A Stable Mixed Disulfide between Thioredoxin Reductase and Its Substrate, Thioredoxin: Preparation and Characterization[†]

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ABSTRACT: The flavoenzyme thioredoxin reductase (TrR) catalyzes the reduction of the small redox protein thioredoxin (Tr) by NADPH. It has been proposed that a large conformational change is required in catalysis by TrR in order to visualize a complete pathway for reduction of equivalents. The proposal is based on the comparison of the crystal structures of TrR and glutathione reductase, the latter being a well-understood member of this enzyme family [Waksman, G., *et al.* (1994) *J. Mol. Biol. 236*, 800−816]. Bound NADPH is perfectly positioned for electron transfer to the FAD in glutathione reductase, but in TrR, these two components are 17 Å apart. In order to provide evidence for the proposed conformational change, a complex between TrR and its substrate Tr involving a mixed disulfide between TrR and Tr was prepared. The redox active disulfide of TrR is composed of Cys^{135} and Cys^{138}, and the redox active disulfide of Tr is made up of Cys^{32} and Cys^{35}. The complex C135S−C32S is prepared from forms of TrR and Tr altered by site-directed mutagenesis where Cys^{138} and Cys^{35} are remaining in TrR and Tr, respectively. The purified C135S−C32S presents a band on a nonreducing sodium dodecyl sulfate−polyacrylamide gel electrophoresis corresponding to a molecular weight sum of one subunit of TrR and one of Tr. Several observations indicate that C135S−C32S can adopt only one conformation. It was reported previously that TrR C135S can form a charge transfer complex in the presence of ammonium cation in which the donor is the remaining thiolate of Cys^{138} [Prongay, A. J., *et al.*, (1989) *J. Biol. Chem. 264*, 2656−2664], while titration of C135S−C32S with NH_4Cl does not induce charge transfer, presumably because Cys^{138} is participating in the mixed disulfide. Reduction of C135S−C32S with dithiothreitol (DTT) results in a decrease of ϵ_{454} to a value similar to that of TrR C135S, and subsequent NH_4Cl titration leads to charge transfer complex formation in the nascent TrR C135S. Reductive titrations show that approximately 1 equiv of sodium dithionite or NADPH is required to fully reduce C135S−C32S, and treatment with NH_4Cl and DTT demonstrates that the mixed disulfide remains intact. These results indicate that C135S−C32S is a stable mixed disulfide between Cys^{138} of TrR C135S and Cys^{35} of Tr C32S that locks the structure in a conformation where FAD can be reduced by NADPH, but electrons cannot flow from $FADH_2$ to the mixed disulfide bond.

Thioredoxin reductase is a member of the pyridine nucleotide−disulfide oxidoreductase family of flavoenzymes. The family also includes glutathione reductase and lipoamide dehydrogenase. These enzymes catalyze electron transfer between pyridine nucleotide and disulfide/dithiol compounds via a FAD prosthetic group and an active site disulfide (Schirmer & Schulz, 1987; Williams, 1992). *Escherichia coli* thioredoxin reductase (TrR)[1] is a dimer of identical subunits. Each subunit with a M_r of 35 300 includes one redox active disulfide and one FAD and catalyzes the reduction of thioredoxin by NADPH (Moore *et al.*, 1964; Zanetti & Williams, 1967; Williams, 1995). The substrate thioredoxin is a small monomeric protein with a M_r of 12 000 and contains one redox active disulfide which is composed of Cys^{32} and Cys^{35} (Holmgren, 1985).

Glutathione reductase and lipoamide dehydrogenase are very closely related, while thioredoxin reductase has a distinct structure and catalytic properties. Glutathione reductase is a well-understood member of this enzyme family and can serve as a prototype for the description of the mechanism and structure (Arscott *et al.*, 1981; Schulz *et al.*, 1978; Karplus & Schulz, 1987; Rietveld *et al.*, 1994). Like other members of the family, glutathione reductase is a homodimer where each subunit consists of three domains: FAD, NADPH, and interface domains (Chart 1A). The nicotinamide ring of the bound NADPH is in close contact with the *re* face of the flavin ring, and the active site disulfide is adjacent to the *si* face of the flavin ring. The substrate glutathione binds in a crevice between the FAD domain and the dimer interface, within bonding distance of the active site disulfide. Electrons flow from NADPH to glutathione via the isoalloxazine ring and the redox active disulfide

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[1] Abbreviations: TrR C135S, thioredoxin reductase in which Cys^{135} has been changed to Ser; TrR C138S, thioredoxin reductase in which Cys^{138} has been changed to Ser; Tr C32S, thioredoxin in which Cys^{32} has been changed to Ser; Tr C35S, thioredoxin in which Cys^{35} has been changed to Ser; C135S−C32S, mixed disulfide between TrR C135S and Tr C32S; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB^-, 5-thio-2-nitrobenzoate anion; DTT, 1,4-dithiothreitol; Tr C32S−TNB, mixed disulfide between Tr C32S and TNB; $APyADP^+$, oxidized form of 3-acetylpyridine adenine dinucleotide phosphate.

