General Acid/Base Catalysis in the Active Site of *Escherichia coli* Thioredoxin†

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ABSTRACT: Enzymic catalysts of thiol:disulfide oxidoreduction contain two cysteine residues in their active sites. Another common residue is an aspartate (or glutamate), the role of which has been unclear. *Escherichia coli* thioredoxin (Trx) is the best characterized thiol:disulfide oxidoreductase, and in Trx these three active-site residues are Cys32, Cys35, and Asp26. Structural analyses had indicated that the carboxylate of Asp26 is positioned properly for the deprotonation of the thiol of Cys35, which would facilitate its attack on Cys32 in enzyme−substrate mixed disulfides. Here, Asp26 of Trx was replaced with isologous asparagine and leucine residues. D26N Trx and D26L Trx are reduced and oxidized more slowly than is wild-type Trx during catalysis by *E. coli* thioredoxin reductase. Stopped-flow spectroscopy demonstrated that the cleavage of the mixed disulfide between Trx and a substrate is slower in the D26N and D26L enzymes. Buffers increase the rate of mixed disulfide cleavage in these variants but not in wild-type Trx. These results indicate that Asp26 serves as an acid/base in the oxidation/reduction reactions catalyzed by Trx. Specifically, Asp26 protonates (during substrate oxidation) or deprotonates (during substrate reduction) the thiol of Cys35. A similar role is likely filled by the analogous aspartate (or glutamate) residue in protein disulfide isomerase, DsbA, and other thiol:disulfide oxidoreductases. Moreover, these results provide the first evidence for general acid/base catalysis in a thiol:disulfide interchange reaction.

Thiol:disulfide oxidoreduction reactions are prevalent in biology (Gilbert, 1990). Enzymic catalysts of this reaction are found in both eukaryotic and prokaryotic cells, and some of these enzymes are known to be essential for the viability or other phenotypes of their host organisms (Bardwell et al., 1991; Scherens et al., 1991). For example, protein disulfide isomerase (PDI[1]) is found in the endoplasmic reticulum of eukaryotic cells and is a necessary catalyst of the unscrambling of non-native protein disulfide bonds (Laboissière et al., 1995). DsbA is found in the periplasm of *Escherichia coli* cells and is a required catalyst for the oxidation of some cysteine residues to cystines (Bardwell et al., 1991).

Thiol:disulfide oxidoreductases have a common active-site sequence: Cys-Xaa-Xaa-Cys (CXXC). The two cysteine residues in this active site can form a disulfide bond. *E. coli* thioredoxin (Trx) is the best characterized thiol:disulfide oxidoreductase. Trx is a good reducing agent, having a CXXC motif with a reduction potential ($E°'$) of −0.270 V (Moore et al., 1964). Moreover, Trx reduces disulfide bonds quickly. For example, Trx reduces the three disulfide bonds of insulin 10^3-fold faster than does dithiothreitol (Holmgren, 1979).

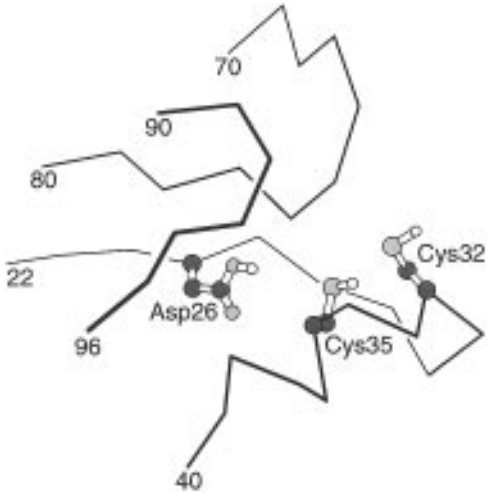

FIGURE 1: Functional groups in the active site of reduced *E. coli* thioredoxin (Jeng et al., 1994). This figure was created using MOLSCRIPT v1.2 (Kraulis, 1991).

The two active-site cysteine residues in Trx are Cys32 and Cys35. The side chain of Cys32 is solvent-accessible and has a microscopic pK_a value close to physiological pH (Chivers et al., 1997b). Such a pK_a value provides the optimal balance between the fraction of the cysteine side chain that exists in the active thiolate form and the nucleophilicity of that thiolate (Szajewski & Whitesides, 1980; Bednar, 1990; Gilbert, 1990).

