

Chapter 7: DNA Binding Shifts the Redox Potential of the Transcription Factor SoxR

Adapted from: Gorodetsky, A. A., Dietrich, L. E. P., Lee, P. E., Demple, B., Newman, D. K., Barton, J. K. (2008) *Proc. Natl. Acad. Sci. U. S. A.* **105**, 3684–3689.

SoxR was expressed and purified by Lars Dietrich.

ABSTRACT

Electrochemistry measurements on DNA-modified electrodes are used to probe the effects of binding to DNA on the redox potential of SoxR, a transcription factor that contains a [2Fe-2S] cluster and is activated through oxidation. A DNA-bound potential of +200 mV versus NHE (normal hydrogen electrode) is found for SoxR isolated from *Escherichia coli* and *Pseudomonas aeruginosa*. This potential value corresponds to a dramatic shift of +490 mV versus values found in the absence of DNA. Using Redmond Red as a covalently bound redox reporter affixed above the SoxR binding site, we also see, associated with SoxR binding, an attenuation in the Redmond Red signal compared with that for Redmond Red attached below the SoxR binding site. This observation is consistent with a SoxR-binding-induced structural distortion in the DNA base stack that inhibits DNA-mediated charge transport to the Redmond Red probe. The dramatic shift in potential for DNA-bound SoxR compared with the free form is thus reconciled based on a high-energy conformational change in the SoxR–DNA complex. The substantial positive shift in potential for DNA-bound SoxR furthermore indicates that, in the reducing intracellular environment, DNA-bound SoxR is primarily in the reduced form; the activation of DNA-bound SoxR would then be limited to strong oxidants, making SoxR an effective sensor for oxidative stress. These results more generally underscore the importance of using DNA electrochemistry to determine DNA-bound potentials for redox-sensitive transcription factors because such binding can dramatically affect this key protein property.

7.1 Introduction

SoxR belongs to the MerR family of transcriptional regulators. The members of this family are defined by an N-terminal helix–turn–helix DNA binding motif, a coiled-coil dimerization region, and a C-terminal sensory domain (1–3). Although the DNA binding and dimerization regions are conserved among MerR-type regulators, their sensory domains are diverse (2). Typically, MerR type transcription factors occupy suboptimally spaced 19 ± 1 -bp promoter elements in the inactivated state, often inducing a slight bend of the promoter DNA. Upon activation, these proteins are thought to undergo a conformational change that unwinds the promoter region, thereby allowing RNA polymerase to initiate transcription (2).

SoxR regulates an oxidative stress response to superoxide in the enterics *Escherichia coli* and *Salmonella enterica* (4, 5). This unique transcription factor is a 17-kDa polypeptide that binds DNA as a dimer and contains a [2Fe-2S] cluster in each monomer (4). Loss of this cluster does not affect protein folding, DNA binding, or promoter affinity (6–8), but oxidation of this cluster by either oxygen or superoxide-generating agents (e.g., methyl viologen) triggers expression of the transcription factor SoxS (8, 9). Subsequently, SoxS controls the expression of > 100 genes in the SoxRS regulon that collectively act to repair or avoid oxidative damage (10).

The role of SoxR appears to vary dramatically across organisms. Whereas SoxR is conserved in both Gram-negative and Gram-positive bacteria, *soxS* is exclusively found in enterics, indicating that SoxR can be part of different regulatory networks (11, 12). Indeed, *Pseudomonas putida* and *Pseudomonas aeruginosa* do not rely on SoxR for

an oxidative-stress response (13, 14). Instead, *P. aeruginosa* SoxR responds to phenazines, endogenous redox-active pigments, and activates transcription of two probable efflux pumps and a putative monooxygenase (15) that might aid in phenazine transport and modification. Considering that SoxR shows functional diversity between pseudomonads and enterics, it is surprising that the transcription factor is biochemically conserved: (i) Expression of *P. putida* SoxR in *E. coli* can complement a *soxR* deletion mutant (14), and (ii) the redox potentials of soluble SoxR from *E. coli* and *P. aeruginosa* *in vitro* are both approximately -290 mV (7, 8, 15).

That SoxR requires oxidation for its transcriptional activity seems biologically reasonable but also leads to a conundrum. Under normal physiological conditions, it is assumed that SoxR is kept in its reduced, inactive state by the intracellular NADPH/NADP⁺ redox potential of approximately -340 mV versus NHE (16, 17). Furthermore, it has been reported that NADPH-dependent SoxR reduction is enzyme-mediated, allowing for a rapid adjustment to changes in cellular conditions, although direct enzymatic interaction with SoxR has not yet been demonstrated (18, 19). The conundrum does not lie in the mechanism of SoxR reduction but rather in the specificity of its oxidation: At a low redox potential of -290 mV versus NHE (7, 8, 15), many cellular oxidants could react with SoxR, in particular glutathione (20), and therefore SoxR would be primarily in an oxidized form, even without imposing oxidative stress. Since this is not the case, how is SoxR maintained in its reduced and transcriptionally silent form?

The mechanism underlying the oxidation/activation of SoxR is also not well understood. For *E. coli* SoxR, it was first suggested that superoxide directly oxidizes the

iron-sulfur cluster, but this has not been established (21, 22). Alternatively, the redox state of SoxR might be coupled to changes in the equilibrium of biologically relevant redox couples, such as NADPH or glutathione (16, 23). Recently, we have shown that the activation of SoxR in *P. aeruginosa* can occur in an oxygen-independent manner (14). Considering that both *E. coli* and *P. aeruginosa* SoxR can transfer electrons to the mediator safranin O, a phenazine derivative (7, 8, 15), it seems reasonable that endogenous phenazines may oxidize SoxR in pseudomonads. Alternatively, given that pseudomonad phenazines can also modulate the intracellular NADH/NAD⁺ ratio, the possibility that phenazines activate SoxR indirectly must also be considered (24).

One interesting possibility that has been suggested but never addressed experimentally is the effect of DNA binding on the redox potential of SoxR. The published redox potentials for SoxR were measured in the absence of DNA (4, 7, 8). This is particularly significant because SoxR activates transcription only in its DNA-bound state, so determining the redox potential of the DNA-bound form of SoxR becomes critical.

We have previously explored DNA-modified electrodes as flexible platforms for the study of DNA-mediated charge transport chemistry (25–27). Typically, self-assembled DNA monolayers on gold or graphite are interrogated electrochemically with the efficiency of charge transfer to an electroactive probe yielding information on the integrity of the intervening base pair stack. In fact, duplexes that are covalently modified with redox-active reporters at a fixed position provide particularly well defined systems for study of DNA charge transport at electrode surfaces, allowing for the electrochemical detection of even small perturbations in the intervening base pair stack (28–31).

