Oxidation-Reduction Properties of *Escherichia coli* Thioredoxin Reductase Altered at Each Active Site Cysteine Residue*

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The abbreviations used are: TRR(Ser135,Cys138), site-directed mutation of thioredoxin reductase with cysteine-138 replaced with a serine; TRR(Cys135,Ser138), site-directed mutation of thioredoxin reductase with cysteine-138 replaced with a serine; [E], total enzyme concentration; [Eo], concentration of reduced enzyme; [EoO2], concentration of oxidized enzyme; DTNB, 5,5'-dithiobis(nitrobenzoic acid).

Thioredoxin is a small oxidation-reduction (redox) mediator protein. Its reduction by NADPH is catalyzed by the flavoenzyme thioredoxin reductase. Site-directed mutagenesis has provided forms of the reductase in which Cys135 and Cys138 have each been changed to a serine residue (Prongay, A. J., Engelke, D. R., and Williams, C. H., Jr. (1989) *J. Biol. Chem.* 264, 2656–2664). Cys135 and Cys138 form the redox-active disulfide in the oxidized enzyme. The redox properties of the two altered forms of *Escherichia coli* thioredoxin reductase have been determined from pH 6.0 to 9.0. Photooxidation of TRR(Ser135,Cys138) produces the blue, neutral semiquinone species, which disproportionate (K = 0.73) to an apparent maximum of 29% of the total enzyme as the semiquinone. In contrast, the semiquinone formed on TRR(Cys135,Ser138) during a photoreductive titration does not disproportionate and 70% of the enzyme is stabilized as the semiquinone. Reductive titrations have demonstrated that 1 mol of sodium dithionite (2 electrons)/mol of FAD is required to fully reduce TRR(Ser135,Cys138) whereas 2 mol of dithionite/mol of FAD are required to fully reduce TRR(Cys135,Ser138). The midpoint potentials for the 1-electron and 2-electron reductions of TRR(Ser135,Cys138) have been determined by NADH/NAD+ titrations in the presence of a mediator, benzyl viologen. The midpoint potential for the 2-electron reduction of TRR(Ser135,Cys138) is −280 mV at pH 7.0 and 20 °C. Thus, the redox potential is similar to that of the FAD/FADH2 couple in the dithiol form of wild type enzyme. −270 mV (corrected to 20 °C) (O'Donnell, M. E., and Williams, C. H., Jr. (1983) *J. Biol. Chem.* 258, 13795–13805). The ΔEo/ΔpH is −57.1 mV, which corresponds to a proton stoichiometry of 2 H+/2 e−. A maximum of 19% of the enzyme forms a stable semiquinone species during the titration, and the potential for the oxidized enzyme/semiquinone couple, $E_o$, and the semiquinone/reduced enzyme couple, $E_0$, are −296 and −256 mV, respectively, at pH 7.0 and 20 °C. These studies provide evidence that the residue at position 138 exerts a greater effect on the FAD than does the residue at position 135.

*This work was supported by the the Health Services and Research Administration of the Department of Veterans Affairs and by Grant GM21444 from the National Institute of General Medical Sciences, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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and confirm these conclusions (Kuriyan et al., 1991). The effects of replacing these cysteine residues with serines on the oxidation-reduction properties of the FAD are presented.

MATERIALS AND METHODS

Reagents—TRR(Ser135,Cys138) and TRR(Cys135, Ser138) were purified as previously described (Prongay et al., 1989). NAD and NAD+ (Grade V) were purchased from Sigma. Sodium dithionite was obtained from the Fluka Chemical Corp. Lumiflavin-3-acetic acid and 3,10-dimethyl-5-deaza-isoalloxazine (Massey and Hemmerich, 1977; Massey et al., 1978) were the generous gifts of Dr. Vincent Massey, University of Michigan, Ann Arbor, MI. All other reagents and buffer sets were of the highest quality available.

Absorbance spectra were recorded with a Cary 118C spectrophotometer interfaced to an IBM-AT personal computer using a DATA Translation 2800 multipurpose board and the ASYST software package purchased from the Macmillian Software Co., and adapted by Glenn Piot and L. D. Arscoat, Department of Veterans Affairs Medical Center, Ann Arbor, MI.

