Thioredoxin reductase from *Escherichia coli*: Evidence of restriction to a single conformation upon formation of a crosslink between engineered cysteines

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Abstract

Thioredoxin reductase is a flavoprotein which catalyzes the reduction of the small protein thioredoxin by NADPH. It contains a redox active disulfide and an FAD in each subunit of its dimeric structure. Each subunit is further divided into two domains, the FAD and the pyridine nucleotide binding domains. The orientation of the two domains determined from the crystal structure and the flow of electrons determined from mechanistic studies suggest that thioredoxin reductase requires a large conformational change to carry out catalysis (Williams CH Jr, 1995, *FASEB J* 9:1267–1276). The constituent amino acids of an ion pair, E48/R130, between the FAD and pyridine nucleotide binding domains, were mutagenized to cysteines to form E48C,R130C (CC mutant). Formation of a stable bridge between these cysteines was expected to restrict the enzyme largely in the conformation observed in the crystal structure. Crosslinking with the bifunctional reagent N,N',1,2-phenylenedimaleimide, spanning 4–9 Å, resulted in a >95 % decrease in thioredoxin reductase and transhydrogenase activity. SDS-PAGE confirmed that the crosslink in the CC-mutant was intramolecular. Dithionite titration showed an uptake of electrons as in wild-type enzyme, but anaerobic reduction of the flavin with NADPH was found to be impaired. This indicates that the crosslinked enzyme is in the conformation where the flavin and the active site disulfide are in close proximity but the flavin and pyridinium rings are too far apart for effective electron transfer. The evidence is consistent with the hypothesis that thioredoxin reductase requires a conformational change to complete catalysis.

Keywords: conformation; dithiol crosslinking; flavoprotein; thioredoxin reductase

Thioredoxin reductase belongs to the pyridine nucleotide-disulfide oxidoreductase flavoprotein family that includes lipoamide dehydrogenase and glutathione reductase (Williams, 1992). It catalyzes the transfer of electrons from NADPH to the small protein thioredoxin (M_r 12 kDa). Like other members of this family, thioredoxin reductase is a homodimeric protein where each monomer includes one redox active disulfide and one FAD. In *Escherichia*

coli thioredoxin reductase, each subunit (M_r 35 kDa) is further divided into two domains, the FAD and pyridine nucleotide binding domains.

The structures of thioredoxin reductase and glutathione reductase are known (Karplus & Schulz, 1987; Waksman et al., 1994). There are several distinct differences between these related enzymes. Whereas thioredoxin reductase has only two domains, glutathione reductase consists of three domains: the FAD- and pyridine nucleotide-binding domains and an interface domain. In glutathione reductase, the NADPH binding site is on the *re* side of the flavin and the active site disulfide on the *si* side of the flavin. The location of the flavin ring between the source of electrons and the redox active disulfide enables an efficient as well as an obvious electron pathway. In thioredoxin reductase, the structure reveals that the active site disulfide is on the *re* side of the FAD between the bound NADPH and the FAD thus eliminating the close contact needed for electron transfer between the nicotinamide and isoalloxazine rings and effectively burying the active site disulfide.

The path of electron flow in glutathione reductase between NADPH and glutathione via the flavin and the active site disulfide

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Abbreviations: E48C,R130C (CC mutant), thioredoxin reductase in which Glu48 and Arg130 have been changed to Cys; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); TNB⁻, 5-thio-2-nitrobenzoate anion; DTT, 1,4-dithiothreitol; ApYADP⁺, oxidized form of 3-acetylpyridine adenine dinucleotide phosphate; oPDM, N,N'-*o*-phenylenedimaleimide; eq, equivalents relative to 1 FAD-containing subunit.

This paper is dedicated to the memory of Finn Wold, a Robert A. Welch Professor of Biochemistry and Molecular Biology at the University of Texas, Houston, third President of the Protein Society and a pioneer in the use of bifunctional crosslinking reagents.

is supported by structural analyses. In the crystal structure of thioredoxin reductase, however, the pathway of electron transfer inferred from mechanistic studies cannot be followed. Therefore, a conformational change has been proposed to correlate known mechanistic data with the structure (Waksman et al., 1994; Williams, 1995; Wang et al., 1996).

