Formation and properties of mixed disulfides between thioredoxin reductase from *Escherichia coli* and thioredoxin: Evidence that cysteine-138 functions to initiate dithiol-disulfide interchange and to accept the reducing equivalent from reduced flavin

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Abstract

Mutation of one of the cysteine residues in the redox active disulfide of thioredoxin reductase from *Escherichia coli* results in C135S with Cys¹³⁸ remaining or C138S with Cys¹³⁵ remaining. The expression system for the genes encoding thioredoxin reductase, wild-type enzyme, C135S, and C138S has been re-engineered to allow for greater yields of protein. Wild-type enzyme and C135S were found to be as previously reported, whereas discrepancies were detected in the characteristics of C138S. It was shown that the original C138S was a heterogeneous mixture containing C138S and wild-type enzyme and that enzyme obtained from the new expression system is the correct species. C138S obtained from the new expression system having 0.1% activity and 7% flavin fluorescence of wild-type enzyme was used in this study. Reductive titrations show that, as expected, only 1 mol of sodium dithionite/mol of FAD is required to reduce C138S. The remaining thiol in C135S and C138S has been reacted with 5,5'-dithiobis-(2-nitrobenzoic acid) to form mixed disulfides. The half time of the reaction was <5 s for Cys¹³⁸ in C135S and approximately 300 s for Cys¹³⁵ in C138S showing that Cys¹³⁸ is much more reactive. The resulting mixed disulfides have been reacted with Cys³² in C35S mutant thioredoxin to form stable, covalent adducts C138S–C35S and C135S–C35S. The half times show that Cys¹³⁸ is approximately fourfold more susceptible to attack by the nucleophile. These results suggest that Cys¹³⁸ may be the thiol initiating dithiol-disulfide interchange between thioredoxin reductase and thioredoxin.

Keywords: dithiol-disulfide interchange; flavoprotein; thioredoxin reductase

Thioredoxin reductase from *Escherichia coli* is a pyridine nucleotide-disulfide oxidoreductase containing FAD and a disulfide in each active site. The redox active disulfide is composed of Cys¹³⁵ and Cys¹³⁸. Reducing equivalents move from NADPH to FAD, from

reduced flavin to the active site disulfide; dithiol-disulfide interchange effects reduction of the substrate thioredoxin. The redox active disulfide of thioredoxin is composed of Cys³² and Cys³⁵. Modification by site-directed mutagenesis of the active site in the enzyme has produced the single thiol mutants C138S (with Cys¹³⁵ remaining from the active site disulfide) and C135S (with Cys¹³⁸ remaining) (Prongay et al., 1989). The spectral characteristics of C135S and flavin analog studies have determined that the free thiol of Cys¹³⁸ interacts more closely with the flavin than does Cys¹³⁵ of C138S (Prongay et al., 1989; Prongay & Williams, 1990, 1992). The crystal structure confirms that Cys¹³⁸ is closer to the flavin (Kuriyan et al., 1991; Waksman et al., 1994).

The disulfide bond in the related enzyme glutathione reductase is virtually perpendicular to the plane of the flavin ring so that only one thiol can interact with the flavin while the other thiol is clearly positioned to initiate dithiol-disulfide interchange with glutathione disulfide (Karplus & Schulz, 1987); a similar arrangement is seen

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Abbreviations: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); TNB anion, 5-thio-2-nitrobenzoate anion; DTT, 1,4-dithiothreitol; DPDS, 4,4'-dithiodipyridine; PDS, 4-thiopyridone; IPTG, isopropyl β -D-thiogalactoside; C138S and C135S, thioredoxin reductase in which Cys¹³⁸ or Cys¹³⁵ has been changed to Ser, respectively; C32S and C35S, thioredoxin in which Cys³² or Cys³⁵ has been changed to Ser, respectively; C138S–C35S, a covalent adduct where the remaining active site thiol of C138S is linked via a disulfide to the remaining active site thiol of C35S, analogous designations are used for the three other possible combinations of mutated thioredoxin reductases and thioredoxins.

in lipoamide dehydrogenase (Mattevi et al., 1991). While it is clear the Cys¹³⁸ interacts with the flavin in thioredoxin reductase, the thiol initiating dithiol-disulfide interchange is not known. A large conformational change is required to relocate the nascent dithiol from its buried position (the FO conformation) to the surface (FR conformation) where it can react with thioredoxin (Waksman et al., 1994). The two conformations, the FO from the crystal structure and the FR model, are diagrammed in Figure 1. A conformational change is not required in glutathione reductase or lipoamide dehydrogenase. Examination of the relative positions of the two thiols suggests that in the FR model, the thiol of Cys¹³⁸ is more exposed than is the thiol of Cys¹³⁵. The caveat required for this suggestion is that the FO to FR conformational change involves nothing more than rotation of the pyridine nucleotide-binding domain relative to the flavin-binding domain (Waksman et al., 1994).