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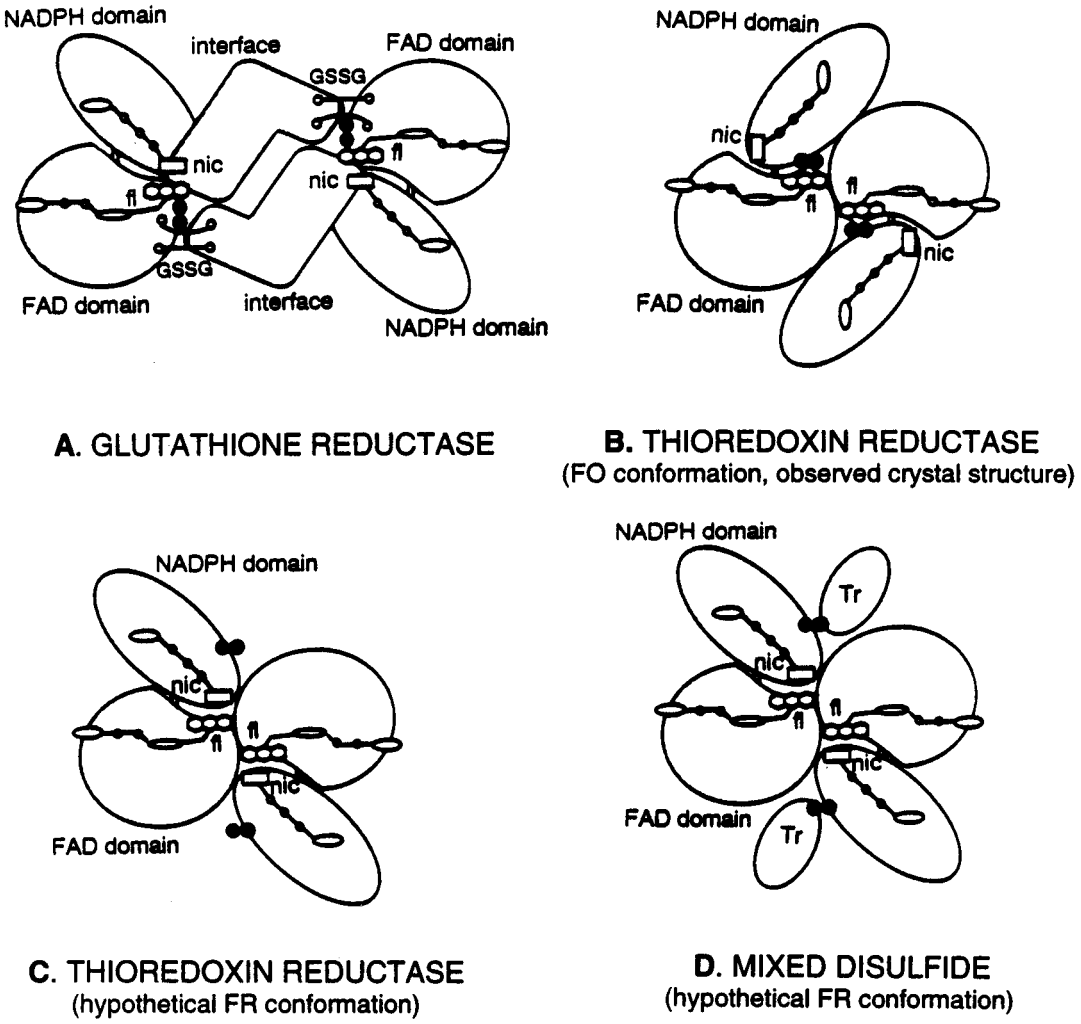
Chart 1: Cartoons Depicting the Domain Juxtapositions from the Crystal Structures of Glutathione Reductase (A) and Thioredoxin Reductase (B), the Proposed FR Conformation of Thioredoxin Reductase (C), and the Tr−TrR Mixed Disulfide C135S−C32S (D)[a]



[a] Modeled after Waksman *et al.* (1994).

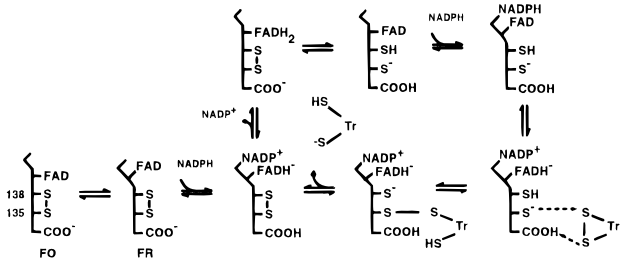
without a major conformational change. In the case of thioredoxin reductase, each subunit consists of only the NADPH and FAD domains and lacks an interface domain. Both the FAD and NADPH domains have structures similar to the corresponding domains in glutathione reductase, but the relative orientation of the two domains is different (Kuriyan *et al.*, 1991; Waksman *et al.*, 1994). The active site disulfide is in the NADPH domain rather than in the FAD domain as in glutathione reductase (Russel & Model, 1988). Thus, the location of the active site disulfide is adjacent to the *re* side of the flavin ring, interposed between the bound NADPH and the FAD. The large substrate thioredoxin appears too bulky to fit into the space near the active site disulfide, and there is no close contact between the nicotinamide ring of NADPH and the isoalloxazine ring of FAD (Chart 1B).

It is proposed that a large conformational change is required during catalysis by thioredoxin reductase (Kuriyan *et al.*, 1991; Waksman *et al.*, 1994). If the FAD domains of thioredoxin reductase and glutathione reductase are first aligned, the NADPH domains can be superimposed by rotating one of them by 66°. If such a rotation is applied to the NADPH domain of thioredoxin reductase, leaving its FAD domain fixed, the nicotinamide ring of the NADPH would be moved into close contact with the isoalloxazine ring, and the redox active disulfide would be moved to the surface of the protein where it is accessible to the substrate thioredoxin as shown in the cartoon, (Chart 1C). Reverse rotation would be blocked by the bound Tr as indicated in Chart 1D.

We have referred in two previous publications to the conformation observed in the crystal structure as the unrotated conformation and to the putative conformation as the rotated conformation (Lennon & Williams, 1995; Williams, 1995). We regret this choice which will have relevance only, we hope, until the putative conformation is observed. We propose therefore to refer to the presently observed conformation in which the flavin oxidation occurs by the nascent disulfide as the FO conformation and to the putative conformation in which flavin reduction occurs as the FR conformation. Thus, in the FR conformation, the pyridinium ring of NADPH is aligned with the isoalloxazine ring of FAD and the redox active dithiol is near the surface where it can interchange with the disulfide of the substrate, thioredoxin.

The mechanism of thioredoxin reductase shown in Scheme 1 is our current working hypothesis (Williams, 1995). The redox active disulfide of thioredoxin reductase is composed of Cys^{135} (presumed to initiate interchange) and Cys^{138} (closer to and interacting with the flavin). In the putative FR conformation (right to left in the lower row), the thiolate of

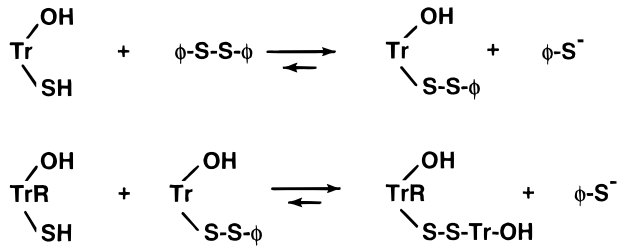
Scheme 1: Mechanism of Thioredoxin Reductase[a]



[a] From Williams (1995). The FO conformation is that observed in the crystal structure with the FAD and redox active disulfide juxtaposed, while the FR structure has the FAD and NADPH juxtaposed. The two conformations are designated by the position of the crook in the backbone. The acid catalyst is Asp^{139} (Mulrooney & Williams, 1994). Catalysis is from two-electron-reduced to four-electron-reduced to two-electron-reduced enzyme (Lennon & Williams, 1996).