In contrast to Cys32, Cys35 has a pK_a that is at least 4 units higher than physiological pH (LeMaster, 1996; Chivers et al., 1997b), making this residue relatively unreactive. The effectiveness of Cys35 as a nucleophile could, however, be enhanced by general base catalysis. A good candidate for that base in Trx is Asp26, which is proximal to Cys35 in the native enzyme (Figure 1). An aspartate residue at

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; $NADP^+$, nicotinamide adenine dinucleotide diphophosphate, oxidized form; NADPH, nicotinamide adenine dinucleotide diphophosphate, reduced form; NTB, 2-nitro-5-thiobenzoic acid; PDI, protein disulfide isomerase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TCEP, tris-(2-carboxyethyl)phosphine hydrochloride; TR, *E. coli* thioredoxin reductase; Tris, tris(hydroxymethyl)aminomethane; Trx, *E. coli* thioredoxin.

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Protein	Residues	Sequence	Reference
E. coli Trx	26 – 37	D F W A E W C G P C K M	Höög et al. (1984)
human Trx	26 – 37	D F S A T W C G P C K M	
human PDI	29 – 40	E F F A P W C G H C K A	Pihlajaniemi et al. (1987)
	373 – 384	E F V A P W C G H C K Q	
rat PDI	29 – 40	E F Y A P W C G H C K A	Edman et al. (1985)
	373 – 384	E F Y A P W C G H C K Q	
murine PDI	49 – 60	E F Y A P W C G H C K A	Mazzarella et al. (1990)
	393 – 404	E F Y A P W C G H C K Q	
murine ERp72	78 – 89	E F Y A P W C G H C K Q	Mazzarella et al. (1990)
	193 – 204	E F Y A P W C G H C K K	
	542 – 553	E F Y A P W C G H C K Q	
S. cerevisiae PDI	33 – 44	E F F A P W C G H C K N	Scherens et al. (1991)
	378 – 389	L Y Y A P W C G H C R R	
S. cerevisiae Eug1p	33 – 44	E F F A P W C L H S Q I	Tachibana and Stevens (1992)
	377 – 388	K Y Y A T W C I H S K R	
S. cerevisiae Mpd1p	32 – 43	E F Y A P W C G H C K K	Tachikawa et al. (1995)
E. coli DsbA	24 – 35	E F F S F F C P H C Y Q	Bardwell et al. (1991)
E. coli DsbB	35 – 46	V M L L K P C V L C I Y	Bardwell et al. (1993)
E. coli DsbC	112 – 123	V F T D I T C G Y C H K	Missiakas et al. (1994)
E. coli DsbD	398 – 409	D L Y A D W C V A C K E	Missiakas et al. (1995)
E. coli Grx	5 – 16	I F G R S G C P Y C V R	Björnberg and Holmgren (1991)
T4 Grx	8 – 19	D S N I H K C V Y C D N	LeMaster (1986)

FIGURE 2: Active-site sequences of thiol:disulfide oxidoreductases. Boxes enclose the common aspartate (or glutamate) and cysteine residues.

position 26 is conserved in thioredoxins from different species (Eklund et al., 1991). In other thiol:disulfide oxidoreductases, a glutamate residue is found at the analogous position in the amino acid sequence relative to the CXXC motif (Figure 2).

Here, we have used site-directed mutagenesis and a variety of kinetic assays to reveal the role of the common Asp/Glu residue in catalysis by a thiol:disulfide oxidoreductase. Our data indicate that Asp26 acts as an acid in the oxidation reactions and a base in the reduction reactions catalyzed by Trx. This finding enables us to propose a detailed mechanism for catalysis by Trx and other thiol:disulfide oxidoreductases.

EXPERIMENTAL PROCEDURES

General Methods. All enzymes for the manipulation of DNA were from Promega (Madison, WI). *E. coli* thioredoxin reductase was a generous gift of C. H. Williams, Jr. (University of Michigan). All other chemicals and reagents were of commercial or reagent grade, or better, and were used without further purification.

Creation of Asp26 Variants. pTRX, a pET vector (Novagen, Madison, WI) containing *E. coli trxA*, was constructed as described previously (Chivers et al., 1997b). Site-directed mutagenesis was performed using the method of Kunkel (1985). Oligonucleotides for site-directed mutagenesis were from IDT Technologies (Coralville, IA). Oligonucleotide p-ASN (5′ CTCTGCCCAGAAGTTAACGAGGATCGC 3′) and oligonucleotide p-LEU (5′ CTCTGCCCAGAAGAGTACTAGGATCGC 3′) were used to create the D26N and D26L variants of *E. coli* Trx, respectively. The underlined sequences correspond to restriction sites (p-ASN, *Hpa*I; p-LEU, *Sca*I) used to screen for mutated DNA. Dideoxynucleotide sequencing of plasmids identified by restriction screens was used to verify the DNA sequence coding for the Trx variants. The wild-type, D26L, and D26N enzymes were produced and purified as described previously (Chivers et al., 1997b). The protein preparations were of >95% purity as judged by SDS−PAGE.