Anaerobiosis—All titrations were performed under anaerobic conditions at 20 °C, except where noted. Anaerobiosis was performed as previously described (Williams et al., 1979; O'Donnell and Williams, 1983). In an attempt to minimize the amount of turbidity resulting from the denaturation of TRR(Ser135,Cys138) and TRR(Cys135, Ser138) during the anaerobic process, the buffer containing either a redox mediator (3,10-dimethyl-5-deaza-isoalloxazine, methyl viologen, or benzyl viologen) or NAD+ was made anaerobic by 15 alternating cycles of vacuum of 10-mm Hg and oxygen-free nitrogen or argon gas at 4 °C with vigorous vortexing. The volume loss was quantified by changes in absorbance at wavelengths below 300 nm. Enzyme solutions (20-100 μl, 1-4 μM) were added under positive pressure of either nitrogen or argon gas to the anaerobic buffer through an injection port in the anaerobic cell and the solution subjected to five additional alternating cycles of 10-nm Hg vacuum and nitrogen or argon gas at 4 °C, without vortexing, but with the vessel on its side in order to create maximal surface area for gas exchange. The solutions were scrupulously protected from light. Hamilton syringes containing anaerobic titrants were fitted to the anaerobic cuvette under positive pressure of either nitrogen or argon gas (Williams et al., 1979).

Photoreduction—The enzymes, in 1 ml of 50 mM sodium/potassium phosphate buffer, pH 7.6, containing 30 mM EDTA and 2 ml of 3,10-dimethyl-5-deaza-isoalloxazine were made anaerobic and reduced at 4 °C by exposure for varying time intervals to bright light from a Sun Gun (Smith-Victor Corp., Griffith, Ind., model Q-1U) at a distance of 7 cm from the sample. The intensity of the illumination was controlled by a rheostat (Massey et al., 1978). The absorbance spectra were recorded after each exposure as in Fig. 1. During the photoreduction of TRR(Cys135, Ser138), the maximum absorbance for the semiquinone formation was 70% of the total enzyme concentration. Therefore, the spectrum of the semiquinone was obtained by extrapolation. The first four spectra recorded during the photoreduction were composites of different ratios of oxidized and semiquinone forms of the enzyme. Therefore, in order to obtain the spectrum of the semiquinone species, the contribution of the oxidized enzyme to the spectra was removed by multiplying the spectrum of the initial oxidized enzyme by a factor representing the fraction of oxidized enzyme in each of the mixed spectra, and subtracting this manipulated spectrum from each of the mixed spectra. The resultant spectra were divided by a factor representing the fraction of semiquinone present in each of the mixed spectra and subtracting this manipulated spectrum from each of the mixed spectra. The resultant spectra were divided by a factor representing the fraction of reduced enzyme present in each of the mixed spectra, and the average of the three extrapolated spectra yielded the extrapolated spectrum of 2-electron reduced enzyme (Figs. 1 and 2).

A blue, neutral semiquinone also forms upon photoreduction of TRR(Ser135,Cys138), but the semiquinone is unstable and slowly disproportionate. Thus, a phototitration was not obtained. The absorbance at 530 nm of the semiquinone species of wild type thioredoxin reductase and TRR(Cys135, Ser138) are nearly equal. This is true also of NAD+. Therefore, the values for these extinction coefficients calculated for TRR(Ser135,Cys138) were used to quantify the amount of semiquinone stabilized on TRR(Ser135,Cys138).

