Properties of Lipoamide Dehydrogenase and Thioredoxin Reductase from *Escherichia coli* Altered by Site-Directed Mutagenesis[a]

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Lipoamide dehydrogenase and thioredoxin reductase are members of the pyridine nucleotide–disulfide oxidoreductase family of flavoenzymes, which is distinguished by an oxidation-reduction–active disulfide.[1] Other members of the family, glutathione reductase and mercuric reductase, are homologous with lipoamide dehydrogenase in all domains.[2–5] Thioredoxin reductase is homologous with the others only in its two adenosine binding regions; the remainder of the protein, including its active-site disulfide region, appears to have evolved convergently.[6]

Catalysis takes place in two half-reactions, as shown in FIGURE 1 for lipoamide dehydrogenase.[7–9] In the first, dithiol-disulfide interchange effects reduction of the oxidized enzyme (E) to the 2-electron reduced form of the enzyme (EH_2); and in the second, the reoxidation of EH_2 to E, electrons pass very rapidly via the FAD to NAD^+. The distinct roles of the two nascent thiols of EH_2 have been demonstrated.[10,11] The thiol nearer the amino terminus reacts almost exclusively with iodoacetamide, and it is this thiol that interchanges with the dithiol substrate; the sulfur nearer the carboxyl terminus interacts with the FAD. Similar results are seen with glutathione reductase[12] and mercuric reductase.[13] The assignment of roles to the two nascent thiols in

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thioredoxin reductase has been more difficult because both nascent thiols are reactive with all reagents tried.[14] This problem is being solved by site-directed mutagenesis.[15]

In addition to the FAD and the redox-active disulfide, a base is essential for catalysis (FIG. 1). Its function is to deprotonate the dihydrolipoamide for nucleophilic attack on the enzyme disulfide.[16] Glutathione reductase catalysis is in the chemically opposite direction. In this enzyme, the function of the base is to stabilize the nascent interchange thiol as an anion for attack on the glutathione disulfide.[12] The amino acid sequence[17] and X-ray crystallographic structure[18] of human glutathione reductase show that the base is a histidyl residue strongly hydrogen-bonded to a glutamate.[19] The thiol nearer to the amino terminus is positioned for interchange with glutathione, and the other thiol is nearer to the flavin. Thus, the crystal structure and other types of data agree. The structure of *Azotobacter vinelandii* lipoamide dehydrogenase, recently derived, appears to be very similar.[21] It has been suggested that the positive charge of a lysyl residue whose side chain reaches under the flavin ring, from the disulfide compartment to the pyridine nucleotide compartment, modifies the redox potential of the FAD.[20] Three of the mutations described below were used in an attempt to alter slightly the position of this positive charge relative to the flavin ring.

Earlier studies showed that the *Escherichia coli* enzyme is somewhat more complex than the pig heart enzyme (FIG. 2). Whereas in the latter species EH_2 is predominantly the charge transfer complex, in the *E. coli* enzyme at neutral pH, two additional forms are present in significant amounts: in one of these, a proton shift has left the charge transfer thiol protonated and this form is fluorescent; electrons are on the FAD in the other form.[22]

METHODS

Site-directed mutagenesis of *E. coli* lipoamide dehydrogenase[23] and thioredoxin reductase[15,24,25] has been described. The purification and assay of thioredoxin reductase followed established procedures.[15] An *E. coli* strain, JRG1342, having an Lpd^- phenotype (*ΔaroP-lpd*)[26] and transformed with pJLA504,[27] expressed the wild-type or mutated lipoamide dehydrogenases from λ promoters[23] and was grown in 6 × 1 l of TB broth containing 50 μg/ml ampicillin by shaking in a Lab Line rotary incubator at 30°C. When the A_{600} of the cultures reached 0.6, the temperature was raised to 42°C and incubation was continued for 16–17 hr. The yield of cells was 5.5, 6.0, 7.0, and 5.0

