration was calculated from the OD$_{280}$ based on an extinction coefficient of 25,590 M$^{-1}$cm$^{-1}$. Glycerol was added to 20% and aliquots of the enzyme were snap-frozen and stored at -20°C.

**Equilibrium DNA Binding assays**

Equilibrium DNA binding assays were performed to examine the binding of MUG glycosylase to the 5'-hexachlorofluorescein-labeled oligonucleotides. In order to measure fluorescence anisotropy, a Fluoromax 3 spectrofluorometer fitted with automated polarization filters was utilized. Data was recorded using an excitation wavelength of 535nm and an emission wavelength of 556nm. The binding assays were conducted at 25°C in 400ul reaction volume in MUG reaction buffer (50mM Tris-HCl, pH8.0; 1mM EDTA; 50mM NaCl). Small amounts of the enzyme were titrated into a fixed concentration of the fluorescent DNA. The excitation was at 535nm and emission was detected through a 556 nm cut-off filter. Five measurements of anisotropy were made and averaged and each protein titration repeated at least in duplicate. The observed anisotropy was plotted against MUG concentration and the data fitted using Grafit Version 5.0 to the Hill equation with the following equation (below). In order to investigate the effect of ionic strength on equilibrium binding, MUG binding was monitored in MUG reaction buffers containing 50mM, 150mM and 300mM NaCl.

Competition titration experiments were conducted using the Fluoromax 3 spectrofluorometer. MUG and DNA were mixed and incubated in standard MUG reaction buffer at 25°C for 30 minutes for equilibrium binding. Unlabeled competitor DNA was added in small increments. Change in anisotropy was monitored as described above.
**Binding models**

When MUG binding data was fitted to a hyperbolic binding curve, a systematic deviation in the data was observed indicating that a single site binding (Eq 7.1) does not describe the equilibrium. Instead, the binding data fit best fit to the Hill equation (Eq 7.2).

### One-site binding model

$$y = \frac{[L] \times \text{Cap}}{[L] + K_d} + \text{background} \quad \text{Eq 7.1}$$

### Hill equation

$$y = \frac{[L]^n \times \text{Cap}}{[L]^n + K_d^n} + \text{background} \quad \text{Eq 7.2}$$

Where $[L]$ is the concentration of the free ligand, $K$ is equilibrium dissociation constant, $n$ is the cooperativity constant, Cap is the capacity, background is the anisotropy of free DNA.
Time-resolved fluorescence anisotropy analysis

Fluorescence decays were collected using a time-resolved spectrofluorometer, previously described in Manning et al. (Manning et al. 2008). Fluorescence decays were monitored by titrating small amounts of MUG glycosylase into 100nM of HexG•AP. The assay was conducted at 25°C in 400ul reaction volume in standard MUG binding buffer (50mM Tris-HCl, pH8.0; 1mM EDTA; 50mM NaCl). Excitation was set to 530 nm with emission collected at 550 nm. Decays were measured at polarizations angles parallel, perpendicular and at the magic angle to the excitation. Typical acquisition times were 30 seconds at each polarization. An instrument response function (IRF) was measured using a scattering solution of LUDOX and a G-factor was measured using rhodamine B in methanol.

Analysis of the fluorescence anisotropy decays was performed using a TRFA data processor (Scientific Software Technologies Center, Minsk, Belarus). The data were fitted using the anisotropy model shown in Equation 7.3. In this equation \( m(t) \) is the polarization intensity at time \( t \). \( A \) is the parameter that accounts for the different detection efficiencies of the system at different polarization angles and in this case the measured G-factor was used. \( \alpha_j \) is the pre-exponential associated with the lifetime value \( \tau_j \), \( \phi \) is the polarization angle, \( r_\infty \) is the limiting anisotropy and \( \beta_k \) the pre-exponential associated with the rotational correlation time \( \theta_k \).

\[
m(t) = A \times \left\{ \sum_j \alpha_j \exp\left(-t/\tau_j\right) \times \left[ 1 + \left(3 \cos^2 \phi - 1\right) (r_\infty + \sum_k \beta_k \exp\left(-t/\theta_k\right)) \right] \right\}
\]

Eq. 7.3

The goodness of fit was judged based on the reduced \( \chi^2 \) value and visual inspection of the residuals. The data was best fitted using two fluorescence lifetime components and two rotational correlation times.
Mismatch DNA glycosylase activity assays

