Chapter 6

Summary and Outlook

Summary: Pathways for Protein-DNA Charge Transfer

The experiments described in the preceding chapters examined the charge-transfer (CT) properties of several site-directed mutants of three base-excision repair enzymes: UDG, MutY, and EndoIII. These proteins are thought to participate in DNA-mediated long-range charge transfer in order to scan the genome for DNA damage. The mutants that were created and characterized were chosen in order to answer four questions about how amino-acid changes in the proteins affect protein-to-DNA CT: 1) How do perturbations in the [4Fe-4S] cluster binding domain affect the CT properties of DNA repair enzymes? 2) Which types of amino acid residues best mediate protein-to-DNA CT? 3) What possible amino acid pathways may exist for DNA-protein CT? 4) What are the biological consequences of having an impaired CT pathway?

The first of these questions, how the [4Fe-4S] cluster binding domain affects CT, was addressed by studying the UDG mutants C17H, C85S, C101S, and the EndoIII mutants W178A and Y185A. Altering the coordination environment could greatly decrease CT, as was the case with C85S UDG. However, C101S UDG produced an electrochemical signal that was within error of that of WT UDG. The UDG mutant C17H and the EndoIII mutants W178A and Y185A produced signals larger than those of the corresponding WT proteins. This signal increase was attributed to structural perturbations in the mutants' secondary structures that may have permitted water molecules to enter the protein and accelerate CT [1, 2].

The C17H mutation may have also placed a more CT-active residue closer to the cluster than that present in the native protein.

Mutagenesis studies of MutY Y82 position mutants and EndoIII Y82 position mutants investigated which types of residues best facilitate CT in their respective proteins. In MutY, long residues such as tyrosine and leucine that can intercalate into the DNA helix were the best CT mediators. In EndoIII, aromatic residues proved most CT-proficient. Several aromatic amino acids in EndoIII, including Y82, Y75, Y55, and F30, form what may be a partial pathway between the DNA and the [4Fe-4S] cluster.

The fourth question, the biological consequences of CT impediments has been addressed through colorectal cancer research. Mutations at residues Y114, Y165, and Y166 in human MUTYH have appeared in colorectal cancer patients [3, 4]. These residues align with F30, L81, and Y82, respectively, in *E. coli* EndoIII, and Y165 aligns with Y82 in *E. coli* MutY. EndoIII F30 and Y82, and MutY Y82 facilitate CT in their proteins. Therefore, the equivalent MUTYH residues may possibly mediate protein to DNA CT in human cells. Without this CT capability, MUTYH may be unable to detect and repair DNA damage, causing mutations to accumulate that would lead to colorectal cancer. CT deficiencies in biological systems could produce very deleterious consequences for the cell.

As a whole, the BER mutants examined here helped elucidate the properties that create a CT-active enzyme capable of participating in DNA-mediated long-range signaling. The protein must contain a well-protected, well-coordinated, redox

active cofactor (such as a [4Fe-4S] cluster). It must contain amino acids capable of intercalating into the DNA helix to allow charge to flow from the DNA into the protein, and it must contain a pathway of CT-active amino acids that can convey this charge from the DNA to the redox cofactor. Future experiments, such a time-resolved spectroscopy, will be necessary to determine how mutations in the CT pathway affect CT rates. Other ongoing *in vivo* experiments will determine how mutations in the CT pathways of these DNA repair proteins affect *in vivo* mutagenesis rates.

Outlook: Beyond DNA Repair

The majority of the experiments performed for this thesis investigated DNA-mediated charge-transfer (CT) for DNA-repair using *in vitro* characterization of proteins that may participate in this process. However, recent research is developing *in vivo* experiments to examine long-range electrochemical signaling as means of searching for DNA damage [5]. Such experiments include the "helper function" assay that investigated whether EndoIII (and variants) could help MutY detect and repair its substrates [5]. Another way to study the *in vivo* activity of EndoIII would be to investigate the role of the *rnf* operon in DNA repair. The *rnf* operon is a seven-gene operon of which the *nth* gene, which encodes EndoIII, is the final component [6] (Figure 6.1). This operon has not been fully characterized in *E. coli,* but could possibly regulate redox processes within the cell.

The notion that *rnf* genes control cellular redox processes is supported by several lines of evidence. First, the "*rnf*" operon is so named because its operon structure and gene sequences bear similarity to "rhodobacter nitrogen fixation" genes, or genes used by *Rhodobacter capsulatus* and *Azotobacter vinelandii* to reduce dinitrogen [7–9]. In *R. capsulatus, rnf* operon genes are thought to donate electrons to the nitrogenase complex [8, 9]. In *A. vinelandii*, *rnf* genes are thought to assist in iron-sulfur cluster assembly [7]. Similar gene products are also hypothesized to supply electrons for 2,4-dinitrophenol reduction in *R. capsulatus* [10], encode a ferredoxin:NAD^+ oxidoreductase for caffeate reduction in *Acetobacterium woodii* [11–13], and form a Na^+ translocating NADH:quinone oxidoreductase complex in *Vibrio alginolyticus* [14]. The fact that homologous genes participate in redox processes in so many other organisms makes it likely that they mediate redox chemistry in *E. coli.*