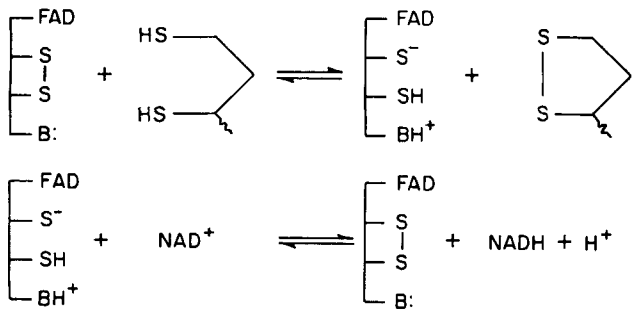

FIGURE 1. Lipoamide dehydrogenase half-reactions: reduction of E to EH_2 by dihydrolipoamide (**upper panel**) and reoxidation of EH_2 to E by NAD^+ (**lower panel**).

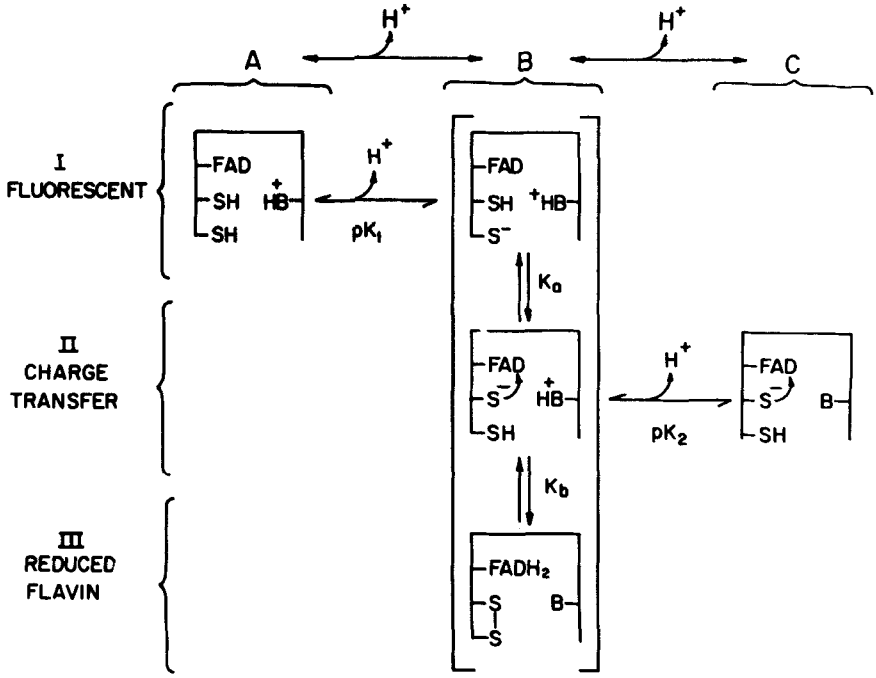

FIGURE 2. Equilibria between species contributing to EH_2 in *E. coli* lipoamide dehydrogenase and the effect of pH. **A**, low pH; **B**, neutral pH; **C**, high pH. (Adapted from Wilkinson & Williams.[22])

g/l for wild type and mutants Glu-188→Asp,[g] His-444→Gln, and Lys-53→Arg/Glu-188→Asp, respectively. (The numbering for the *E. coli* lipoamide dehydrogenase amino acid sequence is derived from the nucleotide sequence.[28]) Cell were harvested by centrifugation at 6500 rpm for 10 min, homogenized in a minimal volume of 100 m*M* phosphate buffer, 0.3 m*M* EDTA, pH 7.6, containing 25 μM phenylmethylsulfonyl fluoride, and diluted with the same buffer to give 5 ml of suspension/g wet cell weight. Cells were broken in a Branson D610 sonifier using a rosette cell immersed in an ice-salt mixture for 5 bursts of 3 min each. Streptomycin sulfate was added to give a final concentration of 2%, and the suspension was spun in a Beckman L8-70M ultracentrifuge, using the VTI-50 rotor, initially at 25,000 rpm for 20 min and then at 45,000 rpm for an additional hour. The supernatant was fractionated with ammonium sulfate from 35 to 85% saturation, and the precipitate was dissolved in 100 m*M* phosphate buffer, 0.3 m*M* EDTA, pH 7.6. Following extensive dialysis against 10 m*M* phosphate buffer, 0.3 m*M* EDTA, pH 7.6 (buffer A), the solution was applied to a DEAE-TRISACRYL (Pharmacia LKB Biotechnology) column, 2.5 × 11 cm, equilibrated with buffer A. A fraction of protein was eluted by washing with buffer A; elution was continued with a gradient formed from 250 ml each of buffer A, without and with 1 *M* NaCl. The fraction eluting between 0.16 and 0.2 *M* NaCl contained most of the enzyme. After concentration with ammonium sulfate and dialysis against