The first thiol-disulfide interchange between reduced thioredoxin reductase and thioredoxin results in the formation of a mixed disulfide between the more reactive Cys³² of thioredoxin and one of the thiols of thioredoxin reductase (Kallis & Holmgren, 1980). The mixed disulfide breaks down in the subsequent thiol-disulfide interchange that forms reduced thioredoxin. These reactions can be arrested at the mixed disulfide stage if mutants of thioredoxin and thioredoxin reductase are involved. Thus, C32S, having Cys³⁵ present as a mixed disulfide with TNB, will react with the free thiol of Cys¹³⁸ in C135S to form the stable mixed disulfide C135S–C32S that is restricted to the FR conformation (Wang et al., 1996). The preparation and characterization of two of the three remaining mixed disulfides, C135S–C35S and C138S–C35S are reported here. C138S–C32S has been prepared but it appears unstable, possibly due to base catalyzed hydrolysis of the mixed disulfide (Andersson, 1970). Crystals of C135S–C35S diffract to 3.5 Å and analysis of the structure is in progress (Lennon et al., 1997a).

The expression system for the gene encoding thioredoxin reductase and its active site mutations C135S and C138S has been re-engineered to allow for greater yields of protein (Mulrooney, 1997). The gene sequences of the expression vectors and the properties of the expressed proteins were compared with previous reports (Prongay et al., 1989; Prongay & Williams, 1990, 1992). Wild-type enzyme and C135S were found to be as previously

reported, whereas discrepancies were detected in the characteristics of C138S necessitating a re-examination of this enzyme. The original C138S enzyme was isolated from cells grown in a lengthy 200 L fermentation run where ampicillin was used to maintain the plasmid. The resolution of two distinct enzyme components from that C138S led to the conclusion that the preparation was contaminated by wild-type enzyme (Veine & Williams, 1997). Experiments that involved the suspect C138S preparation (Prongay et al., 1989; Prongay & Williams, 1990, 1992) have been repeated using C138S from several preparations employing the new expression system, and are reported here.

Results and discussion

Construction and analysis of enzymes from the new expression system

The properties of the expressed proteins of C138S, C135S, and wild-type enzyme from the re-engineered plasmids (Mulrooney, 1997) were compared with previous reports (Prongay et al., 1989; Prongay & Williams, 1990, 1992). Wild-type enzyme and C135S were found to be as described, whereas discrepancies were detected in the characteristics of C138S. The previous report indicated that C138S retained 50% activity and 65% flavin fluorescence compared with wild-type enzyme. The high activity was not as surprising a result as might first appear, in light of the related flavoenzyme NADH peroxidase, which has a single redox active thiol that is oxidized to the sulfoxide in catalysis and is very active (Poole & Claiborne, 1989). However, mutation of the cysteine residues comprising the redox active disulfide has been effected in lipoamide dehydrogenase, glutathione reductase, and mercuric reductase, and have demonstrated a near complete loss of catalytic activity (Williams, 1992). The resolution of two distinct enzyme components from C138S(pAJP2) enzyme briefly described in a preliminary report led to the conclusion that the original preparation was contaminated by wild-type enzyme (Veine & Williams, 1997).

The fluorescence excitation spectrum shows that the fluorescence of C138S is 7% that of wild-type enzyme. The overall shape of the excitation spectrum is similar to that of its absorption spectrum. Examination of the absorbance spectrum shows that the extinction at 448 nm is essentially independent of pH and ionic strength. Fluorescence as a function of pH was determined with enzyme at 100 mM ionic strength. It was found that samples behaved as in wild-type enzyme where above pH 8.3 the samples were nearly twice as fluorescent as those at a lower pH. The pH dependence of the fluorescence of wild-type enzyme indicates a $pK_a = 7.0$ in 55 mM phosphate, 12 °C (O'Donnell & Williams, 1983).

C138S has very low activity (Table 1), approximately 0.1% that of wild-type enzyme at a fixed concentration of NADPH and thioredoxin. Considering the extremely low level of activity, a full series of steady-state assays was not performed.

Separation and analysis of two distinct species from C138S(pAJP2)

It is known that wild-type thioredoxin reductase contains four cysteine residues: two are buried in the protein structure and the other two form the active site disulfide, thus separation of a disul-

Fig. 1. Representations of the C138S mutant of TrxR in the FO and postulated FR conformers. The FAD and pyridine nucleotide domains are indicated and connected by lines depicting the antiparallel double-stranded β -sheet. The three circles represent the FAD, and PN indicates bound pyridine nucleotide. Although the difference between the FO and FR is shown as a 180° flip in this illustration, it is postulated actually to be 66°. The same situation exists for the C135S mutant with the positions of the thiol and serine reversed. It is assumed that only the FR conformation can react to form mixed disulfides with TNB or mutant thioredoxin.

Table 1. Comparison of C138S thioredoxin reductase fractions

	Wild-type enzyme	C138S	C138S (pAJP2)	Fraction 1	Fraction 2
% of sample from column	—	—	—	57	43
Vis max nm	456	448	453	456	448
Vis max ϵ mM ⁻¹ cm ⁻¹	11.3	11.8	11.0	11.3	11.5
Activity (%)	100	0.1	50	100	7
Relative fluorescence (%)	100	7	65	100	38
Thiols (native)	0.1	1.1	0.4	0.1	0.8
Thiols (denatured)	2.0	3.0	2.4	2.0	2.8

fide (wild type) and a thiolate (C138S) was achieved using a Pierce TNB agarose column (see Methods). With the column in the TNB-mixed disulfide form, the free thiol of Cys¹³⁵ in C138S(pAJP2) formed a mixed disulfide with the column matrix displacing TNB, and allowed the contaminating wild-type enzyme to pass through. The two fractions eluted from the column, Fraction 1, noncovalently bound to the column and Fraction 2, covalently bound, were characterized and compared to wild-type enzyme, C138S and C138S (pAJP2) (Table 1).