Dithionite Titrations—Solutions of sodium dithionite were prepared in anaerobic 50 mM sodium pyrophosphate buffer, pH 8.5, and standardized by titrating a solution of lumiflavin-3-acetic acid. In typical titrations of the enzymes, 20 μM TRR(Ser135,Cys138) and 0.15 mM methyl viologen in 1 ml of 0.1 M sodium pyrophosphate buffer, pH 7.6, or 40–45 μM TRR(Cys135, Ser138) and 1.5–2.5 mM methyl viologen in 2–3 ml of the same buffer were anaerobically reduced with aliquots of sodium dithionite added from a gas-tight Hamilton syringe attached to the anaerobic cell. The progress of the reductions were followed by recording spectra at each stage of the titration (Figs. 3 and 8). The rates of the reductions of these proteins were slow; therefore, a catalytic amount of methyl viologen was included in the titrations. In addition, the appearance of the spectrum of reduced methyl viologen at 395 nm (subtracted in Fig. 3 but prominent in Fig. 8) is an indicator of completion of the titration, since the midpoint of the reduced methyl viologen is considerably more positive than the potential of the methyl viologen. The extinction coefficients for absorbance at 585 and 454 nm of the fully reduced TRR(Ser135,Cys138) at pH 6.5, 7.6, and 8.5 were obtained from single additions of excess sodium dithionite. The extinction coefficients for absorbance at 585 nm of the fully reduced TRR(Cys135, Ser138) were extrapolated from several titrations.

Potentiometric Titrations—The midpoint potentials of TRR(Ser135,Cys138) were determined in the pH range 6.0–9.0 at 20 °C by anaerobic NADH titrations. The concentration of the NADH titrant solution was determined by measuring the absorption at 340 and 259 nm of diluted samples, using the extinction coefficients ε259 = 6.320 M⁻¹ cm⁻¹ and ε550 = 14,900 M⁻¹ cm⁻¹. The concentration of the NAD⁺ stock solution was determined by measuring the absorbance at 259 nm of diluted samples, using the extinction coefficient ε259 = 18,000 M⁻¹ cm⁻¹. Anaerobic solutions of TRR(Cys135, Cys138) (80–90% reduced) were added to 1.5 ml of 0.1 M sodium/potassium phosphate-KCl buffer at varying pH, containing between 44 nmol and 4.6 μmol of NAD⁺, were titrated with NADH at 20 °C. Buffer solutions in the pH range 6.0–8.0 were citric acid–dibasic sodium phosphate mixtures; the ionic strength was adjusted to approximately 0.1 by the addition of KCl, and the buffering strength was 32–56 mM. Buffer solutions in the pH range 8.0–9.0 were prepared by mixing together a solution of 50 mM Tris base, 50 mM KCl with a solution of 100 mM HCl, 100 mM KCl. To compensate for an increasing difference in the midpoint potentials of the enzyme-FAD/FADH₂ and the NAD⁺/NADH couples as the pH is lowered, the amount of NAD⁺ initially present was increased as the pH was lowered. The quantitative determination of the equilibrium at less than 30% reduction of the enzyme.

Potentiometric titrations were performed on TRR(Cys135, Ser138) as described above. However, the presence of a spectrally silent second reducible center on this protein created a problem of quantifying the various species present at any point during a reductive potentiometric titration. Additionally, the increasing degree of turbidity of the solution throughout the titration decreased the accuracy of spectrally determining the equilibrium concentration of NADH after each addition (see below). These results combined to make meaningful potentiometric titrations of TRR(Cys135, Ser138) difficult. Therefore, solutions of TRR(Cys135, Ser138) (64 nmol of enzyme in 1.46 ml of 0.1 M sodium/potassium phosphate buffer, pH 7.6, containing 102 and 112 nmol of NAD⁺) were made anaerobic and reduced by the addition of 51 and 219 nmol of NADH. At equilibrium, the spectra were recorded and the macroscopic distributions determined (Table I).

Corrections for Turbidity—The anaerobic process results in denaturation of a small amount of the enzymes, giving rise to turbidity. To correct for the turbidity component in the spectra, the enzyme solution following the titration was centrifuged in an Eppendorf microcentrifuge at 15,000 rpm for 5 min. The supernatant was discarded and an aliquot of sodium dithionite added from a gas-tight Hamilton syringe and the spectrum recorded. The contribution of turbidity to the spectra recorded during the titrations was subtracted by multiplying the turbidity spectrum by a factor so that the absorbance at 800 nm of the turbidity spectrum equals the absorbance at 800 nm of the enzyme spectrum. The turbidity spectrum was subtracted from each enzyme spectrum resulting in zero absorbance at 800 nm. In the worst case, this correction was 25% of the initial absorbance at 454 nm; most turbidity spectrum was then subtracted from the enzyme spectrum and the average of the three extrapolated spectra yielded the extrapolated spectrum of 2-electron reduced enzyme (Figs. 1 and 2).
RESULTS