The structure of glutathione reductase was used as a model for the conformational change proposed for thioredoxin reductase. If a 66° rotation of the pyridine nucleotide binding domain relative to the FAD binding domain occurred in thioredoxin reductase, then the orientation of the two domains would be as seen in glutathione reductase. The nicotinamide ring and the isoalloxazine ring would be in close contact and the active site disulfide would move to the surface of the protein where it would be accessible to its substrate thioredoxin.

The conformation of thioredoxin reductase observed by X-ray diffraction analysis enables flavin oxidation by the disulfide and is referred to as the FO conformation (Scheme 1). The proposed conformation referred to as FR, enables flavin reduction by NADPH and positions the nascent dithiol near the enzyme surface for reaction with its protein substrate. It is proposed that if the enzyme was restricted to either one of the conformations then catalysis would be disrupted. Enzyme in the FO conformation would be unable to transfer electrons from NADPH to the flavin whereas enzyme in the FR conformation would be unable to transfer electrons from reduced flavin to the active site disulfide.

The FR conformation was the focus of another study (Wang et al., 1996). Thioredoxin and thioredoxin reductase were altered by converting one active site cysteine in each protein to a serine residue leaving a single thiol in each protein active site (Russel & Model, 1986; Prongay et al., 1989). The thioredoxin and thioredoxin reductase mutants were oxidized with DTNB to form a stable mixed disulfide bond between enzyme and substrate thus restricting the enzyme to the putative FR conformation. As predicted NADPH was able to reduce the flavin but the flavin was unable to transfer electrons to the active site disulfide. Crystallization of the enzyme-substrate complex is in progress and has yielded crystals that diffract to ca. 4 Å (Lennon et al., 1997).

In the present study, it has been proposed to restrict the enzyme to the known FO conformation by introduction of a bridge between the two domains (Scheme 1). An ion pair, consisting of E48 in the

FAD domain and R130 in the pyridine nucleotide binding domain, is observed in the crystal structure (Waksman et al., 1994). E48 and R130 were mutated to cysteine residues to form the E48C,R130C mutant (CC mutant). A disulfide can be formed between these engineered cysteines using DTNB or a crosslink can be introduced using a sulfhydryl specific bismaleimide. The properties of the crosslinked CC mutant indicate that it is indeed restricted largely in the FO conformation.

Results

An ion pair between E48 in the FAD domain and R130 in the pyridine binding domain brings these residues into close proximity, as observed in the crystal structure (Waksman et al., 1994). Each was mutated to a cysteine residue to form E48C,R130C (CC mutant). In view of the enzyme structure presented in the Introduction, the engineered thiols should be close to one another in the FO conformation and far apart in the FR conformation (Scheme 1).

The number of thiols in the CC mutant was verified using both native and denaturing conditions. The CC mutant has a total of six cysteines, two form the active site disulfide, two are buried in the protein structure and are detectable only under denaturing conditions, and the remaining two are the engineered cysteines which are readily detectable in the native protein. The CC mutant was further examined for autooxidation of the engineered thiols. Non-reducing SDS PAGE and the thiol determinations showed that the engineered thiols in the CC mutant were capable of slowly forming both inter- and intramolecular bonds. Therefore, our data refer to the CC mutant after treatment with DTT and subsequent removal of excess reagent and products by gel filtration (see Materials and methods).

The enzyme kinetic parameters for the CC mutant both as a thioredoxin reductase and as a transhydrogenase (with APyADP⁺ and NADPH as substrates) are given in Table 1. It can be seen that the CC mutant retains about half the activity of wild-type enzyme and that the K_m values for pyridine nucleotides are unchanged. The K_m value for thioredoxin is slightly increased.

Analysis of the crystal structure indicates that the original distance between the ion pair OE2 of Glu48 and NH1 of Arg130 is ca. 3.2 Å. Substitution of cysteine at both sites increases the distance to be bridged to ca. 9.2 Å. A disulfide bond whose typical length

Scheme 1. A cartoon representation of the CC mutant in the FO conformation observed in the crystal structure, in the putative FR conformation with the substrate bound, and in the crosslinked FO conformation. The flavin is represented by three circles on the FAD binding domain. The redox active disulfide is shown and the position of the pyridinium ring of NADPH is represented by PN. The engineered cysteines are designated and the crosslink is indicated by an X.