The thiol content of wild-type enzyme, C138S, C138S(pAJP2), and the two fractions were determined using DTNB, in the absence (native) and in the presence of 6 M guanidinium-Cl (denatured). As expected, a total of three thiols were detected in the denatured protein of C138S. One thiol was detected in the native C138S, corresponding to the conversion of the active site disulfide to a single thiol (Table 1). Comparing the properties of C138S(pAJP2) with those of wild-type enzyme or Fraction 1 on the one hand, and with those of C138S or Fraction 2 on the other, it is clearly seen that C138S(pAJP2) was a heterogeneous mixture of wild-type and C138S enzyme (Table 1). It is of interest to note that a spectrum constructed by adding 50% of the wild-type enzyme spectrum and 50% of the C138S spectrum has a wavelength maximum, extinction coefficient and shoulder shape very similar to the spectrum of C138S(pAJP2) (data not shown). In the original study of C138S(pAJP2) (Prongay et al., 1989) three thiols were found, verifying the mutation of the active site cysteine. The value of 2.4 thiols found in the present study would directly support the theory that C138S(pAJP2) is a mixture of wild-type and C138S enzyme. However, the lower thiol titer could be due to the aging of the C138S(pAJP2).

The pAJP2 DNA, encoding the C138S mutation, was verified by sequencing following the original construction (Prongay et al., 1989). A recent reread of this sequence is consistent with Ser at position 138. Upon incorporation of the gene into the new plasmid (Mulrooney, 1997), the sequence of the entire thioredoxin reductase gene was verified. After detection of a significant difference in characteristics of the enzyme from the new plasmid system with the original enzyme, the DNA from both plasmids was verified again. Lacking evidence of contamination of the plasmid DNA preparations with wild-type enzyme sequences, it is conceivable that some type of plasmid reversion or recombination event early in the long culture growth (ca. 30 h), possibly unique to the pAJP2-host system, may have resulted in the observed wild-type contamination. The source of C138S(pAJP2) was the single 200 L fermenter run that produced 0.7 g of enzyme. All published experiments were performed with enzyme from this single large preparation (Prongay et al., 1989; Prongay & Williams, 1990, 1992; Kuriyan et al., 1991); experiments involving the C138S(pAJP2) enzyme have been repeated using C138S enzyme from the new expression system and are reported here. It is worthwhile to note that multiple preparations of C138S from the new pTrR336/*E. coli* A326 expression system have yielded enzyme with consistent and reproducible characteristics. At present the source of the wild-type enzyme contamination in C138S(pAJP2) remains unknown, and we have not directed our efforts toward diagnosing its cause. The data obtained for C138S from the new expression system (100% C138S) have been methodically compared with data for C138S(pAJP2) (50% C138S). Only a few significant differences in the characteristics of C138S have been found, notably the decreased catalytic activity and spectral properties. These do not in any way alter the conclusion reached in the earlier work that Cys¹³⁸ (in C135S) influences the flavin more strongly than does Cys¹³⁵ in C138S. C138S served largely as a control in these studies (Prongay et al., 1989; Prongay & Williams, 1990, 1992) reporting the characterization, flavin analog, and redox potential studies of both C135S and C138S enzymes. The data for C135S are correct as described. However, considering the extent of the contamination, of C138S(pAJP2) enzyme, the data for C138S require the following corrections.

Spectral changes in anaerobic, reductive titrations of C138S enzyme

Figure 2 shows the changes in the C138S spectrum after exposure to light under anaerobic conditions in the presence of high concentrations of EDTA. The spectra demonstrate the characteristic appearance of a long wavelength band associated with the forma-

Fig. 2. Photoreduction of C138S enzyme. A 40 μ M solution of enzyme in 50 mM Na/K phosphate, pH 7.6, containing 30 mM EDTA and 2.5 μ M 5-deaza-FAD was photoreduced by exposure to bright light at 14 °C. The spectra are of oxidized enzyme and enzyme photoreduced for 3 min, 20 min, 2 h, 5 h, 12 h. Inset, plot of the extinction at 585 nm versus time. The data were fit to a 2 exponential curve giving rate constants of 0.183 and 0.0048 min⁻¹.

Fig. 3. Dithionite titration of C138S enzyme. A solution of 25 μM C138S enzyme was titrated anaerobically with sodium dithionite in the presence of a catalytic amount of methyl viologen (1 μM). The buffer conditions of the titration were 0.1 M Na/K phosphate pH 7.6, 20°C. Inset. Plot of the percent oxidized enzyme versus eq of dithionite. The fraction of oxidized enzyme has been corrected for semiquinone using the equation $[E_{ox}] = [E_t] - (\frac{1}{2}[E_{sq}] + [E_{red}])/[E_t]$.

tion of a blue, neutral flavin semiquinone. The rate of semiquinone formation is as seen in wild-type enzyme (Zanetti et al., 1968) where semiquinone forms biphasically (Fig. 2, inset) and reduction beyond semiquinone requires extensive exposure to light (data not shown).

Reductive titrations of C138S with sodium dithionite were performed in the presence of a catalytic quantity of methyl viologen at pH 7.6 and 20°C (Fig. 3). The increasing absorbance at 585 nm indicates the formation of the same blue, neutral semiquinone observed during photoreduction. Extrapolation of the absorbance values at 585 and 448 nm indicates that nearly 80% semiquinone was formed. It was determined, as originally expected, that after correction for semiquinone, 1 mol of dithionite/mol of FAD was required to fully reduce the enzyme (Fig. 3, inset). The previous report (Prongay & Williams, 1992) that 1.8 eq of dithionite was required to fully reduce this enzyme provides, in retrospect, corroborating evidence that the original enzyme contained some wild-type enzyme.