Assays Using Thioredoxin Reductase. In *E. coli*, the oxidized form of Trx is converted to the reduced form in a reaction catalyzed by thioredoxin reductase (TR):

$$\mathrm{Trx_S^S + NADPH + H^+ \xrightleftharpoons{\text{thioredoxin reductase}} Trx_{SH}^{SH} + NADP^+} \quad (1)$$

In this reaction, TR forms a mixed disulfide with Trx, and oxidized TR is reduced in a separate step by NADPH (Williams, 1976; Holmgren, 1985). The kinetics of the TR-catalyzed reduction of the wild-type, D26N, and D26L enzymes were assessed by using the assay of Moore (Moore et al., 1964). In this assay, the reduction of oxidized Trx is monitored by the decrease in A at 340 nm due to the concomitant oxidation of NADPH [ϵ = 6220 M^{-1} cm^{-1} (Horecker & Kornberg, 1948)]. Here, TR (1 μL of a 0.94 μg/μL solution) was added to a solution (1.0 mL) of 0.10 M Tris−HCl buffer, pH 7.0, containing Trx (0.10−0.12 mg), NADPH (25 μM), and EDTA (1 mM).

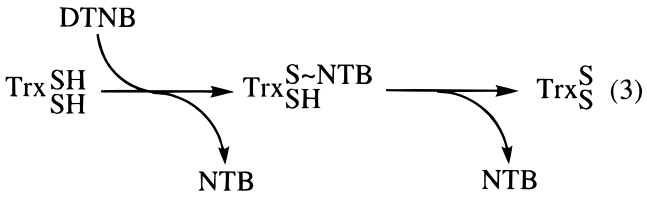
The kinetics of the TR-catalyzed oxidation of the wild-type, D26N, and D26L enzymes were also assessed. After equilibrium had been reached in the reaction of eq 1, $NADP^+$ (10 μL of a 0.138 M solution, as determined by A at 260 nm and ϵ = 18 000 M^{-1} cm^{-1}) was added to generate a new equilibrium.

Finally, the equilibrium data were used to compare the values of $E^{\circ\prime}$ for the three enzymes. The values of $E^{\circ\prime}$ were determined from the equation

$$E^{\circ\prime} = E^{\circ\prime}_{NADP^+} + \frac{RT}{nF} \ln \frac{[\mathrm{Trx(SH)_2}][NADP^+]}{[\mathrm{TrxS_2}][NADPH]} \quad (2)$$

where $E^{\circ\prime}_{NADP^+} = -0.315$ V (Clark, 1960), $R = 8.314$ J/(K·mol), $T = 298$ K, $n = 2$, and $F = 96\,485$ C·mol^{-1}. The concentration of NADPH (and hence of $NADP^+$) was evident from A at 340 nm. The concentrations of $Trx(SH)_2$ and $TrxS_2$ were derived from the total concentration of Trx and the concentrations of $NADP^+$ and NADPH. Three different starting concentrations of Trx were used to obtain a mean value of $E^{\circ\prime}$ for each enzyme.

Assays Using Stopped-Flow Spectroscopy. Reduced thioredoxin reacts in two chemical steps with its substrates (Holmgren, 1985). For example, the reaction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to give two molecules of 2-nitro-5-thiobenzoic acid (NTB) proceeds via a mixed disulfide intermediate:



(3)

The rate of each step in this reaction was determined by using stopped-flow spectroscopy.

The reduced forms of wild-type, D26N, and D26L enzymes were produced by the addition of a 10-fold molar excess of tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (Pierce, Rockford, IL). After 30 min, the reducing agent was removed by injecting the solution onto a Pharmacia Fast Desalt FPLC column that had been equilibrated with buffer purged of O_2(g) (*vide infra*). Protein concentrations after buffer exchange were typically 1.5−2.0 mg/mL, as determined by A at 280 nm [ϵ = 13 700 M^{-1} cm^{-1} (Krause et al., 1991)]. The reduced forms of the enzymes were used immediately after their preparation.

Reactions were performed in 0.010 M potassium phosphate buffer, pH 6.5, containing EDTA (1 mM). Unless noted otherwise, the ionic strength was held constant at 0.20 M by the addition of potassium chloride. To purge buffers of O_2(g), which could oxidize reduced Trx, buffer solutions were degassed (by vacuum for 30 min) and then saturated with N_2(g) (by bubbling for 30 min). If necessary, the pH of the buffer was adjusted after purging. A stock solution of DTNB (7.5 mg/mL, 12.6 mM) was prepared in O_2(g)-purged potassium phosphate buffer, and its pH was adjusted to 6.5.