Table 1. Enzymatic properties of the CC mutant

	Wild-type enzyme	CC mutant
K_m for NADPH (μM)	4.55 ± 0.9	6.0 ± 1.86
K_m for thioredoxin (μM)	1.67 ± 0.4	7.55 ± 1.43
Thioredoxin reductase activity (min^{-1})	2,400	1,200
K_m for ApyADP ⁺ (μM)	31 ± 6.1	28 ± 6.1
Transhydrogenase activity (min^{-1})	550 ± 22	309 ± 15

is 1.8–2.4 Å may be difficult to envisage without assuming considerable flexibility of the protein structure. Therefore, crosslinking of the engineered cysteines was attempted in two ways. DTNB was used to promote formation of a disulfide, and a bismaleimide (oPDM), suitable for bridging a distance of 4–9 Å, was inserted between the cysteines.

The chemistry and accessibility of the engineered thiols allow both intra- and intermolecular bonds to form as well as false disulfides to remain from incomplete reactions with either DTNB or oPDM (Scheme 2). As detailed under Materials and methods, the byproducts of the reaction (species III, IV and V in Scheme 2) as well as unmodified enzyme (species I) were removed by TNB-thiol columns. Concentration, temperature, and reaction time were optimized to minimize the formation of undesired intermolecular bonds (species V and VI in Scheme 2), and to maximize the formation of the desired intramolecular bond (species II).

The purified oPDM-crosslinked CC mutant was further analyzed structurally for the presence of the bisuccinimide crosslink. Following denaturation, reduction and alkylation of the remaining thiols according to procedures previously described (O'Donnell &

Williams, 1985), the protein was digested with trypsin and examined by liquid chromatography/mass spectroscopy. The results showed the absence of both tryptic peptides containing an engineered thiol (3,016 and 1,775 Da) and the appearance of a new peptide (5,092 Da), which approximates the combined masses of the missing fragments and the addition of the crosslinker (3,016 + 1,775 + 302 = 5,093 Da). Figure 1 shows the predicted structure. All other expected tryptic peptides having masses greater than 500 were identified by liquid chromatography/mass spectroscopy. In addition, a fragment of 6,325 Da potentially corresponding to an intermolecular crosslink (3,016 + 3,016 + 302 = 6,334 Da) was observed, although in low yield.

The oPDM-crosslinked CC mutant was found to crystallize under the same conditions as wild-type enzyme (FO conformation) (B. Lennon & M. Ludwig, pers. comm.).

The two enzyme species obtained by crosslinking are compared in Table 2. As shown, the thiol titer (under native conditions) is diminished to zero by either treatment. Further analysis by SDS-PAGE shows that the DTNB-treated CC mutant and the oPDM-crosslinked CC mutant migrate as the uncrosslinked control with virtually no material indicating double the molecular mass. This shows that both crosslinks are intramolecular. Visual estimates are given in the last line of Table 2.

If Cys48 and Cys130 in a subunit of the CC mutant are bridged, then the NADPH domain will be restricted in its rotation and electrons will not be transferred efficiently from NADPH to FAD. This restricted rotation should markedly diminish the thioredoxin reductase and transhydrogenase activities and also affect the number of electrons taken up by the mutant enzyme in reductive titrations. As predicted, the activities of the crosslinked enzyme species were substantially reduced (Table 2). Thioredoxin activity was less than 1% for the oPDM-crosslinked CC mutant whereas the CC mutant treated with DTNB retained some activity which suggests that the putative disulfide bond is not stable.

Titration of the CC mutant with NADPH shows a rapid uptake of electrons comparable to that of wild-type enzyme (data not shown) whereas the CC mutant crosslinked with oPDM undergoes reduction by excess NADPH only very slowly (less than 50% in

Scheme 2. Reaction of crosslinking reagent with the engineered thiols of the CC mutant. Species II–IV represent the possible thiol reactions of crosslinking reagent (X) on each monomeric unit (A and A' but shown specifically only on A). Species II is the desired product on each monomeric unit. Species V and VI are two of several possible intermolecular reactions that can occur.

Fig. 1. Proposed structure of the hydrolyzed bisuccinimide (302 Da) crosslinking two tryptic peptides (3,016 and 1,775 Da). The redox active dithiol has been reacted with iodoacetic acid (CM is carboxymethyl). Under the conditions used, the bisuccinimide would be hydrolyzed as shown (Knight, 1979).