Flavin analog replacement experiments of C138S enzyme

C138S reconstituted with 6-thiocyanato-FAD had an absorbance spectrum nearly identical to that previously found with a maximum at 448 nm, which decreased slightly as the pH was changed from 5.54 to 9.93, and a shoulder at 385 nm, which became more pronounced at higher pH values (data not shown). There was no evidence of conversion of the 6-thiocyanato-FAD to 6-mercapto-FAD, confirming previous results that the remaining thiol Cys¹³⁵ is too distant to catalyze this reaction (Prongay & Williams, 1990). The absorbance spectrum of 1-deaza-FAD C138S was similar to

wild-type enzyme with 1-deaza-FAD bound, except that the maximum is at 535 nm instead of 548 nm. The maximum previously reported for C138S(pAJP2) was 544 nm. As previously reported, there is no indication of a 414 nm absorbance from a C4a-thiolate adduct (data not shown); this adduct involves Cys¹³⁸ and, thus, forms only with C135S.

Potentiometric titrations of C138S enzyme

The midpoint potential for C138S E_{ox}/E_{red} was redetermined from NADH/NAD⁺ titrations in the pH range 6.0–9.0 and 20°C. Nernst plots show that the data correspond to theoretical lines determined for 2-electron reduction (Fig. 4A). The E_m was –309 mV at pH 7.0 and 20°C. In comparison, the midpoint potential of C135S was –280 mV, similar to that of the FAD/FADH₂ couple in the dithiol form of wild-type enzyme, –270 mV at pH 7.0 and 20°C (Prongay & Williams, 1992; O'Donnell & Williams, 1983). The $\Delta E_m/\Delta\text{pH}$ for C138S is –56 mV, which corresponds to a proton stoichiometry of $2\text{H}^+/2e^-$ (Fig. 4B). Detailed explanations of the calculations are in the previous paper (Prongay & Williams, 1992). It was not possible to determine the midpoint potentials of the two 1-electron couples, E_{sq}/E_{red} and E_{ox}/E_{sq} , associated with the formation of a semiquinone intermediate. This may have been due to the difficulty in reducing the semiquinone. K_{eq} in the relationship $K_{eq} = [E_{sq}]^2/[E_{red}][E_{ox}]$ did not remain constant.

Thiol reactivities and properties of C135S–C35S and C138S–C35S

Several previous studies have examined the reactivity and pK_a values of the thiols of thioredoxin (Kallis & Holmgren, 1980; Li et al., 1993; Jeng et al., 1995), and have found that Cys³² has a lower pK_a and is more reactive than Cys³⁵. Reactions of protein thiols with low molecular weight disulfides in a thiol-disulfide interchange reaction are known to correlate with the thiol pK_a more than the particular protein tertiary structure (Shaked et al., 1980). The free thiol of C138S, C135S, C32S, and C35S readily forms a TNB-mixed disulfide when reacted with excess DTNB, and the relative rates are given in Table 2. As expected in thioredoxin, the thiol-disulfide interchange thiol Cys³² reacts more rapidly than Cys³⁵. Table 2 also shows that Cys¹³⁸ is more reactive with DTNB than Cys¹³⁵. After purification on a gel filtration column, the TNB-mixed disulfides can be reduced with DTT and the TNB anion released shows that one mixed disulfide is present per mole of protein monomer. It is of interest that the relative rates of these reactions are the inverse of the rates of reaction of the thiols with DTNB (Table 2). This indicates that the sulfur of Cys³⁵ is more electrophilic than that of Cys³² and that the sulfur of Cys¹³⁵ carries less negative charge than that of Cys¹³⁸ in the TNB-mixed disulfides, remembering that in all four reactions, the attacking nucleophile, DTT is the same and the leaving group, TNB anion is the same.

In comparing the reactivities of the free thiols with DTNB and of the mixed disulfides with DTT (Table 2), it is assumed that the structure of Cys³² in C35S is exposed and that Cys³⁵ in C32S is buried as they are in wild-type thioredoxin. Comparing the structures of reduced wild-type human thioredoxin with that of the C32S/C35S double mutant shows that positions of the oxygen atoms in the mutant are essentially the same as the sulfur atoms (to within 1.1 Å for S/O at position 32 and 0.3 Å for S/O at position 35, RMS = 0.20 Å for all C_α atoms) in the wild-type thio-

Fig. 4. Dependence of the redox potential of the FAD/FADH₂ couple in C138S enzyme on pH. **A:** C138S (35 μ M) was equilibrated with mixtures of NADH and NAD⁺ in buffers described in Methods; top to bottom pH ([NAD⁺] in parentheses) 6.0 (123 μ M), 6.45 (127), 6.8 (84), 7.25 (48), 7.7 (2 data sets, 96 and 34), 8.2 (0), 8.5 (0). The lines through the data points assume a slope consistent with 2 electron reduction. **B:** The data from A have been plotted as a function of pH. The line drawn through the data points indicates a slope of 56 mV/pH unit (2 protons/2 electrons). The dotted line gives the slope of 29 mV/pH unit (1 proton/2 electrons). $E_m = -309$ mV for the NAD⁺/NADH couple at 20 °C.

redoxin (Weichsel et al., 1996). This makes it likely that in the reaction with DTNB, the colinearity of the three sulfurs involved in thiol-disulfide interchange would be more easily maintained with the more exposed Cys³² than with Cys³⁵. This might not be the case in the reaction of the mixed disulfides with DTT, and care must be exercised in ascribing greater electronegativity to Cys³².