Cys^{135} attacks the disulfide of thioredoxin, to form the mixed disulfide between the enzyme and the substrate. Next, the thiolate of Cys^{138} attacks the mixed disulfide to form the active site disulfide again and release the reduced thioredoxin. Therefore, preparation of a stable mixed disulfide between thioredoxin reductase and thioredoxin would be important for the understanding of the proposed conformational change during the catalysis. However, for wild type thioredoxin reductase and wild type thioredoxin, the second thiol/disulfide interchange occurs rapidly after the formation of the mixed disulfide and precludes the accumulation of the mixed disulfide.

The altered forms TrR C135S and TrR C138S (Prongay *et al.*, 1989), with one of the active site cysteines replaced by a serine and a single active thiol remaining, make it possible to stop the reaction after the first interchange. Altered forms of the substrate, thioredoxin with a single active thiol remaining (Tr C32S and Tr C35S) (Russel & Model, 1986), can be modified by a reaction of DTNB with the remaining thiol to form a mixed disulfide between thioredoxin and TNB (Tr−S−S−Φ). The mixed disulfide between altered TrR and altered Tr can be prepared by the reaction of altered TrR with the mixed disulfide, Tr−S−S−Φ.[2] The release of a highly colored TNB^- would signal the formation of the mixed disulfide. The preparation is described by the following reactions, where Φ-S-S-Φ represents DTNB:



In the present work, we have prepared and characterized a mixed disulfide between the altered enzyme TrR C135S and altered substrate Tr C32S (C135S−C32S). The choice of this pair, C135S and C32S, was totally pragmatic in that the yield of mixed disulfide was highest. Four observations indicating that the disulfide of C135S−C32S prevents rotation will be presented and thus provide support for the putative conformational change.

[2] The chemistry was suggested by Dr. Colin Thorpe, Department of Chemistry, University of Delaware.

MATERIALS AND METHODS

Reagents. TrR C135S was purified as previously described (Prongay *et al.*, 1989). Plasmids containing the gene coding for Tr C32S or Tr C35S were obtained from Dr. Marjorie Russel (Russel & Model, 1986). The expressed proteins were purified as for wild type thioredoxin (Lennon & Williams, 1995). DTNB, NADPH, and $APyADP^+$ were purchased from Sigma. DTT was purchased from ICN biochemical. Sodium dithionite was from Baker Inc. All other reagents and buffer salts were of the highest quality available.

Solutions of 100 mM DTT in 50 mM Na/KPO_4 at pH 7.6 were made fresh daily. DTNB solutions (25 mM) were made in 100 mM sodium acetate buffer at pH 5.0 and stored at 4 °C. Stock solutions of DTNB show a slow rate of increasing A_{412}, and assays using DTNB required background correction at 412 nm. Assays and desalting columns were performed with 50 mM Na/KPO_4 at pH 7.6, except where noted. All experiments for the preparation and characterization of C135S−C32S were performed in the absence of ethylenediaminetetraacetic acid (EDTA) to reduce the formation of enzyme semiquinone.

Preparation of the Mixed Disulfide Tr C32S−TNB. An aliquot of Tr C32S was treated with DTT by incubation with a 25-fold excess of fresh DTT at room temperature for 30 min to assure that the thiol was fully reduced. The reaction mixture was concentrated with Amicon Centriplus 3 filtration units to a volume of about 3 mL and applied to a 1.5 × 25 cm BioRad Biogel P-6DG desalting column to remove the excess DTT. Fractions of 1.5 mL were collected, and the A_{280} was recorded. The first peak off the column contained the thioredoxin. The fractions containing the thioredoxin were pooled, and the protein was quantitated by using the equation (Laurent *et al.*, 1964)

$$\text{milligrams of thioredoxin per milliliter} = (A_{280} - A_{310}) \times 0.9$$

The sample was then reacted with an 80-fold excess of DTNB and monitored at 412 nm for the completion of the reaction. The volume of the reaction mixture was reduced again to about 3 mL with the Amicon Centriplus 3 apparatus and then applied a second time to the desalting column to remove excess DTNB and released TNB^-. The fraction of the mixed disulfide Tr C32S−TNB after the desalting column process was used directly in the reaction with TrR C135S as described in the following section. Tr C32S−TNB can be reduced by DTT to free Tr C32S and TNB^-. The quantitation of Tr C32S−TNB was performed by reaction of an aliquot of Tr C32S−TNB with a 100-fold excess of DTT and monitoring the release of TNB^- at 412 nm. The concentration of TNB^- was calculated by using an extinction coefficient of 13 600 M^{-1} cm^{-1} at 412 nm.

The reaction of Tr C32S with DTNB gives the mixed disulfide Tr C32S−TNB which shows a characteristic shoulder peak around 337 nm and a ratio of A_{280}/A_{337} of approximately 2.6. It was found that the mixed disulfide Tr C32S−TNB was stable at 4 °C by monitoring the peak at A_{337} and A_{412} for release of TNB^-.