Photoreduction of TRR(Cys153, Ser158) in the presence of 3,10-dimethyl-5-deaza-isosaloxazine2 and 30 mM EDTA (Massey and Hemmerich, 1977, 1978; Massey et al., 1978), as described under "Materials and Methods," results in the formation of a stable 1-electron reduced species having a spectrum of a blue, neutral semiquinone, Ez (Fig. 1). During such a phototitration of TRR(Cys153, Ser158) a maximum of 70% of the enzyme is present as the semiquinone. The absorbance values at 585 and 454 nm of 100% semiquinone were extrapolated from a plot of A585 versus A454, revealing extinction coefficients of 3,800 M⁻¹ cm⁻¹ and 3,500 M⁻¹ cm⁻¹, respectively (Fig. 1, inset). The computer manipulations of spectra to obtain the spectrum of the semiquinone are described under "Materials and Methods." The extrapolated spectrum of the semiquinone confirmed the values of these extinction coefficients (Fig. 2), which are nearly equal to the values of the semiquinone of wild type thioredoxin reductase (Zanetti et al., 1968; O'Donnell and Williams, 1983). The value of e585 for the fully reduced enzyme, 1,900 M⁻¹ cm⁻¹, was extrapolated from the same plot, assuming an e585 of zero.

Reduction of TRR(Ser135, Cys158) by phototitration has revealed that this enzyme does not stabilize as much of the blue, neutral semiquinone; instead, the semiquinone that is formed initially disproportionate slowly to oxidized and 2-electron

2 This compound is named as an isosaloxazine as in the reference, but note that it lacks the 7- and 8-methyl groups.
TRR(Cys$^{135}$,Ser$^{138}$) are nearly equal, the values determined for TRR(Cys$^{135}$,Ser$^{138}$) were used to quantify the amount of semiquinone formed during reductive titrations of TRR(Ser$^{136}$,Cys$^{138}$). The value of $e_{454}$ for fully reduced TRR(Ser$^{136}$,Cys$^{138}$) was determined to be 1,800 M$^{-1}$ cm$^{-1}$ after complete reduction of the enzyme with sodium dithionite (see "Materials and Methods"). When an anaerobic solution of TRR(Ser$^{135}$,Cys$^{138}$) in 50 mM sodium/potassium phosphate buffer, pH 7.6, 30 mM EDTA was exposed to brilliant light for 45 s (see "Materials and Methods"), approximately 60% of the enzyme was reduced to the semiquinone, which slowly disproportionated with a half-time of 18 min to a mixture of 45% oxidized, 29% semiquinone, and 26% 2-electron reduced enzyme. Thus, TRR(Cys$^{135}$,Ser$^{138}$) stabilizes 2.4 times as much semiquinone as TRR(Ser$^{136}$,Cys$^{138}$).

Reductive titration of TRR(Ser$^{135}$,Cys$^{138}$) with sodium dithionite in the presence of a catalytic quantity of methyl viologen at pH 7.0 and 20 °C demonstrated that, as expected, 1 mol of dithionite/mol of FAD was required to fully reduce the enzyme (Fig. 3). The 2-electron reduced enzyme has an absorbance spectrum identical to the spectrum of 4-electron reduced wild type thioredoxin reductase (Zanetti and Williams, 1967; O'Donnell and Williams, 1983), and has an $e_{454} = 1,700-1,900$ M$^{-1}$ cm$^{-1}$. During the course of the titrations the maximum formation of semiquinone is 5–12% of the total enzyme, and the absorbance peak of reduced methyl viologen at 395 nm does not appear until the enzyme is completely reduced. The absorbance spectra of the enzyme at various stages of the titration reveal an isosbestic at 340 nm (Fig. 3).

