FLAVOPROTEIN STRUCTURE AND MECHANISM 6

Mechanism and structure of thioredoxin reductase from *Escherichia coli*

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The family of flavoproteins with redox-active cystein/cystine residues that link the pyridine nucleotides with sulfur-containing substrates is an ever-growing one. In the case of thioredoxin reductase, the second substrate is the small protein thioredoxin, itself containing a redox-active disulfide, which in its reduced form is the substrate for ribonucleotide reductase. Like other members of this family, thioredoxin reductase catalysis involves the reduction of the enzyme-bound FAD by NADPH, followed by electron transfer from the reduced flavin to the redox–active disulfide, which in turn is responsible for the reduction of thioredoxin. The crystal structure of the enzyme raises interesting mechanistic questions about how this internal transfer of reducing equivalents takes place. The possible answers to these questions provide the principal theme of this article.

ABSTRACT **The flavoprotein thioredoxin reductase catalyzes the reduction of the small redox protein thioredoxin by NADPH. Thioredoxin reductase contains a redox active disulfide and is a member of the pyridine nucleotide-disulfide oxidoreductase family of flavoenzymes that includes lipoamide dehydrogenase, glutathione reductase, trypanothione reductase, mercuric reductase, and NADH peroxidase. The structure of thioredoxin reductase has recently been determined from X-ray crystallographic data. In this paper, we attempt to correlate the structure with a considerable body of mechanistic data and to arrive at a mechanism consistent with both. The path of reducing equivalents in catalysis by glutathione reductase and lipoamide dehydrogenase is clear. To envisage the path of reducing equivalents in catalysis by thioredoxin reductase, a conformational change is required in which the NADPH domain rotates relative to the FAD domain. The rotation moves the nascent dithiol from its observed position adjacent to the *re* surface of the flavin ring system toward the protein surface for dithiol-disulfide interchange with the protein substrate thioredoxin and moves the nicotinamide ring of NADPH adjacent to the flavin ring for efficient hydride transfer. Reverse rotation allows reduction of the redox active disulfide by the reduced flavin. This requires that the enzyme pass through a ternary complex; the kinetic evidence for such a complex is discussed.—Williams, C. H., Jr. Mechanism and structure of thioredoxin reductase from *Escherichia coli*. *FASEB J.* 9, 1267–1276 (1995)**

Key Words: thioredoxin reductase · flavoprotein · disulfide reductase · pyridine nucleotide · thiol-disulfide interchange

THIOREDOXIN REDUCTASE CATALYZES THE REDUCTION of the 12,000 M_r redox protein thioredoxin by NADPH, as shown below, where $Tr(S_2)$ is thioredoxin and $Tr(SH)_2$ is reduced thioredoxin.[2]

$$NADPH + H^+ + Tr(S_2) \rightleftharpoons NADP^+ + Tr(SH)_2$$

Thioredoxin reductase is a member of the pyridine nucleotide-disulfide oxidoreductase family of flavoenzymes. The best-studied members of the family are lipoamide dehydrogenase, glutathione reductase, mercuric reductase, and NADH peroxidase. Trypanothione reductase is the subject of another review in this series (1). All members of the family are homodimers except NADH peroxidase, which is a homotetramer. Lipoamide dehydrogenase, glutathione reductase, trypanothione reductase, and mercuric ion reductase are very closely related whereas thioredoxin reductase and NADH peroxidase are more distant relations. Thioredoxin reductase contains a redox active disulfide in addition to the FAD. The flow of electrons in catalysis by thioredoxin reductase is the same as in glutathione reductase: from NADPH to the FAD, from re-

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[2]Abbreviations: $Tr(S_2)$, thioredoxin; $Tr(SH)_2$, reduced thioredoxin; E_{ox}, oxidized thioredoxin reductase; $E(FAD)(SH)_2$, $E(FADH_2)(S_2)$ and EH_2, 2-electron reduced forms of thioredoxin reductase; $E(FADH_2)(SH)_2$ and EH_4; 4-electron reduced thioredoxin reductase; C135S, thioredoxin reductase in which Cys^{135} as been changed to Ser, with Cys^{138} remaining; C138S, thioredoxin reductase in which Cys^{138} has been changed to Ser, with Cys^{135} remaining; DTNB, 5,5′-dithiobis(2-nitrobenzoate); TNB, 5-thio-2-nitrobenzoate; $AADP^+$, aminopyridine adenine dinucleotide phosphate; PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

[3]In ref 24, the amino acid numbering included the initial methionine that is removed from the mature protein. Thus, Cys^{135} and Cys^{138} are referred to as Cys^{136} and Cys^{139}, respectively, in that paper.

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duced FAD to the active site disulfide, and finally, from the nascent active site dithiol to the disulfide of thioredoxin by two sequential thiol-disulfide interchange reactions. The mode of electron transfer from reduced FAD to the disulfide may be via a sulfur adduct at the flavin C4a position (2). The distribution and possible roles of thioredoxin as a dithiol-disulfide redox mediator alone and in concert with glutaredoxin and glutathione have been reviewed (3, 4). These three systems function generally to maintain the thiol-disulfide poise of the cell. The structure of thioredoxin is known (5, 6).

Thioredoxin reductase was reviewed in the context of the family in 1992 in the CRC series edited by Franz Müller, *Chemistry and Biochemistry of Flavoenzymes* (7). However, the structure of thioredoxin reductase has appeared since then (8, 9). The structures of lipoamide dehydrogenase and glutathione reductase largely confirmed and augmented the mechanistic information available in that they revealed the structural pathway of electrons predicted by the mechanism (10, 11). The structure of thioredoxin reductase, on the other hand, does not reveal a clear path for reducing equivalents nor does it show a binding site for thioredoxin. One suggested solution to this paradox involves a large conformational change as an integral part of catalysis (9). Very little mechanistic data supporting this suggestion are yet available.

PROPERTIES OF THIOREDOXIN REDUCTASE

Thioredoxin reductase has a subunit M_r of 35,300 including the FAD. The equilibrium constant of the reaction ($[Tr(SH)_2][NADP^+]/[Tr(S_2)][NADPH]$) is pH-dependent, having values of 44 at pH 7, 5 at pH 8, and 0.7 at pH 9 (12). The pH optimum under a standard set of assay conditions is centered at about pH 7.7 in both phosphate and Tris, but the absolute activity is approximately twofold higher in phosphate (12). The extinction coefficient of the FAD at 456 nm is 11.3 mM^{-1} cm^{-1} and the spectral ratios are A(271 nm):A(456 nm) = 5.8; A(380 nm):A(456 nm) = 1.03 (13).

