Structures of Nitroreductase in Three States

EFFECTS OF INHIBITOR BINDING AND REDUCTION

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The crystal structure of the nitroreductase enzyme from Enterobacter cloacae has been determined for the oxidized form in separate complexes with benzoate and acetate inhibitors and for the two-electron reduced form. Nitroreductase is a member of a group of enzymes that reduce a broad range of nitroaromatic compounds and has potential uses in chemotherapy and bioremediation. The monomers of the nitroreductase dimer adopt an α+β fold and together bind two flavin mononucleotide prosthetic groups at the dimer interface. In the oxidized enzyme, the flavin ring system adopts a strongly bent (16°) conformation, and the bend increases (25°) in the reduced form of the enzyme, roughly the conformation predicted for reduced flavin free in solution. Because free oxidized flavin is planar, the induced bend in the oxidized enzyme may favor reduction, and it may also account for the characteristic inability of the enzyme to stabilize the one electron-reduced semiquinone flavin, which is also planar. Both inhibitors bind over the pyridimine and central rings of the flavin in partially overlapping sites. Comparison of the two inhibitor complexes shows that a portion of helix H6 can flex to accommodate the differently sized inhibitors suggesting a mechanism for accommodating varied substrates.

Nitroaromatic compounds are pervasive pollutants whose toxicity is generally the result of their enzymatic reduction to more reactive species (1–3). There is considerable interest in the flavin-containing nitroreductases that catalyze the reductive activation of nitrated aromatics, because of their central role in mediating nitroaromatic toxicity (4–9), their potential use in bioremediation (1–3, 10), and their utility in activating prodrugs in directed anticancer therapies (11, 12).

The nitroreductase from Enterobacter cloacae (NR) catalyzes two-electron reduction of a variety of nitrated aromatics as well as quinones and flavins (13–16). Indeed, this enzyme was first isolated from bacteria growing in a weapons storage dump, and can reduce trinitrotoluene (80). NR reduces nitrobenzene to the corresponding hydroxylamine and derives reducing equivalents from reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamidase adendine dinucleotide phosphate (NADPH), or other nicotinamides (15) by means of a flavin mononucleotide cofactor (FMN) (13, 14).

NR follows ping-pong bi-bi kinetics, and its FMN groups cycle between the oxidized neutral and reduced anionic states with an E\text{ox/hq} of ~190 mV, near that of free FMN (81). Single-electron redox chemistry and associated formation of the semiquinone are not stabilized. This results in NR’s activity being “oxygen-insensitive,” in that the enzyme does not readily transfer one electron to molecular oxygen to form the superoxide radical (13, 15).

Several other FMN-containing oxidoreductases have been identified that have similar broad substrate specificity ranges and also do not stabilize the semiquinone state of the bound flavin. They are homodimers that share a similar fold and key amino acids, although the amino acid identities conserved over the whole group are few (17). The minor O2-insensitive nitroreductase of Escherichia coli (NTR or NfsB), shares 88% sequence identity with NR (18). The major NADPH:FMN oxidoreductase of Vibrio Fischeri (FRase I) shares only 33% sequence identity (17, 19) whereas NADH oxidase from Thermus thermophilus (NOX) is even more remotely related (20, 21). Additional homologues include the minor O2-insensitive nitroreductase of Escherichia coli (NfsA) (22) and NADPH:flavin oxidoreductase of Vibrio harveyi (FRP) (23). Crystal structures have been deposited and/or published for several members of this family in the oxidized state, and all show an FMN bound at the interface between monomers in each of two symmetry-related but independent active sites (24–29).

We have determined crystal structures of oxidized NR in complex with two different inhibitors of the first half reaction as well as the crystal structure of the reduced enzyme. This work represents the most extensive comparison of states yet published for an NR homologue and includes the first crystal structure of one of these enzymes in the reduced state. Details
of the flavin conformation and inhibitor binding interactions provide novel insights into the bases for the oxygen insensitivity and substrate specificity range of this important family of enzymes.

**EXPERIMENTAL PROCEDURES**

**Protein Crystalization**—*Escherichia coli* nitroreductase was overexpressed in *Escherichia coli* and purified according to published methods (30). The purified enzyme was stored at a concentration of 4.75 mg/ml in 50 mM KH$_2$PO$_4$ (pH 7.0), 0.02% (w/v) NaN$_3$. The protein buffer was exchanged with 10 mM HEPES (pH 7.0) and 50 mM KCl prior to crystallization.

