Crystal structure of reduced thioredoxin reductase from *Escherichia coli*: Structural flexibility in the isoalloxazine ring of the flavin adenine dinucleotide cofactor

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(RECEIVED April 20, 1999; ACCEPTED August 12, 1999)

Abstract

Catalysis by thioredoxin reductase (TrxR) from *Escherichia coli* requires alternation between two domain arrangements. One of these conformations has been observed by X-ray crystallography (Waksman G, Krishna TSR, Williams CH Jr, Kuriyan J, 1994, *J Mol Biol* 236:800–816). This form of TrxR, denoted FO, permits the reaction of enzyme-bound reduced FAD with a redox-active disulfide on TrxR. As part of an investigation of conformational changes and intermediates in catalysis by TrxR, an X-ray structure of the FO form of TrxR with both the FAD and active site disulfide reduced has been determined. Reduction after crystallization resulted in significant local conformation changes. The isoalloxazine ring of the FAD cofactor, which is essentially planar in the oxidized enzyme, assumes a 34° “butterfly” bend about the N(5)–N(10) axis in reduced TrxR. Theoretical calculations reported by others predict ring bending of 15–28° for reduced isoalloxazines protonated at N(1). The large bending in reduced TrxR is attributed in part to steric interactions between the isoalloxazine ring and the sulfur of Cys138, formed by reduction of the active site disulfide, and is accompanied by changes in the positions and interactions of several of the ribityl side-chain atoms of FAD. The bending angle in reduced TrxR is larger than that for any flavoprotein in the Protein Data Bank. Distributions of bending angles in published oxidized and reduced flavoenzyme structures are different from those found in studies of free flavins, indicating that the protein environment has a significant effect on bending.

Keywords: conformational change; flavin reduction; flavin structure; thioredoxin reductase; X-ray structure

Thioredoxin reductase (TrxR) (EC 1.6.4.5) is a member of the pyridine nucleotide-disulfide oxidoreductase family of flavoenzymes (Williams, 1992). The enzyme from *Escherichia coli* is a homodimer in which each monomer of $M_r = 35,300$ contains one FAD and one redox active disulfide (Moore et al., 1964; Zanetti & Williams, 1967; Russel & Model, 1988). The reaction catalyzed by TrxR is the reduction of a protein substrate, thioredoxin, by NADPH (Moore et al., 1964; Holmgren, 1968). The path of electron transfer from NADPH to thioredoxin (Holmgren, 1985) is shown in Figure 1. Reduced thioredoxin, in turn, is a source of electrons for other cellular processes, such as ribonucleotide reduction.

A crystal structure of oxidized *E. coli* TrxR was determined by Kuriyan and coworkers (Kuriyan et al., 1991; Waksman et al., 1994). Each chain of the dimer folds into two α/β domains with topologies related to those found in all members of the pyridine-nucleotide disulfide oxidoreductase family (Figs. 2A, 3). *E. coli* TrxR is a unique member of the family because the active site disulfide resides in the same domain as the pyridine nucleotide binding site, rather than in the domain that binds FAD. As a result, each of the half reactions in turnover (Fig. 1) occurs at the same

Fig. 1. The reactions catalyzed by thioredoxin reductase and thioredoxin, showing (from left to right) the sequence of oxidation-reduction steps.

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Abbreviations: FAD_{ox}, the oxidized (quinone) form of FAD; FAD_{red}, the 2 electron-reduced (hydroquinone) form of FAD; FAD_{ox}-(SH)₂, 2 electron-reduced enzyme containing oxidized FAD and a reduced disulfide; FAD_{red}-(S-S), 2 electron-reduced enzyme containing reduced FAD and a disulfide; FMN_{ox}, the oxidized form of FMN; FMN_{red}, the 2 electron-reduced (hydroquinone) form of FMN; PDB, Protein Data Bank.

Fig. 2. The observed and proposed conformations of thioredoxin reductase and how they relate to the catalytic mechanism. **A:** Observed and proposed conformations of dimeric thioredoxin reductase (from Waksman et al., 1994). nic, nicotinamide ring of NADPH; fl, isoalloxazine ring of FAD; filled circles, active site cysteines. Domain arrangements are depicted for the FO conformation of thioredoxin reductase (upper left panel), for the proposed FR conformation of thioredoxin reductase (upper right panel), and for glutathione reductase (lower panel). To construct a model for the FR conformation, the NADPH domain of thioredoxin reductase was rotated 66° so that the relative positions of the NADPH and FAD domains resembled those observed in glutathione reductase, the prototypical member of this enzyme family. The interface domain of glutathione reductase is missing from thioredoxin reductase. **B:** The catalytic cycle of thioredoxin reductase. The FO conformation is signified by a straight backbone and the proposed FR conformation by a kinked backbone. For a more complete description of the events in turnover, see Lennon and Williams (1996).

Fig. 3. Ribbon diagrams of thioredoxin reductase. **A:** The alpha carbons of the oxidized structure were matched to those of the reduced structure (RMSD = 0.4 Å) by the least-squares fitting routine in O (Jones et al., 1991). The ball and stick model of the FAD cofactor is visible at the interface between the FAD and NADPH domains. Silver: oxidized enzyme (Waksman et al., 1994) (PDB accession code 1tde). Cyan: reduced enzyme. **B:** A more detailed view of reduced TrxR showing the contacts between the FAD and NADPH domains. In this figure, as well as in Figures 6, 7, and 9, the isoalloxazine ring protons on N(1), N(3), and N(5) have been modeled based on geometry supplied in the X-PLOR topology and parameter files (see Materials and methods). The FAD domain and flavin from the first chain (cyan) is at the upper left; the C-terminus of the second chain (magenta) is at the lower left. The NADPH domain at the lower right makes both intra- and interchain contacts with FAD domains. The contact near the FAD involves residues from the same chain. Sequences/structure elements that participate in each interface are colored gray and spans of residues that contribute to the interfaces are labeled. Both of these interdomain interfaces will be broken in the transition to the FR conformation.