The midpoint potentials for the 1-electron and 2-electron reductions of TRR(Ser$^{135}$,Cys$^{138}$) were determined from NADH/NAD$^+$ titrations in the pH range 6.0–9.0 at 20 °C. The detailed explanations of the quantification of the distributions of enzyme species throughout the titrations and the calculation of the midpoint potentials are presented under "Materials and Methods." At pH 7.0 and 20 °C the value of $E_m$ for the 2-electron reduction of TRR(Ser$^{135}$,Cys$^{138}$) is $-280$
mV. The spectra of a titration performed at pH 7.2 are presented in Fig. 4 (after subtracting the absorbance contribution of the NADH as described under “Materials and Methods”). Profiles of the distributions of the three redox forms of the enzyme reveal that the equilibrium titration of TRR(Ser<sup>135</sup>,Cys<sup>138</sup>) results in a pH-independent maximum of 18–20% semiquinone formation (Fig. 5). The values of the potentials for the two 1-electron couples (\(E_1 = E_{on}/E_{eq}; E_2 = E_{red}/E_{eq}\)) have been calculated, and at pH 7.0 and 20 °C these values for TRR(Ser<sup>135</sup>,Cys<sup>138</sup>) are -306 and -256 mV, respectively. In contrast, during a reductive titration of TRR(Cys<sup>138</sup>,Ser<sup>138</sup>) with NADH in the presence of NAD<sup>+</sup> and a mediator, benzyl viologen, the maximum amount of semiquinone (1-electron reduced and 3-electron reduced forms combined) formed increases from 30% at pH 8.9 to 53% at pH 6.0. Thus, the relative redox potentials, \(E_1\) and \(E_2\), and consequently, the amount of semiquinone stabilized, as well as the pH dependence of the relative potentials, are dependent on the polarity of the residue at position 138.

The plots of the system potential, \(E_m\), as a function of the log ([Em]/[Eox]) are presented for TRR(Ser<sup>135</sup>,Cys<sup>138</sup>) in Fig. 6. The measured values of \(E_m\) during titrations of this enzyme show excellent agreement with the theoretical lines determined with \(E_m\) values calculated from data between 30 and 70% reduction; deviations from the theoretical lines below

**FIG. 5.** 
Profiles of the distribution of TRR(Ser<sup>135</sup>,Cys<sup>138</sup>) species during a NADH titration. The fractions of oxidized, semiquinone, and reduced forms of TRR(Ser<sup>135</sup>,Cys<sup>138</sup>) are plotted against the equivalents of NADH oxidized to NAD<sup>+</sup> during a potentiometric titration at pH 7.2 and 20 °C. Circles, oxidized enzyme; squares, semiquinone; triangles, 2-electron reduced enzyme.

**FIG. 4.** 
NADH titration of TRR(Ser<sup>135</sup>,Cys<sup>138</sup>). A solution of 55.2 nmol of TRR(Ser<sup>135</sup>,Cys<sup>138</sup>) in 1.2 ml of 17.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.3 mM citrate, 54.2 mM KCl, pH 7.2, containing 430 nmol of NAD<sup>+</sup>, was anaerobically titrated with 1.5 and 4.8 mM NADH solutions at 20 °C. The spectra, recorded after equilibrium was attained, are of oxidized enzyme and enzyme after addition of 11.8, 26.6, 53.3, 101, 189, and 251.8 nmol of TRR(Ser<sup>135</sup>,Cys<sup>138</sup>) in 1.2 ml of 17.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.3 mM citrate, 54.2 mM KCl, pH 7.2, containing 430 nmol of NAD<sup>+</sup>, was subtracted from the spectra.

**FIG. 6.** Nernst plots of potentiometric titrations of TRR(Ser<sup>135</sup>,Cys<sup>138</sup>). The system potentials, \(E_m\), of several potentiometric titrations of TRR(Ser<sup>135</sup>,Cys<sup>138</sup>) at 20 °C and the indicated pH are plotted as a function of the log ([Em]/[Eox]). The solid lines are theoretical lines calculated for 2-electron reductions using the experimentally determined values of \(E_m\). Solid squares, 52.4 μM enzyme, 1.86 mM NAD<sup>+</sup>, pH 6.0; open triangles, 53.0 μM enzyme, 1.67 mM NAD<sup>+</sup>, pH 6.4; closed circles, 47.9 μM enzyme, 1.49 mM NAD<sup>+</sup>, pH 6.7; open diamonds, 44.7 μM enzyme, 440 μM NAD<sup>+</sup>, pH 7.2; closed triangles, 41.9 μM enzyme, 188 μM NAD<sup>+</sup>, pH 7.5; open circles, 43.8 μM enzyme, 104 μM NAD<sup>+</sup>, pH 7.7; solid diamonds, 42 μM enzyme, 50.4 μM NAD<sup>+</sup>, pH 8.2; open squares, 43.5 μM enzyme, 31.4 μM NAD<sup>+</sup>, pH 8.5.