Table 2. Properties of CC mutant species containing an intramolecular crosslink

	Wild-type enzyme	CC mutant	oPDM crosslinked CC mutant	DTNB treated CC mutant
Titrateable thiols per subunit (native conditions)	0	1.9	0.08	0
Visible absorption maxima (nm)	456	456	458	453
Thioredoxin reductase activity (%)	100	50	0.4	23
Transhydrogenase activity (%)	100	56	3	16
Intramolecular crosslinks per subunit	0	0	>0.95	>0.90

2 h, Fig. 2). The titrations show the presence of a new absorbance band typical of the blue neutral flavin semiquinone (Fig. 2). It has been shown previously that semiquinone is not a catalytic intermediate (Zanetti et al., 1968). Formation of semiquinone is believed to result from the catalytic action of active oxygen species coming from very small amounts of oxygen contamination. It can also arise from irradiation of enzyme partially reduced with NADPH (Zanetti et al., 1968). Addition of the substrate thioredoxin to the reduced oPDM-crosslinked CC mutant enzyme failed to reoxidize the enzyme indicating that in addition to the impaired reductive half reaction the enzyme active site was entirely inaccessible to the substrate thioredoxin (Scheme 1).

In the FO conformation where the FAD and the disulfide are juxtaposed for efficient transfer of reducing equivalents, and the pyridine nucleotide binding site is too far away for efficient electron transfer to the flavin (Fig. 2 and Scheme 1), any reduction of the FAD would result in reduction of the disulfide, and reduced flavin is expected to accumulate only as the dithiol is formed (O'Donnell & Williams, 1983). oPDM-crosslinked CC mutant in the presence of 4 eq. NADPH for 16 h indeed exhibited nearly full reduction of the flavin (Fig. 2). Since the structure of the oPDM-crosslinked CC mutant indicates that it is in the FO conformation, we propose that free FAD is a catalyst for the reduction of the oPDM-crosslinked CC mutant rather than direct reduction by NADPH. It was found upon addition of 0.025 and 0.10 eq of free FAD to the above reaction, that the rate of reduction increased by 2.4- and 3.2-fold, respectively. We propose that reduced FAD is produced on a small fraction of intermolecularly crosslinked enzyme and is subsequently released. The free reduced FAD upon binding to the intramolecularly crosslinked fraction would subsequently transfer electrons to the bound FAD thereby reducing the active site disulfide. The effect of added FAD on the kinetics of this process is large, as would be expected given the low K_d for FAD on the enzyme.

DTNB-treated CC mutant also exhibited impaired reduction by NADPH as would be predicted from its diminished transhydrogenase activity. Equilibration time for each addition of NADPH was slower than untreated CC mutant but was complete within 15 min (data not shown). The number of reducing equivalents exceeded the amount required for the untreated CC mutant (2 eq) by ca. 1 equivalent. Thioredoxin activity measured following removal of NADP(H) by gel filtration was found to have been restored to the full activity of CC mutant prior to crosslinking which strongly suggests that the putative disulfide bridge was reduced by the third equivalent of NADPH. The proximity of the engineered disulfide to the active site dithiol suggests that dithiol-disulfide interchange between them is possible. This was further examined using dithionite.

Dithionite titrations are known to deliver electrons directly to the FAD in either the FR or the FO conformation (Williams, 1995 and Scheme 1). Titrations of oPDM-crosslinked CC mutant and CC mutant (Fig. 3A) gave similar results. Extrapolation of the first six data points to an apparent end point at $\epsilon = 1.5 \text{ mM}^{-1} \text{ cm}^{-1}$, the extinction coefficient of 4 electron-reduced enzyme, indicates an uptake of 2.6 equivalents per FAD. The excess apparent uptake of 0.6 equivalents indicates that the reaction is not quantitative even for the first six data points. The propensity of the CC-mutant to form semiquinone and the fact that dithionite does not appear to reduce the semiquinone quantitatively in the mutant mean that the quantitation particularly in the latter stages of these titrations is imprecise. The DTNB-treated CC mutant required ca. 1 additional equivalent of dithionite (Fig. 3B). The increased uptake of dithionite by the DTNB-treated CC mutant compared to the CC mutant and the oPDM-crosslinked CC mutant is obvious, in spite of the imprecision in the latter stages of the titrations. This indicates that the engineered disulfide in the DTNB-treated CC mutant is reduced via dithiol-disulfide interchange during dithionite titration, albeit slowly. This is confirmed by the observation that reoxidation of flavin in the DTNB-treated CC mutant by exposure to air shows that the visible maximum has shifted from 453 to 456 nm (Spec-

Fig. 2. Reduction of oPDM-crosslinked CC mutant (29 μM) with 4 eq of NADPH. Spectrum 1, oPDM-crosslinked CC mutant without NADPH; spectrum 2–6 were recorded at 2, 20, 60, 120 min and 16 h, respectively. Inset, absorbance at 458 nm.