In addition, DNA-modified electrodes have proven useful for probing redox centers within proteins bound to DNA (32–34). We have used DNA monolayers to probe the redox potential of MutY and Endonuclease III, base excision repair glycosylases that contain a [4Fe-4S] cluster. Initial studies of these enzymes had found no clear role for the clusters because, in the absence of DNA, they did not display redox activity within a physiologically relevant range of potentials (35–37). We found, however, that at DNA-modified Au surfaces, these repair enzymes display reversible, DNA-mediated electrochemistry with redox potentials of ≈ 90 mV (33). Moreover, experiments comparing directly the electrochemistry of Endonuclease III on bare and DNA-modified graphite demonstrated that binding to DNA shifts the redox potential of the protein by ≈ 200 mV into a physiologically relevant range, activating the cluster for oxidation (34). DNA binding thus changes the redox properties of the enzymes from being similar to ferredoxins to instead resembling high potential iron proteins. Based on these data, we have proposed a redox role for the [4Fe-4S] clusters in long-range DNA-mediated signaling as a first step in detecting damaged sites that are to be repaired in the genome (32–34, 38). Our ability to alter the redox states of these proteins in a DNA-mediated manner further suggests that DNA may be a medium through which oxidation/reduction reactions occur. This mechanism may also be important to consider in the context of SoxR.

Given the sensitivity of DNA-modified electrodes in probing redox centers of proteins bound to DNA and the precedent that DNA binding can alter redox potentials of the bound protein, here we explore the redox properties of the DNA-bound form of SoxR. Model studies have shown repeatedly the sensitivity of redox potentials of iron-sulfur

clusters to environmental perturbations, which are expected to be significant for SoxR (39). Here, using self-assembled DNA monolayers on highly oriented pyrolytic graphite (HOPG), we address the effect of DNA binding on the redox potential of both *E. coli* and *P. aeruginosa* SoxR. The DNA-bound potential provides convincing evidence for the mechanism the cell uses to maintain SoxR in its reduced form *in vivo*.

7.2 Experimental Procedures

7.2.1 Materials

All phosphoramidites and reagents for DNA synthesis were purchased from Glen Research. 1,6-Diaminohexane was obtained from Acros Organics. All organic solvents and other reagents were purchased from Aldrich in the highest available purity.

7.2.2 Oligonucleotide Synthesis

Oligonucleotides were prepared using standard phosphoramidite chemistry on an ABI 394 DNA synthesizer. DNA was purified by HPLC on a reverse-phase C18 column with acetonitrile and ammonium acetate as eluents. The desired products were characterized by HPLC, UV-visible spectroscopy, and MALDI-TOF mass spectrometry. For experiments on HOPG, DNA was modified with pyrene at the 5' terminus by following the procedure reported in reference 27. In brief, oligonucleotides were prepared by solid phase synthesis using standard reagents with an unprotected hydroxyl group at the 5' terminus. The 5'-OH was treated with a 120 mg/ml solution of carbonyldiimidazole in dioxane for 2 hours followed by an 80 mg/ml solution of 1,6-diaminohexane for 30 minutes. Subsequently, the free amine was treated with 1-pyrenebutyric acid, *N*-

hydroxysuccinimide ester, resulting in the desired pyrene moiety linked to the 5' terminus. The oligonucleotides were deprotected with concentrated NH₄OH at 60 °C for 8 hours.

DNA modified with Redmond Red at the 3' terminus or 3 bases in from the 5' terminus was prepared according to the ultra-mild protocols outlined on the Glen Research web site (www.glenres.com). Pac-protected bases and ultra mild reagents were used. The oligonucleotides were deprotected in 0.05 M potassium carbonate in methanol at room temperature for 12–14 hours to prevent degradation of the Redmond Red moiety under harsh conditions.

7.2.3 Expression and Purification of SoxR

E. coli SoxR was prepared as described in reference 66. N-terminally histidine-tagged SoxR from *P. aeruginosa* PA14 were expressed from plasmid pET16b in *E. coli* strain BL21 (DE3). Cells were grown in 1 liter of LB medium with 100 µg/ml ampicillin at 37 °C. At an OD_{600nm} of 0.3, protein expression was induced by the addition of 1 mM IPTG and the cultures were incubated for an additional 10 hours at 16 °C. All subsequent steps were performed at 4°C. Cells were pelleted, resuspended in buffer A [50 mM NaH₂PO₄ (pH 8.0), 300 mM NaCl, 10% glycerol] containing 10 mM imidazole and PIC (protease inhibitor mixture without EDTA; Roche), and lysed using a French Press. The cell extract was centrifuged at 14,000 × g for 20 minutes. The supernatant was incubated with Talon-beads (Clontech) for 30 minutes and then transferred to a column. The beads were washed with buffer A containing 50 mM imidazole and PIC. Histidine-tagged SoxR was eluted from the column with buffer A containing 250 mM imidazole and PIC. Peak fractions and purity were determined by SDS/PAGE with Coomassie Blue staining.

Purified protein was dialyzed against SoxR storage buffer [50 mM Pi (pH 8.0), 500 mM NaCl, 20% glycerol].

To generate expression plasmid pET16b-soxR, *soxR* (PA14_35170) was PCR-amplified from genomic DNA of *P. aeruginosa* PA14 using primers A (CGC *catatg* AAG AAT TCC TGC GCA TC) and B (GGC *gga* tcc CTA GCC GTC GTG CTC G). Primer A contains an NdeI restriction site (small letters) and *soxR*'s start codon (italicized). Primer B contains a BamHI site (small letters) and *soxR*'s stop codon. The PCR fragment was ligated into NdeI/BamHI-digested pET16b.

7.2.4 Formation of DNA Monolayers and Electrochemical Measurements

DNA films were self-assembled on SPI-1 grade HOPG electrodes (SPI) with an estimated surface area of 0.08 cm² defined by an *o*-ring. Duplex DNA was formed in (pH 8) 50 mM Pi/500 mM NaCl/20% glycerol buffer (SoxR storage buffer) by combining equimolar amounts of the pyrene-modified strand with its complement. Loosely packed DNA monolayers were allowed to form over a period of 24–48 hours. The electrodes were then thoroughly rinsed with SoxR storage buffer before being backfilled for 2–4 hours with 10% by volume octane or decane solutions in SoxR storage buffer. The electrodes were then thoroughly rinsed with SoxR storage buffer again and moved into a nitrogen atmosphere for electrochemistry experiments.