[g]Designation of mutants: Glu-188→Asp, mutation of Glu-188 to Asp.

50 m*M* phosphate buffer, 0.3 m*M* EDTA, pH 7.6 (buffer B), it was further purified by adsorption chromatography on a 2.5 cm × 27 cm calcium phosphate gel column equilibrated with buffer B. The column was washed successively with buffer B and buffer B containing 0.1 *M* ammonium sulfate, and the enzyme was eluted with buffer B containing 0.3 *M* ammonium sulfate. In the case of the wild-type enzyme, the A_{280}/A_{450} ratio was 6.4 and the yield was 85 mg.

Since the Glu-188→Asp enzyme did not bind well to calcium phosphate gel, further purification was attempted, following the DEAE step, by apolar chromatography, first on phenyl-Sepharose and then on a hydrophobic interaction column (TSK phenyl-5PW). The yield was poor and the improvement in purity was marginal in both steps; loss of FAD was evident. The A_{280}/A_{455} ratio of the Glu-188→Asp enzyme used in these studies was 11. The His-444→Gln enzyme was not retained by DEAE. Following chromatography on calcium phosphate gel, it was further purified by apolar chromatography on phenyl-Sepharose. The A_{280}/A_{455} was 7.0 and the yield was 95 mg.

Lipoamide dehydrogenase was assayed by following the reduction of acetylpyridine adenine dinucleotide ($APAD^+$) by dihydrolipoamide, measured by a change in absorbance at 363 nm, at 25°C. The assay volume was 2.5 ml of 60 m*M* phosphate buffer, pH 7.6, containing 0.8 mg/ml bovine serum albumin, 1.4 m*M* EDTA, 400 μM $APAD^+$, and 80 μM dihydrolipoamide. The turnover number of the wild type enzyme was approximately 500 mol $APAD^+$ oxidized/mol FAD. The turnover number of the Glu-188→Asp mutated enzyme was 1550 mol $APAD^+$ oxidized/mol FAD, or approximately 3 times that of wild-type enzyme.

RESULTS AND DISCUSSION

The nine mutageneses of *E. coli* lipoamide dehydrogenase effected thus far are described in another paper in this volume.[29] Two of the altered enzymes, the Glu-188→Asp and the His-144→Gln, have been extensively purified. For purified enzyme, it is necessary to use the dihydrolipoamide–acetylpyridine adenine dinucleotide assay. Use of the higher-potential $APAD^+$, rather than of NAD^+, overcomes the extreme mixed inhibition of the *E. coli* enzyme by NADH; in addition to product inhibition, NADH reduces the active intermediate EH_2 to the inactive EH_4, the 4-electron reduced form of the enzyme. In crude extracts, NAD^+ can be used as the acceptor, presumably because of a very active NADH oxidase. Recently, we have shown with wild-type enzyme that assays using NAD^+ as the acceptor are possible in the rapid reaction spectrophotometer, where rates can be established before there is significant buildup of NADH.[30] Steady-state, stopped-flow traces monitoring NADH production at increasing levels of NAD^+ showed a pronounced lag at the lowest NAD^+ level, while at the highest level, inhibition was apparent within 1.5 s. However, initial rates estimated from the linear phase conformed approximately to a Michaelis-Menten model and quite well to a cooperative binding model. The apparent V_{max} of 400 s^{-1} was approximately half that observed with the pig heart enzyme, where the NADH inhibition is much less severe. These studies examining the steady-state kinetics of catalysis in the physiological direction of the reaction, together with earlier investigations of the kinetics in the opposite direction,[31] as well as the spectral properties of *E. coli* lipoamide dehydrogenase,[22] will form the basis of our comparisons with the enzyme altered by site-directed mutagenesis.