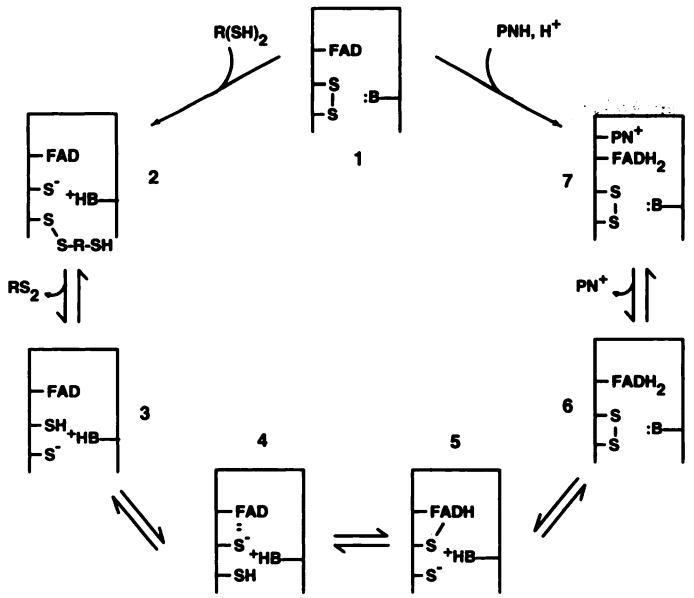
The small separation between the $FAD/FADH_2$ and disulfide/dithiol redox potentials in thioredoxin reductase is in contrast to the separation of more than 50 mV in lipoamide dehydrogenase and glutathione reductase between the E_{ox}/EH_2 and EH_2/EH_4 potentials (7). Thioredoxin reductase has four microforms: E_{ox}, $E(FAD)(SH)_2$, $E(FADH_2)(S_2)$, and $E(FADH_2)(SH)_2$. The microscopic redox potentials are -254 mV for the $E_{ox}/E(FAD)(SH)_2$ couple, -243 mV for the $E_{ox}/E(FADH_2)(S_2)$ couple, -271 mV for the $E(FADH_2)(S_2)/E(FADH_2)(SH)_2$ couple, and -260 mV for the $E(FAD)(SH)_2/E(FADH_2)(SH)_2$ couple. Thus, the potential of the disulfide/dithiol couple is 11 mV lower than that of the $FAD/FADH_2$ couple and there is a 17 mV negative cooperativity, i.e., the potential of the $FAD/FADH_2$ couple depends on the redox state of the disulfide/dithiol and vice versa (14).

The gene (*trxB*) encoding thioredoxin reductase has been cloned into an f1 filamentous phage vector (15) and a plasmid expression vector pPMR14 under the control of the *trxB* promoter for expression in an *Escherichia coli* B cell line, A304, deficient in thioredoxin reductase (16). The gene has been sequenced. The two thiols in thioredoxin reductase that form the active center disulfide are in the pyridine nucleotide domain, rather than in the FAD domain, as they are in the other members of this enzyme family (17). The derived amino acid sequence also showed homology between the 30 carboxyl-terminal residues of thioredoxin reductase and the central domain of the other members of the family, especially mercuric reductase (7). This is important because it defines the carboxyl terminus of thioredoxin reductase within the central domain of glutathione reductase. Thus, thioredoxin reductase lacks an interface domain. This is confirmed by the X-ray structure (see below) showing that elements of the FAD binding domain serve most of the interface function. The *trx* gene has also been cloned into a high copy plasmid vector, pTrR1, resulting in a 10-fold improvement in enzyme yield (18).

Distinct functions of the nascent thiols

Specific alkylation of one of the nascent thiols in the 2-electron reduced state of lipoamide dehydrogenase and glutathione reductase allowed the assignment of the thiol nearer the carboxyl terminus, as the electron transfer (or flavin-interacting) thiol and the other was assumed to be the thiol-disulfide interchange thiol in the mechanism of both these enzymes (19, 20). The structure of glutathione reductase demonstrated that the assumption had been correct (21). Alkylation studies with 2-electron reduced thioredoxin reductase resulted in an equal alkylation of both nascent thiols (22, 23). Consequently, assignment of the particular catalytic roles to each thiol was not possible.

Cys^{135} and Cys^{138} form the redox active disulfide in thioredoxin reductase. Cys^{138} interacts more closely with the FAD than does Cys^{135}, but the thiol functioning in interchange is a matter of speculation (24–26). By analogy to glutathione reductase and lipoamide dehydrogenase, where each nascent thiol has a distinct task, Cys^{135} is assumed to be the interchange thiol in thioredoxin reductase. The close interaction of Cys^{138} with the flavin is based on three lines of evidence. The properties of two altered forms of the enzyme have been compared: C135S with Cys^{138} remaining and C138S with Cys^{135} remaining. First, spectral analyses of C135S as a function of pH and ionic strength have revealed a strong dependence of the flavin spectrum on both parameters. On the other hand, the spectra of wild-type enzyme and C138S are pH-independent. A new band tentatively identified as revealing a charge-transfer complex (ε_{530} = 1300 M^{-1} cm^{-1}) unique to C135S has been observed under conditions of high ammonium ion concentration. These results indicate the assignment of Cys^{138} as the FAD-interacting thiol in the reduction of thioredoxin by NADPH via thioredoxin reductase (24). Second, when the FAD is replaced with 1-deaza-FAD in each mutated enzyme, only C135S forms a



Scheme 1. Mechanism of glutathione reductase or lipoamide dehydrogenase. Catalysis starts with species 1 and proceeds clockwise for glutathione reductase and counterclockwise for lipoamide dehydrogenase. Species 2 is the mixed disulfide; species 4 is the thiolate-FAD charge-transfer complex, and species 3 is its prototropic tautomer; species 7 is the $FADH_2$-PN^+ charge-transfer complex; species 5 is a sulfur adduct at the flavin C4a position observed when lipoamide dehydrogenase is monoalkylated on the interchange thiol or when thioredoxin reductase is substituted with 1-deaza-FAD and reduced; the flavin in species 7 (and 6) may be $FADH^-$. The Scheme as written applies formally to lipoamide dehydrogenase. One molecule of glutathione reductase departs as species 2 goes to species 3 and the second molecule as species 3 goes to species 4.

pH-dependent flavin-C4a adduct (25). A similar pH-dependent adduct forms when 1-deaza-FAD wild-type enzyme is 2-electron reduced (2). Third, the absorbance spectrum of 4-thio-FAD-C135S after 12 h at 4°C is indicative of a mixture of approximately half 4-thio-FAD and half FAD, suggesting a reaction between the C4 position of the flavin and the remaining Cys^{138}, perhaps only on one subunit. In contrast, 4-thio-FAD-C138S resembles the spectrum of 4-thio-FAD. (The binding of 6-thiocyanato-FAD to the mutant apoproteins showed no evidence for a reaction between either of the thiols and the C6 position of the flavin.) Thus, at least three lines of spectral evidence support the placement of Cys^{138} nearer the FAD (see below, upper thiol in Scheme 3). If, as with lipoamide dehydrogenase and glutathione reductase, the two distinct catalytic functions are each carried out by a different nascent thiol, then Cys^{135} would be the interchange thiol (lower thiol in Scheme 3) (24, 25).