Preparation of the Mixed Disulfide between TrR C135S and Tr C32S. TrR C135S was treated with DTT (25-fold excess for 30 min) before the reaction with Tr C32S−TNB to make sure the remaining active site thiol (Cys^{138}) was in the reduced state. TrR C135S contains three free thiols, Cys^{138}, and two buried thiols that do not participate in catalysis. The free thiol (Cys^{138}) content of TrR C135S was determined by reaction of an aliquot of TrR C135S with an 80-fold excess of DTNB and monitoring the release of TNB^- at 412 nm. Without DTT treatment, the thiol titer of TrR C135S was 0.7; it increased to 1.0 after DTT treatment, suggesting that some of the active site thiol of TrR C135S is in a higher oxidative state. The reaction mixture, following DTT treatment, was concentrated with an Amicon Centriplus 30 apparatus to about 3 mL and applied to a 1.5 × 25 cm BioRad Biogel P-6DG desalting column to remove the excess DTT. The fractions containing enzyme were pooled and spectrally quantitated. TrR C135S was reacted with equimolar mixed disulfide Tr C32S−TNB at room temperature. The reaction was monitored at 412 nm for the release of TNB^- and quantitated. Upon completion of the reaction (about 30 min), the mixture was dialyzed overnight at 4 °C against 20 mM histidine buffer at pH 5.6. The dialyzed solution was adjusted to pH 5.0 with 20 mM 1-methylpiperazine buffer at pH 4.5 and centrifuged at 11 000*g* for 20 min to remove a yellow precipitate containing unreacted TrR C135S (see below). The supernatant was applied to a 1 × 9 cm Waters Protein Pak Q HR15 anion exchange column equilibrated with 20 mM 1-methylpiperazine at pH 4.5 and eluted with a 5 mM per column volume gradient of NaCl. Unreacted Tr C32S does not bind to the column, and any remaining unreacted TrR C135S eluted around 45 mM NaCl. Mixed disulfide C135S−C32S eluted around 80 mM NaCl. The fractions containing C135S−C32S were pooled and adjusted to pH 7.0 with 1.0 M Na/KPO_4 at pH 7.6 and then concentrated with an Amicon Centriplus 30 apparatus and applied to the desalting column. The fraction of C135S−C32S after the desalting column process was precipitated in 80% saturation of ammonium sulfate and stored at 4 °C.

Measurement of Solubility of C135S−C32S and TrR C135S at Low pH. C135S−C32S (80 μL, ca. 0.6 mM) or TrR C135S in 50 mM Na/KPO_4 at pH 7.6 was added to 1.0 mL of 40 mM methylpiperazine at pH 5.0. The mixture, which had a final pH of 5.4, was centrifuged, and the pellet was dissolved in 50 mM Na/KPO_4 at pH 7.6 and quantitated spectrally. The solubility was calculated by subtraction of the amount of pellet from the starting amount. The solubilities at pH 4.9 and 4.3 were determined in a similar manner by using 40 mM methylpiperazine at pH 4.4 and 100 mM acetate at pH 4.1.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE) Gels. The purity of proteins and the presence of C135S−C32S were verified by SDS−PAGE using the Laemmli method. The thioredoxin purity was examined with 15% gels. Enzyme and mixed disulfide formation were examined with 10 or 12% gels. Detection of the mixed disulfide C135S−C32S was performed under nonreducing conditions. The high- and low-M_r bands sometimes seen in C135S are not observed under reducing conditions. Gels containing reducing agent required addition of fresh 2-mercaptoethanol to the sample buffer for complete dissociation. All samples were heated for 3 min at 90 °C in sample buffer prior to loading.

Steady State Kinetic Analysis. Thioredoxin reduction activity and NADPH−$APyADP^+$ transhydrogenase activity were examined using previously described methods (Mulrooney & Williams, 1994). Thioredoxin reduction activity was measured at a fixed NADPH concentration (10 μM) and varying thioredoxin concentrations by monitoring the disappearance of NADPH at 340 nm. The concentration of C135S−C32S in the assays was 10 nM. NADPH−$APyADP^+$ transhydrogenase activity was measured at a fixed NADPH concentrations (80 μM) and varying $APyADP^+$ concentration by monitoring the increase of APyADPH absorbance at 395 nm. The enzyme concentration in the assays was 15 nM.

Extinction Coefficient Determination. The extinction coefficient of C135S−C32S at 454 nm was determined by using the previously described method of SDS denaturation on the basis of the amount of released FAD (Prongay *et al.*, 1989). The concentration of free FAD was calculated using an extinction coefficient of 11 300 M^{-1} cm^{-1} at 450 nm.

NH_4Cl Titration and DTT Titration. The NH_4Cl and DTT titrations of C135S−C32S (∼20 μM) were done aerobically at 25 °C in 0.1 M Na/KPO_4 at pH 7.6. The solution of 4.6 M NH_4Cl was prepared in 0.1 M Na/KPO_4, and adjusted to pH 7.6 with an aqueous NaOH solution. The 32 mM DTT solution was prepared fresh daily in 0.1 M Na/KPO_4 at pH 7.6.

NADPH Titration. NADPH titrations were performed as previously described (Prongay & Williams, 1992). Protocatechuic acid (PCA, 80 mM) and protocatechuic acid dioxygenase (PCD, 0.04 u/mL) were used in the NADPH titration to remove trace oxygen.

The NADPH solution was prepared as follows. In an anaerobic tube with PCD in a side arm, a solution of NADPH (∼1.5 mM) in 0.1 M Na/KPO_4 at pH 7.6 containing PCA was made anaerobic by 10 cycles of vacuum/nitrogen with vortexing. The anaerobic solution was tipped into the side arm to mix with PCD. Aliquots were withdrawn with an anaerobic syringe for calibration and titration of the anaerobic enzyme. The concentration of NADPH was calculated by using an extinction coefficient of 6220 M^{-1} cm^{-1} at 340 nm.

C135S−C32S was titrated with NADPH anaerobically in 0.1 M Na/KPO_4 at pH 7.6 at 25 °C in the presence of PCA and PCD. Anaerobiosis was performed as previously described (Williams *et al.*, 1979; O'Donnell & Williams, 1983).

Sodium Dithionite Titration. Sodium dithionite titrations were performed as previously described (Prongay & Williams, 1992). Solutions of sodium dithionite were prepared anaerobically in 50 mM pyrophosphate buffer at pH 9.0 and quantitated by titration of a solution of lumiflavin-3-acetic acid. C135S−C32S was titrated anaerobically in 0.1 M Na/KPO_4 at pH 7.6 at 25 °C with sodium dithionite, in the presence of 5% methyl viologen.