Electrochemical data were collected with a Bioanalytical Systems CV-50W potentiostat using the inverted drop cell configuration. All measurements reported for the working electrode were taken versus a platinum (Pt) auxiliary and a silver/silver chloride (Ag/AgCl) reference. The Ag/AgCl reference was frequently standardized versus SCE, and all reported potentials have an experimental uncertainty of < 40 mV. Electrochemical

experiments were performed at ambient temperature and under an anaerobic atmosphere in SoxR storage buffer. In a typical experiment, background electrochemical scans were performed before SoxR was added to the storage buffer, resulting in an $\approx 15\text{--}35\ \mu\text{M}$ monomer concentration within the cell. Further scans were then performed in the presence of SoxR, typically over a period of 30–45 minutes.

7.3 Results

7.3.1 *Experimental Strategy Used to Probe SoxR Electrochemically*

Figure 7.1 illustrates the experimental strategy used to investigate the electrochemistry of DNA-bound SoxR from *E. coli* and *P. aeruginosa* PA14. DNA duplexes are prepared by hybridizing pyrene-modified single-stranded DNA with its complement (with or without covalently attached Redmond Red). The duplexes are then self-assembled on HOPG in the absence of Mg^{2+} to form a loosely packed DNA monolayer, leaving room for SoxR to bind (32–34). The surface is backfilled with octane or decane to prevent direct charge transfer from the surface to the protein (40, 41). The electrode is subsequently incubated with protein, and electrochemical experiments are performed before and after protein addition. The DNA binding sites for SoxR are 18-bp symmetrical sequences that are conserved across species (15). For *P. aeruginosa* experiments, we chose the SoxR binding site found upstream of an operon that encodes the efflux pump MexGHI-OpmD in *P. aeruginosa* PA14.

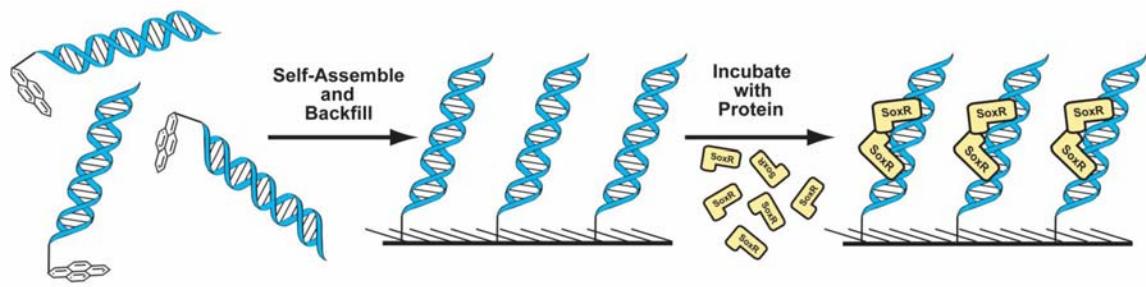


Figure 7.1: Schematic illustration of the self-assembly/backfilling of a DNA monolayer followed by incubation with protein

7.3.2 SoxR Binding Is Reported Through the Redmond Red Electrochemical Signal

We can observe protein binding in electrochemistry experiments by monitoring DNA-mediated transport between the electrode and the redox active probe Redmond Red that is attached at either end of the DNA duplex (Figure 7.2). The midpoint potential of Redmond Red is -160 mV versus NHE, and the linearity of the plot of peak current as a function of scan rate indicates that Redmond Red behaves as a surface-bound species (42). Although small potential shifts (≈ 20 mV) in the Redmond Red signal are occasionally observed upon addition of SoxR, Redmond Red provides a convenient and reliable internal standard.

It is expected that a redox-active probe located at the top of the DNA monolayer will report on perturbations of the base pair stack that intervene between the redox probe and the electrode, whereas the same probe located at the bottom of the monolayer near the electrode surface will not be affected by disruptions in base stacking above the probe. Previously, we have reported attenuation in charge accumulation by chronocoulometry for daunomycin covalently attached near the top of a DNA film due to perturbations in the intervening DNA structure by the base-flipping methylase M.HhaI and TATA binding protein (43). Here, as shown in Figure 7.2, when Redmond Red is incorporated above the SoxR binding site, a 16% decrease in the integrated cathodic charge of Redmond Red is observed upon addition of SoxR. In contrast, when Redmond Red is incorporated at the bottom of the DNA duplex below the SoxR binding site, there is little detectable change in the Redmond Red signal in the presence of SoxR. Although the loss of signal observed upon addition of SoxR is far smaller than that found for TATA

binding protein or M.HhaI, the decrease in electrochemical signal when the binding site is positioned between the probe and the electrode does provide evidence for SoxR binding.

7.3.3 *Electrochemistry of *P. aeruginosa* SoxR*

As is evident in Figure 7.2, besides the Redmond Red probe, we also observe a second distinct and quasi-reversible electrochemical signal at +200 mV versus NHE upon addition of SoxR. The signal is observed only after SoxR addition and is not affected by Redmond Red because it is also present in the absence of the probe. Note that no redox signature is observed at -290 mV versus NHE, the potential previously reported for SoxR in solution (Figure 7.2). Incubation of the DNA-modified surface with PA2274, a control protein that lacks an iron-sulfur cluster, does not result in the appearance of any redox signature. Furthermore, experiments with SoxR stocks featuring low iron-sulfur content after purification do not lead to appreciable cyclic voltammetric signals (data not shown). Therefore, we can assign the new signal observed to the [2Fe-2S] cluster of SoxR.

In a typical experiment, the observed SoxR signal increases over a period of \approx 15 minutes and is stable for a minimum of 18 scans before slowly decaying (Figure 7.3), although we have found a high variability in electrode stability upon addition of protein. High concentrations of protein ($> 10 \mu\text{M}$) are required for these experiments, certainly concentrations higher than is required for site-specific binding, and both the high protein concentrations and long DNA sequences used make DNA/protein film formation difficult. It is important to note that the Redmond Red signal is highly stable and exhibits no noticeable degradation during typical electrochemistry experiments.