In order to determine the optimal MUG: DNA ratio for maximum glycosylase activity, 200, 400, 600nM HexG•U was incubated with increasing amounts of MUG for 15 minutes. The substrate was reacted at 25°C with MUG in reaction buffer (50mM Tris-HCl, pH 8.0; 50mM NaCl, 1mM EDTA and 0.1mg ml⁻¹ bovine serum albumin). At selected time points, 10ul samples were removed and quenched with 10ul- aliquots of 0.1M NaOH. The quenched samples were then heated to 90°C for 30 minutes to cleave the abasic site. An equal volume of formamide loading buffer (95% formamide, 0.04% bromphenol blue, 0.04 xylene cyanol and 20mM EDTA) was added and incubated at 90°C for 5 minutes before loading onto a 20% PAGE with UREA. The bands were visualized by FLA-5000 fluorescent image analyzer (Fujifilm) and quantified using PhoretixTM 1D software. In activity assays, the reaction rate determined by fitting the data to the first order rate equation (Eq. 7.4) using GraFit 5 (Erithacus Software). $A_t$ is product at time $t$, $A_{\infty}$ is product at saturation, $t$ is time, and $k$ is the rate constant.

$$A_t = A_{\infty}(1 - e^{-kt}) \quad \text{Eq. 7.4}$$

In order to investigate the effect of an increase in ionic strength on MUG’s glycosylase activity, 100nM HexG•U was reacted with 5μM MUG at 25°C in MUG reaction buffer (50mM Tris-HCl, pH8.0; 1mM EDTA and 0.1mg ml⁻¹ bovine serum albumin) containing 50, 150 or 300mM NaCl. At selected time points, 10ul samples were removed and quenched with 10ul- aliquots of 0.1M NaOH. The quenched samples were treated and analyzed as described above.

In order to investigate whether Exonuclease III increases the turnover rate of MUG, which would lead to an increase in glycosylase activity, 20nM MUG was incubated with
200nM HEX-U·G-Hairpin for 30 minutes at 25°C. Increasing concentrations of Exonuclease III were added to the MUG & DNA complex and incubated at 25°C for one hour. The samples were loaded, ran and visualized as described above.

**Analytical ultracentrifugation measurements**

Sedimentation equilibrium analytical ultracentrifugation experiments were performed using a Beckman XLA ultracentrifuge. The experiments were performed at 25°C in MUG reaction buffer 50mM Tris-HCl, pH 8.0; 1mM EDTA containing either 50mM NaCl or 150mM NaCl or 300mM NaCl. For measuring the oligomerization states of MUG, absorbance at 280nm was monitored. Protein concentrations used were 33, 17 and 8 μM.

**Fluorescence Resonance Energy Transfer**

**MUG labeling with Alexa488**

Alexa488 was purchased from Invitrogen (California, USA). Labeling was performed according to the manufacturer’s recommendations. MUG after expression and purification is stored in Buffer C (20mM Tris-HCl, pH8, 1mM EDTA, 200mM NaCl) and 20% Glycerol after purification. When labeling with amine-reactive reagents, it is recommended to avoid buffers that contain primary amines such as Tris as these will compete for conjugation with amine-reactive compounds with proteins. Therefore, MUG was dialyzed into standard phosphate saline buffer first to avoid Tris interfering with labeling. Once in PBS, sodium bicarbonate (pH: 7.5) was added to have a 0.1M final concentration. Alexa488 was added to MUG in a 9:1 ratio and incubated at room temperature for 1 hour. Unincorporated dye was separated from labeled protein by using Sephadex G-25 gel filtration columns from GE Healthcare (Buckinghamshire, UK),
which were equilibrated in Buffer C (20mM Tris-HCl, pH8, 1mM EDTA, 200mM NaCl).

Alexa488 and HEX have following excitation and emission characteristics:

<table>
<thead>
<tr>
<th></th>
<th>Excitation Maximum (nm)</th>
<th>Emission Maximum (nm)</th>
<th>$\varepsilon$ (M$^{-1}$cm$^{-1}$)</th>
<th>CF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa488</td>
<td>494</td>
<td>518</td>
<td>71000</td>
<td>0.11</td>
</tr>
<tr>
<td>HEX</td>
<td>535</td>
<td>556</td>
<td>98000</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Table 7-6 Excitation and emission maxima of Alexa488 and HEX

Table shows excitation and emission maxima of Alexa488 and HEX as well as their Extinction Coefficient ($\varepsilon$) and Correction Factor (CF) ($A_{280}$ free dye/ $A_{max}$ free dye).