(*re*) face of the isoalloxazine ring of FAD. In the structure solved by Waksman et al. (1994), the redox active disulfide formed by residues Cys135 and Cys138 is located near the flavin ring with the S γ of Cys138 only 3.1 Å from C(4a) of the isoalloxazine (see Fig. 4 for FAD atom numbering). This conformation permits elec-

tron transfer between the flavin and the disulfide (Fig. 2A, upper left panel), and is referred to as the FO conformation because it allows flavin oxidation by the enzyme disulfide. However, the active site disulfide is buried in this structure and is not available for reaction with thioredoxin; the nicotinamide ring of NADP⁺ is

Fig. 4. Chemical structure and standard numbering for relevant portions of FAD_{ox} . When FAD atoms are referenced in the text, the atom number is placed in parentheses, e.g., N(10).

distant from the FAD (Waksman et al., 1994), in a position precluding hydride transfer between the pyridine nucleotide and the flavin. An alternate conformation has been derived by model building (Waksman et al., 1994), as shown schematically in Figure 2A. Rotation of the NADPH domain brings the nicotinamide ring of NADPH into a position that is favorable for hydride transfer to the flavin. In the same rearrangement, the active site cysteines move from the interior of the enzyme to a site on the surface where they can react with thioredoxin. The model conformation is denoted the FR conformation because it allows flavin reduction by pyridine nucleotide. Catalysis of the overall reaction, in which FAD mediates both NADPH oxidation and disulfide reduction, requires the enzyme to alternate between the FO and FR conformations.

During turnover, TrxR from *E. coli* cycles between 2 electron-reduced and 4 electron-reduced forms (Lennon & Williams, 1996). Participation of the 4 electron-reduced forms in the reaction cycle is a special feature of catalysis by *E. coli* TrxR; glutathione reductase does not utilize the fully reduced form in catalysis (Williams, 1992). The catalytic cycle of TrxR, represented in Figure 2B, begins with the 2 electron-reduced $\text{FAD}_{\text{ox}}(\text{SH})_2$ (Species 1) in the FO conformation. The enzyme rearranges to the proposed FR conformation (Species 2), and the FAD is rapidly reduced by NADPH to form 4 electron-reduced enzyme (Species 3) (Lennon & Williams, 1996). Oxidized thioredoxin binds and is reduced, yielding Species 4, after which the enzyme rearranges to the FO conformation, this time as $\text{FAD}_{\text{red}}(\text{S-S})$ (Species 5). Species 5 rapidly undergoes an intramolecular electron transfer to regenerate Species 1. At high concentrations of thioredoxin, the predominant 4 electron-reduced form is Species 3, because thioredoxin binds and stabilizes that conformation much more rapidly than Species 3 can convert to Species 3a (Lennon & Williams, 1995, 1997). At low concentrations of thioredoxin, however, the 4 electron-reduced enzyme can exist to some extent as the FO Species 3a, a form in which it cannot react with thioredoxin.

As part of a study of intermediate states and conformations of TrxR, we have determined the structure of the 4 electron-reduced Species 3a, which can be obtained by reducing crystals of the known FO form of the enzyme. Reduction of crystalline FO lets us examine a species that is otherwise difficult to characterize. Local structural changes elicited by reduction of flavodoxins (Ludwig

et al., 1997) and glutathione reductase (Karplus & Schulz, 1989) are confined to the protein and to small adjustments in the flavin. In contrast, we describe here some unusual and unexpected changes in the FAD cofactor that accompany reduction of the flavin and the Cys135–Cys138 disulfide in the FO form of thioredoxin reductase.

Results and discussion

The structure of reduced TrxR

The structure was determined by refinement, using the coordinates for oxidized TrxR (PDB accession code 1tde) as the starting model. X-ray data to 2.5 Å were collected from a frozen crystal that had been reduced with excess dithionite (see Materials and methods). Minimization and simulated annealing using X-PLOR (Brünger, 1992b) were alternated with manual adjustments of the coordinates, and led to a model with an R -factor of 0.200 and an R_{free} of 0.292 (Table 1). The enzyme does not exhibit any global conformational changes upon reduction (Fig. 3A); the root-mean-square deviation (RMSD) of the $\text{C}\alpha$ atoms, relative to oxidized TrxR, is 0.4 Å for all residues. However, reduction does induce local changes in the conformation and interactions of the FAD cofactor and in the positions of the sulfur/side-chain atoms of the redox active disulfide group.

Visual inspection indicated that the flavin cofactor in the crystalline enzyme was fully reduced. The deep yellow color typical of oxidized FAD bleached during the reaction with dithionite. The frozen crystal was initially pale yellow, consistent with full reduction of FAD to the hydroquinone, and its appearance was unchanged at the conclusion of data collection. The midpoint potentials of the disulfide/dithiol and $\text{FAD}_{\text{ox}}/\text{FAD}_{\text{red}}$ couples in TrxR are nearly the same (O'Donnell & Williams, 1983) and predict that the disulfide should be reduced under conditions where the flavin is fully reduced.

Table 1. Summary of structure refinement

	Reduced enzyme (this work)	Oxidized enzyme (Waksman et al., 1994)
Resolution range (Å)	10–2.5	6–2.1
No. of reflections	12,009	18,508 ($I > 2\sigma I$)
No. of atoms	3,332	3,494
No. of waters	109	164
R -factor ^a	0.200	0.192
R_{free} ^b	0.292	NR ^c
Average isotropic B -factors (Å ²) ^d		
Backbone atoms	19.3	22.8
Side-chain atoms	26.9	28.1
FAD (isoalloxazine)	28.9 (41.6)	15.9 (18.8)
RMSD from target values		
Bond lengths	0.006 Å	0.008 Å
Bond angles	1.33°	2.3°

^a $R = \sum ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum |F_{\text{obs}}|$.

^b R -factor calculated using 6% of the total reflections, which were not used in refinement (Brünger, 1992a).

^cNR, Not reported.

^dExcluding the FAD and residues in the disordered region with a $\text{C}\alpha$ B -factor >50 (residues 224–230 in reduced TrxR, residues 223–232 in oxidized TrxR).

The separation of the sulfurs of cysteines 135 and 138, together with tests of their scattering contributions, established that the disulfide was reduced. A difference map with amplitudes ($|F_o| - |F_c|$), calculated after simulated annealing and refinement in which FAD and residues 131–140 were omitted, is displayed in Figure 5 (the $|F_o| - |F_c|$ omit map). In the superimposed model resulting from refinement with the cysteines modeled as thiols (Fig. 5A), the sulfur atoms are separated by 3.1 Å. There are no significant peaks at the 3σ contour level in ($|F_o| - |F_c|$) maps calculated after refinement of the complete structure, and no systematic sets of peaks at the 2σ level to indicate contributions from a disulfide species. Mixtures of conformers with a substantial contribution from the disulfide should not be fit this well by a single model. In contrast, refinement of a model in which Cys135 and Cys138 were connected by a 2.0 Å disulfide bond leads to final ($|F_o| - |F_c|$) maps with large positive and negative density peaks of magnitudes 4–6 σ (Fig. 5B). Negative density between the S γ atoms of the active site residues and positive density on the opposite sides of the

Fig. 5. Redox-active cysteine residues of reduced thioredoxin reductase. Residues 133–140 and the FAD were omitted from the model in a round of simulated annealing and positional refinement. The resulting ($|F_o| - |F_c|$) omit map is shown at the 1σ contour level in cyan. ($|F_o| - |F_c|$) maps from refinement with the complete model are shown at the -3σ contour level (magenta) and $+3\sigma$ contour levels (blue). **A:** Active site residues Cys135 and Cys138 modeled as thiols. **B:** Active site residues Cys135 and Cys138 modeled as a disulfide. The positive peak to the right of S γ 135 is greater than 6σ in magnitude and the one to the left of S γ 138 is greater than 4σ .