Three solution parameters were varied in the stopped-flow spectroscopy experiments: ionic strength, buffer type, and buffer concentration. Ionic strength was varied by adjusting the concentration of potassium chloride in the DTNB stock solution. Buffer type was varied by adding acetate, imidazole, or Tris. Buffer concentration or ionic strength was adjusted by making 4× stocks of the appropriate solution in 0.010 M potassium phosphate buffer, pH 6.5, containing EDTA (1 mM). Each 4× stock was mixed with an equal volume of DTNB stock solution, generating a 2× stock solution in 0.010 M potassium phosphate buffer, pH 6.5, containing EDTA (1 mM).

Stopped-flow spectroscopy was performed with an OLIS RSM1000 rapid-scanning spectrometer equipped with a stopped-flow injection device (On-Line Instrument Systems; Bogart, GA). Experiments were performed at 22 °C. A solution (75 μL) of enzyme was placed in one injection chamber. An identical solution (75 μL) containing substrate rather than enzyme was placed in the other chamber. The contents were mixed at $t = 0$. The concentrations of DTNB and protein in the reaction chamber were 3.2 mM and 62−83 μM, respectively. Data were fitted to a two-exponential equation describing the appearance of a single species (that is, NTB). At least six experiments were performed for each reaction condition. The spectrometric data in different experiments varied by <10%.

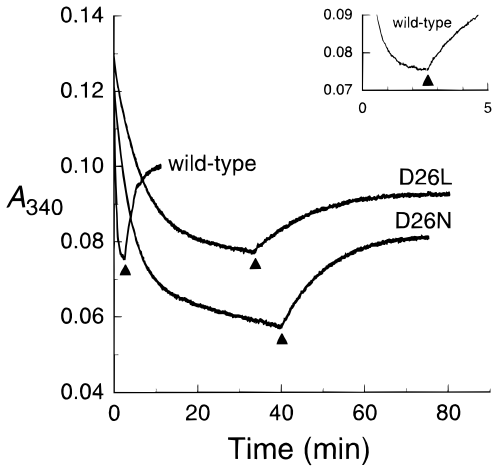


FIGURE 3: Time-course of the thioredoxin reductase-catalyzed reduction of wild-type and the D26N and D26L variants of thioredoxin. The decrease in A at 340 nm results from the reduction of thioredoxin reductase by NADPH. The inset is a magnification of the wild-type data, showing the approach to equilibrium. Arrowheads indicate the addition of $NADP^+$, which generates a new equilibrium. Assays were performed at 298 K in 0.10 M Tris−HCl buffer, pH 7.0, containing EDTA (1 mM). The value of ΔA_{340} varies because the concentrations of thioredoxin were similar, but not identical.

RESULTS

Three enzymes were prepared from *E. coli* by a recombinant DNA system: wild-type Trx, D26N Trx, and D26L Trx. The yield of pure enzymes was 100−150 mg per L of culture. The purification scheme made no attempt to remove endogenous wild-type Trx from the samples of the D26N and D26L enzymes. Any such contamination, which was estimated to be <0.1%, would have had no significant effect on the interpretation of the results reported herein. The three enzymes were subjected to two types of kinetics assays.

In the first type of assay, a decrease in the concentration of NADPH was used to report on the rate of reduction of Trx by TR (eq 1). In this reaction, Asp26 could act as an acid, donating a proton to the thiolate of Cys35. This assay is reversible, and an increase in the concentration of NADPH was used to report on the rate of reoxidation of Trx by TR.

In the second type of assay, a stopped-flow instrument was used to monitor the oxidation of Trx by DTNB. In this reaction, Asp26 could act as a base, removing a proton from the thiol of Cys35. The oxidation reaction proceeds in two steps (eq 3). Each step proceeds with the release of one molecule of NTB.