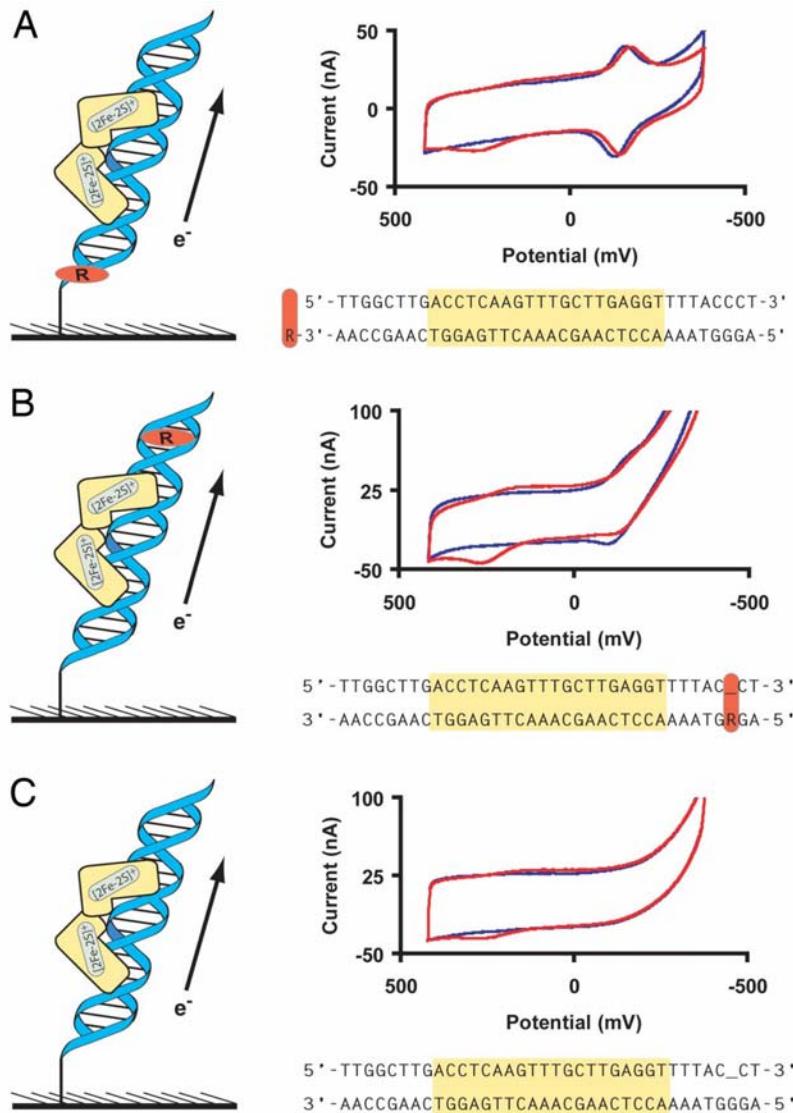


Figure 7.2: Cyclic voltammetry at 50 mV/s of electrodes modified with DNA featuring Redmond Red at the bottom (A), Redmond Red above the binding site (B), and no Redmond Red (C). Voltammograms before addition of SoxR are blue whereas those after addition of SoxR are red. The sequences used in the course of these experiments are illustrated with the binding sequence for SoxR highlighted, the location of Redmond Red indicated by an “R,” and the location of abasic sites underlined.

As can be seen in Figures 7.2 and 7.3, the cathodic and anodic waves observed for SoxR are asymmetric: The oxidation wave is pronounced and substantially less broad compared with the reduction wave. In an ideal quasi-reversible system, the anodic to cathodic peak current ratio is unity (42, 44), but this is certainly not the case for SoxR. We find an anodic to cathodic peak current ratio of 3.0 for SoxR in Figure 7.2, strongly indicative of a non-ideal and quasi-reversible electrochemical response. In contrast, the anodic to cathodic peak current ratio is 1.3 for the 3'-Redmond Red on the same film, far closer to the ideal value for a fully reversible system. These data show that the electrochemistry of SoxR is complicated, hardly surprising given that SoxR binds DNA as a dimer.

Interestingly, the asymmetries in the reduction and oxidation waves of SoxR are qualitatively distinct from those previously observed for the DNA repair enzymes MutY and Endo III; the electrochemistry of those enzymes featured a reduction wave that was somewhat more pronounced than the oxidation wave (32). The better resolved anodic wave of SoxR integrates to very low surface coverages of 0.5 pmol/cm². This apparent low coverage is comparable to that of 2 pmol/cm² previously found for MutY at DNA monolayers on gold (33) and may reflect poor coupling of the iron-sulfur cluster with the base pair stack. However, the Redmond Red probe at the bottom of the DNA monolayer integrates to surface coverages of 1 pmol/cm² whereas the Redmond Red probe at the top of the film integrates to coverages that are 3-fold lower (over sample sizes of at least 10 electrodes). These values are far less than the ideal DNA surface coverage of 10 pmol/cm² expected for a loosely packed DNA monolayer and indicate that the amount of DNA on the surface is the main determinant of the size of the SoxR signal.

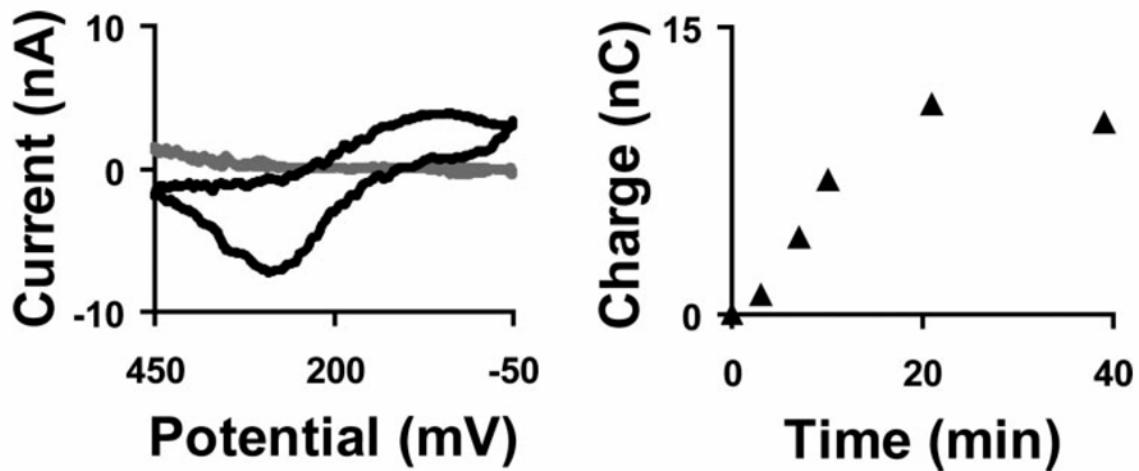


Figure 7.3: Binding of SoxR to the DNA-modified film. (*Left*) Background subtracted cyclic voltammetry of *P. aeruginosa* SoxR at DNA-modified graphite electrodes at a 50 mV/s scan rate immediately after addition of SoxR (light) and 20 min after addition of SoxR (dark) revealing the signal observed. (*Right*) Integrated anodic charge for SoxR showing the growth of the signal as a function of time.