Table 2. Selected interatomic distances in the active sites of oxidized and reduced thioredoxin reductase

Distance (Å)	Reduced enzyme	Oxidized enzyme ^a
Cys135 S γ to Cys138 S γ ^b	3.09	2.01
Cys138 S γ to FAD C(4a)	3.40	3.12
Cys138 S γ to FAD N(5)	4.03	3.30

^aWaksman et al. (1994) (PDB accession code 1tde).

^bDisulfide bond distance = 2.03 Å (derived from the Cambridge Structural Database (Engh & Huber, 1991), as provided in the X-PLOR 3.851 parameter file parhcsdx.pro).

S γ atoms indicate that a disulfide is a poor fit to the data. Refinement with the dithiol and disulfide as alternate conformers with fractional occupancies confirms the conclusion that the disulfide has been reduced. Occupancies of the disulfide sulfurs, initially set at 0.5, refine to less than 0.1.

Separation of the reduced sulfurs is accomplished partly by movement of the S γ of Cys138 by 0.6 Å toward the pyrimidine ring of the isoalloxazine and partly by movement of S γ of Cys135 away from the flavin. However, the distance between the S γ atoms of Cys135 and Cys138 in the refined model, 3.1 Å (Table 2), is significantly less than the sum of the van der Waals radii, 3.6 Å (Bondi, 1964). A short sulfur to sulfur distance could result from steric constraints in the structure or from hydrogen bonding. Thiol–thiolate hydrogen bonding in reduced thioredoxin and related proteins is suggested by NMR and crystallographic studies. NMR chemical shifts provide strong evidence for the existence of this interaction in reduced glutaredoxin (Nordstrand et al., 1999) and measurements of pK_a s support the assignment of an $\text{SH} \cdots \text{S}^-$ interaction in reduced *E. coli* thioredoxin (Jeng et al., 1995). Unfortunately, the NMR and X-ray studies do not agree on a characteristic distance for the thiol–thiolate hydrogen bond. Sulfur to sulfur distances derived from the X-ray structures are 3.4–3.5 Å in reduced DsbA (Guddat et al., 1998) and 3.9 Å in reduced human thioredoxin (Weichsel et al., 1996). One NMR study finds a sulfur to sulfur separation of 3.1 Å for reduced human thioredoxin (Qin et al., 1994), but another NMR analysis assigns a distance of 3.8 Å between the interacting active site sulfurs of reduced *E. coli* thioredoxin⁴ (Jeng et al., 1994, 1995). Measurements of the thiol pK_a s in reduced TrxR would provide supporting evidence for our proposed $\text{SH} \cdots \text{S}^-$ hydrogen bond.

The most striking changes resulting from reduction occur at the flavin itself. As shown in Figure 6A, the density in simulated annealing omit maps reveals that in the reduced enzyme the isoalloxazine ring of the FAD is not planar, whereas in the oxidized enzyme it is planar (Waksman et al., 1994). N(5) and N(10) of the central flavin ring are displaced toward the core of the FAD domain in the reduced enzyme, while the outer ends of the isoalloxazine shift only slightly. The model of the reduced flavin shown in

⁴Jeng et al. (1994) attributed the short sulfur to sulfur distance found by Qin et al. (1994) to the soft potentials assigned to sulfur by the X-PLOR parameters. Similar potentials were employed in our study. Increasing the van der Waals coefficients for steric repulsion by a factor of 20 increases the separation of the sulfurs by 0.1 Å.

Fig. 6. Views of the isoalloxazine ring system and part of the ribityl side chain of FAD in reduced thioredoxin reductase. **A:** The model of FAD derived by refinement is compared with electron density from the $(|F_o| - |F_c|)$ omit map (see caption, Fig. 5). Contours in cyan are drawn at the 1σ level. The stereoview is from N(5) to N(10), showing the “butterfly” bend (see Fig. 4 for FAD chemical structure and numbering). The FAD from oxidized thioredoxin reductase (orange ball and stick model) (Waksman et al., 1994) was oriented by superimposing the α atoms of the two structures. Displacement of the pyrimidine wing of the flavin and of the central N(5) and N(10) atoms from their positions in oxidized thioredoxin reductase is indicated by well-defined density, and the location of the dimethylbenzene wing of reduced FAD is determined by densities at C(9) and at the methyl groups at C(7) and C(8). **B:** The model derived by refinement is compared with electron density from a $(2|F_o| - |F_c|)$ map based on the complete structure and contoured at 1σ . Positive and negative peaks from the final $(|F_o| - |F_c|)$ map are contoured at -3σ (magenta) and 3σ (blue). The view is at an angle to the isoalloxazine face and shows the positions of the first three ribityl side-chain carbons. The atoms from the oxidized structure (orange ball and stick model) are superimposed for comparison. Rearrangement of the ribityl atoms can be seen clearly in this view.

Figure 6 was derived by fitting the isoalloxazine ring to the X-ray data in rounds of simulated annealing refinement. The computations began with the planar flavin from oxidized TrxR, shown in

Figure 6. Initial rounds used restraints intended to maintain a flat flavin; the bending angle nevertheless increased to 32° during these refinements. Later rounds were carried out with restraints

constructed to allow butterfly bending (Materials and methods). Figure 6B displays the density in $(2|F_o| - |F_c|)$ maps based on the complete refined model. The bending angle between the dimethylbenzene and pyrimidine wings of the isoalloxazine, calculated as described in Materials and methods, is 34° .

Temperature factors for the atoms of the flavin ring are significantly larger than the average for the protein and larger than those found for FAD in the structure of oxidized TrxR (Table 1). *B*-values from X-PLOR refinement vary from 37 to 40 for the pyrimidine ring atoms and from 41 to 48 Å² for the outer atoms of the dimethylbenzene ring. Unrestrained refinement of the individual thermal factors in SHELXL-97 (Sheldrick & Schneider, 1997) yields a similar average *B* for the outer atoms in the dimethylbenzene ring. The thermal factors correlate with the electron densities, which are particularly weak for the dimethylbenzene wing. There is no density for the peripheral atoms of the dimethylbenzene ring that would correspond to an alternative planar conformation of the isoalloxazine. We conclude that the *B*-factors indicate mobility and flexibility of the reduced flavin in TrxR rather than a mixture of conformers. Some distortions or motions of the isoalloxazine ring that are more complicated than bending around the N(5)–N(10) axis may affect the shapes and magnitudes of the electron density but have not been allowed for in our construction of restraints. Manual adjustment of the dimethylbenzene ring in omit densities suggests that the angle between the wings of the isoalloxazine moiety cannot be less than 30° .