Catalytic activity of C135S and C138S

Both C135S and C138S have some catalytic activity. Steady-state kinetic analyses of C135S revealed a turnover number of 10% of the value of the wild-type enzyme and no changes in the apparent K_m values of thioredoxin or NADPH. All three enzymes show parallel line kinetics when thioredoxin is the varied substrate, indicating that the mechanism is unchanged in the mutated enzymes. However, in the secondary plots where NADPH is the varied substrate (at infinite thioredoxin), the expected linear behavior is not observed with C138S as it is with wild-type enzyme and with C135S. This suggests a change of mechanism in C138S (24).[3]

The catalytic activity possessed by the two mutated proteins was initially surprising. The proposed mechanism for this family of enzymes (**Scheme 1**), including thioredoxin reductase (see below), involves sequential thiol-disulfide interchange reactions in which the interchange thiol of 2-electron reduced enzyme attacks the disulfide of the substrate, forming a mixed disulfide (species 3 to species 2). This mixed disulfide is then attacked by the electron-transfer thiol of 2-electron reduced enzyme, reforming the active site disulfide and releasing the dithiol form of the substrate (species 2 to species 1). It was thought that the removal of either of the thiols of this active site disulfide should completely remove the catalytic activity with the disulfide containing substrate. Indeed, the active site Cys to Ser mutations of lipoamide dehydrogenase and glutathione reductase are inactive (27; N. Hopkins, personal communication).

The structure of glutathione reductase shows that the disulfide bond of the protein is perpendicular to the plane of the isoalloxazine ring, the proximal thiol being within bonding distance of the C4a position of the flavin and the interchange thiol being further out on the perpendicular (**Fig. 1*A***) (10, 21, 28). The catalytic activities of C135S and C138S indicated that the stereochemical arrangement may be different in thioredoxin reductase (24, 25). An arrangement analogous to that in glutathione reductase, placing Cys^{138} within bonding distance of the flavin and Cys^{135} further out on a perpendicular, would not allow either electron transfer by $FADH_2$ to a mixed disulfide involving Cys^{135} or interchange with oxidized thioredoxin initiated by Cys^{138} as postulated for catalysis by C138S or C135S, respectively. In explaining the catalytic activities of C135S and C138S in structural terms, it was easier to picture the enzyme disulfide axis as more nearly parallel to the flavin ring than perpendicular. In this way, interchange initiated by Cys^{138} and electron transfer from $FADH_2$ to a mixed disulfide involving Cys^{135} might be possible, albeit inefficient (24, 25). The prediction that the disulfide axis would be more parallel to the flavin ring has been confirmed by the X-ray crystal structure (8, 9) (Fig. 1*B*). An alternative explanation for the activity in C135S and C138S will be offered later when the mechanism that is our current working hypothesis has been presented.

Contrasts between thioredoxin reductase and lipoamide dehydrogenase, glutathione reductase, and mercuric reductase

Table 1 lists major contrasts between thioredoxin reductase and the other members of the family. Some charac-

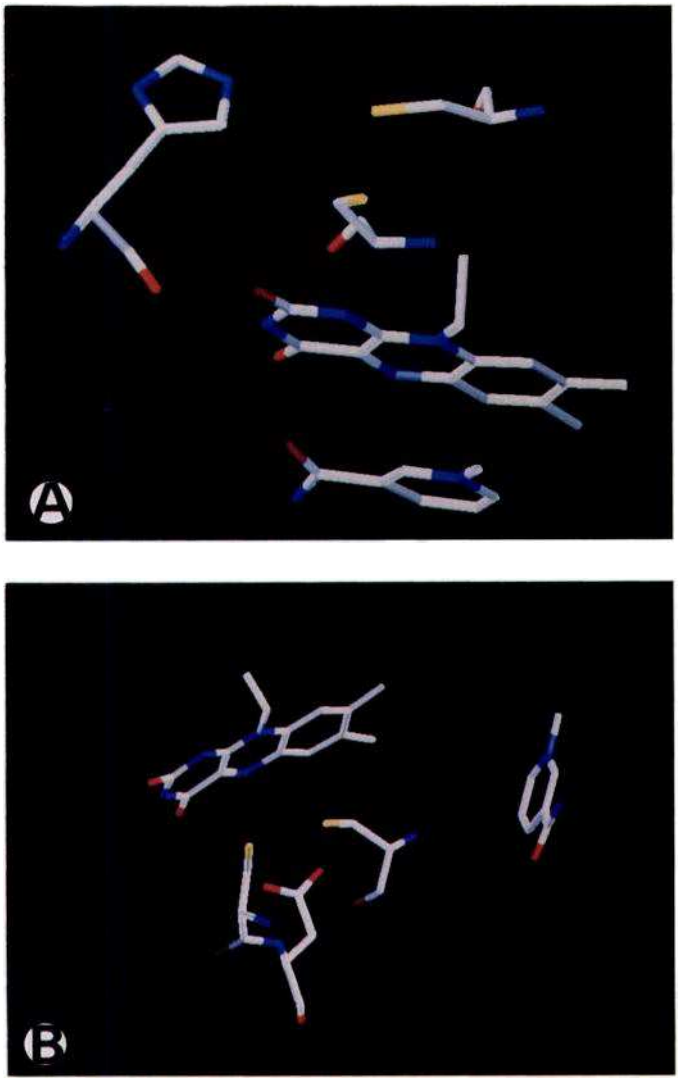


Figure 1. Active sites. *A*) Glutathione reductase (or lipoamide dehydrogenase). View is of the *re* face of the flavin ring system with the nicotinamide ring of NADPH in the foreground, the electron transfer thiol proximal to the flavin, the interchange thiol distal, and N-3 of the imidazole side chain slightly closer to the interchange thiol. *B*) Thioredoxin reductase. View is of the *re* face of the flavin ring system with the nicotinamide ring of NADPH off to the right in the foreground, the flavin-interacting thiol 3.0 Å from the flavin C4a, the sulfur of Cys^{135} 4.4 Å from the flavin C5a, and the carboxylate of Asp^{139} below the flavin N-5, 6.3 Å from the Cys^{135} sulfur and 5.1 Å from the Cys^{138} sulfur.

teristics covered in Table 1 have not yet been mentioned in the text. Although there are many similarities, extensive work on the steady state, presteady state (see below) kinetics, the identity of the base, and certain features of the structure make the impressive differences obvious (9, 12, 14, 18, 24, 31–33). An important similarity between thioredoxin reductase and lipoamide dehydrogenase is the propensity to form a covalent bond between the C4a position of the flavin and the flavin-interacting thiol (2).