The data in the last four columns of Table 2 indicate that when the attacking nucleophile is the free thiol of a thioredoxin mutant or of a thioredoxin reductase mutant, the reactivity of the thiol determines the rate of reaction with the TNB-mixed disulfide. The relative rates reinforce the fact that Cys¹³⁸ is the more available thiol in thioredoxin reductase and confirms that Cys³² is the more

available thiol in thioredoxin. These comparisons follow principles summarized in a study by Whitesides et al., 1983.

The suggestion that the sulfur of Cys¹³⁵ is more electrophilic than that of Cys¹³⁸ in a TNB-mixed disulfide is based on the twofold lower reactivity of Cys¹³⁸ with DTT. This twofold difference may be due to the proximity of Asp¹³⁹, which is adjacent to Cys¹³⁸ and was found to serve as the acid/base catalyst for this enzyme (Mulrooney & Williams, 1994). The nearest oxygen of Asp¹³⁹ is 5.1 Å from the sulfur of Cys¹³⁸, while the closest approach to Cys¹³⁵ is 6.3 Å. The influence of a negative charge of Asp¹³⁹ on Cys¹³⁸ would, therefore, be slightly larger than its influence on Cys¹³⁵. Thus, Cys¹³⁸ may initiate thiol-disulfide interchange in thioredoxin reductase catalysis. If this is the case, the "working hypothesis" mechanism proposed earlier (Lennon et al., 1997b) will have to be modified as shown in Figure 5, where the roles previously ascribed to Cys¹³⁵ and Cys¹³⁸ have been reversed.

Several previous studies, as well as the present work, provide evidence for the existence of the FR conformation of thioredoxin reductase. The TNB-mixed disulfide of C32S reacting with the free thiol of Cys¹³⁸ in C135S forms the stable mixed disulfide C135S-C32S (Wang et al., 1996). The properties of this enzyme-substrate complex are consistent with the enzyme being restricted to a conformation in which the pyridine nucleotide and the flavin can interact but the flavin and the redox active disulfide are not juxtaposed (Fig. 1).

Three other mixed disulfides are possible and the present work establishes that two of them have properties that are consistent with the enzyme component being restricted to the FR conformation. C135S-C35S has been prepared by the same procedure used for C135S-C32S (Wang et al., 1996). Table 2 shows that the reaction of Cys¹³⁸ in C135S with either C32S or C35S TNB-mixed disulfide is a fairly facile reaction. The spectral properties and characteristics of the reductive titrations with NADPH and dithionite are nearly identical to those observed for C135S-C32S (Wang et al., 1996) (data not shown). In addition, crystals of C135S-C35S diffract to 3.5 Å, and analysis is in progress (Lennon et al., 1997a). In an analogous study, site directed mutagenesis has been used to engineer a thiol into each of the two domains of the enzyme; when they are crosslinked with a bifunctional reagent, the enzyme is restricted to the FO conformation (Veine et al., 1998).

C138S-C35S cannot be prepared in high yield using the method of reacting enzyme with substrate TNB-mixed disulfide that works well for both C135S-C32S and C135S-C35S, because the reaction time is long (last column, Table 2) thus allowing side reactions to occur. C138S-C35S was, therefore, obtained by the more favorable reaction of Cys³² in C35S with the TNB-mixed disulfide of C138S (Table 2). C138S-C35S was also prepared using the mixed disulfide between C138S and 4,4'-dithiodipyridine (see Methods). Figure 6 shows the spectrum of C138S-C35S compared to that of C138S and wild-type thioredoxin reductase. It is observed that upon formation of the enzyme-substrate adduct the visible maximum is shifted from 448 nm to 453 nm, the extinction is enhanced from 11.8 to 13.2 mM⁻¹ cm⁻¹, and the relative fluorescence is increased by 20-fold.

Previous studies of thioredoxin reductase involving rapid reaction kinetics on wild-type and several mutants (Lennon & Williams, 1997) and on native and thiol reagent-treated C138S (Mulrooney & Williams, 1997) have lead to the hypothesis that the FO and FR forms of the enzyme are in an equilibrium as depicted in Figure 1. C138S is mostly in the FO conformation with characteristic low fluorescence due to proximity of Ser¹³⁸ to the flavin fluorophore.