Despite the broad cathodic wave, we can calculate an upper bound for the number of electrons transferred for the oxidation of SoxR. For an ideal surface-bound species, the slope derived from the plot of peak current as a function of scan rate divided by the integrated charge Q at any scan rate is equal to $nF/4RT$, where F is Faraday's constant, R is the gas constant, and T is the temperature (44); performing this operation for the Redmond Red probe at the bottom of the monolayer ($Q_{\text{anodic/cathodic}} = \approx 17 \text{ nC}$ at any scan rate) yields a value of $n = 2$, as expected for 2 e^- transfer to the resorufin moiety. The integrated charge for the anodic wave of SoxR on the same film varies from 3 to 9 nC, indicating that SoxR receives at most half the number of electrons transferred to the Redmond Red. If we assume that all of the DNA is bound and that the Redmond Red signal at the bottom of the monolayer corresponds perfectly to the number of DNA molecules on the surface (highly likely for the sparse films obtained), we can estimate that each DNA-bound SoxR dimer undergoes at most a one electron oxidation/reduction. In fact, all of these observations are consistent with titrations of free SoxR, which deviate from ideal reductions, but appear also to yield values of $n = 1$ (8, 45).

7.3.4 Comparison of the Voltammetry of *E. coli* and *P. aeruginosa* SoxR

To expand our work to multiple organisms, we directly compared the electrochemistry of *E. coli* and *P. aeruginosa* SoxR. Only weak cyclic voltammetry for *E. coli* SoxR is obtained, irrespective of the source, likely because of poor solubility. Therefore, the comparison was made using square wave voltammetry, which is a more sensitive technique and allows for better discrimination of small signals. As can be seen in Figure 7.4, the potentials, referenced to the Redmond Red internal standard, are nearly identical for *P. aeruginosa* SoxR and *E. coli* SoxR. This observation is consistent with

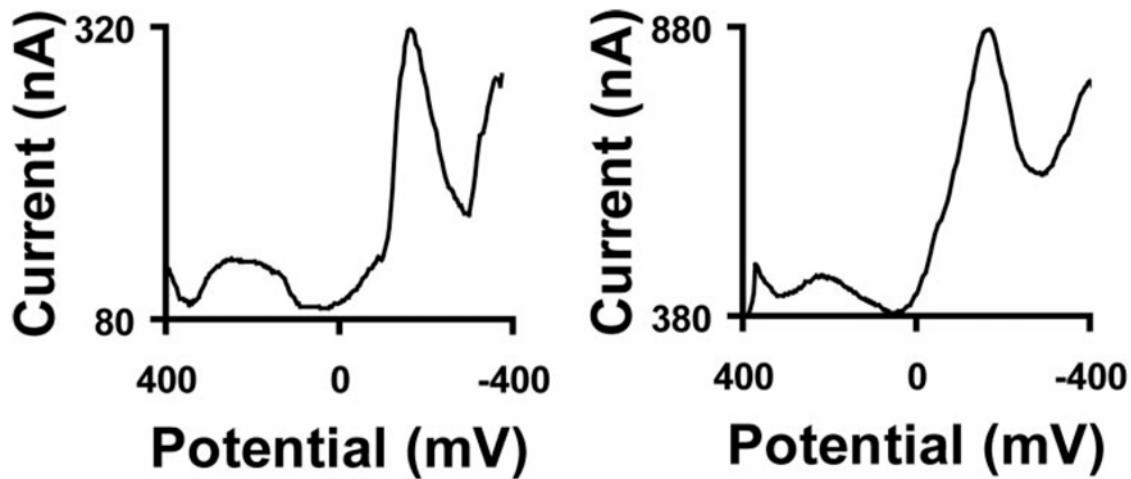


Figure 7.4: Square wave voltammetry of *P. aeruginosa* SoxR (Left) and *E. coli* (Right) at DNA-modified graphite electrodes at a frequency of 15 Hz showing both the Redmond Red and SoxR signals. The 5' Redmond Red-modified sequence was 5'-AGR GTA AAA CCT CAA GCA AAC TTG AGG TCA AGC CAA-3' plus pyrene-modified complement, where "R" indicates the position of the probe.

the *in vitro* redox titrations of SoxR in the absence of DNA that found their potentials to differ from one another by ≈ 10 mV (7, 8, 15). Here, we see that the DNA-bound potentials are indistinguishable. The identical redox potentials of the free and DNA-bound *E. coli* and *P. aeruginosa* SoxR should allow both to activate transcription upon oxidation. In fact, previous work has shown that *P. putida* SoxR is functional and can complement an *E. coli* SoxR deletion mutant, lending credence to the *in vivo* importance of these observations (13).

7.3.5 Electrochemistry of the *P. Aeruginosa* S95L SoxR Mutant

To further explore the surrounding environment of the iron sulfur cluster of *P. Aeruginosa* SoxR, the voltammetry of a S95L SoxR mutant was also investigated at a DNA-modified surface (Figure 7.5). The serine 95 residue (or its neighbor) may be important for the *in vivo* regulation of SoxR activity since it is conserved for SoxR homologues in over one hundred organisms (46). As can be seen in Figure 7.5, the redox signal of the S95L mutant is very qualitatively and quantitatively similar to that of wild type *P. aeruginosa* SoxR in Figure 7.2. When referenced to the Redmond Red internal standard, the midpoint potential of the S95L mutant is shifted by ≈ -15 mV, and its cathodic wave is shifted by ≈ -40 mV (relative to the wild type, DNA-bound SoxR). These observations are consistent with the *in vitro* reductive titrations of wild type SoxR and a transcriptionally active S95L mutant in *E. coli*; the potentials of the two proteins differed from one another by ≈ -60 mV (47). The data is also consistent with higher transcriptional activity by the S95L *E. coli* SoxR mutant (due to an enhanced sensitivity to oxidation), again supporting the *in vivo* relevance of these observations (47).

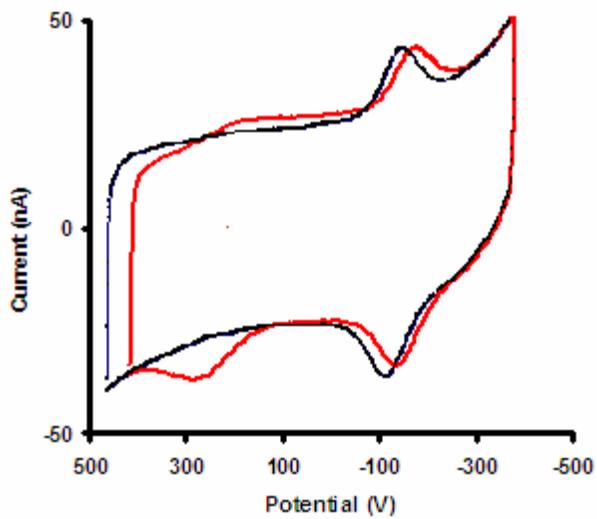


Figure 7.5: Cyclic voltammetry at 50 mV/s of an electrode modified with DNA featuring Redmond Red above the binding site. Voltammograms before addition of the *P. aeruginosa* SoxR S95L mutant are black whereas those after addition of S95L SoxR are red. The sequence context is the same as in Figure 7.2.