Crystals of oxidized NR were grown by hanging-drop vapor diffusion at 4 °C against well solution containing 100 mM homopipes (pH 4.8), 25 mM sodium benzoate, 25% PEG-4000, and 18% glycerol for 18% (25). They grew to full size (0.5 mm) within 30 days. Prior to data collection, crystals were dialyzed against cryoprotectant solution containing 100 mM HEPES (pH 4.8), 25 mM sodium acetate, 25% PEG-4000, and 18% glycerol for 16 h. The crystals were mounted in nylon loops and flash cooled by plunging into liquid nitrogen (31).

**Formation of the Benzoate Complex**—Oxidized NR crystals were dialyzed against cryoprotectant solution containing 100 mM HEPES (pH 4.8), 600 mM sodium benzoate, 25% PEG-4000, and 18% glycerol for 20 h prior to flash cooling.

**Chemical Reduction of Oxidized Crystals**—A fresh solution of sodium dithionite was added to cryoprotectant, and 25 μl of the resulting solution was gently mixed with oxidized NR crystals in 25 μl of cryoprotectant buffer containing dithionite. After ~10 min, 25 μl of cryoprotectant buffer was removed and replaced with 25 μl of cryoprotectant buffer containing dithionite. This procedure was repeated two more times. The crystals lost their deep yellow color over the course of the treatment. They were then immediately flash cooled and stored in liquid nitrogen prior to data collection.

**Data Collection and Structure Determination**—Crystals were held at 115 K for data collection on a CuKα source with an R-AXIS IV+ image plate detector. Data were reduced with the HKL (32) and CCP4 packages (33). The crystal and data parameters are given in Table I. In all crystals there are four monomers, or two dimers, in the asymmetric unit.

Initial structures were determined by molecular replacement using the CNS software package (34) and the coordinate set of unliganded nitroreductase as a search object. Geometry restraint files for acetate provided novel insights into the bases for the oxygen insensitivity and substrate specificity range of this important family of enzymes.

**RESULTS AND DISCUSSION**

**Flavin Mononucleotide Binding and Geometry in the Oxidized State**—NR (13, 14) and homologous enzymes (17, 18, 21, 38, 39) are dimers of 24-kDa subunits that share a characteristic α+β fold (Fig. 1). A central sheet consists of four antiparallel strands, with a fifth, parallel strand arising from the C terminus of the other dimer subunit. Surrounding the sheet are two large helices on one side, three smaller helices on the other, and two helices that pack against one end of the sheet. Several small helices or helical turns are also present. The FMN prosthetic groups bind in deep pockets at the dimer interface and interact with elements from both monomers (see Fig. 2 for FMN structure and numbering). In NR, each flavin group packs up against one end (S3) of the central sheet and is surrounded by helices H4 and H7. Helix H6 and the loop between H2 and S1 (particularly residues 36–43) from the other subunit form a cap over the cofactor binding site. H7 from that monomer also contributes to the pocket.

Affinity for the FMN cofactor is high (10 nm), and within this structural family, binding typically involves extensive interactions with the protein, some of which are conserved or similar across the group. The polar groups in the isoalloxazine ring of FMN typically participate in a number of hydrogen bond interactions with protein, and seven hydrogen bonds with the flavin are present in the NR crystal structure. One group of contacts involves the N1, O4, and N3 positions of the ring system, including donation of a hydrogen bond by the amide group of Glu-165 to N5, which is thought to be the site of hydride transfer (40). This set of interactions is remarkably well conserved across the entire group of homologues, although the identities of some residues vary. One interacting residue, Gly-166 in NR, is absolutely conserved within the group. Main-chain torsion angles for this residue are either near the β-strand region of the Ramachandran plot (NR, NTR, FRase I, and NOX) or in the helical region (FRP and NfsA), both allowed positions for residues with side chains. Glycine is not, therefore, required at this position because of its greater backbone flexibility. Instead it may be conserved, because any side-chain atoms would intrude on space over the flavin reserved for substrate binding.

The other group of contacts with the isoalloxazine ring involves the O2 and in some cases N1 atoms, which largely interact with basic residues. In NR and NTR, for example, there are two strong interactions between O2 of the pyrimidine ring and lysines 14 and 74. Unlike the first set of contacts, however, these interactions vary substantially across the group of related enzymes. This variation within the second group of interactions is particularly interesting, because the anionic forms of both the one- and two-electron reduced flavins develop resonance stabilized negative charge at the N1–C2 = O2 locus. Positive electrostatic potential from the protein near this region is a common feature of flavoenzymes, and the degree of positive charge is thought to be a strong factor in determining the redox potential of the system (40, 41). Variations in the exact nature of the contacts within the related group may then modulate the redox characteristics of the enzymes.

