Chapter 9

Summary and Perspective

The importance of [4Fe4S] clusters in DNA-processing enzymes is difficult to understate. [4Fe4S] clusters have now been reported in base excision repair (BER), nucleotide excision repair (NER), and DNA replication pathways in organisms from bacteria to man (*1 - 9*). This is underscored by the strict conservation of such a metabolically expensive complex when, in many cases, structural integrity and basic enzymatic activity can be achieved with zinc or even bulky amino acids (*10-12*). In addition, many of these proteins have disease-relevance, in particular human NTHL1 and MUTYH BER glycosylases (*13, 14*). Thus, understanding the function of the [4Fe4S] cluster in these proteins is a matter of great importance.

Although their response to oxidants and reductants in solution varies (*1 - 9*), previous work in the Barton lab has demonstrated that DNA binding activates the cluster for oxidation, making the $[4Fe4S]^{3+/2+}$ couple accessible at biological potentials (*15*). Furthermore, once oxidized, the DNA-binding affinity is increased by ~500-fold at least in *Escherichia coli* endonuclease III (EndoIII) (*15, 16*). In addition to activating these proteins for redox activity, DNA has been shown to serve as a means of long-range communication through the process of DNA-mediated charge transport (DNA CT) (*17*). Bacterial BER proteins use DNA CT as a means of localizing to their target lesions (*17*). In this model, proteins are initially oxidized to the $[4Fe4S]^{3+}$ state by species like the guanine radical cation that are generated under conditions of oxidative stress (*18*). Once oxidized, these individual proteins can then be reduced by a distally protein in the reduced state; at this point, the reduced protein diffuses away from the DNA, while the oxidized protein remains tightly bound. This effectively confirms that the DNA stretch between the two proteins is undamaged. If an intervening lesion is present between two proteins, DNA CT is attenuated and this search process cannot occur. In this case, the oxidized proteins remain bound in the vicinity of the lesion and are poised to resolve the damage. Overall, this

model serves to explain how BER proteins find scarce targets on a biologically relevant time scale.

While the work leading up to this DNA repair model has provided great insight into the function of [4Fe4S] clusters in DNA, several details remained to be elucidated. In particular, the precise cause of the potential shift seen upon DNA binding was not clear, and the relevance of redox activity and DNA CT to human disease had not been addressed. Indeed, eukaryotic repair proteins had not been directly investigated using the electrochemical and spectroscopic techniques typically used in the Barton lab. Moving beyond repair, the recently reported occurrence of [4Fe4S] clusters in eukaryotic DNA replication brought to light a set of proteins whose activity could not be easily understood using the model developed for BER.

In this thesis work, we have addressed each of these questions using a combination of electrochemical, spectroscopic, and biochemical techniques. First, the molecular cause of the potential shift was investigated using a combination of electrochemistry on a pyrolytic graphite edge (PGE) electrode and S K-edge X-ray absorbance spectroscopy (XAS) (*Chapter 2, 3*). Second, we carried out the first complete characterization of a disease-relevant mutant of a human [4Fe4S] protein, the BER glycosylase MUTYH (*Chapter 4*). Third, the role of the [4Fe4S] cluster in yeast DNA polymerase (Pol) δ, the eukaryotic lagging strand DNA polymerase, was assessed using a variety of complementary *in vitro* methods (*Chapter 5-7*).

Experiments with EndoIII and MutY on PGE electrodes in the presence and absence of DNA revealed a potential shift similar to that seen on highly-oriented pyrolytic graphite (HOPG) in earlier studies (*15, Chapter 2*). Simply considering electrode surfaces, this work represented an advance by making the electrochemistry of DNA-free proteins simpler and more reproducible. HOPG, which was used for this type of work in the past, is a very flat, hydrophobic

surface ill-suited to protein adsorption, and is furthermore quite challenging to prepare in a reproducible manner (*15*). PGE, in contrast, is a rough surface with plenty of adsorption sites for proteins; these effects can be enhanced by drying the protein on in a thin film and applying highly conductive carbon nanotubes (*Chapter 2*). With respect to the role of nearby amino acid residues in tuning [4Fe4S] cluster potential, we used PGE electrochemistry to study a series of EndoIII point mutants in which single charges near the cluster were altered. No significant potential shift was observed in the absence of DNA, supporting the notion that the negatively charged DNA backbone is the dominant effect in tuning the [4Fe4S] redox potential.