In three of the mutations, we were seeking to alter the position of the Lys-53 side chain charge relative to the isoalloxazine ring: by shortening the ion-pair side chain, as

in the Glu-188→Asp mutation, by dispersing the positive charge, as in the Lys-53→Arg mutation, or by using a double mutation which should maintain the overall length of the ion pair. This strategy was worked out in a conversation between one of us (C. H. W.) and Professor Georg E. Schulz, University of Freiberg. There are at least three ways the protein might adjust to accommodate such changes: the ion pair might be weakened or broken, the polypeptide chain carrying Glu-188 might move, or the polypeptide chain containing the Lys-53 might move.

The Glu-188→Asp enzyme has been extensively purified. FIGURE 3 shows the absorbance spectrum of this altered enzyme compared with that of the wild-type enzyme. Changes to the absorbance in the visible region are minimal, primarily a lessening of the resolution of the shoulder at 480 nm. Purification of the other two altered enzymes in this group, the Lys-53→Arg and the Lys-53→Arg/Glu-188→Asp, is in progress.

Lipoamide dehydrogenase in which the base, His-444, has been changed to Gln has been purified to virtual homogeneity and has very low activity in the $APAD^+$ assay—about 0.3 to 0.4% that of wild type. Given that the pH of the assay is 1.7 units below the first pK_a of dihydrolipoamide,[32] it would be expected that the activity would be diminished by 50 to 100 times, if the only function of the base is to deprotonate the

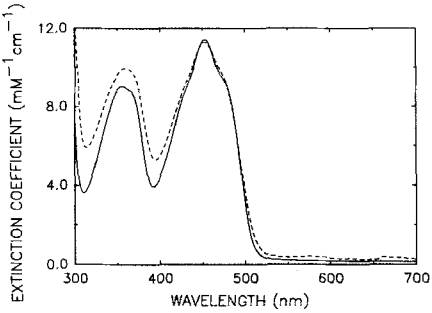

FIGURE 3. Absorbance spectra of wild-type and Glu-188→Asp lipoamide dehydrogenases. The proteins were in 10 m*M* Na/K phosphate buffer, pH 7.6, 0.3 m*M* EDTA, at 20°C. *Solid line,* 39.0 μM wild-type enzyme; *dashed line,* 13.6 μM mutated enzyme.

substrate. But, it has been postulated that the base also stabilizes the thiols as thiolates. Absence of this latter function should lead to an additional diminution of activity. Similar levels of activity have been reported for the analogous mutation in glutathione reductase.[33] FIGURE 4a shows spectra resulting from titration of this enzyme by dihydrolipoamide. Even in the early phases of the reduction, no detectable charge transfer is seen. Charge transfer is a direct indication of stabilization of the electron transfer thiol as a thiolate.[34] In a similar equilibrium experiment with the wild-type enzyme (shown in FIGURE 4b),[35] the charge transfer band at 530 nm is obvious; but, as already discussed, in the *E. coli* enzyme, the charge transfer complex is only one of several species present in EH_2.[22] When the mutated enzyme is reduced in a stopped flow apparatus, (FIG. 5a) sizable charge transfer builds up over the first 30 s. In a similar experiment with the wild-type enzyme,[1] a high level of charge transfer is almost fully formed in the 3-ms dead time of the rapid-reaction spectrophotometer (FIG. 5b). Thus, in the mutated enzyme, the charge transfer band forms far more slowly and decays more extensively, demonstrating the crucial role of the base in stabilizing the charge transfer complex and, thus, its role in catalysis where the thiolate attacks the FAD.