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**Table I**

**Summary of crystallographic data**

|   | NR-oxidized acetate | NR-oxidized benzoate | NR reduced |
|---|-------------------|---------------------|------------|
| Space group | P2$_1$ | P2$_1$ | P2$_1$ |
| Unit cell | a = 52.79, b = 79.61, c = 97.14 | a = 52.92, b = 79.39, c = 97.18 | a = 52.83, b = 79.98, c = 97.25 |
| β | 93.63 | 93.66 | 93.62 |
| Wavelength (Å) | 1.5418 | 1.5418 | 1.5418 |
| Resolution (Å) | 20.0–1.8 | 20.0–1.8 | 20.0–1.9 |
| Last shell (Å) | 1.88–1.80 | 1.88–1.80 | 1.99–1.90 |
| Average redundancy (last shell) | 3.74 (3.51) | 3.74 (3.51) | 4.00 (3.09) |
| R$_{sym}$ (last shell) (%) | 5.2 (27.8) | 6.5 (16.2) | 5.4 (14.3) |
| I/$\sigma$(last shell) | 28.3 (4.8) | 20.3 (8.3) | 27.6 (7.5) |
| Completeness (last shell) (%) | 92.0 (87.5) | 97.5 (93.5) | 97.7 (88.9) |

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* R$_{sym}$ = Σ$|I_i| - (I/2)I_j$. 

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Other relevant points include:

- The purified enzyme was stored at a concentration of 4.75 mg/ml in 50 mM KH$_2$PO$_4$ (pH 7.0), 0.02% (w/v) NaN$_3$. The protein buffer was exchanged with 10 mM HEPES (pH 7.0) and 50 mM KCl prior to crystallization.

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- Formation of the Benzoate Complex—Oxidized NR crystals were dialyzed against cryoprotectant solution containing 100 mM HEPES (pH 4.8), 600 mM sodium benzoate, 25% PEG-4000, and 18% glycerol for 20 h prior to flash cooling.

- Chemical Reduction of Oxidized Crystals—A fresh solution of sodium dithionite was added to cryoprotectant, and 25 μl of the resulting solution was gently mixed with oxidized NR crystals in 25 μl of cryoprotectant. After ~10 min, 25 μl of cryoprotectant buffer was removed and replaced with 25 μl of cryoprotectant buffer containing dithionite. This procedure was repeated two more times. The crystals lost their deep yellow color over the course of the treatment. They were then immediately flash cooled and stored in liquid nitrogen prior to data collection.

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in inducing the bent flavin conformation. The dimethylbenzene ring and a portion of the central ring are held tightly by structural elements on both faces. Hydrogen bonding groups interacting with the pyrimidine end of the isoalloxazine arise largely from the st side of the flavin, the same direction as the bend. On binding, then, the interactions with the pyrimidine ring are optimized at the cost of distorting the flavin. Bending of the flavin cofactor has also been attributed to steric aspects of the binding site in pyruvate oxidase (55), NADH oxidase (24), and in the reduced form of thioredoxin reductase (54).

In addition, specific features of the protein-flavin interaction in NR may affect flavin conformation. Among the NR homologues, the interactions that stand out are: 1) the presence of an electron rich group (main-chain carbonyl oxygen in NR and homologues) interacting with the \( \pi \) system of the flavin at or near the central ring, 2) a hydrogen bond donor (main-chain amide in NR and homologues) interacting with N5 of the flavin, 3) the presence of positively charged residues near the central ring of the isoalloxazine ring and a portion of the central ring are held tightly by aromatic interactions involving stacking, at least in part, with the central ring of the isoalloxazine.

Because this ring becomes distorted in inducing the bent flavin conformation. The dimethylbenzene ring and a portion of the central ring are held tightly by structural elements on both faces. Hydrogen bonding groups interacting with the pyrimidine end of the isoalloxazine arise largely from the st side of the flavin, the same direction as the bend. On binding, then, the interactions with the pyrimidine ring are optimized at the cost of distorting the flavin. Bending of the flavin cofactor has also been attributed to steric aspects of the binding site in pyruvate oxidase (55), NADH oxidase (24), and in the reduced form of thioredoxin reductase (54).