In parallel with PGE electrochemistry, S K-edge XAS was used to study the effects of DNA binding and solvation on the potential of EndoIII and MutY (*Chapter 3*). S atom absorption in XAS reports on Fe-S bond covalency, which directly reflects redox potential. Notably, a decrease in absorbance corresponding to a -150 mV shift in potential was observed for EndoIII upon DNA binding; similar results occurred for MutY, although spectra were too small and noisy for accurate quantification. In addition, removal of solvent by lyophilization decreased absorbance, although not to the same extent seen with DNA binding; furthermore, the same effect of DNA-binding was seen in lyophilized samples. These results indicate that solvent hydrogen-bonding has a relatively minor role in defining the [4Fe4S] potential, and, together with PGE electrochemistry, emphasize the importance of DNA in this process.

Base excision repair in humans involves homologues of many of the *E. coli* proteins, including NTHL1 and MUTYH, which are, respectively, the EndoIII and MutY homologues (*13, 14*). Notably, recent years have seen an increasing number of catalogued mutations in the Fe-S region of these proteins in cancer databases, although none had been characterized *in vitro* up to this point. The discovery of a novel MUTYH mutation in one of the cysteine residues that ligate

the [4Fe4S] cluster (C306W) brought an opportunity to advance both the understanding of the cluster in human disease and the role of CT in human cells (*Chapter 4*). MUTYH C306W was determined to be pathological, and biochemical assays showed low activity levels and poor DNA binding relative to wild type MUTYH and the mutants Y179C and G396D, which are cancer-relevant but have no defects in the Fe-S domain. UV-visible spectroscopy confirmed that MUTYH C306W could still bind cluster as effectively as wild type, but electrochemistry showed that this mutant is susceptible to cluster degradation in the presence of oxygen. Using EPR spectroscopy, we identified the degradation product as the $[3Fe4S]^{+}$ cluster. Taken as a whole, these results indicate that MUTYH C306W tends to lose an iron upon aerobic oxidation, resulting in a $[3Fe4S]^{+}$ cluster. This species would already be expected to have less electrostatic attraction to the DNA than the $[4Fe4S]^{3+}$, or even the $[4Fe4s]^{2+}$ states, and reduction to the $[3Fe4S]^{0}$ state exacerbates this problem even further. Ultimately, the cluster degrades in the course of the redox search, impairing DNA binding and, as a consequence, enzymatic activity. In the context of work in the Barton lab, this study was the first confirmation that the human homologues of bacterial [4Fe4S] BER proteins have comparable redox activity when DNA-bound, and represents a first step toward assessing the pathology of numerous cancer-relevant MUTYH and NTHL1 mutations.

While CT in eukaryotic DNA repair was reasonably assumed to work similarly to the bacterial version, no explanation existed for the role of [4Fe4S] clusters in DNA replication proteins until recently. A characterization of the cluster in yeast Pol δ over the course of this thesis work shed some light on this situation, and led to a more general expansion of the CT signaling model to incorporate diverse protein activities (*Chapter 5*). Electrochemistry on DNA-modified gold electrodes demonstrated that Pol δ is redox active with a midpoint potential in the

same range as previously studied proteins. Incorporation of an abasic site or CA mismatch into the DNA led to signal attenuation, confirming that Pol δ is capable of DNA-mediated signaling. EPR spectroscopy confirmed the identity of the relevant redox couple as $[4Fe4S]^{3+/2+}$, as expected. Anaerobic oxidation by either electrochemical methods or photooxidation with anthraquinone (AQ) resulted in a stalling of DNA synthesis as seen on alkaline agarose gels. Critically, re-reduction using bulk electrolysis restored activity to near-untreated levels. As a whole, these results show that the oxidation state of the [4Fe4S] cluster acts as a reversible switch for enzymatic activity, stalling Pol δ upon oxidation and reactivating processive DNA synthesis upon reduction. Oxidative stalling could represent a means to respond to replication stress, especially oxidative stress, on a time scale even more rapid than protein-protein signaling cascades in the standard stress response. This work thus led to a generalization upon the CT model, with cluster oxidation state serving as a switch to turn activity on or off in different metabolic contexts.