Viewed perpendicular to the N(5)–N(10) axis (Fig. 7B), the central ring of the reduced isoalloxazine is seen to adopt a boat conformation with N(5) and N(10) displaced toward the *si* side of the isoalloxazine ring. The change in geometry at N(10) is accompanied by movement of C(1') and by changes in the torsion angle about the N(10)–C(1') bond that move C(2') by 2.9 Å toward the *re* side of the isoalloxazine (Figs. 6B, 7B). These reorientations were not introduced by model building but occurred during refinement (see Materials and methods). They are confirmed by the absence of difference density at O(2') of the oxidized flavin (Fig. 6B). The movement of C(2') in turn permits the formation of new hydrogen bonds between O(2') and the backbone carbonyl of Arg293, and between O(2') and N(1), both of which presumably stabilize the bent conformation of the isoalloxazine ring (Fig. 7A,B). Since OH(2') would serve as donor in the hydrogen bond with the backbone carbonyl of Arg293, O(2') would be an acceptor in the hydrogen bond with N(1), implying that N(1) is protonated. The interaction of OH(3') with Asp286 is also altered, and the Asp286 side chain is displaced from its position in oxidized TrxR. However, the remainder of the FAD cofactor, beyond C(3'), is essentially unchanged by reduction. Comparison with other flavoproteins (see Materials and methods) shows that the altered conformation adopted by the ribityl side chain in reduced TrxR is unusual. The shifts of N(10) and C(1'), combined with the *gauche* arrangement of OH(2') and OH(3'), confer a novel shape on the side chain. The energies associated with the conformation changes in FAD are discussed in the section that treats conformations of protein-bound isoalloxazines.

The positions and hybridization of N(5) and N(10) were not biased by the restraints derived from structures of model reduced flavins with boat conformations in which the nitrogens have partial *sp*³ character (see Materials and methods). As a test, the final model was refined using the topology and parameter files for oxidized FAD, in which restraints are designed to maintain a planar isoalloxazine. The position of N(10) was nearly the same as in

Fig. 7. Comparisons of the interactions of the FAD cofactor in oxidized and reduced thioredoxin reductase. Hydrogen bonds that are affected by the conformation changes of the FAD are shown with dashed lines. **A:** Oxidized enzyme (Waksman et al., 1994) (PDB accession code 1tde). **B:** Reduced enzyme. The FAD from the oxidized structure (thin model) is shown for comparison. The movement of O(2') toward the *re* face of the isoalloxazine allows the formation of two new hydrogen bonds, one with the backbone carbonyl of Arg293 and one with the N(1)H of the FAD. Water 668, which forms a hydrogen bond with N(5) in the reduced structure, is conserved in the oxidized structure as well, and is the likely proton donor to N(5) during reduction of the isoalloxazine. The ribityl side chain approaches an axial position at N(10), while the proton modeled on N(5) is in an equatorial position.

Figures 6B and 7B, and the bending angle was 33° . The displacements in the positions of atoms C(1') and C(2') also serve to verify the shift of N(10) to the boat conformation, since these rearrangements in the ribityl side chain are coordinated with the displacement of N(10), as can be seen in Figures 6B and 7B.

All of the changes in the protein that are induced by reduction occur near an intrachain interface where the NADPH and FAD domains contact one another (Fig. 3B). This interface, unlike most domain interfaces, includes only one salt bridge and one hydrogen bond interaction, and displays little interdigitation of side chains (Waksman et al., 1994). It is lined with waters, presumably in keeping with the functional requirement that it must readily dissociate and reform; the two association/dissociation steps that oc-

cur in each reaction cycle must together be at least as fast as the k_{cat} of $\sim 33 \text{ s}^{-1}$ (Lennon & Williams, 1997). An important contributor from the NADPH domain is the sequence 133–142, which adjoins the flavin ring and includes the active site Cys138. The sulfur of Cys138 moves toward the FAD domain by 0.6 Å upon reduction to the thiol form. Residues 46–51 and 292–296 of the FAD domain are also partly buried in the interface. The side chain of Arg293 from the FAD domain stacks against His245 from the domain connector and is likely to change position in the rearrangement to the FR conformation. Although this side chain itself is not perturbed, reduction of the flavin generates new interactions between the main chain of Arg293 and the ribityl side chain (Fig. 7). Thus, the energetics of the interface interactions may be affected by the ring bending and other conformation changes that accompany reduction. The energy differences are not large enough to drive dissociation of the domain interface in crystalline TrxR. However, the [FR]/[FO] equilibrium constant in solution is ~ 3 (Lennon & Williams, 1997), so small changes concomitant with reduction may be functionally significant.

Conformations of model flavins

Structures and properties of model isoalloxazines, not bound to proteins, provide references for assessing the effects of the protein environment on the conformation and properties of flavin cofactors. Here, we summarize what is known about the conformations of oxidized and reduced isoalloxazines from X-ray, NMR, and computational studies of model flavins.

Crystallographic studies

X-ray crystal structures have shown that the isoalloxazine ring system in free oxidized flavins is essentially planar; the bending angle between the dimethylbenzene and pyrimidine rings is generally not larger than $\sim 4^\circ$ (Tanaka et al., 1969; Trus & Fritchie, 1969; Kierkegaard et al., 1971; Norrestam & Stensland, 1972; Wang & Fritchie, 1973; Kuo et al., 1974; Fritchie & Johnston, 1975; Porter & Voet, 1978; Wouters et al., 1994, 1995). Ring stacking is a frequent feature in crystal structures of model flavins and may help to restrain these isoalloxazines to planar conformations. On the other hand, the ring system is nonplanar in reduced model flavins (Werner & Rönquist, 1970; Kierkegaard et al., 1971; Leijonmarck & Werner, 1971; Werner et al., 1971; Norrestam & Von Glehn, 1972; Porter & Voet, 1978). In six reported structures of reduced isoalloxazines, the rings deviate from planarity by 8.9 to 35.5° with a “butterfly” bending about the N(5)–N(10) axis; the central pyrazine ring generally adopts a boat conformation with N(5) and N(10) out of plane. Some of the reduced model flavins were substituted at N(1) and/or N(5) to prevent oxidation, and these or other substituents can affect the bending of the isoalloxazine (Tauscher et al., 1973; Zheng & Ornstein, 1996). The substantial differences in geometry between oxidized and reduced flavin models are significant despite some bias in the medians of the distribution of bending angles.