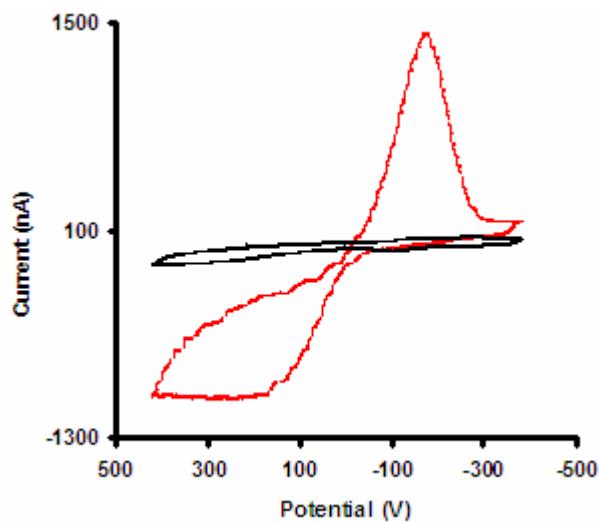
7.3.6 Comparison of the Voltammetry of SoxR to that of Free Iron

As an important control, we explored the electrochemistry of free iron in the presence of excess L-cysteine under conditions identical to those used for SoxR experiments. As can be seen in Figure 7.6, the voltammetry of free iron on either bare or DNA-modified HOPG is vastly different from that found for SoxR; the midpoint potential is 0 mV versus NHE, rather than +200 mV versus NHE. In fact, the backfilled DNA monolayer dramatically broadens and attenuates the electrochemical signal, presumably by providing reduced access to the surface. These data conclusively indicate that we are, indeed, observing the voltammetry of a protein-bound iron sulfur cluster cofactor.

7.4 Discussion and Implications

The activity of SoxR, a transcription factor containing an Fe-S cofactor, is regulated via a redox switch: SoxR triggers transcription in its oxidized state (4, 5). However, the redox potentials of free *E. coli* and *P. aeruginosa* SoxR have previously been determined to be approximately -290 mV in solution (7, 8, 15). Although the redox potential of SoxR can explain how it is maintained in its reduced state by coupling it to the cellular NADPH/NADP⁺ pool (-40 mV), it was unclear how the relatively low potential of -290 mV would allow for specificity *in vivo*. To understand the activation of SoxR at a mechanistic level, it is crucial to determine its redox potential within an appropriate context.

A.



B.

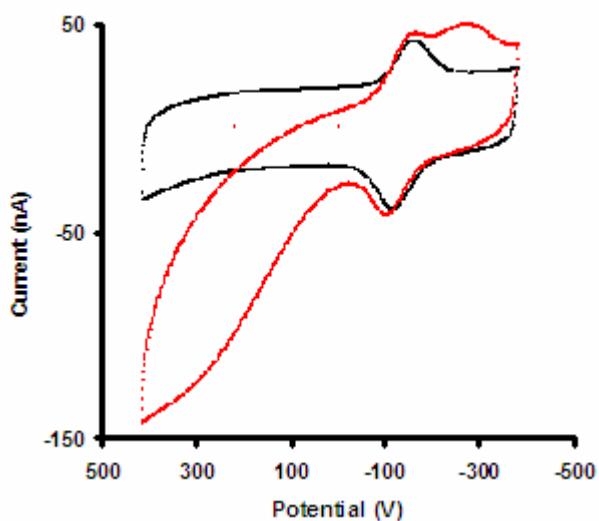


Figure 7.6: Cyclic voltammetry of $\approx 50 \mu\text{M}$ FeCl_3 with an excess of dithionite at 50 mV/s in SoxR storage buffer. An unmodified HOPG electrode (A) and an electrode modified with DNA featuring Redmond Red below the binding site (B) are shown. Voltammograms before addition of free iron are black whereas those after addition of free iron are red. The sequence context is the same as in Figure 7.2.

Here, using DNA-modified HOPG electrodes, we have demonstrated that DNA association positively shifts the redox potential of SoxR to 200 mV versus NHE. The +490-mV shift between the free and DNA-bound states of SoxR is functionally crucial because it keeps SoxR reduced across a range of intracellular potential. For example, Figure 7.7 shows standard and free midpoint potentials for a variety of cellular redox pairs, and where DNA-bound and free SoxR are positioned along this series. Although numerous redox couples, ranging from glutathione to FADH, are oxidants for soluble SoxR, they are reductants to DNA-bound SoxR. In fact, the positive shift in potential associated with DNA binding means that DNA-bound SoxR is primarily in the reduced, transcriptionally silent form *in vivo*. Oxidative stress serves to promote oxidation of DNA-bound SoxR, activating the numerous genes required to protect the organism. This provides a rationale for how DNA-bound SoxR can serve as an effective sensor of oxidative stress in *E. coli*.

In *P. aeruginosa*, the paradigm for SoxR activation may be different. Here, activation may be promoted by pyocyanin. When we consider the standard potential of the phenazine pyocyanin ($E_m = -34$ mV at pH 7 and $E_m = -110$ mV at pH 8), we predict it would also act as a reductant for DNA-bound SoxR (48). However, pyocyanin is an extracellular electron shuttle that reacts readily with oxygen, as indicated by the bright blue color of *P. aeruginosa* cultures. Uptake of oxidized pyocyanin increases the intracellular ratio of the oxidized versus the reduced form and thus favors the oxidation of SoxR. Considering a one-electron transfer under physiological conditions (pH 7 and 37 °C), to shift the redox equilibrium of DNA-bound SoxR toward its oxidized state would