Efforts to elucidate the role of the cluster in Pol δ *in vivo* by using CT-deficient mutants were also begun in this work (*Chapter 6*). We designed and characterized two mutants, W1053A and Y1078A, that were conserved and located sufficiently close to the [4Fe4S] cluster to be involved in the CT pathway. Unfortunately, the complete set of *in vitro* studies revealed structural instability in both mutants, necessitating a switch to less destabilizing mutations such as W, Y → F. While nothing conclusive came out of these efforts, the failures can be used to guide the next round of experiments, and overall this line of investigation remains worth pursuing.

Long-range CT in the context of replication could certainly occur within newly-synthesized DNA stretches, but much of the DNA present at the fork is single-stranded and

coated with the single-stranded binding protein RPA (*Chapter 8*). Single-stranded DNA cannot support CT, so long-distance signaling during replication appears limited. However, given that aromatic amino acid residues can electronically couple into the base stack and facilitate CT, we reasoned that RPA, with its numerous aromatic stacking interactions with DNA, might be able to open up more pathways to this type of communication. To investigate this possibility, we have begun setting up photooxidation experiments to see if RPA can bridge a Rh photooxidant and a GG site on the other side of a single-stranded gap. No significant results have yet been achieved in this work, but the possibility is intriguing and will be continued.

In summary, the work detailed in this thesis has expanded upon previous work in DNA repair and initiated significant advances in understanding the role of [4Fe4S] proteins in DNA replication. Furthermore, several new methods were brought to the Barton lab in the course of these studies, and the work on DNA replication in particular sparked new approaches to the study of [4Fe4S] proteins in the context of DNA CT. As is readily apparent from the leaps in understanding made in the last decade, this work itself is not the final say on these issues as more proteins involved in DNA processing that contain [4Fe4S] clusters are identified, and it is almost a certainty that more critical roles for long range DNA CT will emerge.

References

1) Cunningham, R.P.; Asahara, H.; Bank, J.F.; Scholes, C.P.; Salerno, J.C.; Surerus, K.; Munck, E.; McCracken, J.; Peisach, J.; Emptage, M.H. Endonuclease III is an iron-sulfur protein. *Biochemistry* **1989**, *28*, 4450 – 4455.

2) Porello, S.L.; Cannon, M.J.; David, S.S. A Substrate Recognition Role for the $[4Fe4S]^{2+}$ Cluster of the DNA Repair Glycosylase MutY. *Biochemistry*, **1998**, *37*, 6465 – 6475.

3) Aspinwall, R.; Rothwell, D.G.; Roldan-Arjona, T.; Anselmino, C.; Ward, C.J.; Cheadle, J.P.; Sampson, J.R.; Lindahl, T.; Harris, P.C.; Hickson, I.D. Cloning and characterization of a functional human homolog of *Escherichia coli* endonuclease III. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 109 – 114.

4) Alseth, I.; Eide, L.; Pirovano, M.; Rognes, T.; Seeberg, E.; Bjørås, M. The *Saccharomyces cerevisiae* Homologues of Endonuclease III from *Escherichia coli*, Ntg1 and Ntg2, are both Required for Efficient Repair of Spontaneous and Induced Oxidative DNA Damage in Yeast. *Mol. Cell Biol.* **1999,** *19*, 3779 – 3787.

5) Ren, B.; Duan, X.; Ding, H. Redox control of the DNA damage-inducible protein DinG helicase activity via its iron-sulfur cluster. *J. Biol. Chem.* **2009**, *284*, 4829 – 4835.