THE STRUCTURE OF THIOREDOXIN REDUCTASE

General features of the structure

Four structures of thioredoxin reductase have been solved: two altered forms, C135S and C138S, wild-type enzyme, and C138S with $NADP^+$ bound; these structures were found to be essentially the same (8, 9). From amino terminus to carboxyl terminus, the polypeptide chain of each monomer forms the first part of the FAD binding domain (FAD-1), the NADPH binding domain, and the last part of the FAD binding domain (FAD-2). The part of the FAD binding domain after the NADPH domain is referred to as the central domain in glutathione reductase and lipoamide dehydrogenase (10, 11). The connection between the two domains in thioredoxin reductase is a two-stranded, antiparallel β-sheet (**Fig. 2**). Each domain has a secondary and tertiary structure strongly resembling the analogous domain in glutathione reductase and lipoamide dehydrogenase. The relative orientation of these domains is, however, very different: if the FAD domains of glutathione reductase and thioredoxin reductase are superimposed, the NADPH domain of one is rotated 66° with respect to the other. As a consequence, in thioredoxin reductase the pyridinium ring of bound $NADP^+$ is not adjacent to the isoalloxazine ring as it is in other members of the family. The redox active disulfide is adjacent to the *re* side of the flavin ring and is therefore interposed between the flavin and nicotinamide rings. FAD is bound in the extended conformation with the *re* face of the isoalloxazine ring presented to the NADPH domain and the redox active disulfide.

The dimer interface is formed primarily by the interaction of three α-helices and two loops in each monomer. These are all elements of the FAD domain and constitute almost two-thirds of the interface (9). The two loops of one monomer wrap around the other monomer. The rest of the interface involves interaction between the FAD domain of one monomer and the NADPH domain of the other. This is in contrast to the dimer structures of the other family members, which have a separate domain forming a large part of the interface, resulting in the formation of deep crevices that mediate substrate binding at each interdomain interface. Only shallow depressions are formed on thioredoxin reductase that lacks the interface domain. Fewer solvent molecules stabilize the dimer interface in thioredoxin reductase than in glutathione reductase (28 vs. 104) (9, 10).

A putative conformational change as a part of catalysis

The structure presents several tantalizing features particularly with respect to mechanistic work (12, 14). In the related enzymes glutathione reductase (10) and lipoamide dehydrogenase (11), the structures reveal a clear path of electrons from reduced pyridine nucleotide to the substrate

TABLE 1. *Contrasts between thioredoxin reductase and lipoamide dehydrogenase, glutathione reductase, and mercuric reductase*

Characteristic	Thioredoxin reductase	Other family members
Location of the redox active disulfide	*re* side of the flavin	*si* side of the flavin
Amino acid side chain serving as acid base catalyst	Asp	His[a]
Thiol interacting with the flavin	Carboxyl-terminal Cys	Carboxyl-terminal Cys
Interchange thiol	Not identified	Amino-terminal Cys
Stabilization of thiolate-FAD charge-transfer	Not present[b]	Present
Catalysis by a single thiol	Possible	Not possible[c]
The flavin milieu	Less positive	More positive
Relative orientation of the flavin and pyridine nucleotide domains	Not rotated	Rotated[d]
Mode of dimerization	Via FAD domain	Via interface domain
The relative redox potentials of the $FAD/FADH_2$ and the disulfide/dithol couples	11 mV	>60 mV
Redox states during catalysis	EH_2/EH_4	E/EH_2
Subunit molecular weights	36,000	52,000

[a]A Tyr occupies the homologous position in mercuric reductase and serves as a Hg^{2+} ligand (29) and the need for an acid base catalyst is not obvious. [b]In the presence of high ammonium ion concentration, C135S has a red color and the band at 530 nm is reminiscent of the thiolate-flavin charge transfer band in other family members. [c]Mercuric reductase with one thiol changed to Ser has 5–15% the activity of the wild-type enzyme in a disulfide (DTNB) reductase reaction, but essentially no Hg^{2+} reductase activity (30). [d]"Rotated" indicates the same relative orientation as in glutathione reductase.

disulfide via the isoalloxazine ring and the redox active disulfide (Fig. 1*A*). This is not the case with thioredoxin reductase, because the two thiols in thioredoxin reductase that form the active center disulfide are in the NADPH domain on the *re* side of the flavin ring system, interposed between the NADPH binding site and the flavin (Fig. 1*B*). Displacement of the NADPH binding site from the flavin is a consequence of the juxtaposition of the FAD binding and NADPH binding domains. Figure 1*B* is a view looking down on the N1-N10-C9 edge of the isoalloxazine ring. The sulfurs of Cys^{135} and Cys^{138} (Ser^{138}) have no bond between them, but it can be seen that the bond would be more parallel to the flavin ring than is the case in glutathione reductase or lipoamide dehydrogenase. Cys^{138} is closer to the flavin C4a, 3.0 Å vs. 4.8 Å for Cys^{135}.

Thus, a sizable conformational change appears to be required during catalysis to allow the pyridinium ring to approach the isoalloxazine ring (9). The structure indicates that this conformational change can be accomplished by the unimpeded rotation of the NADPH domain 66° with respect to the FAD domain, resulting in the juxtaposition of the domains as they are in glutathione reductase (10). Such a rotation would move the dithiol out toward the solvent for interchange with the protein substrate thioredoxin, and move the pyridinium ring of the bound NADPH adjacent to the isoalloxazine ring for efficient hydride transfer (Fig. 2). It is hypothesized that the two conformations are in a dynamic equilibrium, but that the crystal structure favors what is referred to as the unrotated conformation. Although the juxtaposition of the FAD binding and the NADPH binding domains is an impressive difference, it must be stressed that each domain in thioredoxin reductase is homologous in three dimensions with the analogous domain in glutathione reductase. Thus, divergent evolution of the domains appears to have preceded the convergent evolution of the active sites.