NMR studies

From NMR studies of ^{15}N - and ^{13}C -substituted oxidized and reduced model flavins, Müller and colleagues (Moonen et al., 1984a, 1984b) concluded that the oxidized flavins are not quite planar in solution, in agreement with the slight deviations from planarity in the crystal structures of oxidized flavins, described above. They also concluded that anionic reduced isoalloxazines are

essentially planar, and that neutral reduced flavins dissolved in polar solvents are only “slightly bent” in accord with the calculations of Dixon et al. (1979) (see next section). The apparent disagreement between the crystal structures of neutral reduced flavins and the interpretations of the NMR data has not been fully resolved. Estimates of the hybridization of the N(10) and N(5) atoms in reduced flavins, based on chemical shifts, could be complicated by fast exchange. If the barrier to ring inversion is very small for free isoalloxazines, then the NMR measurements will sample time averages of conformations that are in rapid equilibrium. Inversion barriers have been variously estimated at 2 kcal/mol (Hall et al., 1987) and 4 kcal/mol (Dixon et al., 1979).

Theoretical studies

Molecular orbital calculations have uniformly concluded that the isoalloxazine ring systems of oxidized flavins are essentially planar, while those of neutral reduced flavins are bent by ~ 15 to $\sim 28^\circ$ (Dixon et al., 1979; Hall et al., 1987; Platenkamp et al., 1987; Zheng & Ornstein, 1996). Interestingly, a recent theoretical study (Zheng & Ornstein, 1996) has calculated that reduced lumiflavin deprotonated at N(1) is essentially planar, which is in agreement with the NMR study of Moonen et al. (1984b). The theoretical studies support the idea that modest distortions of the ring conformations from the optimal bending angles are not energetically expensive, costing 2–5 kcal/mol. Such distortions of protein-bound flavins can be achieved with the energies available from noncovalent interactions like hydrogen bonding. For reduced lumiflavin with an energy minimum at a bending angle of 15° , the calculations and graphs of Dixon et al. (1979) estimate that only 1–2 kcal/mol is needed to increase the bend angle to 30° , and 4 kcal/mol are required to attain planarity. Alternatively, Hall et al. (1987) and Zheng and Ornstein (1996) calculated an energy minimum for reduced lumiflavin at a bending angle of $\sim 27^\circ$, with 2 and ~ 6 kcal/mol required, respectively, to flatten the ring. For the oxidized isoalloxazine ring system, calculations suggest that bending from the minimum energy conformation at 0° to an angle of 10° costs only 1–2 kcal/mol (Dixon et al., 1979; Hall et al., 1987). However, larger perturbations of oxidized isoalloxazines require relatively more energy than corresponding perturbations of reduced flavins. According to the analyses of Dixon et al. (1979), bending an oxidized flavin from 0° to 20° costs 8–9 kcal/mol. Thus, oxidized isoalloxazines appear to be “stiffer” than their neutral reduced counterparts.

Conformations of isoalloxazines in flavoproteins

To examine the bending of isoalloxazines in proteins, we obtained the coordinates for all the crystal structures of flavin-containing proteins from the PDB. After sorting (see Materials and methods), there were 55 unique flavin structures (46 oxidized, 2 semiquinone, 7 reduced). The angles between planes through N(10), N(5), and the pyrimidine or dimethylbenzene rings are shown as a function of frequency of occurrence in Figure 8. The mean of the distribution for oxidized flavoproteins is $7.0^\circ \pm 5.2^\circ$. The bending of oxidized isoalloxazines in flavoproteins disagrees with the theoretical calculations that predict planar structures for oxidized flavins. It is also larger than the $\leq 4^\circ$ bending found in crystal structures of free oxidized flavins, although the distribution of bends in model flavins may be skewed toward small values by intermolecular interactions. Thus, this survey of isoalloxazines in published flavoprotein structures provides support for the intuitive idea that the

Fig. 8. Histogram showing the deviations from planarity of the isoalloxazine rings in flavoproteins. “Butterfly” bending angles were determined as described in Materials and methods. Dark gray bars, oxidized structures; light gray bars, semiquinone structures; open bars, reduced structures; black bar, reduced thioredoxin reductase.

protein environment has a significant effect on the conformation of bound isoalloxazines.

There are not many examples of reduced flavoproteins, but the average bend of $8.2^\circ \pm 5.9^\circ$ (excluding TrxR) seems clearly at odds with expectations of nonplanarity from model structures and computations, which predict larger bending angles. However, proteins bound to anionic reduced isoalloxazines dominate the sampling in the PDB. There are seven unique reduced flavoproteins in the PDB with bending angles from 2° to 18° . Assignments of the ionization states have been made from ^{13}C and ^{15}N NMR chemical shifts for five of these proteins: porcine kidney D-amino acid oxidase (4° bend), human glutathione reductase (5° bend), *Clostridium beijerinckii* flavodoxin (7° bend), *Desulfovibrio vulgaris* flavodoxin (7° bend), and *Saccharomyces carlsbergensis* old yellow enzyme (18° bend) (van den Berg et al., 1984; Beinert et al., 1985; Vervoort

et al., 1985, 1986; Miura & Miyake, 1987). In each case the reduced flavin is anionic with N(1) deprotonated. The computations of Zheng and Ornstein (1996) indicate that the bent conformation is optimal for neutral reduced flavins, whereas the planar conformation is favored for anionic reduced isoalloxazines. Although the anionic isoalloxazines in the PDB are not fully planar, they are significantly less bent than expected for neutral reduced flavins, with the single exception of reduced old yellow enzyme.