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![Fig. 1. Overview of the nitroreductase fold.](image-url)

**Panel a** was prepared with the program RIBBONS (78).
gen bond donated to N5, are more prevalent in the large bend angle group, but the small size of the sample limits the significance of this observation. The prevalence of H-bond donor interactions is particularly attractive, because they might directly affect the resonance characteristics of the central ring, which accounts for a large portion of the distortion associated with butterfly bending.

**Inhibitor Binding**—In earlier work (15), we demonstrated that small negatively charged organic molecules such as acetate and benzoate are inhibitors of nitroreductase. They act competitively with substrate (NAD(P)H) for the first half reaction, flavin reduction, and uncompetitively in the second half reaction, reduction of the nitroaromatic. The binding of these inhibitors is relatively weak with overall $K_i$ values of 0.1 mM for benzoate and 9 mM for acetate, for example (15).

In the two oxidized NR crystal structures, both acetate and benzoate bind over the pyrimidine and central rings of the isoalloxazine system in partially overlapping sites (Fig. 4). The central carbon of the acetate is located above a point midway between flavin atoms C4a and C10a, so that the bulk of the molecule lies over the central ring. The carboxylate group of benzoate is in a similar position, but shifted over toward the pyrimidine ring of the flavin. The benzene ring is not stacked over the isoalloxazine $\pi$ system. Rather it extends out over O4 of the flavin and the gap between the pyrimidine and central rings. Both inhibitors hydrogen bond to the O2’ oxygen of the ribose moiety, and the same carboxylate oxygen of each inhibitor interacts with the main-chain NH of Thr-40, which is located in the loop between H2 and S1 that helps form the flavin binding site. The other carboxylate oxygen of acetate interacts with two water molecules, one of which also hydrogen bonds to O2’ of the ribose. The corresponding benzoate oxygen is shifted toward N1 of the flavin, altering the water structure relative to the acetate complex. It interacts with only a single water molecule, which is not equivalent to either of the acetate complex solvent molecules. In general, the presence of a negatively charged group seems to define this class of NR inhibitors (15). Because both acetate and benzoate bind with their carboxylate groups near lysines 14 and 74, electrostatic interaction with these residues may be a principle reason for the binding of these inhibitors.

Both acetate and benzoate are positioned approximately 3.6 Å above the flavin, making van der Waals contacts with a number of ring atoms. The acetate methyl group also contacts atoms in protein residues, in particular Oy of Ser-40, Cy of Thr-41, and the side-chain atoms of Phe-124, and these interactions presumably contribute to binding affinity. The protein residues and flavin define a cavity that is only large enough to accept the acetate methyl group. Binding of benzoate, therefore, requires rearrangement to accommodate this much larger inhibitor. The cavity is enlarged almost exclusively by side-chain and main-chain shifts in helix H6 (Fig. 5), the large helix that is positioned over the re face of the isoalloxazine ring. The movement occurs primarily at two residues, Phe-124, which directly interacts with the bound inhibitor, and Tyr-123, which contacts the other side of the Phe-124 side chain. For both residues, the Cα atoms shift about 0.6 Å between the two complex structures. Changes in the main-chain torsion angles and chi1 side-chain torsion angles amplify this movement, so that Phe-124 shifts by 2.5 Å at its distal ring carbon. The tighter binding of benzoate over acetate may be due in large part to the favorable interaction (56) between its aromatic ring and the phenyl group of Phe-124.

While preparation of this paper was in progress, the structure of NTR with bound nicotinic acid, a first half reaction substrate analogue, was published (29). Nicotinic acid binds in the same position and orientation as the benzoate in the NR
complex. Structures of flavoenzymes with pyridine nucleotide bound in positions consistent with hydride transfer (57–59), also show the nicotinamide group stacking over either the re or si face of the isoalloxazine system. In NR then the organic acid inhibitors overlap the reducing substrate binding site, consistent with their competitive inhibition of the first half reaction.