The protein concentration was determined using equation 7.7 (Eq 7.7) and MUG-Alexa488 was stored as a 20% glycerol stock at -20°C.

$$\text{ConcentrationMUG} = \frac{A_{280} - (A_{495} \times CF)}{\varepsilon_{\text{MUG}}} \times \text{dilution factor} \quad \text{Eq 7.7}$$

$\varepsilon_{\text{MUG}}$ = Extinction coefficient of MUG = 25590; $C_F$ = Correction factor for Alexa488 = 0.11; $A_{280}$ = Absorbance at 280nm; $A_{495}$ = Absorbance at 495nm.

Degree of labeling (DOL) was calculated using the following formula:

$$\text{DOL} = \frac{A_{495} \times MW_{\text{MUG}}}{c_{\text{MUG}} \times \varepsilon_{\text{Alexa488}}} \quad \text{Eq 7.8}$$

$MW_{\text{MUG}}$ = molecular weight of MUG = 18,000g; $\varepsilon_{\text{Alexa488}}$ = extinction coefficient of Alexa488 at its absorbance maximum (495nm) =71,000 ; $c_{\text{MUG}}$ = MUG concentration (mg/ml)

The change in energy transfer (ΔE) was measured using the relative fluorescence intensity of the donor, by calculating the ratio of fluorescence at the end of the reaction
(in the presence of the acceptor \((F_{DA})\)) and fluorescence in the beginning of the reaction (in the absence of acceptor \((F_D)\)) (Eq. 4.1). The complex was excited at 495nm; the emitted fluorescence was collected through a 520nm filter with 20nm band pass to follow donor emission and a 550nm cut-off filter to monitor acceptor emission.

\[
\Delta E = 1 - \frac{FDA}{FD}
\]

Eq. 7.9

**Fluorescence polarization Stopped-Flow measurements**

Fluorescence anisotropy measurements were also made using a KinetAsyst stopped-flow instrument from Hi-Tech Ltd, equipped with a fluorescence polarization accessory, OPTION-661. In the KinetAsyst stopped-flow instrument, a plane polarized excitation beam is delivered to the sample cell from a single fiber optic system. The sample cell is configured in a T-format for dual channel fluorescence detection and each photomultiplier is fitted with a polarizer. One polarizer passes light in the parallel plane and the other in the perpendicular plane with respect to the parallel plane of the excitation beam. The raw data was collected from the two independent photomultiplier channels and change in anisotropy was calculated using equation 7.10

\[
\text{Anisotropy } (r) = \frac{(I_{//} - G * I_{\perp})}{(I_{//} + 2 * G * I_{\perp})}
\]

Eq 7.10

For anisotropy measurements, fluorescence from hexachlorofluorescein (HEX) was obtained by exciting at 545nm and collecting the emission through a a 550nm cut-off filter (Comar); up to 10 transients were collected and averaged for each condition. The
data from each channel was collected and mathematically converted by the KinetAsyst 3 software (TGK Scientific) to anisotropy and total fluorescence.

In order to monitor fluorescence resonance energy transfer, 100nM of the double stranded oligonucleotide containing the G·U or G·AP mismatch was rapidly mixed with 200nM Alexa488 labeled MUG. Samples were illuminated using a 75 W xenon lamp at 495nm and the emitted fluorescence was collected through a 520nm filter with 20nm band pass and a 550nm cut-off filter. All reactions were performed in MUG reaction buffer (50mM Tris-HCl, pH 8.0; 50mM NaCl, 1mM EDTA) at 25°C, up to 10 transients were collected and averaged for each reaction.

For competition experiments, MUG & DNA complexes were either mixed with 1000nM abasic or with 1000nM non-specific DNA to follow passive diffusion. In order to monitor displacement by Exonuclease III, MUG & DNA complexes were competed mixed with 200nM Exonuclease III.