RESULTS

Characteristics of the Mixed Disulfides, Tr C32S−TNB and Tr C35S−TNB. The reaction of Tr C32S or Tr C35S with DTNB gives the mixed disulfides Tr C32S−TNB and Tr C35S−TNB. Tr C32S−TNB shows a characteristic peak at 337 nm and a ratio of A_{280}/A_{337} of approximately 2.6 (Figure 1). Tr C35S−TNB shows a characteristic peak at 320 nm and a ratio of A_{280}/A_{320} of approximately 2.4. The

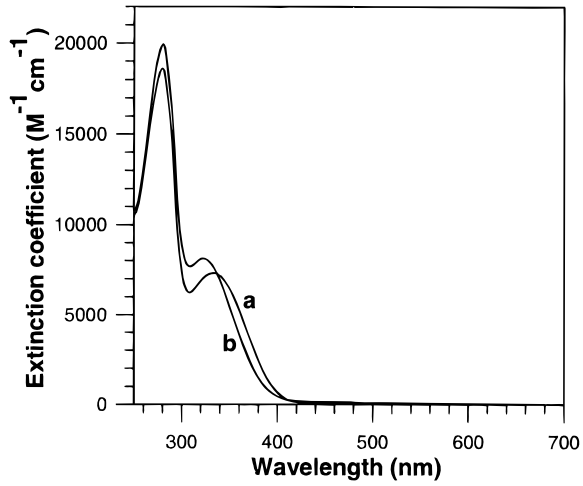


FIGURE 1: Spectra of the mixed disulfides Tr C32S−TNB (a) and Tr C35S−TNB (b). The proteins were in 50 mM Na/KPO_4 at pH 7.6 and 25 °C.

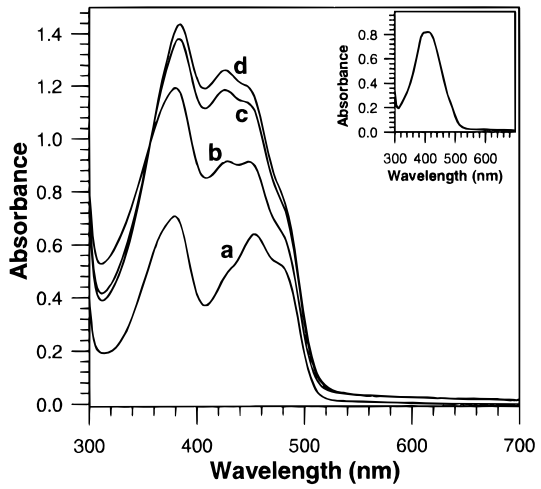


FIGURE 2: Spectra during the reaction of 69.8 μM TrR C135S with 69.4 μM Tr C32S−TNB. The spectrum of TrR C135S in 50 mM Na/KPO_4 at pH 7.6 and 25 °C (a) and the spectra of the reaction mixture at 1 min (b), 6 min (c), and 35 min (d). The inset shows the spectrum produced by subtraction of spectrum a from spectrum d.

absorbance near 330 nm is thought to be due to the disulfide bond, and the position of the maximum reflects the strain of the bond.

Formation of the Mixed Disulfide C135S−C32S. The spectra observed during the reaction of TrR C135S with Tr C32S−TNB are somewhat complex but just as expected. Figure 2 (spectrum a) shows TrR C135S. Spectrum b, recorded at roughly the half-time of the reaction, displays increased absorbance at all wavelengths, including 412 nm, where the product TNB^- absorbs, and at 337 nm, where the reacting Tr C32S−TNB absorbs. As the reaction progresses and Tr C32S−TNB is consumed, there is a decrease at 337 nm and an increase of A_{412}, indicating thiol−disulfide interchange and the release of TNB^-. Incubation at room temperature for 1 h showed release of 0.8−0.9 equiv of TNB^-. If spectrum a is subtracted from spectrum d, the resulting spectrum has a single peak at 412 nm (Figure 2, inset). SDS−PAGE analysis of the reaction mixture under nonreducing conditions confirmed that the product had a molecular mass of 47 kDa, representing one TrR and one Tr (Figure 3).

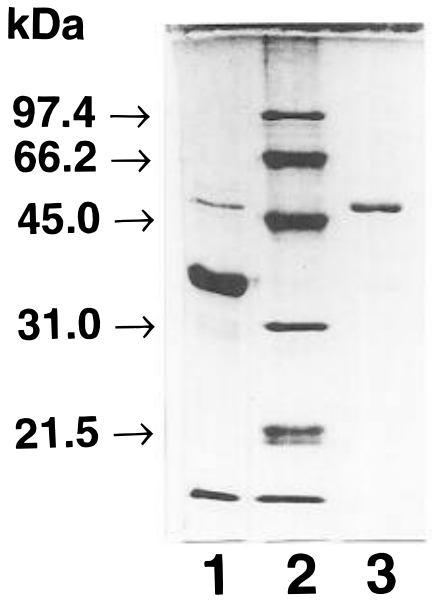


FIGURE 3: Nonreducing SDS−PAGE. Lane 1 is TrR C135S marker. The high- and low-M_r bands sometimes seen in C135S are not observed under reducing conditions. Lane 2 is molecular weight standards. Lane 3 is C135S−C32S.

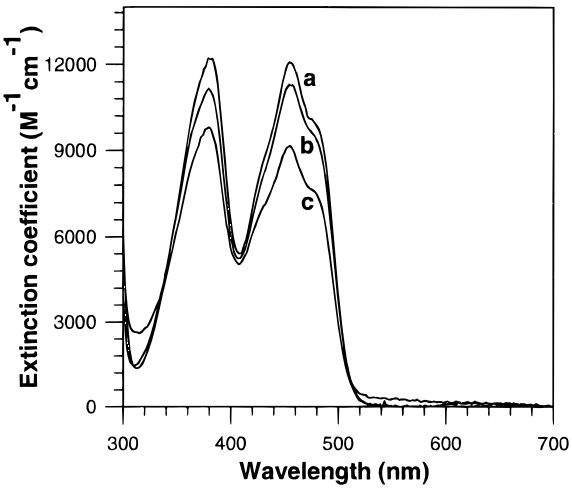


FIGURE 4: Spectra of C135S−C32S (a), wild type TrR (b), and TrR C135S (c). The enzymes were in 50 mM Na/KPO_4 at pH 7.6 and 25 °C.