Conditions for chemical modification of just one of the nascent thiols in *E. coli*

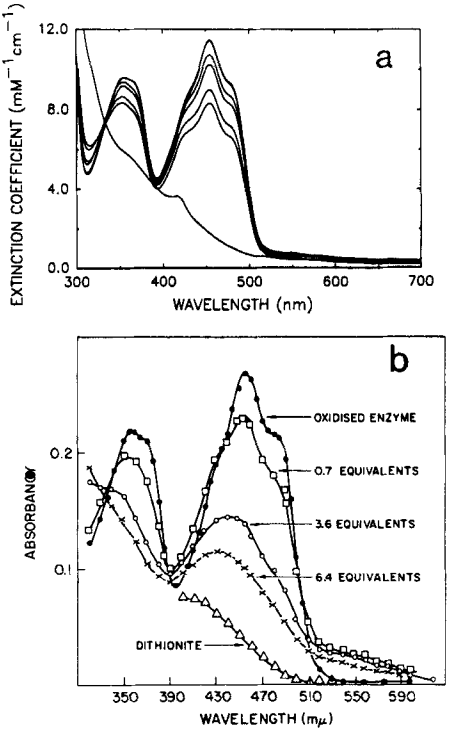

FIGURE 4. Anaerobic titration of lipoamide dehydrogenase with dihydrolipoamide. Spectra were taken at 20°C in conventional spectrophotometers after all changes were complete. (**a**) Spectra of 18.4 μM His-444→Gln enzyme in 0.1 M Na/K phosphate buffer, pH 7.6, 0.3 mM EDTA, titrated with (*top* to *bottom traces*, respectively) 0, 0.21, 0.42, 0.63, 1.05, 1.47, and 9.87 mol dihydrolipoamide per mol enzyme FAD. The spectrum of the oxidized enzyme (*uppermost curve*) overlies that of the enzyme given the first addition of reductant because of incomplete removal of oxygen under these conditions. The small aberration at 550 nm is due to imperfect baseline correction. (**b**) Spectra of 23.5 μM wild-type enzyme in 0.1 M Na/K phosphate buffer, pH 7.6, 1.0 mM EDTA, 30 mM ammonium sulfate, titrated with the indicated amounts (mol) of dihydrolipoamide per mol enzyme FAD or, finally, with dithionite.