**FIG. 7.** pH profile of the midpoint potentials of TRR(Ser<sup>135</sup>,Cys<sup>138</sup>). The values of the midpoint potentials for the \(E_m/E_{red}\) couple, \(E_{on}/E_{eq}\) couple, and the \(E_{red}/E_{eq}\) couple of TRR(Ser<sup>135</sup>,Cys<sup>138</sup>) are plotted as a function of pH. The solid lines are linear regressions of the experimentally determined values. Circles, \(E_1\); triangles, \(E_2\); diamonds, \(E_3\).

25% and above 75% reduction reflect the contribution of the 1-electron reduction. A plot of the pH profiles of the midpoint potentials for the overall 2-electron reduction, \(E_m\), \(E_{on}/E_{red}\) couple, and the two 1-electron reductions, \(E_1\), \(E_{red}/E_{eq}\) couple and \(E_3\), \(E_{red}/E_{eq}\) couple, for TRR(Ser<sup>135</sup>,Cys<sup>138</sup>) is presented (Fig. 7). The slope, \(\Delta E_m/\Delta pH\), of TRR(Ser<sup>135</sup>,Cys<sup>138</sup>) has a value of -57.1 mV. For a proton stoichiometry of 2 H<sup>+</sup>/2 e<sup>-</sup> at 20 °C, this slope has a value of -58.2 mV (Clark, 1960). Thus, the 2-electron reduction of TRR(Ser<sup>135</sup>,Cys<sup>138</sup>) has a proton stoichiometry of 2.0 H<sup>+</sup>/2 e<sup>-</sup>. The slopes, \(\Delta E_m/\Delta pH\), for the 1-electron reductions of TRR(Ser<sup>135</sup>,Cys<sup>138</sup>) have values of -52.4 and -61.7 mV, respectively, indicating proton stoichiometries of 0.9 and 1.1 H<sup>+</sup>/e<sup>-</sup>, respectively. There are no breaks in the slopes of these plots, indicating that the reduction of the FAD is not linked to the ionization of any residues in the pH range 6.0–9.0. Several reductive titrations of TRR(Cys<sup>138</sup>,Ser<sup>138</sup>), in the presence of a catalytic quantity of methyl viologen, with sodium dithionite in the pH 6.5–8.5 range revealed that 1.8–
been tested due to the tendency of sulfenates to further oxidize, and suggesting an apparent equilibrium involving sulfonated cysteine, bisulfite, oxidized and reduced methyl viologen, and the two forms of the enzyme, \(E_{ox}\) and \(E_{red}\). This equilibrium might indicate that the midpoint potential for the \(E_{ox}/E_{red}\) couple is close to the value of the oxidized/reduced couple of methyl viologen (−446 mV). However, the value of the midpoint potential of the \(E_{ox}/E_{red}\) couple of TRR(Cys\(^{35}\), Ser\(^{38}\)) measured by potentiometric titrations with NADH is more positive than predicted from the results of the dithionite titrations. An explanation of this anomaly is not apparent. A value of 1.980±0.190 mV cm\(^{-1}\) for \(e_{454}\) of \(E_{red}\) was extrapolated from several titrations.

The requirement of 1 equivalent (eq) of dithionite to fully reduce TRR(Ser\(^{35}\), Cys\(^{38}\)) is as expected (Fig. 3). The extrapolated requirement of 1.8±0.2 eq to fully reduce TRR(Cys\(^{35}\), Ser\(^{38}\)) is unexpected. However, the appearance of reduced methyl viologen and excess dithionite prior to full reduction of the enzyme, and the requirement of greater than 2 eq to obtain full reduction suggest that the titration may involve a kinetically unreacted equilibrium of the semiquinone and 2-electron reduced forms of the enzyme, oxidized and reduced methyl viologen, and the dithionite and sulfite couple (Fig. 8).