In four structures from the PDB, the flavin ring bending is 18° or greater (Fig. 8; Table 3, Columns 2–5): reduced old yellow enzyme (18°), pyruvate oxidase (18°), NADH oxidase (18°), and trimethylamine dehydrogenase (27°). The largest of these bending angles is 7° less than the value we find for reduced TrxR (cf. Table 3, Columns 1 & 2). Curiously, the significantly bent flavins in pyruvate oxidase, NADH oxidase, and trimethylamine dehydrogenase are oxidized. While it is energetically inexpensive to impart a bend of 10° to an oxidized flavin ring (see above), the analysis of Dixon et al. (1979) estimates an energy of ~ 6 kcal/mol to bend isoalloxazine to the angles found in pyruvate oxidase or NADH oxidase and ~ 15 kcal/mol to bend isoalloxazine to the angle found in trimethylamine dehydrogenase. Muller et al. (1994) report that the isoalloxazine in pyruvate oxidase is surrounded by bulky aromatic residues, with a valine serving as a “backstop” at N(5) to produce the observed bend. The structure of NADH oxidase (Hecht et al., 1995) reveals protein interactions and contacts that act to bend the isoalloxazine, in particular a backbone carbonyl that presses against N(5). Mathews and Lim (1987) note the bent isoalloxazine ring in trimethylamine dehydrogenase but do not discuss the mechanisms that might account for the large distortion of the flavin in this enzyme. The FMN in trimethylamine dehydrogenase is covalently attached to the enzyme via a bond from a cysteine sulfur to C(6) of the flavin. Anchoring one end of the isoalloxazine by a covalent bond may help to force the isoalloxazine into this highly bent conformation. The high energy of an oxidized bent flavin, relative to its reduced form, should be reflected in the values of the flavin midpoint potentials. From the midpoint potentials measured by Barber et al. (1988), the potential for the $\text{FMN}_{\text{ox}}/\text{FMN}_{\text{red}}$ couple in trimethylamine dehydrogenase is found to be +40 mV. This value is 194 mV above the value of

Table 3. Least-squares planes through the dimethylbenzene and pyrimidine rings of flavins from selected flavoproteins^a

Protein	Thioredoxin reductase	Trimethylamine dehydrogenase	NADH oxidase	Pyruvate oxidase	Old yellow enzyme
Organism	<i>E. coli</i>	Methylotrophic bacterium W3A1	<i>Thermus thermophilus</i>	<i>Lactobacillus plantarum</i>	<i>S. carlsbergensis</i>
PDB accession code	1c10	2tmd	1nox	1pow	1oyc
Reference	Present work	Bellamy et al. (1989)	Hecht et al. (1995)	Muller et al. (1994)	Fox and Karplus (1994)
Resolution (Å)	2.5	2.4	1.59	2.50	2.00
Redox state	Reduced	Oxidized	Oxidized	Oxidized	Reduced
RMSD for pyrimidine ring atoms (Å)	0.040	0.085	0.044	0.061	0.131
RMSD for dimethylbenzene ring atoms (Å)	0.063	0.115	0.044	0.104	0.048
Deviation of the two rings from planarity (direction of bend)	34° (re face)	27° (re face)	18° (si face)	18° (re face)	18° (re face)

^aCalculated using the program SHELXL-97 (Sheldrick & Schneider, 1997). Enzymes are listed in order of decreasing bending angle.

–154 mV for free 6-S-cysteiny-FMN (Ghisla et al., 1980), confirming the expectation that bending of the oxidized flavin should favor reduction.

Isoalloxazine bending in reduced TrxR

The magnitude of isoalloxazine bending in reduced TrxR, 34°, is unique among flavoenzyme structures. In contrast to most of the reduced flavoenzymes in our survey, and in contrast to other members of this enzyme family (van den Berg et al., 1984), TrxR appears to be a flavoenzyme in which the reduced isoalloxazine is neutral. The pH dependence of the FAD_{ox}/FAD_{red} potential in TrxR shows that reduction involves uptake of two electrons and two protons (O'Donnell & Williams, 1983). This proton stoichiometry suggests formation of a neutral reduced flavin with N(1) protonated, which is consistent with the N(1)H–O(2') hydrogen bond that we assign in our structure. Based on the crystallographic and theoretical studies described earlier, in the absence of other effects we would expect the reduced flavin in TrxR to be bent by 20–25°, the middle of the range predicted by computations. The distortion energy needed for a neutral isoalloxazine to exhibit the larger 34° bend observed in reduced TrxR is estimated to be ~3 kcal/mol from the graphs of Dixon et al. (1979). We surmise that steric repulsion resulting from the movement of the S γ of reduced Cys138 toward the isoalloxazine ring provides this additional energy. In reduced TrxR, S γ of Cys138 would be only 2.7 Å from C(4a) of the isoalloxazine if there were no compensating displacements of the flavin ring. Adjustments are made by the “butterfly” bending of the isoalloxazine, in which the N(5) and N(10) atoms of the isoalloxazine move 1.0 Å while the outer ends of the pyrimidine and dimethylbenzene rings occupy essentially the same positions as in the oxidized structure (Fig. 9B). Deformability of the reduced flavin ring accommodates the cleavage of the disulfide bond and the separation of the thiols from one another (cf. Fig. 9A,B).

In the structure of the oxidized form of the Cys138Ser mutant (PDB accession code 1trb), in which the active site disulfide is “broken” by mutation but the flavin remains oxidized, the isoalloxazine ring is essentially planar with the Ser138 O γ about 3.1 Å from C(4a). The smaller radius of oxygen compared to sulfur may allow the flavin to remain in its planar minimum energy state in this mutant structure. It would be interesting to examine the extent of isoalloxazine bending in the oxidized and reduced forms of the Cys135Ser mutant. In that mutant, Cys138 S γ would be expected to push against the isoalloxazine ring in both oxidation states, so measurements of the bending could provide additional information about the relative flexibilities of the oxidized and reduced isoalloxazines.

The redox potential for the FAD_{ox}/FAD_{red} couple is higher in TrxR than in other members of the pyridine nucleotide-disulfide reductase family. The ability of the structure to accommodate a bent reduced flavin, demonstrated in the present study, may contribute to the relative ease of reduction. Structure analyses of the oxidized and reduced FR conformers of TrxR, a long-term goal, will be critical to understanding the role of FAD rearrangements in thioredoxin reductase.

Conclusion

By carrying out reduction in the crystal, we have been able to analyze the FO conformer of a 4 electron-reduced species of TrxR.

Fig. 9. Interaction of Cys138 with the isoalloxazine ring of FAD in oxidized and reduced thioredoxin reductase. Surfaces were generated in the program Ribbons (Carson, 1997) using a probe equal to the van der Waals radius for each atom type (Bondi, 1964). The view is from N(10) to N(5) of the isoalloxazine. **A:** Oxidized enzyme (Waksman et al., 1994) (PDB accession code 1tde). **B:** Reduced enzyme.

In this structure the isoalloxazine ring of FAD_{red} adopts a highly bent conformation that is unique among presently studied flavoenzymes. The ribityl side chain rearranges to accommodate the ring structure, and the several changes in FAD are compensated by formation of new hydrogen bonds to the protein. Significant flexing of the flavin is in agreement with theoretical calculations for neutral reduced flavins that show an energy minimum at a high bending angle and a “soft potential” for butterfly bending. The large ring bending appears to be induced partly by steric effects resulting from cleavage of the active site disulfide bond connecting Cys135 and Cys138. The changes that accompany reduction are an intriguing example of the structural rearrangements that are often linked to oxidation/reduction in flavoproteins. TrxR is an unusual case because it is the FAD itself that makes most of the structural adjustments. The solution of additional structures of reduced flavoenzymes would provide a wider sampling of the occurrence and distribution of ring bending and distortion in flavin cofactors, and might better assess the relationship between flavin bending, redox potentials, and reactivities. As illustrated by the correlation of the

bent oxidized isoalloxazine with the elevated flavin midpoint potential for trimethylamine dehydrogenase, the formulation of mechanisms for flavoenzymes should consider the energetics of flavin bending.