Table 2. Reactivity of the remaining thiol in C138S, C135S, C32S, C35S

Mutant	Reactive thiol	$t_{1/2}$ (s) 20× DTNB	TNB-mixed disulfide	$t_{1/2}$ (s) 25× DTT	$t_{1/2}$ C32S	$t_{1/2}$ C35S	$t_{1/2}$ C135S	$t_{1/2}$ C138S
C32S	Cys ³⁵	~60	C32S-TNB	<1	—	—	~1 min	>1 h
C35S	Cys ³²	<1	C35S-TNB	~20	—	—	~1 min	>1 h
C135S	Cys ¹³⁸	<5	C135S-TNB	~50	~10 min	<5 s	—	—
C138S	Cys ¹³⁵	~300	C138S-TNB	~25	>1 h	~20 s	—	—

Reaction of the buried Cys¹³⁵ with a thiol specific reagent, because of steric and hydrophobic effects, forces the equilibrium toward the FR conformation with a concomitant increase in fluorescence as Ser¹³⁸ moves away from the flavin. Use of a larger molecular weight thiol reagent results in a larger fluorescence: methylmethane thiosulfonate gave an 8.5-fold increase in fluorescence, and phenylmercuric acetate a 9.5-fold increase. The disulfide linked C138S–C35S complex has a much larger thioredoxin adduct formed on Cys¹³⁵ resulting in a 20-fold increase in fluorescence over untreated C138S. This is consistent with complete restriction to the FR conformation since no conformational equilibrium could exist with such a large adduct on Cys¹³⁵. This provides further support for a FO ↔ FR equilibrium model.

The shift in conformation is confirmed in reductive titrations with dithionite or NADPH. Monitoring both absorbance and fluorescence, the titrations show that the FAD is reduced by about one equivalent of dithionite or NADPH per FAD (Figs. 7, 8 and insets) and show that the mixed disulfide bond is not able to accept reducing equivalents because it is unavailable in the FR conformation. Reoxidation of the flavin by exposure to air returns the flavin to the high extinction expected for C138S–C35S and restores the high relative fluorescence, confirming that the disulfide bond in the enzyme-substrate adduct remains intact (Figs. 7, 8, insets, open circle). Note that NADPH does not reduce the enzyme stoichiometrically and extrapolation to exactly 1 equivalent/FAD

is not expected (O'Donnell & Williams, 1983). Thus, C138S–C35S is restricted to the FR conformation just as C135S–C32S and C135S–C35S were shown to be.

C138S–C32S cannot be reliably prepared due to the low reactivity of Cys¹³⁵ and Cys³⁵ (Table 2). The relative rates of all of these reactions are consistent with the notion that Cys¹³⁸ is more reactive than Cys¹³⁵ and therefore may be the interchange thiol.

PAGE analysis

C138S–C35S and C138S–C32S were examined on nonreducing SDS-PAGE for the purity of the adduct. A typical gel analysis for the C138S–C35S disulfide-linked complex is shown in Figure 9. Both C138S–C35S and C138S–C32S always showed some degree of reactants remaining, as well as high molecular weight bands associated with incomplete dissociation of the protein in the absence of 2-mercaptoethanol. To test whether the alkaline conditions of the gel were leading to breakdown of the complex, SDS-PAGE modified to low and neutral pH conditions still showed the presence of some reactants. It appears that the adducts formed with C138S decay under the conditions of SDS-PAGE. Sample monitored for fluorescence and absorbance changes at pH 7.6 do not demonstrate the same decay of the adduct. C135S–C35S and C135S–C32S clearly show the disulfide-linked complexes without the presence of the reactants on SDS-PAGE (Wang et al., 1996).

Fig. 5. A "working hypothesis" mechanism of thioredoxin reductase (Williams, 1995). FAD and the redox active disulfide are juxtaposed in the FO conformation and the nicotinamide ring and the flavin ring are juxtaposed in the FR conformation as indicated by the position of the crook in the backbone (lower left). In the redox active disulfide, Cys¹³⁸ is known to interact more closely with the flavin (Prongay & Williams, 1990) and Cys¹³⁸ is also envisaged as the interchange thiol. Asp¹³⁹ is the proposed acid catalyst of dithiol-disulfide interchange (Mulrooney & Williams, 1994). Adapted from Lennon et al. (1997b).

Fig. 6. Spectra of C138S-C35S compared to wild-type and C138S thioredoxin reductase. **A:** C138S-C35S. **B:** C138S. **C:** Wild-type thioredoxin reductase. The enzymes were in 50 mM Na/K phosphate, pH 7.6.

Conclusion

Earlier spectroscopic studies have shown that Cys¹³⁸ interacts more closely with the flavin than Cys¹³⁵ (Prongay et al., 1989; Prongay & Williams, 1990, 1992), and this was subsequently confirmed by the crystal structure (Kuriyan et al., 1991; Waksman et al., 1994). Examination of the model of the FR conformation indicates that Cys¹³⁸ is in a more exposed position to react with the large thioredoxin substrate than is Cys¹³⁵ (Waksman et al., 1994). All of these findings, considered together, suggest that Cys¹³⁸ fulfills a dual role accepting the reducing equivalent from the flavin in the FO conformation and initiating dithiol-disulfide interchange in the FR conformation (Fig. 5). This contrasts with other members of the enzyme family such as glutathione reductase and lipoamide dehydrogenase in which each of the active site thiols performs only one of these roles (Karplus & Schulz, 1987; Mattevi et al., 1991).

Methods

Enzyme and substrate preparation

Purification of C138S(pTrR336) (Mulrooney & Williams, 1997) enzyme was described in Mulrooney (1997). C138S(pAJP2) enzyme was a sample purified from the 200 L fermentation described in Prongay et al. (1989) that has been stored at -20°C . Verification of the sequence for the entire thioredoxin reductase gene of both pTrR336 and pAJP2 was performed by automated methods at the University of Michigan Biomedical Research Core Facility. 1-deaza-FAD and 6-thiocyanato-FAD as previously described (Prongay & Williams, 1990) were gifts from Dr. Vincent Massey of the University of Michigan.