Purification of C135S−C32S. The binding of C135S−C32S to the anion exchange medium Waters Protein Pak Q HR 15 column is stronger than that of TrR C135S. In addition, the solubilities of both TrR C135S and C135S−C32S decrease in the pH range 5.4−4.4, and TrR C135S has a lower solubility than C135S−C32S. This allows some unreacted TrR C135S to be precipitated at pH 5.0 prior to purification on the column. These properties indicate that C135S−C32S has more negative charges on the surface of the protein and a lower isoelectric point than TrR C135S. The yield of C135S−C32S is approximately 50%.

Spectral Characterization. The spectrum of C135S−C32S is compared with the spectra of wild type TrR and TrR C135S in Figure 4. C135S−C32S has a higher ϵ_{454} and a higher A_{379}/A_{314} ratio than TrR C135S. Comparison of the spectral characteristics (Table 1) indicates that the spectrum of C135S−C32S resembles that of wild type TrR rather than that of TrR C135S. The A_{379}/A_{314} ratio is diagnostic.

Steady State Kinetic Analysis. Steady state kinetic analysis demonstrates that the NADPH−APyADP$^+$ transhydrogenase

Table 1: Spectral Characteristics of C135S−C32S, TrR C135S, and Wild Type TrR

	C135S−C32S	TrR C135S	wild type TrR
vis max (nm)	454	453	456
$\epsilon_{\text{vis max}}$ (M^{-1} cm^{-1})	12080	9150	11300
ϵ_{280} (M^{-1} cm^{-1})	65100	49750	51700
$A_{280}/A_{\text{vis max}}$	5.39	5.44	4.58
$A_{379}/A_{\text{vis ma}}$	1.01	1.07	0.99
$A_{\text{vis max}}/A_{408}$	2.23	1.81	2.15
A_{379}/A_{314}	8.88	3.73	7.27

Table 2: NADPH−APyADP$^+$ Transhydrogenase Activities of C135S−C32S, TrR C135S, and Wild Type TrR

	turnover number (mol of substrate min^{-1} (mol of FAD)$^{-1}$)	$K_{\text{m, APyADP}^+}$ (μM)
C135S−C32S	569 ± 26	36.8 ± 6.1
wild type TrR	505 ± 35	37.6 ± 9.4
TrR C135S	378 ± 31	24.6 ± 9.5

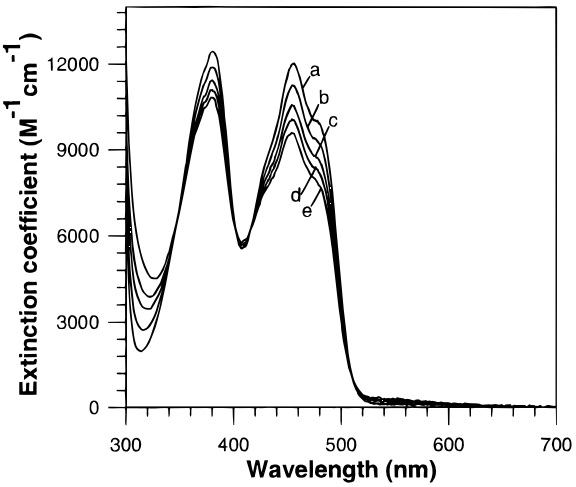


FIGURE 5: Reaction of C135S−C32S with DTT. A solution of 21.9 nmol of C135S−C32S in 1.0 mL of 0.1 M Na/KPO_4 at pH 7.6 was titrated with 32 mM DTT at 25 °C. The spectra are of the mixture after addition of DTT with a final concentration of 0 (a), 0.82 mM (b), 1.54 mM (c), 2.12 mM (d), and 3.47 mM (e).

activity of C135S−C32S is 1.5-fold that of TrR C135S, similar to that of wild type TrR (Table 2). The transhydrogenase activity depends only on the flavin and does not involve the redox active disulfide. On the other hand as expected, C135S−C32S has no detectable thioredoxin reductase activity.

NH_4Cl Titration and DTT Titration. It was reported previously that TrR C135S can form a thiolate−FAD charge transfer complex in the presence of ammonium ion, and it precipitates in ammonium sulfate as a red pellet (Prongay *et al.*, 1989). However, the mixed disulfide C135S−C32S does not possess this property, presumably because it has no thiolate. It precipitates in ammonium sulfate as a yellow pellet. Thus, NH_4Cl can be used to detect the presence of TrR C135S in a solution of C135S−C32S signaled by the formation of the charge transfer complex.

Reaction of C135S−C32S with DTT (Figure 5) results in the decrease of ϵ_{454} from 12 080 to 9600 M^{-1} cm^{-1}, i.e., close to the ϵ_{453} of TrR C135S, as expected. The reaction is kinetically slow, but the lack of further change indicates that it went to completion. C135S−C32S thus treated forms a charge transfer complex in the presence of ammonium ion with a band centered around 530 nm (Figure 6). This is

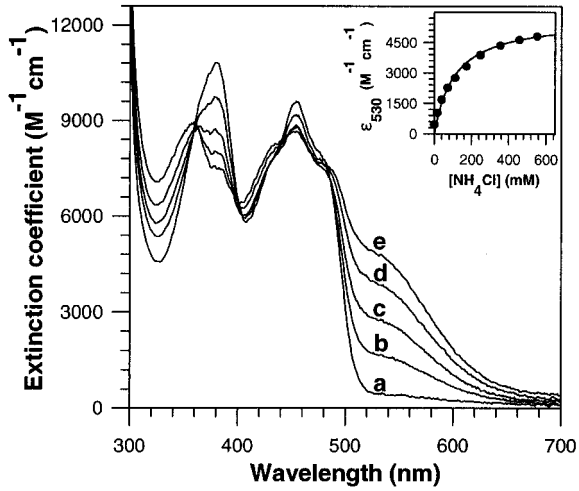


FIGURE 6: NH_4Cl titration of C135S−C32S after reaction with DTT. After the titration of C135S−C32S with DTT (Figure 5), the resulting mixture was titrated with 4.6 M NH_4Cl at 25 °C. The spectra are of the mixture after addition of NH_4Cl with a final concentration of 0 (a), 41 mM (b), 112 mM (c), 248 mM (d), and 554 mM (e). The inset is a plot of ϵ_{530} *versus* ammonium chloride concentration.