Fig. 3. Reductive titrations with dithionite. **A:** Spectrum 1, CC mutant (24 μ M); spectrum 2–8 are representative spectra after additions of dithionite. Inset, absorbance at 456 nm. The titration was not carried to completion. **B:** Spectrum 2, starting spectrum of the DTNB-treated CC-mutant (28 μ M); spectrum 3–9 are representative spectra after additions of dithionite; spectrum 1, reoxidized enzyme following completion of titration. Inset, absorbance at 453 nm.

trum 1, Fig. 3B; Table 2, line 2), i.e., reverted to the maximum of the CC mutant.

Discussion

It has been proposed that thioredoxin reductase must undergo a conformational change in order to complete catalysis, but that a single conformation (FO) is favored in the crystal packing (Waksman et al., 1994). In this scenario, NADPH reduces the FAD in the FR conformation, which then rotates to the FO conformation for transfer of the electrons from the flavin to the redox active disulfide, and then returns to the FR conformation before dithiol-

disulfide interchange between the nascent redox active dithiol and the substrate thioredoxin takes place (Waksman et al., 1994). In the present study, site directed mutagenesis followed by chemical modification is used to restrict the enzyme to the FO conformation so as to examine the effects on catalysis. In a separate study, the enzyme has been restricted to the FR conformation (Wang et al., 1996). According to our hypothesis the enzyme restricted in either conformation would be unable to carry out a complete catalytic cycle.

Glu48 in the FAD binding domain forms an ion pair with Arg130 in the pyridine nucleotide binding domain that stabilizes the FO conformation (Waksman et al., 1994). Site directed mutagenesis of this ion pair to Cys48 and Cys130 results in a 50% loss of catalytic activity (Table 1). This is not due to the partial formation of a disulfide bond because the activity does not change upon treatment with DTT. The engineered dithiol is approximately 12 Å from the active site making rapid dithiol-disulfide interchange unlikely. The substantial drop in activity of the CC mutant, without the introduction of a disulfide, indicates that stabilization of the FO conformation by the ion pair is important.

Observations made during the reductive titrations of the DTNB-treated CC mutant indicate that the engineered disulfide is close enough to the redox active dithiol (12.3 Å from engineered Cys48 to the redox active Cys138) to envisage slow dithiol-disulfide interchange given that proteins are not rigid bodies. As expected, the introduction of a chemical crosslink or a disulfide between the engineered cysteines decreased the catalytic activity, to a very low level in the case of the succinimide bridge (Table 2). Both thioredoxin reduction and transhydrogenase activity were impaired. From our model (Scheme 1) it can be seen that the transhydrogenase activity between the FAD and pyridine nucleotides occurs when the enzyme is in the FR conformation, whereas thioredoxin reductase activity requires both the FO and FR conformation.

The enzyme activity of the DTNB-treated CC mutant (Table 2) can be attributed to the presence of intermolecular disulfides (species V and VI, Scheme 2). The A' monomers could well have failed to react and therefore contain functional active sites. Moreover, the activity given in Table 2 for the DTNB-treated CC mutant is an initial activity; it increases as the assay progresses. This is presumably due to the slow reduction of the disulfide and release of the enzyme from the FO conformation.

The bis-succinimide bridge in the oPDM-crosslinked CC mutant cannot be reduced and therefore maintains the enzyme in the FO conformation where the active site disulfide is in close proximity to the flavin and inaccessible to the substrate thioredoxin (Scheme 1). The presence of <5% intermolecularly crosslinked enzyme would explain the observed 3% residual transhydrogenase activity and the absence of thioredoxin reducing activity (Table 2). The slow reduction of the flavin and active site disulfide of the oPDM-crosslinked CC mutant with NADPH (Fig. 2) via free reduced FAD eliminates the need for rotation to the FR conformation. However, for oxidation of the redox active dithiol by the substrate thioredoxin the enzyme would have to rotate to the FR conformation (Scheme 1). Addition of thioredoxin did not reoxidize the enzyme which indicates that the enzyme is indeed restricted to the FO conformation where the active site dithiol is inaccessible to thioredoxin.