The quantification of thiols on TRR(Cys\(^{35}\), Ser\(^{38}\)) with DTNB under non-reducing and denaturing conditions and by conversion to cysteic acid (Prongay et al., 1988) have confirmed the replacement of Cys\(^{38}\) by Ser. Oxidation of the thiol group of Cys\(^{35}\) to a higher oxidation state, such as sulfenate would account for the uptake of 2 electrons from dithionite in excess of those required to reduce the FAD. A distantly related enzyme, NADH peroxidase, is thought to cycle in catalysis between the sulfenate and thiol states (Poole and Claiborne, 1989). However, a sulfenate would not be expected to react with DTNB; to our knowledge, this has not been tested due to the tendency of sulfenates to further oxidation (Capozzi and Modena, 1974; Kice, 1980). The thiol of Cys\(^{35}\) is 4.43 Å from the nearest point on the isoalloxazine ring, making it unlikely that the flavin would reduce a sulfenate directly.

However, a sulfenate ester between Cys\(^{35}\) and Ser\(^{38}\) would be reduced by dithionite accounting for an additional equivalent of dithionite (Snyder and Carlsen, 1977). The putative hydrophobic environment of the active site in the vicinity of the FAD may serve to stabilize a sulfenate ester. The location of the hydroxyl group of Ser\(^{38}\) near the C-4a position of the electron deficient isoalloxazine ring may result in a polarization of the oxygen atom of the hydroxyl such that a sulfenyl on Cys\(^{35}\) can form a sulfenate ester with the hydroxyl. Upon denaturation of the protein the polarizing environment would be removed and the ester subject to hydrolysis. The conditions used for crystallization (ammonium sulfate and dithiothreitol) must also lead to the breakdown of the putative ester and reduction of the sulfenate since it is not observed in the structure of TRR(Cys\(^{35}\), Ser\(^{38}\)) (Kuriyan et al., 1991).

The presence of a second reducible center on TRR(Cys\(^{35}\), Ser\(^{38}\)) was confirmed by reduction of the enzyme with NADH in the presence of NAD\(^{+}\) (Table I). The progressive increase in the extent of turbidity formation throughout the course of a reductive titration of TRR(Cys\(^{35}\), Ser\(^{38}\)) with NADH decreased the accuracy of measuring the equilibrium concentration of NADH by its absorbance at 340 nm. Therefore, in two separate experiments the enzyme was partially reduced to approximately 20 and 57% total reduction by single additions of NADH to anaerobic enzyme containing NAD\(^{+}\) at pH 7.6 and 20 °C. After equilibrium was attained, the concentration of NADH was determined by measuring the absorbance at 342 nm, a wavelength at which all the enzyme species are isosbestic; the equilibrium concentrations of oxidized, semiquinone and 2-electron reduced FAD on the enzyme were determined as described under “Materials and Methods.” The data support the presence of a second reducible center on the enzyme, which appears to require 2 mol of electrons/mol of enzyme (Table I). The midpoint potential for the 2-electron reduction of the FAD on this enzyme was estimated from these experiments to be −325 ± 2 mV at pH 7.6 and 20 °C. Assuming a slope of −58.2 mV/pH, this corresponds to −290 mV at pH 7.0 and 20 °C. Thus, reduction of the FAD, especially the addition of the second electron, is more difficult with TRR(Cys\(^{35}\), Ser\(^{38}\)).