**Exonuclease III activity assays**

Processing of HEX-Hairpin-AP·A and HEX-Hairpin-U·A by Exonuclease III (ExoIII) was tested by incubating 100nM of each oligonucleotide at 37°C in Exonuclease III reaction buffer (NEB) with 1nM and 100nM ExoIII. 10μl aliquots were removed at 10s, 20s, 40s, and 60s, 2m, 5m, 10m, and 30m. An equal volume of formamide loading buffer (95% formamide, 0.04% bromphenol blue, 0.04 xylene cyanol and 20mM EDTA) was added and incubated at 90 °C for 5 minutes before loading onto a 20% PAGE with UREA. The bands were visualized by FLA-5000 fluorescent image analyzer (Fujifilm) and quantified using PhoretixTM 1D software.
In vivo DNA repair by MUG glycosylase

SMUG Cloning strategy

pACYC vector carrying hSMUG1 under the control of a trc-promotor was used to amplify hSMUG. A forward primer with the restriction enzyme site XbaI was designed and used for PCR. The PCR product was then ligated into the pGEM-T Easy vector system. Close to the integration site the pGEM vector already contained the restriction site for SalI. Sequences of the primers are given in Table 7-7. Therefore it was not necessary to add this site via the primer. To clone the hSMUG1 gene into pACYC184 SalI and XbaI, were used. This process interrupted the tetracycline resistance gene.

Polymerase Chain Reaction

PCR was carried out in 50 μl reaction volume with 0,5 μM forward primer containing the sequence for the restriction enzymes XbaI, 0,5 μM the reverse primer, 5 Units Taq-Polymerase, 10 mM dNTPs, 1 μl pACYC-trc-hSMUG1, 5 μl 10x buffer and 37,5 μl H2O were mixed and the following PCR cycle was used for amplification:

<table>
<thead>
<tr>
<th>SMUG PCR cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Denaturation</strong></td>
</tr>
<tr>
<td><strong>Denaturation</strong></td>
</tr>
<tr>
<td><strong>Annealing</strong></td>
</tr>
<tr>
<td><strong>Extension</strong></td>
</tr>
<tr>
<td><strong>4°C</strong></td>
</tr>
</tbody>
</table>

Table 7-7 Summary of the PCR cycle to amplify hSMUG
3 μl of the PCR product were run on a 0,8% agarose gel for analysis. PCR product were run on a 0,8% agarose elution gel and extracted with QIAquick Gel Extraction Kit (QIAGEN).

**Ligation in pGEM-T Easy**

The PCR product was ligated into the pGEM-T Easy vector (Promega) in a 10μl reaction volume. The reaction included 5 μl 2x T4 ligase Rapid Ligation Buffer, 50ng pGEM-T easy vector, ~150ng trc-hSMUG1-PCR product and 3 Weiss units T4 ligase as recommended by Promega. The incubation time was 1 h at room temperature. After the incubation 10 μl of the ligation were heat-shock transformed into JM109 cells.

**Restriction digest**

**Vector digestion**

The vector pACYC184 was digested using the restriction enzymes XbaI and SalI in a 25μl reaction volume containing buffer 3, BSA and the pACYC184 vector. Digest was incubated for 4 h at 37°C.

**Insert digestion**

Ptrc-hSMUG was cut out of pGEM-T easy vector using XbaI and SalI in a reaction volume of 25μl also containing buffer 3, BSA and the pACYC184 vector. Digest was incubated for 4 h at 37°C. Bands were purified by running a 1.5 % agarose elution gel and using the QIAquick Gel Extraction Kit (QIAGEN).
**Ligation**

For ligation, pACYC184 and hSMUG1 were mixed in a 1:4 ratio with 3 Weiss Units T4 ligase and T4 ligase buffer. The incubation time was 2 h at room temperature. The construct was transformed into JM109 cells via heat-shock transformation. Before transforming into GM31 and BH157 cells, pACYC184-hSMUG was cleaned using MicroSpin G-50 columns (GE Healthcare, Illustra) and was transformed via electroporation.