The evidence provided by this study supports the original hypothesis that *E. coli* thioredoxin reductase requires a conformational change during catalysis. We have shown that the enzyme,

when restricted to the FO conformation observed in the crystal structure, is unable to complete catalysis.

Materials and methods

Site-directed mutagenesis resulting in the CC mutant (E48C,R130C)

The reactions were performed with the Oligonucleotide-directed Mutagenesis Kit version 2.0 from Amersham Life Sciences, Inc. (Arlington Heights, IL) according to the manufacturers instructions. Equal amounts of the E48C and R130C mutagenic oligonucleotides were used in the reactions. Single-stranded wild-type enzyme DNA (pTrR105) served as the template (Mulrooney, 1997). The introduction or deletion of a restriction site allowed for screening of potential mutants. Individual colonies resulting from the reactions were processed as described by Mulrooney (1997). The presence of the mutations was confirmed by digesting the double-stranded DNA with the appropriate diagnostic restriction enzymes and verified by sequencing the entire TrxR gene using automated procedures provided by the University of Michigan Biomedical Research Core Facility.

The sequence revealed that, in addition to the desired mutations, there was an unintended A → C mutation resulting in the substitution of Glu70 by Asp. This mutation was determined to have occurred during the construction of pTrR105 (Mulrooney, 1997). To correct this error the DNA was subjected to a second round of site directed mutagenesis as previously described, reverting residue 70 to a Glu and introducing a silent mutation at His69 which created a diagnostic NsiI restriction enzyme site. The final construct, designated pTrR141, was sequenced across the entire TrxR gene.

Transfection of *E. coli* A326 with pTrR141 resulted in inconsistent expression of the CC mutant enzyme i.e., cultures often exhibited poor growth or low yields of enzyme which was accompanied by unexpectedly low levels of plasmid DNA. In an attempt to increase plasmid stability and thereby improve expression, A326 was first transformed with plasmid pUH7 (Henrich & Plapp, 1986) that contains a *lacI^q* gene. The *lacI^q* gene expresses the lac repressor that would moderate expression of pTrR141. It also has a chloramphenicol resistance gene and a replicon that is compatible with pTrR141. Transformation of *E. coli* A326(pUH7) with pTrR141 resulted in cells which consistently gave high yields of the mutant enzyme. Cultures of 300 ml of A326(pTrR141, pUH7) were grown in 2 × YT medium (Sambrook et al., 1989) supplemented with 50 mM Na/K phosphate, pH 7, 20 mM glucose, 100 µg/mL ampicillin, and 30 µg/mL chloramphenicol. Typical yields were 50 mg of mutant enzyme/5 g cells.

DTT treatment

Prior to using the CC mutant enzyme, it was subjected to reduction by DTT. A 25-fold excess of DTT over flavin was incubated for 20–30 min, then separated by gel filtration using a Bio-gel P-6DG column from Bio-Rad (Hercules, CA).

Thiol quantitation

Thiols were determined with 25-fold excess of DTNB over enzyme in the presence and absence of 6 M guanidinium chloride in

100 mM Na/K phosphate–0.3 mM EDTA, pH 7.6. The titer was determined from the release of TNB anion and quantitated at 412 nm using the extinction value of 13,600 M⁻¹ cm⁻¹.

Enzymatic assays

Steady-state thioredoxin reduction activity was determined using the previously described double-coupled assay (Prongay et al., 1989). Since DTNB and the product TNB anion involved in the double-coupled method react with crosslinked enzyme species (Scheme 2), a thioredoxin assay with fixed concentration of NADPH and thioredoxin was developed. Comparison of $V_{\max}$ values at 25°C using the two methods gave similar values. In this assay the oxidation of NADPH in 0.1 M Na/K phosphate buffer–0.3 mM EDTA, pH 7.6, containing 100 µM NADPH and 100 µM thioredoxin was followed at 340 nm and an extinction value of 6,220 M⁻¹ cm⁻¹ for NADPH at 340 nm. Steady-state transhydrogenase activities were determined from the appearance of APyADP at 395 nm using an extinction change of 3,260 M⁻¹ cm⁻¹ at a fixed concentration of 80 µM NADPH and varying concentrations of APyADP⁺. The assays were performed at 25°C in 0.1 M Na/K phosphate buffer–0.3 mM EDTA, pH 7.6. $V_{\max}$ values (expressed as turnover numbers) were measured at 80 µM NADPH and 400 µM APyADP⁺.