The plasmids containing the gene for the thioredoxin mutants C32S and C35S (Russel & Model, 1986) were obtained from Dr. Marjorie Russel and modified for a high yield of protein. Plasmid

Fig. 7. Dithionite titration of C138S-C35S enzyme-substrate adduct. A solution of 28 μM C138S-C35S was titrated anaerobically with sodium dithionite in the presence of a catalytic amount of methyl viologen (1 μM). The spectra are of the mixture after addition of 0, 0.32, 0.65, 0.97, 1.4, and 1.8 eq of dithionite. **Inset A:** Plot of the extinction at 453 nm versus eq of dithionite. **Inset B:** Relative fluorescence emission at 540 nm and excitation at 450 nm versus eq of dithionite. The open circle represents the fluorescence value after reoxidation by air. The buffer conditions of the titration were 50 M Na/K phosphate pH 7.6, 25°C . Note that both the fluorescence and absorbance data indicate a small amount of residual oxygen at the beginning of the titration.

pPMR5-trxA6 (C35S) and pPMR5-trxA3 (C32S) were digested with restriction enzyme *Hind*II and the fragment containing the mutant thioredoxin gene was isolated and purified using published procedures (Mulrooney, 1997). In parallel, expression vector pSE420 (Brosius, 1992) was digested with *Sma*I, which cuts once within the multiple cloning site downstream of a *trp/lac* promoter, treated with calf intestinal phosphatase, and purified. The fragments of thioredoxin mutant and plasmid DNA were ligated and transformed into *E. coli* XL1-Blue for screening. Clones that showed increased expression of mutant thioredoxin upon IPTG induction were selected and designated ptrxA6-4 (C35S) and ptrxA3-4 (C32S). The new plasmids were transformed into *E. coli* strain A306 (*trxA*⁻) (Russel & Model, 1986), grown and purified as described for wild-type enzyme (Lennon & Williams, 1995). Typical yields: 100 mg/20 g cells.

The plasmid pDL59 (LeMaster & Richards, 1988) designed to express both *E. coli* and T4 thioredoxins was modified to remove a portion of the T4 thioredoxin gene and, thus, allowed easier purification of *E. coli* thioredoxin. Plasmid pDL59 was digested with *Cvn*I followed by appropriate reactions with *Bal*31 exonuclease, T4 DNA polymerase, and T4 DNA ligase. A plasmid that had about 200 base pairs deleted from the T4 thioredoxin gene was isolated and designated pDL59DT4. Thioredoxin was purified as previously described (Lennon & Williams, 1995) from cells grown overnight at 37°C in modified 2 $\times$ YT medium containing

Fig. 8. NADPH titration of C138S-C35S enzyme-substrate adduct. A solution of 28 μ M C138S-C35S was titrated anaerobically with NADPH. The spectra are of the mixture after addition of 0, 0.40, 0.73, 1.2, 1.9, and 2.3 eq of NADPH. **Inset A:** Plot of the extinction at 453 nm versus eq of NADPH. **Inset B:** Relative fluorescence emission at 540 nm and excitation at 450 nm versus eq of NADPH. The open circle represents the fluorescence value after reoxidation by air. The buffer conditions of the titration were 50 M Na/K phosphate pH 7.6, 25 $^{\circ}$ C.

100 μ g/mL ampicillin (Mulrooney, 1997). Typical yields of thioredoxin: 400 mg/40 g of cells.

All mutant enzyme and substrate samples were first pretreated with a 25-fold excess of DTT over flavin and incubated for 20–30 min at 25 $^{\circ}$ C to eliminate oxidation products. The products of the reaction were then separated by gel filtration using a Bio-gel P-6DG resin from Bio-Rad (Hercules, California).

Fig. 9. 12% SDS-PAGE of C138S-C35S adduct at high pH. Lane 1 contains molecular weight standards, beginning with the fastest band, 14.4, 21.5, 31, 45, 66.2, and 97.4 kDa; Lane 2, C138S-C35S under reducing conditions, 7.6 μ g of protein, C138S is 36 kDa and C35S is 12 kDa; Lane 3–5, C138S-C35S, nonreducing conditions, 5.1, 7.6, and 10.1 μ g of protein, respectively.

Enzyme characterization

The extinction coefficient of both C138S(pTrR336) and C138S(pAJP2) was determined from release of the protein bound FAD using SDS as a denaturant as described in Prongay et al. (1989). Thiol titer was determined with 25-fold excess DTNB over flavin in the presence or absence of 6 M guanidinium chloride in 0.1 M Na/K phosphate, 0.3 mM EDTA, pH 7.6. Titer was determined from the release of TNB anion and quantitated at 412 nm using the extinction value of 13,600 $M^{-1} cm^{-1}$.

Activity assays were performed with 50 μ M thioredoxin and 50 μ M NADPH using the coupled assay described in Prongay et al. (1989), without the addition of the NADPH regeneration system of glucose-6-phosphate dehydrogenase and its substrate. The spectral properties of C138S(pTrR336) were examined as a function of pH and ionic strength using methods previously described (Prongay et al., 1989).

TNB: Thiol column chromatography

A Pierce TNB-agarose column (Rockford, Illinois) was used to effect the separation of the proposed enzyme mixture of active site disulfide (wild type) and thiolate (mutant). A 4 mL column containing resin with TNB anion bound was equilibrated with 0.1 M Na/K phosphate, 0.3 mM EDTA, pH 7.6. C138S(pAJP2), after DTT treatment and separation by gel filtration, was applied to the column and further washed with buffer containing 1 M NaCl followed by elution with buffer containing both 1 M NaCl and 20 mM DTT. Fractions were collected, and volume was reduced using Amicon (Beverly, Massachusetts) Centricon-30 filtration units then washed with 0.1 M Na/K phosphate, pH 7.6. Only mutant enzyme containing a free thiol bound initially to the column while putative wild-type enzyme passed through the column.