Substrates for the second half reaction, the oxidizing substrates, probably also bind over the re face of the reduced flavin to effect hydride transfer. This location of the nitroaromatic binding site is consistent with the ping-pong kinetic mechanism of NR (15). Also, mutation of the phenylalanine in NfsA equivalent to Phe-124, which interacts with bound organic acids, affects nitroaromatic substrate specificity (60). NR is capable of reducing a variety of nitroaromatics and isoalloxazine derivatives (15), and other members of this structural group also show broad substrate specificity (18, 20, 22, 61). If the organic acid inhibitors bind at the same site as oxidizing substrates, then the crystal structures with the inhibitors suggest a mechanism for accommodating substrates of different size. The plasticity of helix H6, demonstrated by its response to the two different organic acid inhibitors, creates a variable volume cavity capable of conforming to different bound substrates. The helices equivalent to H6 in crystal structures of other members of the group also show elevated thermal factors and variability in position indicative of inherent plasticity (24–29). These enzymes probably use a similar mechanism for adapting to different substrates. Depending on the type and size of oxidizing substrate, other nearby aromatic residues (Tyr-68, Phe-70, Tyr-123) may also participate in the binding

**FIG. 4.** Inhibitor binding to nitroreductase. a, binding of acetate over the isoalloxazine ring system; b, binding of benzoate over the isoalloxazine ring system. Averaged omit density for both inhibitors is contoured at three times the r.m.s. deviation of the map.

**FIG. 5.** Shifts in helix H6 to accommodate the larger benzoate inhibitor. H6 bonds are blue in the acetate structure and red in the benzoate complex. The density and structure of the bound benzoate are shown.
interaction. Interestingly, the identity of these residues varies among the related flavoenzymes, suggesting that they may play a role in determining substrate preferences (15, 18, 20, 22, 61).

The Reduced Form of the Enzyme—Although a number of crystal structures have been determined for members of the nitroreductase group, no structures have been reported for reduced enzymes. Upon reduction of NR, there is an increase in the butterfly bend angle of the isoxaloxazine ring system (Fig. 6a). Although both oxidized forms have a bend angle of ~16°, the bend in the si direction of the reduced flavin increases to 25°. This extreme conformation places it among the three largest flavin bend angles found in a recent survey of unique flavoprotein structures in the Protein Data Bank (54). The change in bend angle in NR occurs primarily as a rigid motion about the N5–N10 axis, so that each half of the ring system independently superimposes well (r.m.s. of ~0.03 Å) on the oxidized form, but the angle between the two halves has changed. There is considerable disagreement about the equilibrium conformation of the fully reduced anionic flavin (36, 48, 50, 52, 53, 62–64), the form found in reduced NR. Recent theoretical work, however, has predicted a large butterfly bending associated with reduction. The amide NH group of Glu-165, which hydrogen bonds with N5 in the oxidized flavin, still participates in this interaction in the reduced form. Glu-165 does not follow the shift in N5, however, and the distance between the two nitrogens increases to 3.22 ± 0.03 Å (average for four monomers in the crystal asymmetric unit) from values of 3.11 ± 0.03 Å and 3.13 ± 0.03 Å for the oxidized acetate and oxidized benzoate complex structures, respectively.

With only this modest accommodation of hydride transfer to N5 in reduced NR, the amide proton of Glu-165 remains in close proximity to the ring nitrogen, well stabilized by a network of interactions that prevent this region of the protein from rearranging. A consequence of this steric restriction is that hydride transfer must occur in the axial position at N5, which adopts sp³ hybridization. Although the overall effect of hydrogen bond donation may be to disfavor protonation at N5 (39, 60, 84), participation of the N5 lone pair in the hydrogen bond in the oxidized state should somewhat deshield the axial position, making N5 more susceptible to nucleophilic attack by a reducing substrate positioned above the re face of the flavin. The axial position would also be the correct orientation for direct hydride transfer to an oxidizing substrate located in a similar position. No difference density is present for the hydrogen on N5, but hydrogen density is not expected in a 1.9-Å structure.

Finally, although crystals of NR were grown and reduced in the presence of the same acetate concentration as the acetate complex crystals, the acetate bound over the re side of the flavin is displaced and two ordered solvent molecules now occupy a portion of that volume (Fig. 7). One water molecule is located roughly above N5 near the position of the ligand methyl group in the acetate complex. The other water is located over N10 and C10a, largely overlapping the position of one of the ligand carboxylate oxygens in the acetate complex. The two
new solvent molecules in the reduced structure participate in a hydrogen bond network that includes several interactions with nearby protein residues and the flavin (Fig. 7). The solvent molecule above N5 is positioned to make a hydrogen bond interaction via the axial hydrogen at N5, further supporting this position for hydride transfer. This solvent in turn is hydrogen-bonded to the second new water and to the Oy of Ser-40. The second water interacts with the amide group of Thr-41 and O2’ of the FMN ribose.