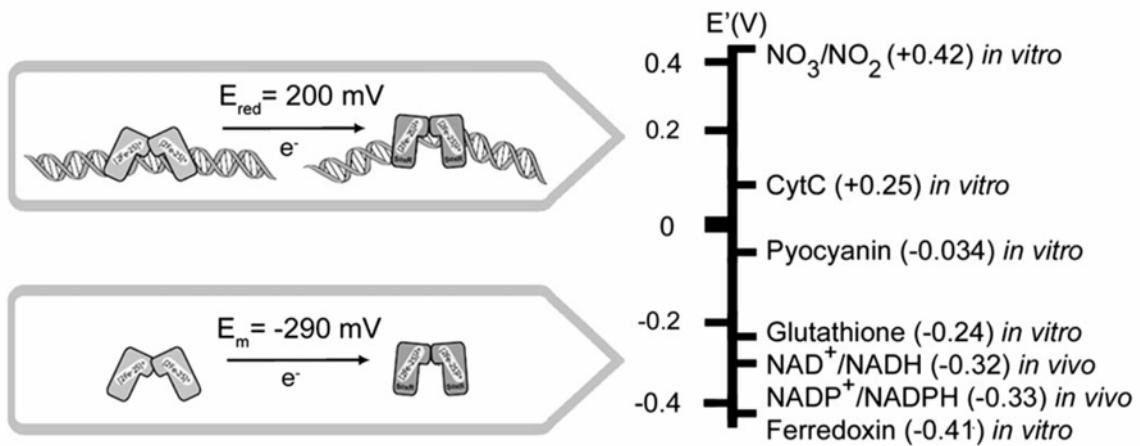


Figure 7.7: Redox potentials of free and DNA-bound SoxR along with those of cellular oxidants/reductants at pH 7.

require a ratio of oxidized-to-reduced pyocyanin of at least 6,500:1. It remains to be determined whether this is of physiological relevance in *P. aeruginosa*.

The substantial shift in SoxR potential on DNA binding of ≈ 500 mV is striking but understandable. The significance of the molecular environment for tuning the redox potentials of Fe-S clusters is well documented (49–51): Each hydrogen-bonding interaction with the cluster can cause a potential shift of ≈ 80 mV. Moreover, for [4Fe-4S] clusters in proteins, all with the same ligating residues, cluster potentials vary from approximately -600 mV for ferredoxins to approximately $+400$ mV for high potential iron proteins (52). We have previously observed a negative shift of at least ≈ 200 mV for Endo III in the presence of DNA (34). Because the structures of Endo III with and without DNA were known and showed no significant distortion in the protein (53–55), thermodynamically this shift was interpreted as a favorable shift in the binding affinity of the protein in the oxidized form relative to the reduced form (34), perhaps not so surprising on binding to the DNA polyanion.

By contrast, although the binding affinities of oxidized and reduced SoxR are comparable (6, 7), SoxR and other MerR-type transcriptional regulators have been shown to induce conformational changes of the promoter region (1, 56–61). In particular, copper phenanthroline footprinting studies have provided strong evidence that SoxR significantly distorts its promoter sequence (62, 63). Although this experiment only reports on the reduced form of SoxR, the observed loss of signal for Redmond Red found here strongly supports a DNA-distortion mechanism. We propose that the more positive reduction potential for DNA-bound SoxR yields a higher energy complex, which may

drive a conformational change in the protein/DNA complex. If this were the case, it would constitute an effective means of allosteric regulation *in vivo*.

It is important to note that the crystal structure of SoxR in any form has not been reported (64). Therefore, the structural difference between the free (low energy) and DNA-bound (high energy) complexes is not clear, but a positive shift of the magnitude we observe has been associated with bulk folding of other metalloproteins from *P. aeruginosa* (65). These energetic differences have been attributed to burying the cofactor in a more hydrophobic environment. Consequently, we predict a large structural difference between the free and DNA-bound SoxR to provide a rationale for the dramatic shift in potential associated with binding.

Within a broader context, these data illustrate that it is critical to take the effect of DNA binding into account when considering the redox characteristics of DNA binding proteins. It is also likely that it is the DNA-bound potential of these proteins that is most relevant within the crowded environment of the cell, and this potential may be altered even further upon recruitment of RNA polymerase. Therefore, in many cases, as with SoxR, it is perhaps the redox characteristics within a multiprotein/DNA complex that must be considered. In fact, because several transcription factors feature iron-sulfur clusters as sensor elements, a change in the redox potential of these cofactors upon binding DNA may generally be an important trait to consider.

REFERENCES

- (1) Brown, N. L., Stoyanov, J. V., Kidd, S. P., Hobman, J. L. (2003) *FEMS Microbiol. Rev.* 27, 145–163.
- (2) Hobman, J. L., Wilkie, J., Brown, N. L. (2005) *Biometals* 18, 429–436.
- (3) Giedroc, D. P., Arunkumar, A. I. (2007) *J. Chem. Soc. Dalton Trans.* 3107–3120.
- (4) Pomposiello, P. J., Demple, B. (2001) *Trends Biotechnol.* 19, 109–114.
- (5) Demple, B., Ding, H., Jorgensen, M. (2002) *Methods Enzymol.* 348, 355–364.
- (6) Hidalgo, E., Demple, B. (1994) *EMBO J.* 13, 138–146.
- (7) Gaudu, P., Weiss, B. (1996) *Proc. Natl. Acad. Sci. U.S.A.* 93, 10094–10098.
- (8) Ding, H., Hidalgo, E., Demple, B. (1996) *J. Biol. Chem.* 271, 33173–33175.
- (9) Ding, H., Demple, B. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 8445–8449.
- (10) Pomposiello, J., Bennik, M. H. J., Demple, B. (2001) *J. Bacteriol.* 183, 3890–3902.
- (11) Ha, U., Jin, S. (1999) *Infect. Immun.* 67, 5324–5331.
- (12) Stover, C. K., et al. (2000) *Nature* 406, 959–964.
- (13) Park, W., Pena-Llopis, S., Lee, Y., Demple, B. (2006) *Biochem. Biophys. Res. Commun.* 341, 51–56.
- (14) Dietrich, L. E. P., Price-Whelan, A., Petersen, A., Whiteley, M., Newman, D. K. (2006) *Mol. Microbiol.* 61, 1308–1321.
- (15) Kobayashi, K., Tagawa, S. (2004) *J. Biochem.* 136, 607–615.
- (16) Zheng, M., Storz, G. (2000) *Biochem. Pharmacol.* 59, 1–6.

(17) Liochev, S. I., Fridovich, I. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 5892–5896.

(18) Kobayashi, K., Tagawa, S. (1999) *FEBS Lett.* **451**, 227–230.

(19) Koo, M.S., Lee, J. H., Rah, S. Y., Yeo, W. S., Lee, J. W., Lee, K. L., Koh, Y. S., Kang, S. O., Roe, J. H. (2003) *EMBO J.* **22**, 2614–2622.

(20) Hwang, C., Lodish, H. F., Sinskey, A. J. (1995) *Methods Enzymol.* **251**, 212–221.

(21) Kobayashi, K., Tagawa, S. (1997) *J. Inorg. Biochem.* **67**, 258.

(22) Liochev, S. I., Benov, L., Touati, D., Fridovich, I. (1999) *J. Biol. Chem.* **274**, 9479–9481.