Anaerobic spectral and potentiometric titrations

The photoreduction of C138S(pTrR336) enzyme (modified to 14 $^{\circ}$ C) and anaerobic titrations with sodium dithionite or NADPH were performed using methods previously described (Prongay & Williams, 1992). The E_{ox}/E_{red} midpoint potential of C138S(pTrR336) was determined in the pH range 6.0–9.0 at 20 $^{\circ}$ C. Buffer solutions and calculations were as previously described (Prongay & Williams, 1992). The problems of increasing turbidity and a spectrally silent second reducible center reported for C138S(pAJP2) (Prongay & Williams, 1992) were absent during titrations with C138S(pTrR336) enzyme; therefore, the experiments were performed as titrations instead of single point equilibrations. It is now presumed that the second redox center detected was the disulfide of the wild-type enzyme contaminant. Since turbidity was not detected during the anaerobic titrations, the titrations did not require the correction previously described (Prongay & Williams, 1992).

Flavin analog replacement

The apoprotein form of C138S(pTrR336) was prepared as described (Prongay & Williams, 1990), with the exception that NADP⁺ was omitted from the final dialysis. The apoenzyme was reconstituted by adding a slight molar excess of 6-thiocyanato-FAD or 1-deaza-FAD and unbound flavin was removed by washing in a Centricon-30 following published procedures (Prongay & Williams, 1990). The spectra of 6-thiocyanato-FAD C138S(pTrR336)

were measured as a function of pH using methods described (Prongay & Williams, 1990). The extinction coefficient of 1-deaza-FAD bound to C138S(pTrR336) was determined by adding excess apoenzyme to a solution of 1-deaza-FAD and observing the spectral changes.

Thiol reactivity experiments

After treatment with DTT to remove oxidation products as described above, the relative rates for formation of the mixed disulfides of C138S, C135S, C32S, and C35S were determined with about 20-fold excess DTNB in 50 mM Na/K phosphate, pH 7.6, at a concentration of 20–30 mM of reactive thiol. Reactions were observed starting at approximately 5 s. After completion of the reaction, the TNB-mixed disulfides were applied to a second Bio-gel P-6DG gel column to remove the products of the reaction. Relative rates of release of TNB from the purified TNB-mixed disulfides were examined with about 25-fold excess DTT. Reactions of TNB-mixed disulfides with enzyme (or substrate) were determined at near stoichiometric conditions. Stock solutions of 100 mM DTT in 50 mM Na/K phosphate at pH 7.6 were made fresh daily; DTNB solutions (25 mM) were prepared in 100 mM acetate buffer, pH 5.0.

Preparation and purification of C138S–C35S

The formation of C138S–C35S was found to be very slow using the procedure of reacting enzyme with substrate TNB-mixed disulfide as described in Wang et al. (1996). Therefore, the method was modified so that enzyme was the mixed disulfide, and substrate was the attacking thiolate. To compensate for the relatively slow reaction of Cys¹³⁵ with DTNB, the thiol reagent 4,4'-dithiodipyridine (DPDS) (Grassetti & Murray, 1967) was substituted. DPDS was found to react rapidly with Cys¹³⁵. The product 4-thiopyridone (PDS) absorbs at 324 nm and could be used to quantitate the reaction using the extinction coefficient of 19,800 M⁻¹ cm⁻¹. Another advantage of this reagent was that the absorbance of the product was far removed from the flavin peak of the enzyme and thus did not obscure spectral changes, allowing for direct determination of the extinction coefficient of the enzyme-substrate adduct.

The PDS-mixed disulfide of C138S was prepared in 50 mM Na/K phosphate, pH 7.6, and reacted with a slight excess of C35S (in the same buffer), monitoring at 324 nm for the release of PDS. Upon completion of the reaction ammonium sulfate was added to achieve a concentration of 1.7 M, the mixture was applied to a 2 × 6 cm phenyl sepharose CL-4B column from Pharmacia (Piscataway, New Jersey) equilibrated with 50 mM Na/K phosphate, pH 7.6 with 1.7 M ammonium sulfate. After washing with equilibrating buffer, buffer containing no ammonium sulfate was applied. Thioredoxin and unreacted C138S were eluted from the column until the absorbance at 280 nm returned to zero, then a solution of 50 mM Na/K phosphate, pH 7.6 with 10% acetonitrile was applied to elute the cross-linked material. Immediately following elution, cross-linked material was concentrated using an Amicon Centricon-30 filtration unit and applied to a gel filtration column equilibrated with 50 mM Na/K phosphate, pH 7.6. The purity of the adduct was examined using 12% PAGE under low, neutral, and high pH conditions (Blackshear, 1984). Denaturation of the samples in the low pH gels was achieved with cetylpyri-

dinium chloride (CPC), and at neutral and high pH was achieved with sodium dodecyl sulfate (SDS). Samples of the adduct were prepared without a reducing agent; standards contained 2-mercaptoethanol for complete dissociation. All samples were heated for 90 s at 90 °C in sample buffer prior to loading.

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