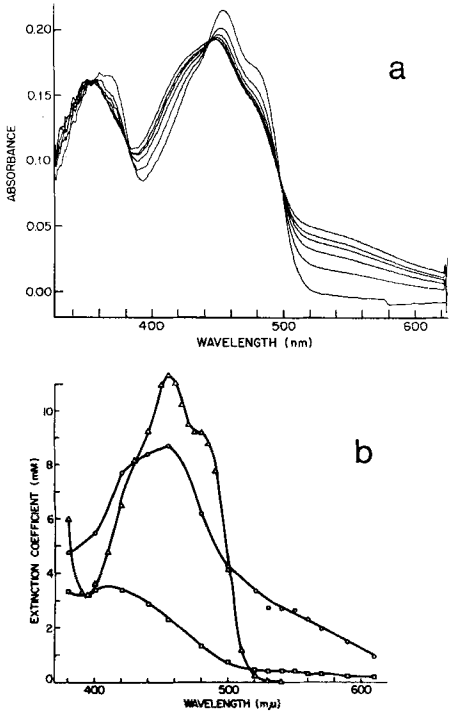

FIGURE 5. Anaerobic reduction of lipoamide dehydrogenase with dihydrolipoamide. (**a**) Spectra of His-444→Gln enzyme were taken, after rapid mixing (ca. 5 ms), using a Tracor Northern diode array spectrometer as the detector. Buildup of charge transfer absorbance (EH_2) at 550 nm at (*bottom* to *top traces*, respectively) 0, 3, 6, 9, 12, and 30 s upon reduction with 5.6 mol of dihydrolipoamide per mol enzyme FAD; enzyme was 9.5 μM in 0.12 M Na/K phosphate buffer, pH 7.6, 0.3 mM EDTA, at 20°C after mixing (2-cm light path). (**b**) Spectra of wild-type enzyme: (Δ) oxidized enzyme, (○) EH_2, (□) EH_4. Spectra of the oxidized and EH_4 forms were taken in a conventional spectrophotometer. The composite spectrum of the EH_2 form was generated in a series of identical mixings of enzyme with 11 mol dihydrolipoamide per mol enzyme FAD in a rapid reaction spectrophotometer with the detector set at the indicated wavelengths. The enzyme was 18 μM in 0.1 M Na/K phosphate buffer, pH 7.6, 0.3 mM EDTA, at 1°C after mixing (2-cm light path).

thioredoxin reductase (TRR) were not found,[14] and it was therefore not possible to assign distinct functions to these thiols as had been done with lipoamide dehydrogenase and glutathione reductase.[10,12] Therefore, site-directed mutagenesis has been used to singly modify each thiol to a serine. The properties of the mutated enzymes have allowed us to assign distinct functions to each of the thiols, at least tentatively.[15] FIGURE 6 shows the spectra of the two mutants compared with that of the wild-type enzyme. The differences between the traces may appear unimpressive, but they suggest which cysteine is nearer the FAD. The resolved character of the spectrum of the wild-type enzyme is due to the apolar milieu of the FAD.[36] For the mutated enzyme in which Cys-136 has been changed to serine [TRR(Ser-136,Cys-139)], the spectrum still shows the resolution characteristic of the wild-type enzyme. But in the enzyme

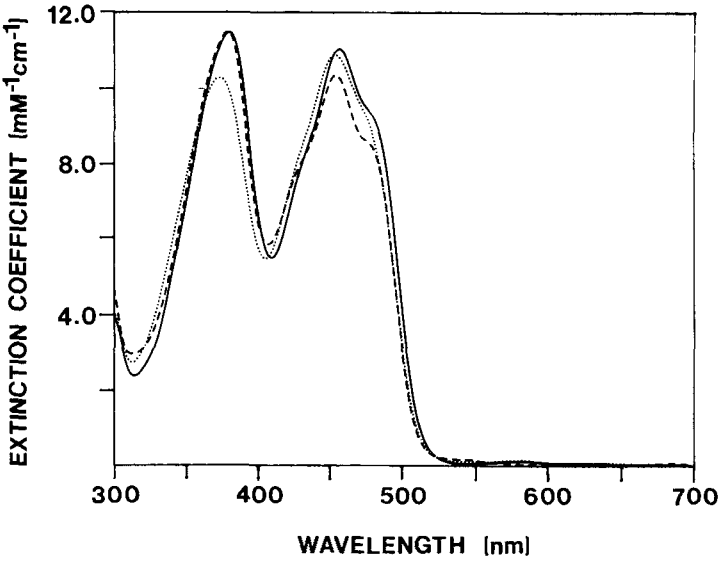

FIGURE 6. Absorbance spectra of wild-type thioredoxin reductase (TRR) and of proteins singly mutated at each thiol group. The proteins were in 0.1 *M* Na/K phosphate buffer, pH 7.6, 0.3 m*M* EDTA, at 12°C. *Solid line*, 18.0 μM (wild type) TRR(Cys-136,Cys-139); *dashed line*, 18.8 μM TRR(Ser-136,Cys-139); *dotted line*, 18.5 μM TRR(Cys-136,Ser-139).

which contains Cys-139 substituted with serine [TRR(Cys-136,Ser-139)], the resolved character is lost. This suggests that Cys-139 is closer to the FAD, since the change of this residue to the more polar serine is reflected in an alteration of the flavin spectrum to one characteristic of a more polar milieu.