The titration of TRR(Cys\(^{35}\), Ser\(^{38}\)) by NADH is shown in Fig. 9. The reduction of a putative second redox center at Cys\(^{38}\) by electrons from NADH provides additional evidence that although the electrons are passed from the FAD to Cys\(^{38}\) during catalysis by the wild type enzyme, the altered enzyme is capable of reducing the second redox center and, presumably, a mixed disulfide with the substrate at Cys\(^{38}\), albeit less efficiently (Prongay et al., 1988).

\[\text{Table I}\]

| Reduction of TRR(Cys\(^{35}\), Ser\(^{38}\)) with NADH |
|---|---|
| Experiment 1 | Experiment 2 |
| nmol | nmol |
| Total enzyme | 64 | 63 |
| NADH added | 51 | 219 |
| NADH at equilibrium | 26 | 164 |
| NAD\(^{+}\) initial | 110 | 112 |
| FAD | 29 | 17 |
| FADH | 28 | 35 |
| FADH\(_2\) | 7 | 11 |
| Second redox center | 4 | 37 |

\(^{3}\) Personal communication, Dr. John Kuriyan, The Rockefeller University.
The three-dimensional structures of TRR(Cys135,Cys138) and TRR(Ser135,Cys138) have been solved, and from this information, it is likely that the structure of the wild type enzyme will become available shortly. The structure of TRR(Cys135,Cys138) was refined at 2Å resolution, with an R-factor of 17.7%, including the placement of approximately 270 solvent molecules (Kuriyan et al., 1991). The structures confirm the prediction (Prongay et al., 1989) that the active center thiols are juxtaposed almost parallel to the isoalloxazine ring rather than perpendicular to it, as in glutathione reductase and lipoamide dehydrogenase (Schulz et al., 1978; Schieberbek et al., 1989). The oxygen of Ser135 (Cys in wild type enzyme) is 3.05 Å from C-4a, and the sulfur of Cys135 is 4.50 Å from N-5a and 4.78 Å from C-4a, across the re face. The environment of the isoalloxazine is apolar as the spectroscopic redox potentials of the FAD/FADH2 and disulfide/dithiol couple is 11 mV more negative than is that of residue 138. Thus, the greater stabilization of semiquinone by TRR(Cys135,Cys138) than by TRR(Ser135,Cys138) provides additional evidence that Cys138 is positioned nearer to the C-4 and C-4a positions of the FAD than is Cys135. It is concluded, therefore, that Cys135 accepts electrons from FADH2 in catalysis.

Potentiometric titrations of wild type thioredoxin reductase have shown that at pH 7.0 and 12 °C the disulfide/dithiol and FAD/FADH2 couples have midpoint potentials separated by only 11 mV with a 17 mV negative interaction (O'Donnell and Williams, 1983). At pH 7.0 and 12 °C the midpoint potential of the FAD/FADH2 couple of the dithiol form of the enzyme has a value of -260 mV, and the ΔE cm/pH of this couple is -60 mV/pH, indicating a 2 H+/2 e- stoichiometry (O'Donnell and Williams, 1983). Applying a temperature correction factor of -1.3 mV/°C (Clark, 1960) to the Ecm of this couple yields a value of -270 mV at pH 7.0 and 20 °C. At pH 7.0 and 20 °C the Ecm values of the FAD/FADH2 couple of TRR(Ser135,Cys138) and TRR(Cys135,Cys138) are -280 and -290 mV, respectively (Fig. 6 and Table I). Thus, the replacement of Cys135 or Cys138 with a serine causes a 10 or 20 mV decrease, respectively, in the midpoint potential of the FAD/FADH2 couple relative to wild type thioredoxin reductase. These results are consistent with the small negative interaction (17 mV) that the dithiol has on the FAD/FADH2 couple relative to the effect of the disulfide on this couple seen with wild type thioredoxin reductase (O'Donnell and Williams, 1983). These results demonstrate that the increased polarity resulting from the replacement of either thiol exerts a similar effect on the redox potential of the FAD/FADH2 couple. On the other hand, the hydroxyl group of Ser135 exerts a far greater effect than Ser135 toward stabilizing the semiquinone species.

Acknowledgments—We are grateful to Dr. Rowena G. Matthews, University of Michigan, and to L. David Arscott, Department of Veterans Affairs Medical Center, for many helpful discussions, to Dr. Vincent Massey, University of Michigan, for advice and for supplying the 3,10-dimethyl-5-deaza-isoalloxazine, and to Dr. John Kuriyan, The Rockefeller University, for communicating the structures to us as soon as they were completed.

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4 This was suggested by a reviewer and replaces our less logical speculation.
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