Materials and methods

Enzyme purification and crystallization

Wild-type TrxR from *E. coli* was overexpressed and purified using published methods (Mulrooney, 1997). After purification, the enzyme was stored at a concentration of 20 mg/mL in 10 mM HEPES, pH 7.0 at -20°C until use. The enzyme was crystallized by a modification of published methods (Kuriyan et al., 1989). Crystals of oxidized TrxR were grown by the hanging drop method at 25°C over reservoirs containing 0.6–0.75 mL of well solution. Bright yellow hexagonal rods formed spontaneously in drops containing 2–5 μL protein (20 mg/mL), 1 μL 19 mM 1,12-dodecanedioic acid, 1–2 μL water, and 2 μL well solution (50 mM HEPES, pH 7.0 + 200 mM $(\text{NH}_4)_2\text{SO}_4$ + 32–40% PEG 3350). These initial crystals were used as a source of microseeds. The 1,12-dodecanedioic acid was included in all crystallizations because it caused crystals to appear more rapidly and to grow with better-defined faces.

In a typical microseeding procedure, 10–30 crystals measuring approximately $0.05 \times 0.05 \times 0.5$ –0.9 mm were crushed with a needle in 20 μL of seed diluent (well solution containing 38% PEG 3350). The slurry of crushed protein crystals was added to 380 μL of seed diluent, and the mixture was spun in a microcentrifuge at $16,000 \times g$ for 15 s. The supernatant microseed stock solution was serially diluted 1:10, 1:10², and 1:10³. Drops containing 2 μL protein (20 mg/mL), 2 μL 19 mM 1,12-dodecanedioic acid, and 4 μL well solution (50 mM HEPES, pH 7.0, 200 mM $(\text{NH}_4)_2\text{SO}_4$, 35% PEG 3350) were allowed to equilibrate at 25°C for two days, then were seeded by adding 0.3 μL of one of the microseed stock dilutions. Crystals appeared within one day, and grew to full size within about five days. Drops seeded with the 1:10² dilution produced ~ 10 crystals 0.05 – 0.1×0.05 – 0.1×0.6 – 1.0 mm that could be used for data collection.

Chemical reduction of oxidized crystals

Oxidized crystals of TrxR were transferred from the mother liquor to a solution of 200 mM HEPES, pH 8.0 + 60% saturated $(\text{NH}_4)_2\text{SO}_4$ by soaking for 15 min in each of a series of seven solutions of gradually increasing concentration of $(\text{NH}_4)_2\text{SO}_4$ and decreasing concentration of PEG 3350. This was necessary because the crystals could not be reduced by $\text{Na}_2\text{S}_2\text{O}_4$ when in the original seed diluent. A 2.5 mL aliquot of 200 mM HEPES, pH 8.0 + 60% saturated $(\text{NH}_4)_2\text{SO}_4$ was transferred to an ignition tube fitted with a side arm and standard taper joint and stopcock. Solid $\text{Na}_2\text{S}_2\text{O}_4$ (13.2 mg) was placed in the side arm and the solution was made anaerobic by 10 cycles of evacuation for ~ 15 s (with vortexing) followed by flushing with argon that had been passed through a gas purifier (OxiClear® model DGP-250-R1, Diamond Tool and Die, Oakland, California). The anaerobic holding solution was tipped into the side arm to dissolve the $\text{Na}_2\text{S}_2\text{O}_4$, and the tube was briefly opened for rapid addition of the oxidized crystals. The tube was resealed and subjected to five cycles of rapid evacuation and argon flushing without mixing. This procedure was adopted because the enzyme crystals become very sticky when transferred from the PEG solution to the

$(\text{NH}_4)_2\text{SO}_4$, and the vigorous mixing needed to make the solution anaerobic would have deposited the crystals on the side wall of the tube above the level of the reduction solution. The mixing would have been mechanically stressful to the crystals as well. After overnight reduction in the dark at room temperature, the crystals were a pale yellow as expected from the small ϵ_{456} ($\sim 2,000 \text{ M}^{-1} \text{ cm}^{-1}$) of reduced FAD in TrxR. They were transferred with several hundred microliters of reduction solution to a depression slide in room atmosphere, briefly passed with a nylon loop through a 300 μL drop of 200 mM HEPES, pH 8.0 + 60% saturated $(\text{NH}_4)_2\text{SO}_4$ + 30 mM $\text{Na}_2\text{S}_2\text{O}_4$ + 17% (w/v) *meso*-erythritol, and were rapidly cooled by plunging into liquid nitrogen.

Data collection, data reduction, and structure refinement

Data were collected with an R-AXIS IV image plate detector system (Molecular Structure Corporation, The Woodlands, Texas), using $\text{CuK}\alpha$ radiation from a Rigaku RUH3R rotating anode source operating at 5 kW, and focused using the MSC/Yale mirror system. Data were processed with the HKL package (v. 1.6) (Otwinowski & Minor, 1997). The space group was unchanged by reduction, with unit cell dimensions slightly smaller than for oxidized TrxR, which was solved at room temperature. Data collection and statistics are summarized in Table 4.