While purifying the TRR(Ser-136,Cys-139) enzyme, we observed that it turned red upon the addition of ammonium sulfate. This was unexpected, since *E. coli* thioredoxin reductase has not previously been observed to stabilize the charge transfer complex.[37] As shown in FIGURE 7, ammonium ion causes the appearance at long wavelengths of a band very similar in shape to that of the lipoamide dehydrogenase and glutathione reductase charge transfer complexes.[1] An apparent K_d of 54 μM can be calculated after correction for non-specific binding (FIG. 7, inset). Only a limited

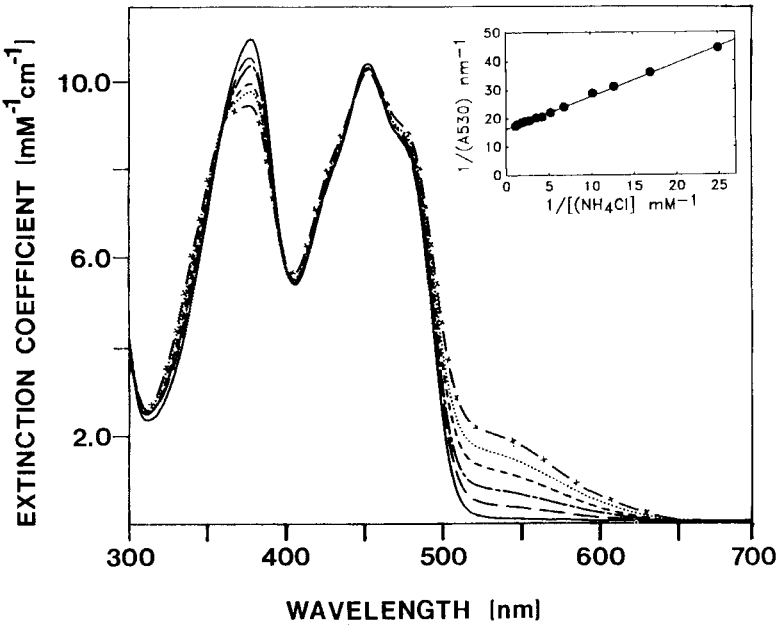

FIGURE 7. Titration of TRR(Ser-136,Cys-139) with 5 *M* NH_4Cl. 42.4 μM TRR(Ser-136,Cys-139) in 0.1 *M* Na/K phosphate buffer, pH 7.6, 0.3 m*M* EDTA, was titrated at 12°C with a 5 *M* NH_4Cl solution in 0.1 *M* Na/K phosphate buffer, 0.3 m*M* EDTA, pH adjusted to maintain the pH of the enzyme solution at 7.6 throughout the titration. (—) no NH_4Cl, (– –) 79 m*M* NH_4Cl, (– – – –) 98 m*M* NH_4Cl, (---) 192 m*M* NH_4Cl; (· · · ·) 455 m*M* NH_4Cl, and (–x–x–) 833 m*M* NH_4Cl. *Inset:* Benesi-Hildebrand plot of the titration; K_d, 54 μM; E_{530}, 1300 M^{-1} cm^{-1}.

number of ions have been tried, but ammonium ion is thus far unique in causing the effect. This result reinforces the suggestion that it is Cys-139 that is interacting with the FAD. If, as with other members of this enzyme family, the two distinct catalytic functions are each carried out by a different nascent thiol, then Cys-136 would perform the initial thiol-disulfide interchange with thioredoxin.

The finding that both thiol-altered enzymes were partially active was totally unexpected.[15] The analogous mutated enzymes in mercuric reductase[38] and lipoamide dehydrogenase[29] were totally without activity in their physiological reactions. The steady-state kinetics of the mutated enzymes have been compared with those of the wild-type enzyme in assays of NADPH-thioredoxin activity linked to 5,5'-dithiobis(2-nitrobenzoic acid) reduction, in which the NADPH concentration was kept constant by glucose-6-phosphate and glucose-6-phosphate dehydrogenase. The K_m values were little changed. The TRR(Ser-136,Cys-139) enzyme had just over 10% activity while the TRR(Cys-136,Ser-139) enzyme appeared to have a V_{max} of about 50% of the wild type. However, with the latter mutant enzyme, the intercept replots of the parallel Lineweaver-Burk plots were not linear, and the V_{max} was extrapolated from the data points for the two highest concentrations. It was therefore an upper limit. It has been suggested, based on the chemical modification studies and from the fact that the substrate for thioredoxin reductase is itself a protein, that the site of thiol-disulfide interchange must be more open in this enzyme than in other members of this enzyme