There appear to be two factors that contribute to the loss of acetate binding in the reduced enzyme. The axial hydrogen at N5 could clash with the methyl group of bound acetate, reducing affinity. Also, electronic restructuring of the reduced flavin may help to change the binding affinity for organic acids. Koder et al. (15) provide evidence for the anionic hydroquinone form of the flavin in reduced NR, and as noted above, much of the negative electrostatic potential in the anionic form would be predicted to reside in the N1–C2–O2 locus. Because the negatively charged group of these inhibitors is positioned over this locus when bound, it is probable that electrostatic repulsion decreases binding affinity.

**NR Redox Properties and Flavin Geometry**—Many aspects of the interaction between flavoproteins and their prosthetic groups are thought to influence the redox properties of the system. In NR, effects that favor reduction must balance those that favor the oxidized form of the flavin, because the $E_{\text{auq}}^{\circ}$ of −190 mV is little different from the −208 mV $E_{\text{auq}}$ of free flavin (81). The most striking feature of NR is the bent flavin conformation in both oxidation states. Some time ago, Massey and Hemmerich (65), proposed that the conformation adopted by the flavin upon binding to the apoprotein may affect the redox potential of the prosthetic group. Although there has been some controversy over the importance of this effect (54), recent work (54, 63, 64, 66, 67) suggests conformational changes may play a role in determining the redox properties of the protein-flavin system. In oxidized NR, the isolobalazine ring system is bound in a conformation that is energetically unfavorable in the oxidized free flavin, and this imposed bend may therefore favor reduction. The energetic cost of bending the ring system in free flavin has been variously estimated, but often-quoted studies (36, 63) indicate a value in the range of 6 kcal/mol for a bend equal to the oxidized NR flavin (16°). If an equal cost is incurred in the protein environment, it would translate to an increase of +130 mV in the two electron reduction potential, a substantial effect.

The hallmark of NR redox chemistry is its inability to stabilize the one electron reduced semiquinone form of the flavin (15, 81). Thus, reduction by the second electron is thermodynamically more favorable than the first one electron reduction, which corresponds to a large positive difference between the second and first one-electron redox midpoint potentials, $E_{\text{sq/hq}}$ and $E_{\text{auq/aq}}$, of at least +581 mV. The lack of a one electron-reduced state is relatively rare among flavoenzymes, which generally act to stabilize the semiquinone (68, 69). The broad substrate specificity of NR may allow it to fully reduce a number of cellular compounds that might otherwise contribute to oxidative stress via one electron reduction (70). Indeed, the related NfsA has recently been demonstrated (71) to be part of the soxRS regulon in *E. coli*, which is induced by redox cycling agents.

The hydrogen bond donated to N5 of the flavin may play a role in the lack of semiquinone stabilization. Donation of a hydrogen bond by the protein may disfavor reduction by increasing the cost of protonating N5. In flavodoxin from *Clostridium beijerinckii*, the interaction of a hydrogen bond acceptor with N5 appears to both increase $E_{\text{auq/aq}}$ and decrease $E_{\text{sq/hq}}$ to account in part for the strongly stabilized semiquinone in this enzyme (72). Donating a hydrogen bond rather than accepting one might have the opposite effect in NR. It is not apparent, however, why this effect should favor the hydroquinone form over the neutral semiquinone, because both are protonated at N5 and probably carry similar atomic charges at that position (63, 73). Also, it does not explain the absence of the anionic semiquinone, which is not protonated at N5. Old yellow enzyme makes a similar hydrogen bond to N5 of its prosthetic group (74) and yet stabilizes the anionic semiquinone state at the level of 10–20% (75, 76). This enzyme, in fact, makes contacts with its isoloalaxazine system that are very similar to those we observe in NR (74). Unlike NR, however, the flavin in old yellow enzyme has only a small butterfly bend in the oxidized state, and this difference may in part explain the different redox properties of the two enzymes. Because all forms of the free semiquinone are believed to be nearly planar (52, 53, 63, 77), the highly bent conformation of the flavin in NR may play a role in its inability to stabilize the one electron reduced form of the prosthetic group. For free flavin, a bend angle of 16°, the conformation in oxidized NR, would destabilize either the neutral or anionic semiquinone forms by ~8 kcal/mol (63), a change in midpoint potential of ~350 mV. This substantial energetic cost may counterbalance the normal tendency toward semiquinone stabilization in the protein environment. The extent of this effect depends on the exact nature of the interaction, however, and a more detailed evaluation of the possible mechanisms of redox tuning in NR raised by the crystal structures reported here must await additional theoretical and experimental studies.

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