Protein coordinates from the published structure of oxidized wild-type enzyme (PDB accession code 1tde) (Waksman et al., 1994) were used as a starting model for refinement with the program X-PLOR version 3.1f (Brünger, 1992b). In the following description, the term “round” of refinement refers to the submission of a script to the program X-PLOR, in which multiple cycles of refinement (e.g., rigid body, positional, *B*-factor, etc.) are performed. One initial round of rigid body refinement followed by one round of positional and overall *B*-factor refinement reduced the *R*-factor for data from 6–2.5 Å to 0.271 ($R_{\text{free}} = 0.341$ for 6% of the reflections) (Brünger, 1992a). Minor rebuilding of the model to place side chains in the $(2|F_o| - |F_c|)$ map was done with the program O version 5.10.2 (Jones et al., 1991). Refinement was continued in X-PLOR version 3.851, implementing a bulk solvent correction (Jiang & Brünger, 1994) and gradually extending the low resolution limit to 10 Å. Each round of refinement involved (in order): overall *B*-factor refinement, positional refinement, simulated annealing, positional refinement, isotropic group *B*-factor refinement, and positional refinement (Brünger et al., 1987, 1990). The thermal parameters from oxidized TrxR were used as starting values for the group refinement of *B*-values. The structure was manually inspected and rebuilt using $(2|F_o| - |F_c|)$ and $(|F_o| - |F_c|)$ maps. To examine local structural changes, omit maps were computed after simulated annealing and refinement of partial models (Hodel et al., 1992). Residues 225–231 were disordered, as evidenced by weak or missing densities in the $(2|F_o| - |F_c|)$ maps. The ϕ and ψ angles for residues Asp224 and Thr225 were adjusted after refinement was complete to bring Asp224 into an allowed region of the Ramachandran plot. Solvent molecules were initially added when the *R*-factor reached 0.254 ($R_{\text{free}} = 0.342$) for data from 8–2.5 Å. Solvents were chosen using three sources of information: the automated water picking protocol in X-PLOR 3.851, the structure of the oxidized enzyme (Waksman et al., 1994), and visual inspection of $(|F_o| - |F_c|)$ maps. Only solvent molecules that were in density in the $(2|F_o| - |F_c|)$ maps, were within 2.4–3.5 Å of a hydrogen bond donor or acceptor, and had *B*-factors $< 60 \text{ Å}^2$ for oxygen have been retained in the deposited coordi-

Table 4. Summary of diffraction data

	Reduced enzyme (this work)	Oxidized enzyme (Waksman et al., 1994)
Space group	P6 ₃ 22	P6 ₃ 22
<i>a</i> = <i>b</i> (Å)	121.3	123.8
<i>c</i> (Å)	81.32	81.56
Molecules per asymmetric unit (Matthews coefficient, Å ³ /Da)	1 (2.45)	1 (2.56)
Temperature	−160 °C	Room temperature
Resolution (Å) (last shell)	50–2.50 ^a (2.59–2.50)	20–2.10
<i>R</i> _{sym} ^b (%) (last shell)	11.9 (33.4)	5.7
Completeness (%) (last shell)	96.3 (80.4)	85
Average <i>I</i> / σ <i>I</i> (last shell)	9.5 (2.1)	NR ^c
No. of reflections (unique)	98,833 (12,681)	54,700 (19,643)

^aThe high resolution limit was determined by adjusting the high resolution cutoff during scaling until the *I*/ σ *I* in the last shell was ≥ 2 .

^b*R*_{sym}, agreement between the intensities of symmetry-related reflections.

^cNR, Not reported.

nates. The final *R*-values and other refinement statistics are shown in Table 1. The coordinates have been deposited in the Protein Data Bank (Protein Data Bank ID 1CL0 and Research Collaboratory for Structural Bioinformatics ID RCSB000998).

In early rounds of refinement, X-PLOR parameter and topology files for the oxidized, planar FAD were used (Waksman et al., 1994). The occupancies of most of the isoalloxazine ring atoms were set to zero to prevent biasing the conformation of the isoalloxazine until more accurate phases were obtained. Rotation of the ribityl side chain around the N(10)–C(1') bond occurred during these stages without the aid of manual model building. X-PLOR parameter and topology files for reduced FAD were then implemented, and refinement continued using the full FAD model. The X-PLOR parameter and topology files for reduced flavin were generated by modifying the files used in the solution of the oxidized structure to reflect the changes brought about by reduction, including protonation of N(1) and N(5) of the isoalloxazine ring system. Bond lengths and angles averaged from six published small molecule structures of reduced flavins and reduced flavin analogs were used (Werner & Rönquist, 1970; Kierkegaard et al., 1971; Leijonmarck & Werner, 1971; Werner et al., 1971; Norrestam & Von Glehn, 1972; Porter & Voet, 1978). The hybridization of the isoalloxazine ring atoms N(10) and N(5) was intermediate between *sp*³ and *sp*², with average bond angles of $116.1^\circ \pm 3.6^\circ$ and $114.9^\circ \pm 2.1^\circ$, respectively. To lessen bias, these bond angle energies were set to 25% of the value in the original files for oxidized FAD. The improper dihedral angle restraints were designed to keep the “wings” of the isoalloxazine planar, including the N(10) and N(5) atoms in each wing. The central pyrazine ring itself was not restrained to be planar. This allowed the “butterfly” bending of the pyrimidine and dimethylbenzene rings about the N(5)–N(10) axis. In the X-PLOR protein topology and parameter files, the default for cysteine residues is the thiol form of the S_Y atoms. This default was used in all refinements except the one that generated Figure 5B. In that refinement a disulfide patch was applied to residues Cys135 and Cys138 to maintain the disulfide bond.

Figures 3, 5, 6, 7, and 9 were generated with the program Ribbons (Carson, 1997).

Analysis of flavin bending angles in published structures

We surveyed the PDB and obtained the coordinates for all the crystal structures of flavin-containing proteins. The database includes 187 structures of flavoproteins containing 190 flavins. Of these flavins, 166 were oxidized, 4 were in the 1 electron-reduced or semiquinone state, and 20 were in the 2 electron-reduced or hydroquinone state. To determine a distribution of the deviations from planarity for the isoalloxazines, the data were sorted according to two criteria. First, all duplicate structures were eliminated, retaining only the highest resolution structure of each protein. Similar flavoproteins from different organisms were treated as separate structures, as were structures of the same flavoprotein solved in different oxidation states. Second, structures containing substrates or mutations were not used, to eliminate the possibility of effects on the planarity of the isoalloxazine. An exception to this rule was allowed if the presence of substrate was necessary for the reduction of the flavin, or if no substrate-free structure of that enzyme was available and the substrate was not bound near the isoalloxazine ring of the flavin. After this sorting procedure, there remained a total of 55 flavins (46 oxidized, 2 semiquinone, 7 reduced). The program SHELXL-97 (Sheldrick & Schneider, 1997) was used to fit least-squares planes separately to the pyrimidine and dimethylbenzene wings. To approximate a butterfly bending about the N(5)–N(10) axis, the N(5) and N(10) atoms were included in both planes for a total of 10 atoms per wing. The angles between the two planes were then calculated by SHELXL-97.

Supplementary material in Electronic Appendix

A table of isoalloxazine bending angles for flavoprotein crystal structures from the PDB is included as Electronic Supplementary Material. Structures used to make Figure 8 are marked in the table.

Acknowledgments

This research was supported by National Institutes of Health grants GM18723 (BWL), GM16429 (MLL), and GM21444 (CHW). B.W.L. would like to thank Brian D. Guenther, Christian H. Weber, and Anita L. Metzger for helpful discussions on structure refinement.

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