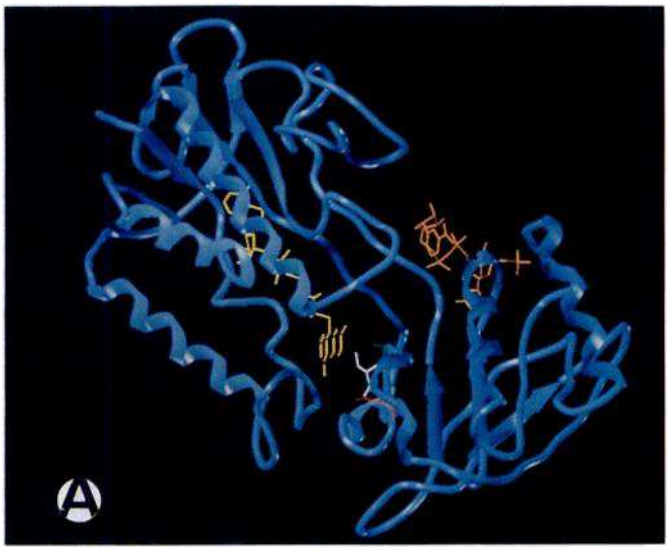
Figure 3*A* shows the dimer structure with one monomer in the conformation observed in the X-ray structure (B, unrotated), whereas the NADPH domain of subunit A has been rotated 66° with respect to the FAD domain. The sulfurs of Cys^{135} and Cys^{138} are colored orange and the oxygens of Asp^{139} are red. In the rotated subunit A, this trio is near the surface of the molecule above the adenine of the $NADP^+$, whereas in the unrotated subunit B they are just above the flavin ring. The view is along the twofold axis, which applies only to the FAD domains because one of the NADPH domains has been rotated.

Figure 3*B* shows the active sites in roughly the same orientation and with the same color code as in Fig. 3*A*. The indole rings of Trp^{52} face each other across the dimer interface. Arg^{26} in the A active site is contributed by the B subunit and vice versa. Asp^{286} and Arg^{293} occupy the same position relative to the flavin as do the homologous residues in glutathione reductase and lipoamide dehydrogenase. The view is of the *re* face of the flavin (FMN portion only) directly in the A subunit (rotated) and looking down on the *re* face from the C9-N10-N1 edge in the B subunit (unrotated). The pyridinium ring of $NADP^+$ is included in the rotated subunit in front of the flavin. Residues that change position relative to the flavin ring upon rotation are those in the NADPH domain (white): Cys^{135}, Cys^{138}, Asp^{139}, and Glu^{159}. His^{245} moves only slightly.

MECHANISM OF THIOREDOXIN REDUCTASE

Acid-base catalysis of dithiol-disulfide interchange

Lipoamide dehydrogenase and glutathione reductase use the imidazole side chain of a histidine residue as a base or acid catalyst, respectively, of dithiol-disulfide interchange between the active site disulfide-dithiol and the substrate (7). It has been proposed that an acid-base catalyst facilitates the reduction of thioredoxin by thioredoxin reductase (14). The X-ray crystal structure reveals two groups that could potentially fulfill this role: His^{245} and Asp^{139} (Fig.



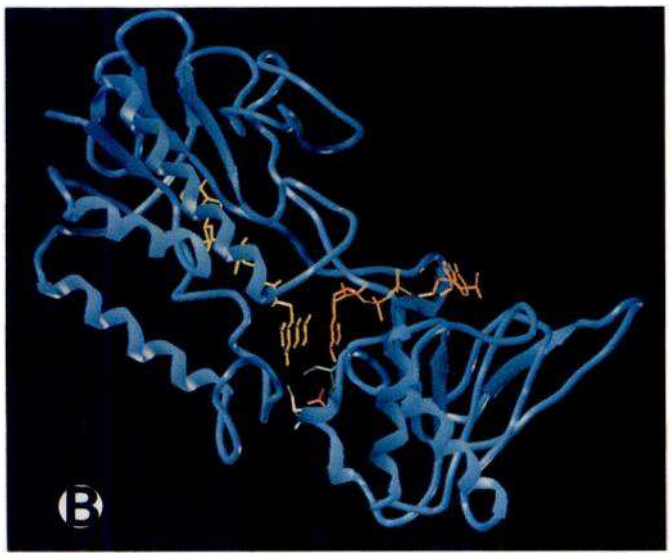
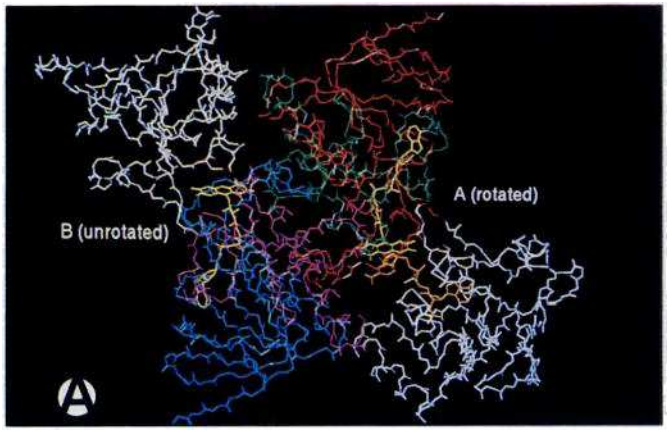


Figure 2. Structures of thioredoxin reductase in the unrotated (*A*) and rotated (*B*) conformations. Helices and sheets are shown as ribbons with arrowheads indicating the polypeptide chain direction. The FAD binding domain is on the left, the NADPH binding domain is on the right, and the *re* face on the flavin ring system is visible. The side chains shown are Cys^{135}, Cys^{138}, and Asp^{139}. In the rotated conformer (*B*), the nicotinamide ring of NADPH (orange) is parallel to the isoalloxazine ring of FAD (yellow).

3*B*, unrotated). Using site-directed mutagenesis, His^{245} was changed to asparagine (H245N) and alanine (H245A), and Asp^{139} was changed to glutamate (D139E), asparagine (D139N), and leucine (D139L) (18). Steady-state kinetic analysis of the His^{245} mutants gave turnover numbers and K_m values similar to those of the wild-type thioredoxin reductase. Other tests confirmed that His^{245} had little effect on activity or the spectral properties of the FAD. Enhancement of the rate of the reductive half-reaction indicated that the alteration had some influence on the interaction of NADPH with the FAD.