(23) Ding, H., Demple, B. (1998) *Biochemistry* **37**, 17280–17286.

(24) Price-Whelan, A., Dietrich, L. E. P., Newman, D. K. (2007) *J. Bacteriol.* **189**, 6372–6381.

(25) Kelley, S. O., Boon, E. M., Barton, J. K., Jackson, N. M., Hill, M. G. (1999) *Nucleic Acids Res.* **27**, 4830–4837.

(26) Boon, E. M., Ceres, D. M., Drummond, T. G., Hill, M. G., Barton, J. K. (2000) *Nature Biotechnol.* **18**, 1096–1100.

(27) Gorodetsky, A. A., Barton, J. K. (2006) *Langmuir* **22**, 7917–7922.

(28) Kelley, S. O., Jackson, N. M., Hill, M. G., Barton, J. K. (1999) *Angew. Chem. Int. Ed.* **38**, 941–945.

(29) Inouye, M., Ikeda, R., Takase, M., Tsuri, T., Chiba, J. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102**, 11606–11610.

(30) Okamoto, A., Kamei, T., Saito, I. (2006) *J. Am. Chem. Soc.* **128**, 658–662.

(31) Gorodetsky, A. A., Green, O., Yavin, E., Barton, J. K. (2007) *Bioconjugate Chem.* *18*, 1434–1441.

(32) Boon, E. M., Livingston, A. L., Chmiel, N. H., David, S. S., Barton, J. K. (2003) *Proc. Natl. Acad. Sci. U. S. A.* *100*, 12543–12547.

(33) Boal, A. K., Yavin, E., Lukianova, O. A., O'Shea, V. L., David, S. S., Barton, J. K. (2005) *Biochemistry* *44*, 8397–8407.

(34) Gorodetsky, A. A., Boal, A. K., Barton, J. K. (2006) *J. Am. Chem. Soc.* *128*, 12082–12083.

(35) 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. (1989) *Biochemistry* *28*, 4450–4455.

(36) Asahara, H., Wistort, P. M., Bank, J. F., Bakerian, R. H., Cunningham, R. P. (1989) *Biochemistry* *28*, 4444–4449.

(37) Fu, W., O'Handley, S., Cunningham, R. P., Johnson, M. K. (1992) *J. Biol. Chem.* *267*, 16135–16137.

(38) Boal, A. K., Yavin, E., Barton, J. K. (2007) *J. Inorg. Biochem.* *101*, 1913–1921.

(39) Stephens, P. J., Jollie, D. R., Warshel, A. (1996) *Chem. Rev.* *96*, 2491–2513.

(40) Kim, Y., Long, E. C., Barton, J. K., Lieber, C. M. (1992) *Langmuir* *8*, 496–500.

(41) Giancarlo, L. C., Flynn, G. W. (1998) *Ann. Rev. Phys. Chem.* *49*, 297–336.

(42) Bard, A. J., Faulkner, L. R. (2001) *Electrochemical Methods*, 2nd ed., John Wiley & Sons: New York.

(43) Boon, E. M., Salas, J. E., Barton, J. K. (2002) *Nature Biotechnol.* *20*, 282–286.

(44) Udit, A. K., Hill, M. G., Bittner, V. G., Arnold, F. M., Gray, H. B. (2004) *J. Am. Chem. Soc.* *126*, 10218–10219.

(45) Hidalgo, E., Ding, H., Demple, B. (1997) *Trends Biochem. Sci.* *22*, 207–210.

(46) Chander, M., Raducha-Grace, L., Demple, B. (2003) *J. Bacteriol.* *185*, 2441–2450.

(47) Hidalgo, E., Ding, H., Demple, B. (1997) *Cell* *88*, 121–129.

(48) Price-Whelan, A., Dietrich, L. E. P., Newman, D. K. (2006) *Nature Chem. Biol.* *2*, 71–78.

(49) Rusling, J. F. (1998) *Acc. Chem. Res.* *31*, 363–369.

(50) Carter, C. W., Jr., Kraut, J., Freer, S. T., Alden, R. A., Sieker, L. C., Adman, E., Jensen, L. H. (1972) *Proc. Natl. Acad. Sci. U. S. A.* *69*, 3526–3529.

(51) Heering, H. A., Bulsink, Y. B. M., Hagen, W. R., Meyer, T. E. (1995) *Biochemistry* *34*, 14675–14686.

(52) Beinert, H. (2000) *J. Biol. Inorg. Chem.* *5*, 2–15.

(53) Kuo, C. F., McRee, D. E., Fisher, C. L., O'Handley, S. F., Cunningham, R. P., Tainer, J. A. (1992) *Science* *258*, 434–440.

(54) Thayer, M. M., Ahern, H., Xing, D., Cunningham, R. P., Tainer, J. A. (1995) *EMBO J.* *14*, 4108–4120.

(55) Fromme, J. C., Verdine, G. L. (2003) *EMBO J.* *22*, 3461–3471.

(56) Ansari, A. Z., Bradner, J. E., O'Halloran, T. V. (1995) *Nature* *374*, 371–375.

(57) Outten, C. E., Outten, F. W., O'Halloran, T. V. (1999) *J. Biol. Chem.* **274**, 37517–37524.

(58) Zheleznova-Heldwein, E. E., Brennan, R. G. (2001) *Nature* **409**, 378–382.

(59) Godsey, M. H., Baranova, N. N., Neyfakh, A. A., Brennan, R. G. (2001) *J. Biol. Chem.* **276**, 47178–47184.

(60) Changela, A., Chen, K., Xue, Y., Holschen, J., Outten, C. E., O'Halloran, T. V., Mondragon, A. (2003) *Science* **301**, 1383–1387.

(61) Newberry, K. J., Brennan, R. G. (2004) *J. Biol. Chem.* **279**, 20356–20362.

(62) Hidalgo, E., Bollinger, J. M., Jr., Bradley, T. M., Walsh, C. T., Demple, B. (1995) *J. Biol. Chem.* **270**, 28908–28914.

(63) Hidalgo, E., Demple, B. (1997) *EMBO J.* **16**, 1056–1065.

(64) Watanabe, S., Kita, A., Kobayashi, K., Takahashi, Y., Miki, K. (2006) *Act. Crystal. F* **62**, 1275–1277.

(65) Wittung-Stafshede P., Hill, M. G., Gomez, E., Di Bilio, A. J., Karlsson, B. G., Leckner, J., Winkler, J. R., Gray, H. B., Malmstrom, B. G. (1998) *J. Biol. Inorg. Chem.* **3**, 367–370.

(66) Chander, M., Demple, B. (2004) *J. Biol. Chem.* **279**, 41603–41610.