Other evidence that the *rnf* gene products facilitate redox processes from sequence predictions and preliminary biochemical characterization of the gene products. RnfB is hypothesized to contain several iron-sulfur clusters, RnfC has several domains that are similar to those found in other redox-active proteins, and RnfG is predicted to contain a FMN-binding domain[1]. RnfB and RnfC from *R. capsulatus* have been purified and were red-brown and brown, respectively [8, 14], colors often indicative of an iron-sulfur cluster. RnfB exhibited an absorbance and

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[1] All predictions based on gene sequence information were either taken from the published literature or made using one of the following online resources: http://www.ncbi.nlm.nih.gov, http://www.uniprot.org, http://ca.expasy.org/.

EPR spectrum characteristic of [2Fe-2S] proteins and RnfC exhibited an absorbance spectrum characteristic of [4Fe-4S] proteins [14]. RnfC from *R. capsulatus* is also predicted to contain binding sites for NAD(H) and flavin mononucleotide (FMN), components of redox-active proteins [15]. However, neither RnfB nor RnfC could be characterized in great detail because the proteins were oxygen labile [14].

Few experiments have been performed on the *rnf* genes of *E. coli*, although one recent breakthrough demonstrated that these genes affect the activity of *E. coli* SoxR [16]. SoxR is a [2Fe-2S] transcription factor that regulates *E. coli* cells' response to oxidative stress [17]. When the iron-sulfur cluster of SoxR becomes oxidized, this protein then upregulates several genes that help the cell remove reactive oxygen species [17]. After the threat of oxidative stress is gone, *rnfC* helps re-reduce SoxR and stop the upregulation of oxidative stress response genes [16]. The mechanism of re-reduction remains unknown. Because SoxR is a DNA-binding protein that can participate in long-range DNA-mediated CT [18], it is possible that the *rnf* operon products that interact with it can also oxidize or reduce other redox-active DNA binding proteins such as MutY and EndoIII.

To better understand the cellular role of rnf proteins, experiments should be performed to address several questions: 1) Where do rnf proteins localize within the cell? 2) When are the *rnf* genes upregulated or downregulated? 3) Does the deletion of any *rnf* genes produce any detectable phenotypes? Regarding their localization, RnfA and RnfE have been shown to be membrane-bound [17, 19], and RnfD is also

predicted to localize to the membrane. RnfB and RnfC are predicted to contain both membrane and soluble domains (see footnote 1). It is possible that the rnf proteins form a membrane-bound complex through which reducing (and/or oxidizing) equivalents are transferred to other cellular components, possibly in an NAD^+/NADH mediated fashion (Figure 6.2). Fractionation and Western blotting experiments could confirm where rnf proteins localize within the *E. coli* cell.