All three Asp^{139} mutants were altered in their overall kinetic properties: D139E had 38% of wild-type activity, D139N had 1.5%, and D139L lacked measurable activity. A series of experiments demonstrated that interaction with pyridine nucleotides was unaffected but that the oxidative half-reaction was severely crippled. Thus, rate constants for the NADPH to 3-acetylpyridine adenine dinucleotide phosphate transhydrogenase activity were similar for both the Asp^{139} mutants and wild-type thioredoxin reductase. Stopped-flow kinetic measurements of the reductive half-reaction of the Asp^{139} mutants had rate constants comparable to those of wild-type. However, the reoxidation of reduced D139N mixed with oxidized thioredoxin occurred at a very slow rate of 0.23 s^{-1}, about 0.3% that of wild-type enzyme (18). It was suggested that Asp^{139} is the active site acid-base catalyst that functions to protonate the nascent thiolate ion of reduced thioredoxin. Thus, the reductive half-reaction is not affected in mutants of Asp^{139}. Only the oxidative half-reaction is slowed, consistent with the pro-



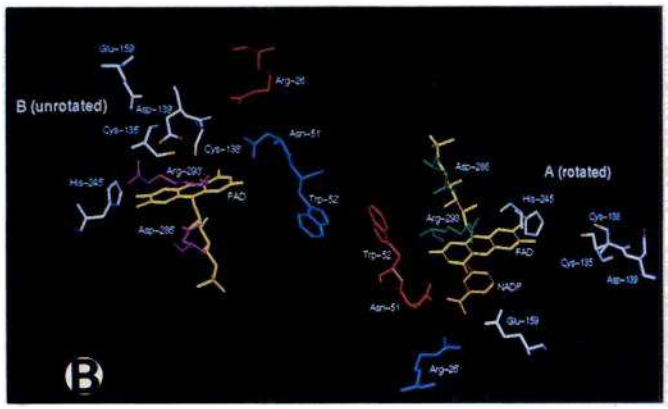


Figure 3. Structure of thioredoxin reductase dimer (*A*) and the active sites in the same orientation (*B*). One monomer is in the conformation observed in the X-ray structure (B, unrotated) whereas the NADPH domain of subunit A has been rotated 66° with respect to the FAD domain in the other monomer. Starting from the amino-terminus of the A subunit, the first part of the FAD domain in shown in red, the lower white and green belong to the NADPH domain and the rest of the FAD domain, respectively, and the cyan, upper white, and magenta designate the same domains of subunit B; this color code applies to both *A* and *B*. The sulfurs of Cys^{135} and Cys^{138} are colored orange and the oxygens of Asp^{139} are red. In the rotated subunit A, this trio is near the surface of the molecule above the adenine of the $NADP^+$ and in the unrotated subunit B they are just above the flavin ring. The view is along the twofold axis, which applies only to the FAD domains.

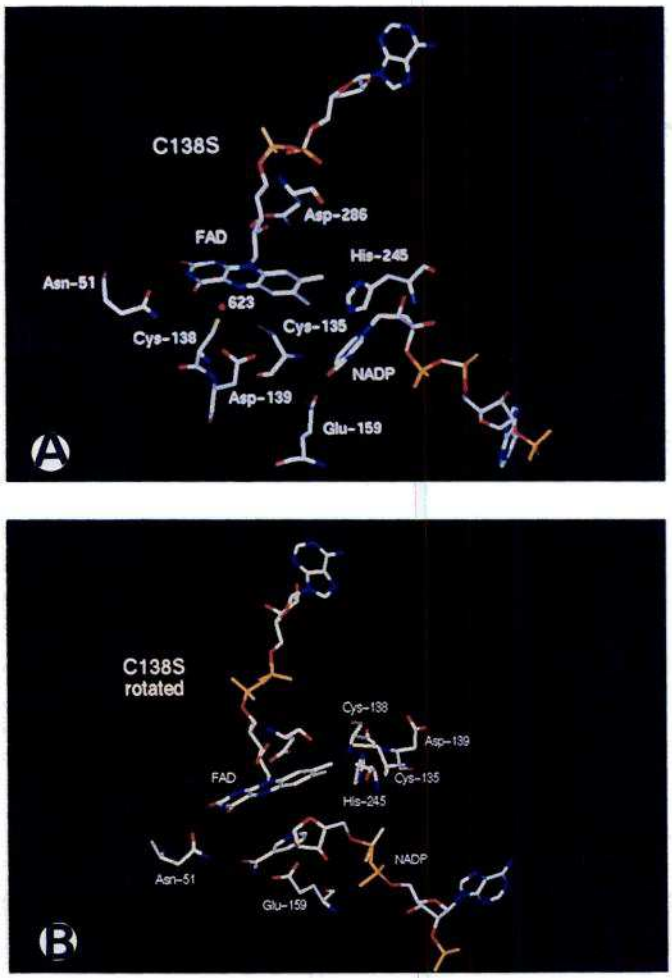


Figure 4. Thioredoxin reductase active site. *A)* Looking at the *re* face of isoalloxazine ring with N10 at the top and N5 at the bottom; solvent-623 is shown in the unrotated conformer just below the flavin N5. *B*) Looking down on the N1-N10-C9 edge with the *re* face of the flavin ring system barely exposed and N10 in the foreground and N5 in the background. Asp^{286}, near the ribityl C-2′, is unlabeled in the rotated conformer.

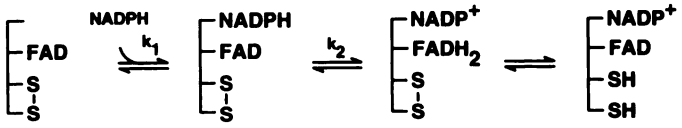
posed function of this residue as an acid-base catalyst (34). The carboxylate of Asp^{139} is 2.9 Å from solvent-623, which is 3.2 Å from the flavin N5; thus, solvent-623 could facilitate proton transfer between the flavin and the acid-base catalyst (**Fig. 4*A***). The carboxylate, in turn, is near other water molecules (solvent-677 and solvent-691).

Modification of the acid-base catalyst in lipoamide dehydrogenase or glutathione reductase markedly affects the dithiol-disulfide interchange reaction between the enzyme and its substrate (species 3 to 2 to 1, for glutathione reductase or species 1 to 2 to 3 for lipoamide dehydrogenase, Scheme 1). Specifically, the base catalyst accepts a proton from dihydrolipoamide (species 1 to 2) or the acid catalyst donates a proton to the first departing molecule of glutathione and is then reprotonated by the flavin-interacting thiol (species 3 to 2) (35, 36). In addition to this expected affect, the rate of electron transfer between the reduced flavin and the disulfide in glutathione reductase (37) or in lipoamide dehydrogenase (38) is markedly diminished. This effect is thought to result from stabilization of the dithiol as a thiolate by the base (species 3 and 4, Scheme 1). This added function of the base is not present in thioredoxin reductase because the rate of the reductive half-reaction is the same in Asp^{139} mutants and wild-type enzyme. The reductive half-reaction is composed of two steps: FAD reduction and electron transfer from the reduced flavin to the disulfide.