family.[14] It would appear, then, that in this more open active site, the remaining thiol in either mutation of thioredoxin reductase can fairly readily form a mixed disulfide with thioredoxin and that either of these can be reduced by $FADH_2$. This is in contrast to lipoamide dehydrogenase, where only one of the sulfurs can be attacked by the dithiol substrate, or to glutathione reductase, where only one of the nascent thiols can interchange with glutathione. Furthermore, in both these enzymes, interaction with FAD is the unique function of only one of the sulfurs. However, in the mutated thioredoxin reductase enzymes, no new chemistry is required.

FIGURE 8 shows mechanisms for the reoxidation by thioredoxin of each of the two mutated enzymes and indicates the step thought to be partially blocked and thus responsible for their reduced catalytic activities. This picture attempts to suggest that whereas in glutathione reductase the electron transfer thiol and the interchange thiol lie on a line perpendicular to the flavin ring—so that only the electron transfer thiol can interact with the FAD[19]—in thioredoxin reductase the thiols may lie on a less perpendicular line. If the proposal that Cys-139 normally interacts with the FAD is correct, the activity of TRR(Cys-136,Ser-139) (lower line in FIG. 8) would be limited by inefficient electron transfer directly from $FADH_2$ to the mixed disulfide, since this step would have to replace the thiol-disulfide interchange normally initiated by Cys-139. The fact that mutation of Cys-136 to serine results in substantial loss of activity suggests that both thiols have distinct functions, leading to a working hypothesis that Cys-136 is the interchange thiol. Then the activity of TRR(Ser-136,Cys-139) (upper line in FIG. 8) would be limited by inefficient thiol-disulfide interchange with oxidized thioredoxin initiated by Cys-139. The suggested mechanisms for activity in each mutated enzyme re-emphasize the more open active site required by thioredoxin reductase compared to related enzymes in order to accommo-

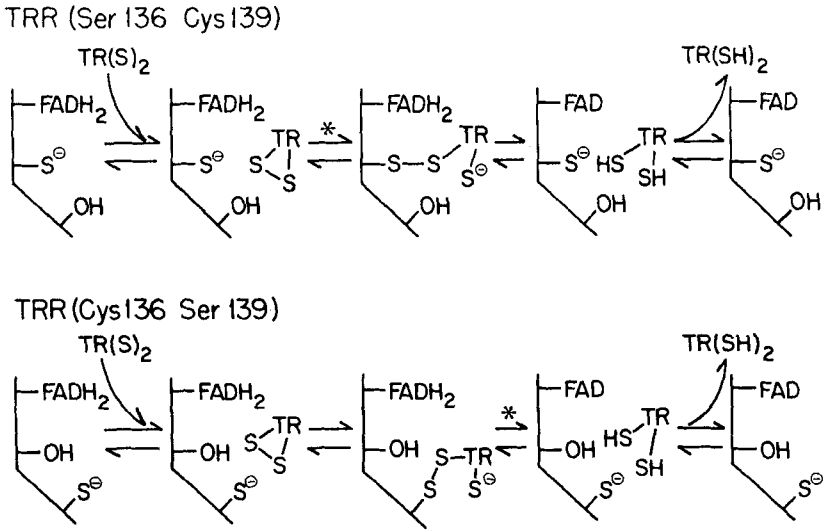

FIGURE 8. Reduction of thioredoxin by reduced forms of **(upper panel)** TRR(Ser-136,Cys-139) and **(lower panel)** TRR(Cys-136,Ser-139). The position of steps possibly responsible for catalytic inefficiencies are indicated by *asterisks*. $TR(S)_2$ and $TR(SH)_2$ are the oxidized and reduced forms of thioredoxin, respectively.

date its protein substrate. However, we are not suggesting that wild-type enzyme is any less specific in dithiol-disulfide interchange than are the other members of this enzyme family.

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