**Oligonucleotides**

For the PCR, the following primers containing the restriction enzyme sites XbaI and BstEII on the forward and BsaI on the reverse primer were used:

<table>
<thead>
<tr>
<th>SMUG PCR Primers</th>
<th>Sequence 5’ →3’</th>
<th>TM (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>fSMUG xba bst F</td>
<td>TCTAGAGGTCACCGTTAGCGCGAATTGATCTGG</td>
<td>75.0</td>
</tr>
<tr>
<td>hSMUG R</td>
<td>GGTCTCGTCAGTCAGATGAATTCTC</td>
<td>68.0</td>
</tr>
</tbody>
</table>

Table 7-8 Primer sequences for PCR amplification of hSMUG

<table>
<thead>
<tr>
<th>SMUG Sequencing Primers</th>
<th>Sequence 5’ →3’</th>
<th>TM (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC/M13 forward</td>
<td>CGCCAGGGTTTCCAGTCACGAC</td>
<td>79.0</td>
</tr>
<tr>
<td>pUC/M13 reverse</td>
<td>TCACACAGGAAACAGCTATGAC</td>
<td>60.0</td>
</tr>
</tbody>
</table>

Table 7-9 Primer sequences for sequencing of hSMUG
Experiments to determine the effect of SMUG and urethane

Addition of urethane

The strains were inoculated in M63 minimal medium and kept shaking in the 37°C incubator over night. After 24 hours incubation, urethane (Fluka) has been added up to 50mM, 100mM, 250mM, 500mM and 1000mM. 1ml of each sample was taken at each time point (Samples were taken before addition of urethane, 4 hours after urethane addition and at 24 hours intervals after urethane addition). Cells were diluted by adding 100μl over night culture into 900μl LB and further dilutions up to $10^{-6}$ were made by adding 100 μl culture into 900 μl LB. For the spotting experiment, 5ul of each dilution was spotted on LB plates.

Spotting to determine urethane’s effects on cell viability

In order to analyze urethane’s effect on the growth of *E. coli* cells, over night cultures of each cell line was diluted up to six fold (up to $10^{-6}$) and 5ul of each dilution was spotted on LB plates at 4 hours, 24 hours, 48 hours and 72 hours after urethane treatment. Formation of colonies at each dilution was recorded to measure urethane’s effect on cell viability. As a control, cells that were not treated with urethane were spotted as well. When a dilution had more than 5 colonies, it was considered as “Growth”.

Measuring the mutation frequency

In order to measure the mutation frequency, 100μl of the $10^{-5}$ dilution of each cell was plated on LB plates. If cells had a plasmid, they were plated on LB plates with the appropriate antibiotic resistance. Cells that had pACYC-SMUG were plated on LB containing chloramphenicol. Cells that had APE1 were plated on LB plates containing ampicillin. Cells that had SMUG and APE1 were plated on LB plates that had chloramphenicol and ampicillin.
10ml of over night the culture of GM31 and BH157 cells were spun down at 3300rpm for 15 minutes and resuspended in 100ul PBS. 100ul suspension was plated on LB plates containing rifampicin. Cells that had pACYC-SMUG were plated on plates containing rifampicin-chloramphenicol, cells containing APE1 were plated on LB plates containing rifampicin and ampicillin, cells containing SMUG and APE1 were plated on LB plates containing rifampicin-chloramphenicol-ampicillin.

All plates were incubated at 37°C, colonies on LB plates were counted 24h after plating, and colonies on rifampicin plates were counted 48 hours after plating. All numbers were adjusted to find the number of colonies in 1ml culture. When cells were plated on LB plates, the number of colonies (*10^8) was multiplied by 10 to find the number of colonies in 1ml of overnight culture (100µl were plated). The number of colonies on rifampicin plates was divided by 10 (BH157, GM31) to find the number of colonies in 1ml. Mutation frequency was calculated as the number of colonies on rifampicin plate divided by the number of colonies * 10^8 on the LB plate using equation 7.11.

\[
\text{Mutation Frequency} = \frac{\text{Number of cells on LB-Rif plate}}{\text{Number of cells on LB plate} \times 10^8} \quad \text{Eq 7.11}
\]

**Determining the number of viable cells**

The number of viable cells per ml was determined as a measure of cell viability. Cells were diluted and 100 µl of the 10^-8 dilution of the cell culture was plated on LB plates. Cell number on the plate was counted after 24 hours incubation at 37°C. The number of viable cells per ml was calculated using Equation 7.12
Number of viable cells (\(10^8\)) = Number of colonies on LB plate \(\times 10^8\) \(\times \) 10  \hspace{1cm} \text{Eq 7.12} \\
ml
Chapter 8

References
Chapter 8: References


O'Neill, R. J. (2004). A kinetic and biophysical analysis of mismatch uracil DNA glycosylase from Escherichia coli: a general or specific mismatch glycosylase? Department of Biochemistry, Imperial College London. PhD.