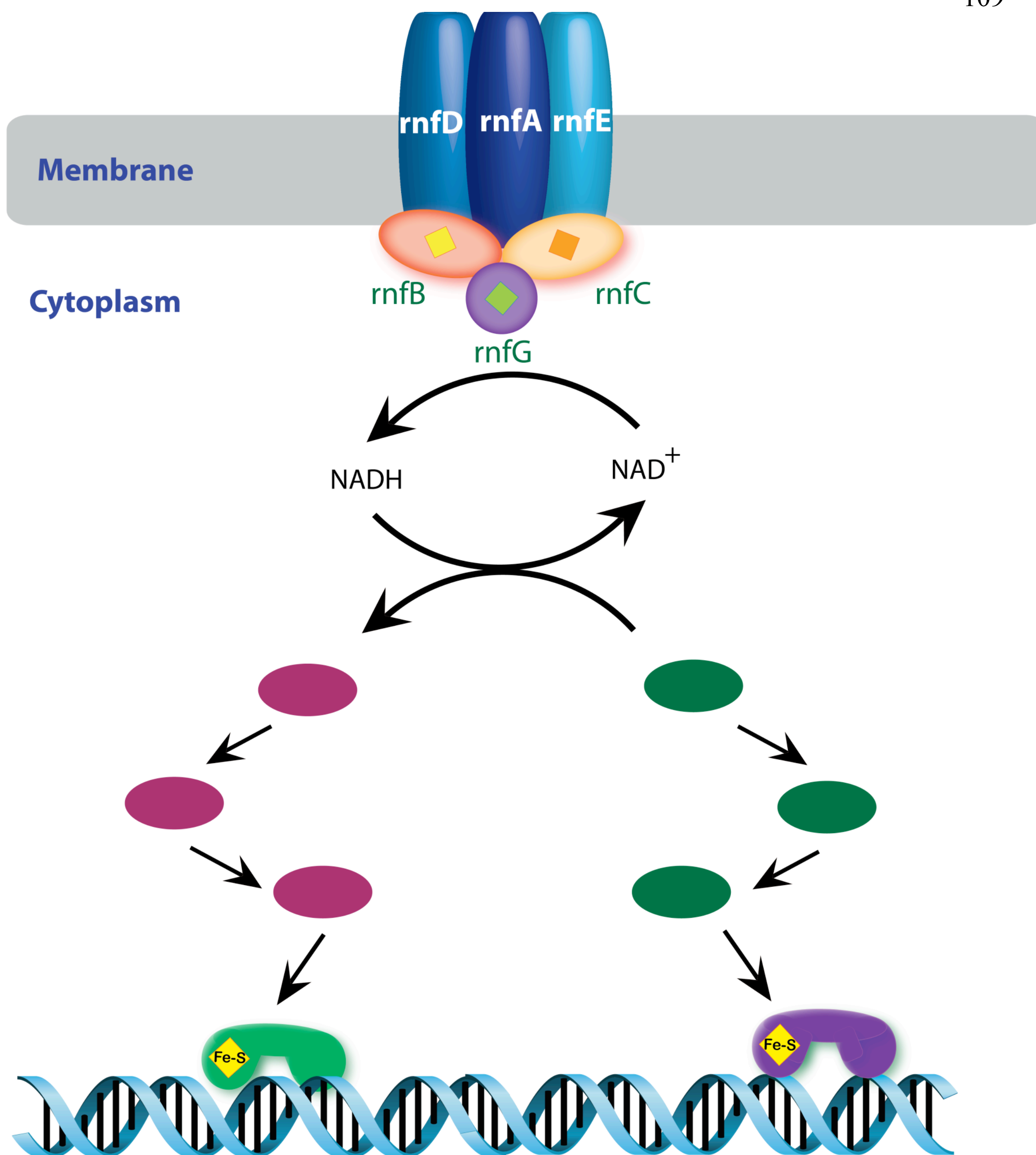
Little is known about what factors alter expression of *rnf* genes. In *R.capsulatus*, their expression is decreased under iron limitation [8]. Addition of paraquat and hydrogen peroxide does not upregulate the expression of *nth* in *E. coli* [20], and so would unlikely upregulate the co-expressed *rnf* genes. Other conditions could be tested using qRT-PCR experiments. Because *rnf* gene products are so important for the activity of the SoxRS regulon, it would also be interesting to test whether they influence the activity of any other redox-active transcription factors in *E. coli*, such as OxyR [15], FNR [15], and MgrA [21]. Experimentally, a link between transcription factors and *rnf* products could be determined by a combination of genetics and molecular biology experiments. First, *rnf* genes could be genetically inactivated, particularly *rnfB* and *rnfC*, since these genes are predicated to contain the most redox-active domains. The activity of OxyR, FNR, and MgrA in these deletion strains could then be measured through qRT-PCR analysis of genes in their regulons or by β-galactosidase assays of *lacZ* fusions to any of these regulon genes. To further investigate whether *rnf* genes are involved in redox regulation, the ratio of a NAD^+ to NADH could also be quantified in *rnf*

deletion strains relative to wild-type *E. coli* strains. Pull-down assays could also be performed to determine which other *E. coli* proteins interact with *rnf*B and *rnfC.*

In total, the experiments performed on mutant BER proteins determined which amino acids mediate protein-to-DNA CT in the [4Fe-4S] glycosylase proteins UDG, MutY, and EndoIII. The experiments mentioned in this final chapter also aim to determine whether these proteins or any of their genomic neighbors play a broader *in vivo* role in regulating DNA repair, and/or other redox processes. EndoIII is part of an operon whose genes encode proteins that may be involved in uptake of oxidizing or reducing equivalents. The operon that encodes MutY also encodes YggX, which has been shown to help protect cells from oxidative stress [22–24]. Consequently, the BER enzymes and their operons, may play an important *in vivo* role beyond DNA repair. These enzymes and their genetic neighbors may be part of much broader system through which the cell maintains redox control and regulates processes that sustain biological activity.

Figure 6.1: *Rnf* operon. The *nth* gene, which encodes EndoIII, is the terminal gene of a seven-gene operon. The upstream genes of this operon play an undetermined role in *E. coli.* The *ydgK* gene is upstream of the *rnf* operon, but contains its own promoter. Figure adapted from reference [25].

Figure 6.2: Possible cellular localization and function of rnf proteins. The products of the *rnfD, rnfA,* and *rnfE* genes are predicted to be membrane-bound. The products of the *rnfB* and *rnfC* genes are predicted to contain both membrane and soluble domains, while the *rnfG* gene product is predicted to be soluble. The latter three genes are predicted to encode binding sites for redox-active cofactors. Collectively, the rnf proteins may form a membrane-bound complex responsible for transporting reducing and/or oxidizing equivalents into the cell and passing them to other molecules *in vivo*. These reducing and/or oxidizing equivalents may be transferred through an NADH/NAD^+ mediated mechanism, since *rnf* genes encode binding sites for these molecules. The downstream recipients of these reducing and/or oxidizing equivalents may include redox-active DNA-binding proteins.


rnfD
rnfA
rnfE
Membrane
Cytoplasm
rnfB
rnfC
rnfG
NADH
NAD+
Fe-S
Fe-S

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