6) Fan, L.; Fuss, J.O.; Cheng, Q.J.; Arvai, A.S.; Hammel, M.; Roberts, V.A.; Cooper, P.K.; Tainer. J.A. XPD Helicase Structure and Activities: Insights into the Cancer and Aging Phenotypes from XPD Mutations. *Cell*, **2008**, *133*, 789 – 800.

7) Pokharel, S.; Campbell, J.S. Cross talk between the nuclease and helicase activities of Dna2: role of an essential iron-sulfur cluster domain. *Nucleic Acids Res.* **2012**, *40*, 7821 – 7830.

8) **a.** Klinge, S.; Hirst, J.; Maman, J.D.; Krude, T.; Pellegrini, L. An iron-sulfur domain of the eukaryotic primase is essential for RNA primer synthesis. *Nat. Struct. Mol. Biol.* **2007**, *14*, 875-877. **b.** Weiner, B.E.; Huang, H.; Dattilo, B.M.; Nilges, M.J.; Fanning, E.; Chazin, W.J. An iron-sulfur cluster in the C-terminal domain of the p58 subunit of human DNA primase. *J. Biol. Chem.* **2007**, *282*, 33444 – 33451.

9) Netz, D.J.A.; Stith, C.M.; Stümpfig, M.; Köpf, G.; Vogel, D.; Genau, H.M.; Stodola, J.L.; Lill, R.; Burgers, P.M.J.; Pierik, A.J. Eukaryotic DNA polymerases require an iron-sulfur cluster for the formation of active complexes. *Nat. Chem. Biol.* **2012**, *8*, 125 – 132.

10) Netz, D. J. A.; Mascarenhas, J.; Stehling, O.; Pierik, A. J.; Lill, R. Maturation of cytosolic and nuclear iron-sulfur proteins. *Trends Cell Biol.* **2014**, *24*, 303 – 312.

11) Klinge, S.; Núñez-Ramírez, R.; Llorca, O.; Pellegrini, L. 3D architecture of DNA Pol α reveals the functional core of multi-subunit replicative polymerases. *EMBO J.* **2009**, *28*, 1978 – 1987.

12) Trasvina-Areñas, C. H.; Lopez-Castillo, L. M.; Sanchez-Sandoval, E.; Brieba, L. G. Dispensability of the [4Fe-4S] cluster in novel homologues of adenine glycosylase MutY. *FEBS J.* **2016**, *283*, 521 – 540.

13) Weren, R.D.A. et al. A germline homozygous mutation in the base-excision repair gene NTHL1 causes adenomatous polyposis and colorectal cancer. *Nat. Genet*. **2015**, *47*, 668 – 671.

14) D'Agostino, V.G., Minoprio, A., Torreri, P., Marinoni, I., Bossa, C., Petrucci, T.C., Albertini, A.M., Ranzani, G.N., Bignami, M., and Mazzei, F. Functional analysis of MUTYH mutated proteins associated with familial adenomatous polyposis. *DNA Repair (Amst)*. **2010**, *9*, 700 – 707.

15) Gorodetsky, A.A.; Boal, A.K.; Barton, J.K. Direct electrochemistry of Endonuclease III in the presence and absence of DNA. *J. Am. Chem. Soc.* **2006**, *128*, 12082-12083.

16) Tse, E.C.M.; Zwang, T.J.; Barton, J.K. The Oxidation State of [4Fe4S] Clusters Modulates the DNA-Binding Affinity of DNA Repair Proteins. *J. Am. Chem. Soc.* **2017**, *139*, 12784 – 12792.

17) Arnold, A. R.; Grodick, M. A.; Barton, J. K. DNA Charge Transport: from Chemical Principles to the Cell. *Cell Chem. Biol*. **2016**, *23*, 183 – 197.

18) Yavin, E.; Boal, A. K.; Stemp, E. D. A.; Boon, E. M.; Livingston, A. L.; O'Shea, V. L.; David, S. S.; Barton, J. K. Protein-DNA Charge Transport: Redox Activation of a DNA Repair Protein by Guanine Radical. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 3546 – 3551.