The reductive half-reaction

The kinetics of the reductive half-reaction have been reexamined in detail and have confirmed the findings of a preliminary study (39) that the reaction proceeds in three phases. NADPH binding and formation of an NADPH-FAD charge-transfer complex take place primarily in the dead time of the rapid reaction spectrophotometer and thus the kinetics are not well observed (k_1, **Scheme 2**). This can happen only in those molecules that are already in the rotated conformation (see below). Flavin reduction in the second phase (k_2) involves the major decrease in A_{456}, comprising about 60% of the total change observed. The rate of the second phase shows a hyperbolic dependence on the concentration of NADPH. Electrons then equilibrate between $FADH_2$ and the disulfide. The third phase is observed as a slower decrease in absorbance at 456 nm accompanied by an increase at 690 nm, which represents the formation of an $FADH^-$ - $NADP^+$ charge-transfer band. The identification of the third phase in chemical terms is not yet completely clear. Because the $FADH^-$ - $NADP^+$ charge-transfer complex is still forming, the third phase cannot simply represent the reduction of thioredoxin reductase by a second equivalent of NADPH, limited by the dissociation of the first $NADP^+$. Nor can it simply be reduction of the flavin by a second molecule of NADPH after oxidation of the flavin by the disulfide, because reduction of C135S, which lacks the redox active disulfide, has three phases. The two conformations of thioredoxin reductase are thought to be in equilibrium. Modeling of the reaction suggests that the third phase is due to reduction of the flavin by NADPH in enzyme molecules that exist initially in the unrotated conformation. In this case, flavin reduction is limited by the rotation of the pyridine nucleotide domain relative to the FAD domain to juxtapose NADPH and FAD for hydride transfer (40; B. W. Lennon, personal communication).

Steady-state assays give the following isotope effects using (4S)-[4-^{2}H]NADPH at 25°C: ^DV of 1.74, a $^DV/K$ for NADPH of 3.51, and a $^DV/K$ for thioredoxin of 2.02 (B. W. Lennon, personal communication).



Scheme 2. Reductive half-reaction of thioredoxin reductase.

The oxidative half-reaction

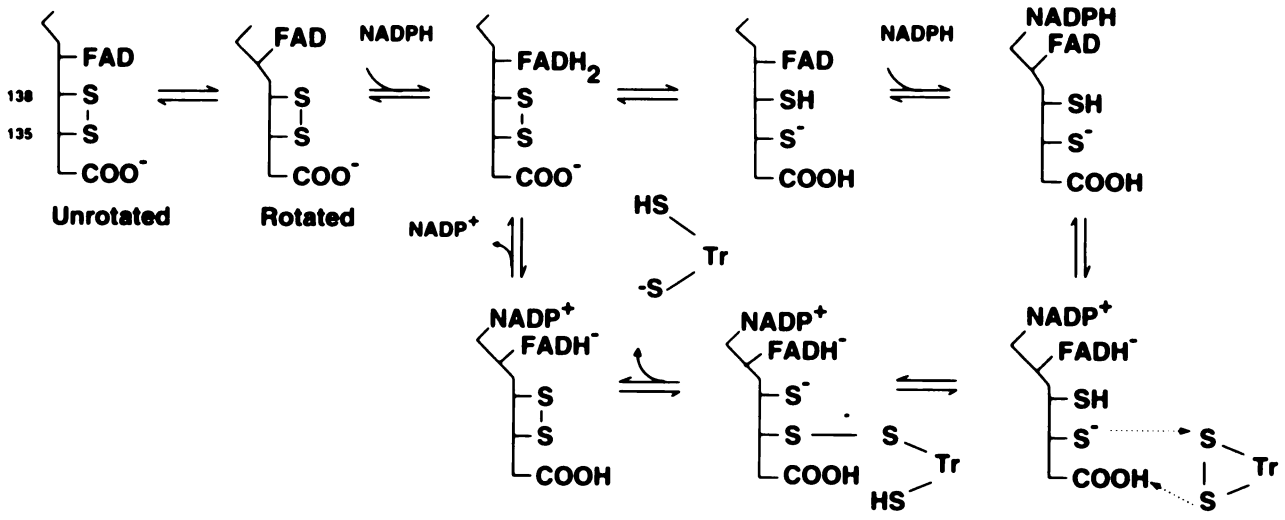
The kinetics of the oxidative half-reaction between reduced thioredoxin reductase and thioredoxin measured in the presence of $NADP^+$ appeared monophasic at a rate essentially equal to k_{cat} and independent of the thioredoxin concentration (41). The kinetics have been reexamined in the presence and absence of pyridine nucleotide. A significant decrease in the rate of the main phase of enzyme reoxidation has been observed in the presence of pyridine nucleotide (34). When one equivalent of NADPH is used as the reductant, $NADP^+$ is present in the oxidative half-reaction. At pH 7.0 or 7.6, the observed rate of the catalytically competent phase of oxidation is decreased by approximately 50%, relative to that with enzyme reduced by the xanthine/xanthine oxidase system (42). The rate with $NADP^+$ present is essentially equal to k_{cat} at each pH, confirming the previous result. It has also been confirmed that there is no dependence on the thioredoxin concentration with NADPH as reductant, nor is there with the xanthine/xanthine oxidase system. Through the use of the nonreducible analog $AADP^+$, it has been shown that the complexation of pyridine nucleotides with reduced thioredoxin reductase is able to effect a change in the rate-limiting steps of the oxidation of flavin by thioredoxin. Thus, there is compelling evidence for a ternary complex mechanism (34).