Formation of a disulfide bond in the CC mutant by oxidation with DTNB

The protein (90 µM in 0.1 M Na/K phosphate–0.3 mM EDTA, pH 7.6) was incubated at 25°C with DTNB added in two portions of 0.5 eq (relative to enzyme FAD, i.e., enzyme subunits) for 30 min following each addition. The mixture was then purified using two Pierce TNB-thiol columns in series as described below. It was subsequently found that a lower enzyme concentration (ca. 40 µM) to decrease the formation of intermolecular disulfides was desirable.

Crosslinking using bifunctional reagent

The crosslinking reagent, oPDM, was obtained from Aldrich Chemical Co (Milwaukee, WI). The concentration of oPDM was determined from the loss of absorbance of TNB anion at 412 nm formed by DTT and excess DTNB in 0.1 M Na/K PO₄ buffer–0.3 mM EDTA, pH 7.6. Enzyme (45 µM) was incubated with 1.3 eq of crosslinker in 0.1 M Na/K PO₄ buffer–0.3 mM EDTA, pH 7.6, at 25°C for 3 h. The reaction mixture was purified using two TNB-thiol columns (see below).

TNB-thiol column

The Pierce Chemical Co. (Rockford, IL) supplies a versatile affinity agarose matrix designed to be used as either a matrix carrying free thiols or as matrix containing disulfide bonded TNB. To effect the purification of the crosslinked enzyme (species II, Scheme 2), both forms of the matrix were used. Two 4 mL columns were prepared as follows: Column 1 (free thiol form)—The column was washed with DTT to remove the bound TNB and then equilibrated with starting buffer. Enzyme containing crosslinker with a free reactive moiety left (Scheme 2, species III and IV) reacts with the thiol column whereas crosslinked enzyme (species II, V, and VI) and thiolates (species I) pass through the columns.

Column 2 (TNB form)—The matrix containing disulfide bonded TNB reacts with enzyme containing free thiol (Scheme 2, species I and V) by releasing TNB anion and retaining the enzyme. The desired intramolecularly crosslinked enzyme (species II) as well as any intermolecularly crosslinked enzyme (species VI) pass through the column. After preparing both forms of the matrix and equilibrating with 0.1 M phosphate–0.3 mM EDTA, pH 7.6, the columns were joined in series and the reaction mixture was applied. Subsequently, buffer containing 1 M NaCl was applied for eluting the crosslinked CC mutant from the tandem columns. The pooled protein fractions were reduced in volume using Amicon Centricon-30 filtration units (Beverly, MA) and washed with 0.1 M phosphate, pH 7.6. The purified material was ca. 50% of the starting material. Enzyme species bound reversibly to the matrix of column 2 was removed with buffer containing 1 M NaCl and 20 mM DTT. Column 1, which had irreversibly bound the oPDM-enzyme species III and IV (Scheme 2), was discarded.

SDS PAGE

Protein samples were analyzed for purity and for the presence of intermolecular bonding using 10% SDS-PAGE according to the Laemmli method. A reducing agent was included in the sample buffer except for analysis concerning the CC mutant reacted with DTNB to form a disulfide.

Reductive titrations

Reductive titrations with NADPH and dithionite were performed using 0.1 M Na/K phosphate, pH 7.6, at 25 °C. Anaerobiosis was achieved as described (O'Donnell & Williams, 1983). A Perkin-Elmer lambda 6 spectrophotometer was used to collect the data. Samples for dithionite titrations contained (relative to enzyme FAD) 0.02 eq methyl viologen as a mediator of reducing equivalents and an oxygen scavenging system consisting of 80 μ M protocatechuate (PCA) and 2 U/mL protocatechuate dioxygenase (PCD).

Structure analysis

Models representing the crystal structure of thioredoxin reductase (Waksman et al., 1994) were analyzed using the computer graphics program SYBYL from Tripos, Inc. (St. Louis, MO). Digestion of oPDM-crosslinked CC mutant by trypsin and identification of the resulting peptides using liquid chromatography and mass spectrophotometry were performed by the University of Michigan Biomedical Research Core Facility.

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