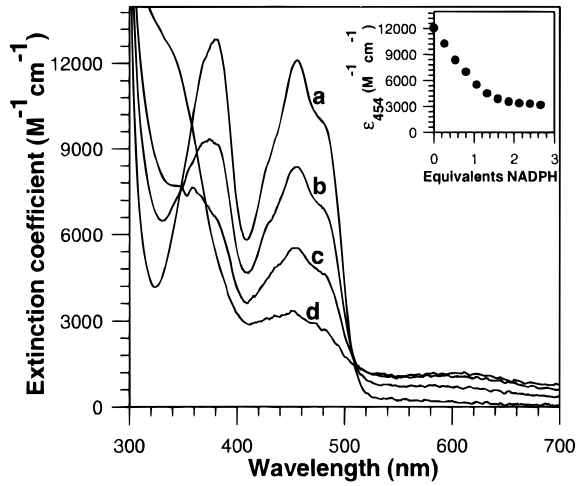


FIGURE 7: Titration of C135S−C32S with NADPH. A solution of 21.3 nmol of C135S−C32S in 1.0 mL of 0.1 M Na/KPO_4 at pH 7.6 in the presence of 80 mM PCA and 0.04 u/mL PCD was titrated anaerobically with 1.4 mM NADPH at 25 °C. The spectra are of the mixture after addition of 0 (a), 0.53 (b), 1.06 (c), and 2.40 (d) equiv of NADPH. The inset is a plot of ϵ_{454} *versus* equivalents of NADPH.

identical to the charge transfer complex band formed in the NH_4Cl titration of TrR C135S (Prongay *et al.*, 1989). These reactions demonstrate that the disulfide bond of C135S−C32S is broken by DTT, and the resulting TrR C135S forms a charge transfer complex in the presence of NH_4Cl. The data fitted to a rectangular hyperbola give an apparent K_d of 108 μM for the NH_4^+ ion, comparable to the value of 54 μM for TrR C135S.

NADPH Titration. The titration of C135S−C32S with NADPH is shown in Figure 7. Addition of NADPH results in a decrease of flavin absorbance, and 1.3 equiv of NADPH is required for essentially full reduction. The isobestic point at 345 nm indicates that only two enzyme species are involved in the titration until excess NADPH begins to accumulate. The reaction mixture was reoxidized by opening to air after the titration. Addition of NH_4Cl to this reoxidized mixture did not induce a charge transfer complex band.

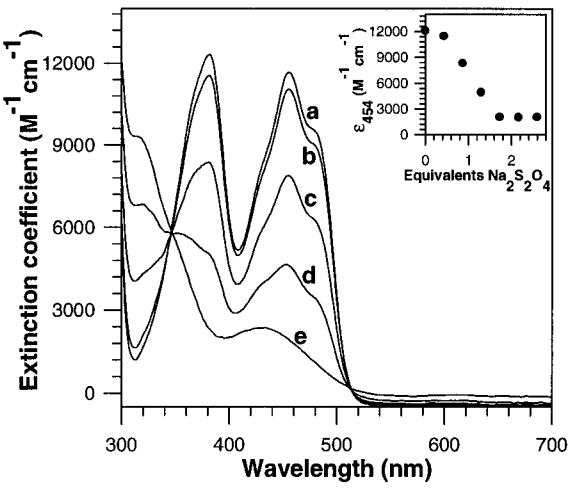


FIGURE 8: Titration of C135S−C32S with sodium dithionite. A solution of 30.4 nmol of C135S−C32S in 1.3 mL of 0.1 M Na/KPO_4 at pH 7.6, containing 1.5 nmol of methyl viologen, was titrated anaerobically with 2.2 mM sodium dithionite at 25 °C. The spectra are of the mixture after addition of 0 (a), 0.43 (b), 0.86 (c), 1.30 (d), and 1.73 (e) equiv of sodium dithionite. The inset is a plot of ϵ_{454} *versus* equivalents of sodium dithionite.

However, following the addition of DTT, a charge transfer complex forms. A control titration of TrR C135S with NADPH shows that 1.5 equiv of NADPH is required for essentially full reduction, and addition of NH_4Cl to the reoxidized mixture results in formation of a charge transfer complex. These experiments show that the FAD of C135S−C32S can be reduced by NADPH, but the resulting $FADH_2$ cannot reduce the mixed disulfide bond between TrR C135S and Tr C32S.

Sodium Dithionite Titration. Figure 8 shows the dithionite titration of C135S−C32S in the presence of a catalytic quantity of methyl viologen, and 1.2 equiv of dithionite is required to fully reduce the C135S−C32S FAD. Again, the isobestic point at 345 nm indicates that only two enzyme species are involved in the titration. As in the NADPH titration, addition of NH_4Cl to the reoxidized mixture does not induce the charge transfer complex band. In the control experiment, titration of TrR C135S with sodium dithionite shows that 1.3 equiv of dithionite is required for full reduction, close to that reported previously (Prongay & Williams, 1992). Addition of NH_4Cl to the reoxidized mixture does not induce the charge transfer complex band. Thus, the mixed disulfide bond of C135S−C32S is still intact after the dithionite titration and cannot be reduced by $FADH_2$ or dithionite directly.

DISCUSSION

The active site thiols have distinct functions in other members of the pyridine nucleotide-disulfide oxidoreductase family, such as glutathione reductase and lipoamide dehydrogenase. The nascent thiol nearer the carboxyl terminus interacts directly with the flavin, and the other nascent thiol interacts directly with the substrate in the thiol/disulfide interchange reaction. The two active site thiols are referred to as the electron transfer thiol and interchange thiol, respectively (Thorpe & Williams, 1976a,b; Arscott *et al.*, 1981; Fox & Walsh, 1982). It has been demonstrated that, in thioredoxin reductase, Cys^{138} interacts more closely with FAD than does Cys^{135} (Prongay & Williams, 1989, 1990). The X-ray crystal structure also shows that Cys^{138} is located closer to the flavin ring (Kuriyan *et al.*, 1991; Waksman *et al.*, 1994); therefore, Cys^{138} is referred to as the electron transfer thiol, and Cys^{135} is assumed to be the interchange thiol. For the substrate thioredoxin, the nucleophilic reactivity of Cys^{32} indicates that it is the interchange thiol (Holmgren, 1985), and indeed, the solution structure of human thioredoxin in a mixed disulfide with its target peptide from the transcription factor NFκB shows that Cys^{32} provides the interchange thiol (Qin *et al.*, 1995).