The redox state of the enzyme in catalysis

During turnover the enzyme cycles between the 2-electron and 4-electron reduced states rather than between the E_{ox} and the 2-electron reduced states (B. W. Lennon, personal communication). Thus, if the enzyme is mixed with a combination of limiting NADPH and excess thioredoxin, almost complete reduction is observed in the first 100 ms, followed by turnover lasting approximately 10 s depending on the amount of NADPH. At the end of turnover (i.e., when the NADPH has been oxidized), the enzyme partially reoxidizes to a level determined by the reduced thioredoxin product. The redox potentials of the flavin and disulfide are nearly equal (14). Thus, because the flavin is largely reduced during turnover, the disulfide must also be reduced. The spectrum of the enzyme at the end of the dead time shows the expected NADPH - FAD charge-transfer complex having a band at 550 nm. This charge-transfer complex disappears as the flavin is reduced and turnover begins. As the enzyme turns over and NADPH is converted to $NADP^+$, a reduced flavin - $NADP^+$ charge-transfer complex forms in increasing amounts, absorbing at 700 nm. The amount of this charge-transfer complex decreases as the enzyme reoxidizes after turnover (B. W. Lennon, personal communication).

A "Working hypothesis" mechanism

Scheme 3 shows our present view of catalysis (18, 34; B. W. Lennon, personal communication; D. Veine, P. F. Wang, S. H. Ahn, personal communication). The oxidized enzyme is represented as an equilibrium mixture of the unrotated and rotated conformers. In the unrotated conformer, FAD and the disulfide are juxtaposed, and in the rotated conformer FAD and NADPH are juxtaposed. NADPH reduces the rotated conformer. This is a "pump priming" reaction because, as can be seen, the catalytic cycle returns to this point. Reducing equivalents equilibrate between the $FADH_2$ and the disulfide via Cys^{138} (the top thiol) in the unrotated conformer. Another NADPH binds and rotation takes place. The NADPH reduces the FAD in the rotated conformer. Now the enzyme is at the 4-electron reduced level. Thioredoxin binds and interchanges with Cys^{135} (the lower thiol) to form the mixed disulfide, as the thiols in the rotated conformation are near the



Scheme 3. Mechanism of thioredoxin reductase. The oxidized enzyme is represented as an equilibrium mixture of the unrotated and rotated conformers. In the unrotated conformer, FAD and the disulfide are juxtaposed; in the rotated conformer FAD and NADPH are juxtaposed. Cys^{138} is the top thiol and Cys^{135} is the lower thiol. The mixed disulfide is depicted as involving Cys^{135}. Note that in presenting inherently 3-dimensional material in 2 dimensions, it has been necessary to place Cys^{135} next to Asp^{139}, cf. Fig. 1*B*.

solvent interface. The thiolate of Cys^{138} attacks the mixed disulfide reforming the active center disulfide, and reduced thioredoxin departs. $NADP^+$ dissociates and reverse rotation takes place. The cycle begins again with 2-electron reduced enzyme. The major function of Asp^{139}, the putative acid-base catalyst, in this mechanism is to protonate the nascent thiolate on thioredoxin as the mixed disulfide forms. Asp^{139} may be reprotonated by Cys^{138}. This role would be the same as the major function of the acid catalyst in glutathione reductase, namely, inhibiting the reverse reaction. Formally, Asp^{139} also accepts a proton during electron equilibration. This cannot be an important function because the rate of this step does not appear to be affected by mutation of Asp^{139}. All of the Asp^{139} mutants had about the same rate in the reductive half-reaction, which includes this step. In contrast, this step is markedly influenced by mutation of the histidine acid-base catalyst in lipoamide dehydrogenase or glutathione reductase (37,38).

This mechanism is at odds with a previous proposal (24). The altered forms of thioredoxin reductase C135S and C138S have residual activity (see above). Analogous mutant forms of lipoamide dehydrogenase and glutathione reductase lack measurable activity. To explain this activity, it was suggested that both sulfurs could perform either role: thiol-disulfide interchange or interaction with the reduced flavin. This would require that Cys^{138} in C135S interchange with thioredoxin and that $FADH_2$ in C138S directly reduce the mixed disulfide formed between Cys^{135} and thioredoxin, albeit inefficiently. The structure precludes the binding of thioredoxin near the FAD. Indeed, the dithiol must rotate away from the FAD in order to react with thioredoxin. An analogy to the work of Claiborne's group offers a possible explanation. They have shown that NADH peroxidase and NADH oxidase of *Streptococcus faecalis*, both with a single active site thiol, which is homologous with the flavin-interacting thiol of the disulfide reductases, catalyze the reduction of hydrogen peroxide and oxygen, respectively, by cycling between the thiol and the sulfenate (43–46). Further, they have demonstrated that the flavin-interacting thiol in glutathione reductase tends to oxidize to the sulfenate when the interchange thiol is alkylated (47). Our data indicate C135S has a low thiol titer, presumably due to oxidation (24), and that Cys^{135} in C138S may form a sulfenate ester with Ser^{138} that would not react with DTNB but would be reduced by dithionite (26). The sulfenates are not observed in the X-ray structures because they would be reduced by the DTT present during crystallization. Thus, the remaining thiol in C135S or C138S could cycle between the thiol and the sulfenate in the reduction of thioredoxin. After rotation, the thiol could form a mixed disulfide with thioredoxin. Attack on the mixed disulfide by hydroxyl ion could release reduced thioredoxin and leave the enzyme thiol as the sulfenate. After rerotation the sulfenate would be reduced by the flavin.

Correlating the mechanism with the structure

As with most dithiol-disulfide interchanges, the mixed disulfide is very unstable. Attempts to capture this intermediate are in progress using altered forms of thioredoxin (redox active disulfide mutants C32S and C35S) and thioredoxin reductase with one of the thiols changed to serine. Thus, the first thiol-disulfide interchange to form the mixed disulfide can take place, but the mutation blocks the second interchange (Scheme 3). If C138S is reacted with C32S in which the remaining thiol has been previously prepared as a mixed disulfide with TNB, no TNB anion is released until NADPH is added. The mixed disulfide is not stable and cannot be demonstrated as a high molecular weight band on nonreducing PAGE. If C135S is similarly reacted, no NADPH is required to release stoichiometric TNB anion and the mixed disulfide can be observed on nonreducing PAGE and isolated on a preparative scale (D. Veine, P. F. Wang, S. H. Ahn, personal communication).

While pyridine nucleotide binding and dissociation are pictured as the triggers of rotation and reverse rotation in the mechanism shown in Scheme 3, binding of thioredoxin would stabilize rotation in the ternary complex and dissociation of thioredoxin would allow reverse rotation as $NADP^+$ dissociates. Reverse rotation should be inhibited with the large thioredoxin molecule-bound (Fig. 3*A*). To write a mechanism involving rotation and assuming ping-pong substrate interactions, two rotations and two reverse rotations per catalytic cycle would be required. The simpler ternary complex mechanism shown here seems more reasonable. The structure of thioredoxin reductase suggesting a large conformation change integral to catalysis is beginning to be complemented by mechanistic data. FJ

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