In this study, the mutants used for the mixed disulfide preparation are TrR C135S and Tr C32S, in which both putative interchange thiols Cys^{135} and Cys^{32} have been changed to serine and the putative electron transfer thiol Cys^{138} of thioredoxin reductase and Cys^{35} of thioredoxin are remaining. The results in this study show that Cys^{138} can form a stable mixed disulfide with Cys^{35}, which is not the putative natural mixed disulfide during the catalysis. The choice of this pair, C135S and C32S, was totally pragmatic in that the yield of mixed disulfide was highest. Mixed disulfides can form from whatever combinations of Tr and TrR are used. This supports the earlier finding that the active site disulfide of TrR is in a more open conformation than in glutathione reductase and lipoamide dehydrogenase (O'Donnell & Williams, 1985). C135S−C32S is the only mixed disulfide to be used in crystallization trials thus far. Microcrystals have been prepared. Exposure to the X-ray beam yielded a powder diffraction pattern with approximately 3 Å resolution.[3] Characterization of C135S−C35S is in progress, and conditions leading to high yields of C138S−C32S and C138S−C35S are being developed.

The ϵ_{280} values (mM^{-1} cm^{-1}) of C135S−C32S and TrR C135S are 65.1 and 49.8, respectively (cf. 51.7 for wild type TrR). The difference of 15.3 corresponds to the absorbance contributed from the thioredoxin moiety in C135S−C32S and is very close to that of thioredoxin (ϵ_{280} = 13.7). Nonreducing SDS−PAGE analysis shows that C135S−C32S presents a molecular mass band around 47 kDa, which is the same as the molecular mass sum of one TrR subunit and one Tr. Moreover, treatment of C135S−C32S with DTT results in the decrease of the ϵ_{454} to a value closer to that of TrR C135S due to reduction of the mixed disulfide. Subsequent addition of NH_4Cl after DTT treatment leads to the formation of a charge transfer complex unique to TrR C135S. Thus, the evidence indicates that the complex C135S−C32S is composed of one TrR C135S and one Tr C32S, which are connected by a mixed disulfide bond which can be reduced by DTT. When C138S−C32S and C138S−C35S are characterized, it will not be possible to use ammonium ion to demonstrate reduction of the mixed disulfide by DTT since Cys^{138} is altered.

Charge transfer complex formation in the presence of ammonium ion is a unique property of TrR C135S and is considered to be the result of an interaction between the thiolate in Cys^{138} and the flavin ring (Prongay *et al.*, 1989). The low ϵ_{453} of TrR C135S relative to those of wild type TrR and TrR C138S may be due to the effect of the Cys^{138} thiolate on the flavin ring. Once the formation of the mixed disulfide between TrR C135S and Tr C32S occurs, the thiolate in Cys^{138} is no longer present. Consequently, Cys^{138} cannot form a charge transfer complex in the presence of

[3] Personal communication from Drs. Brett W. Lennon, Martha L. Ludwig, and Charles H. Williams, Jr., University of Michigan.

ammonium cation. The influence of the Cys^{138} thiolate on the FAD is not present, and the value of ϵ_{454} is similar to that of wild type TrR.

A conformational change has been proposed as an integral step during catalysis by TrR. This suggestion is based on a comparison of the crystal structure of TrR with the structure of glutathione reductase (Kuriyan *et al.*, 1991; Waksman *et al.*, 1994). As shown in the cartoons in Chart 1, in the FR conformation, the active site thiols of TrR are on the surface of the protein where they are accessible to the substrate thioredoxin; thus, dithiol/disulfide interchange can take place. However, after the interchange reaction and the release of the reduced thioredoxin, the active site disulfide of the enzyme cannot be reduced by $FADH_2$ because it is too far from the flavin ring. The reduction of active site disulfide by $FADH_2$ can only take place after it rotates back to the FO conformation, prior to the next cycle of catalysis. Mixed disulfide formed when TrR was in the FR conformation and locked TrR in this conformation, in which the disulfide bond would not be close enough to be reduced directly by $FADH_2$.

Reductive titrations of C135S−C32S indicate that the FAD can be reduced by 1 equiv of either NADPH or dithionite. Treatment with NH_4Cl following reoxidation of the flavin does not result in thiolate−FAD charge transfer, showing that the mixed disulfide is still intact. This indicates that the flavin and the mixed disulfide are not in contact and suggests that the enzyme is in the FR conformation. DTT treatment of the reoxidized complex breaks the mixed disulfide bond of C135S−C32S which had not been reduced by $FADH_2$. As depicted in Chart 1D, the presence of thioredoxin blocks reverse rotation so that electrons cannot flow from $FADH_2$ to the mixed disulfide bond.

The results of the steady state kinetic analysis support the suggestion that the mixed disulfide is held in the FR conformation. The NADPH−$APyADP^+$ transhydrogenase activity of C135S−C32S is about 1.5-fold higher than the value for TrR C135S (Table 2). In the FR conformation, the bound NADPH is in close contact with FAD; that is, the nicotinamide ring is juxtaposed with the isoalloxazine ring, allowing for the efficient hydride transfer. As expected, C135S−C32S has no detectable thioredoxin reduction activity, because there is no electron path from $FADH_2$ to the disulfide in the FR conformation.

The mechanism shown in Scheme 1 presupposes a catalytic cycle from EH_2 to EH_4 to EH_2 as indicated in turnover experiments and a ternary complex as indicated in a study of the reoxidation of reduced TrR by Tr (Lennon & Williams, 1995, 1996). In the first step of the catalytic cycle (after the priming reaction), $FADH_2$ reduces the disulfide. When NADPH binds, rotation is required before hydride transfer to the flavin can occur as depicted in Chart 1B,C. When thioredoxin reductase is in the FR conformation, its flavin and pyridine nucleotide-binding domains are oriented toward each other as are the analogous domains of glutathione reductase (Chart 1A,C). The domains are held in the FR conformation while Tr is bound as indicated in Chart 1D. Dithiol/disulfide interchange can then occur between bound Tr and the dithiol now rotated toward the solvent. $FADH_2$ cannot initiate the second catalytic cycle until reverse rotation has occurred (Scheme 1). All of the data presented in this study are in accord with this picture of the catalytic cycle.

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