Assays Using Thioredoxin Reductase. Replacing Asp26 with an asparagine or leucine residue decreased the rate of reduction of oxidized Trx by TR (Figure 3). The rate of reduction of wild-type Trx was 5- to 10-fold greater than that of D26N Trx or D26L Trx at the same Trx concentration. Doubling the TR concentration did not change these relative rates. Similarly, the rate of reoxidation of the reduced forms of these variants was 5- to 10-fold less than that of the wild-type enzyme (Figure 3). The values of $E^{\circ\prime}$ for the D26N and D26L enzymes were −0.260 (±0.005) and −0.255 V (±0.005), respectively. The observed values of $E^{\circ\prime}$ for each enzyme were independent of the concentration of Trx. At

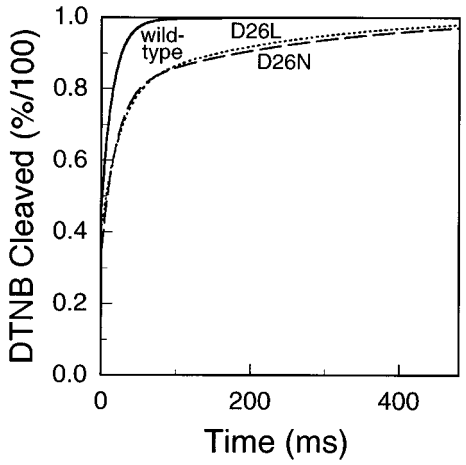


FIGURE 4: Time-course of the reduction of DTNB by wild-type and the D26N and D26L variants of thioredoxin. Assays were performed at 295 K in 0.010 M potassium phosphate buffer, pH 6.5, containing EDTA (1 mM).

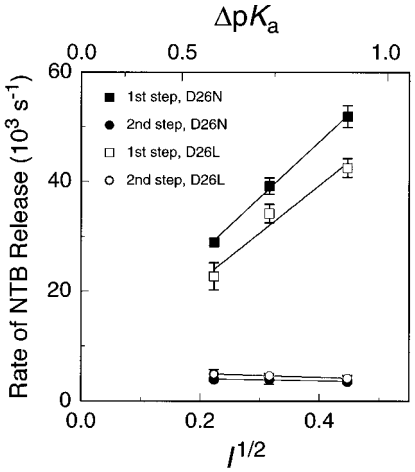


FIGURE 5: Effect of ionic strength on each step of the reduction of DTNB by the D26N and D26L variants of thioredoxin. Reactions were performed at 295 K in 0.010 M potassium phosphate buffer, pH 6.5, containing EDTA (1 mM). Ionic strength was varied by the addition of potassium chloride. Lines are linear least-squares fits of the rate vs $I^{1/2}$ data. The relationship between ΔpK_a ($= pK_a - pK_a°$) and $I^{1/2}$ was calculated with eq 4.

long assay times, evidence for air oxidation of reduced Trx was apparent. The air oxidation was slow relative to the oxidation of NADPH by the Asp26 variants and does not affect our conclusions.

Assays Using Stopped-Flow Spectroscopy. The reduction of DTNB by Trx started during the mixing time of the reaction (~5 ms). The first step in eq 3 occurred at a similar rate for all three enzymes (Figure 4). In contrast, the second step, the release of the second TNB molecule from a mixed disulfide with Cys32, occurs more slowly for D26N Trx and D26L Trx.

Increasing the ionic strength of the reaction medium increased the rate of the first step of DTNB reduction by the enzyme variants but had a negligible effect on the rate of the second step (Figure 5). This result is consistent with the first step (but not the second step) relying on nucleophilic attack by an enzymic thiolate that is fully accessible to solvent. The fraction of Cys32 that exists in the thiolate form depends on the ionic strength (I) of the solution. The relationship between the pK_a of Cys32 and I can be calculated from the simplified Debye−Hückel equation, which relates pK_a, $pK_a°$ (at $I = 0$), and n ($= -7$ for Cys32 of Trx[2]) at 25 °C (Neuberger, 1937; Snyder et al., 1981; Scopes, 1994):

$$pK_a = pK_a° + \frac{0.51n\sqrt{I}}{1 + 1.6\sqrt{I}} \quad (4)$$

As the pK_a of Cys32 decreased, the rate of nucleophilic attack at pH 6.5 increased (Figure 5). The absence of any effect of ionic strength on the second step is consistent with Cys35 being less accessible to solvent in the variants, as it is in wild-type Trx (Figure 1) (Jeng et al., 1994; LeMaster, 1996). This apparent inaccessibility along with experimental data (Kallis & Holmgren, 1980) that reveal Cys35 to be a poor nucleophile suggest that Cys35 does not react directly with DTNB.

Buffer effects on DTNB reduction show that Asp26 acts as an acid and a base. If increasing the buffer concentration increases the rate of a reaction in a solution of constant pH and constant ionic strength, then the reaction occurs (at least in part) by general acid/base catalysis (Bender et al., 1984; Jencks, 1987). Acetate, imidazole, and Tris buffers had no significant effect on catalysis by the wild-type enzyme (Figure 6), nor did these buffers affect the first step of DTNB reduction by either D26N Trx or D26L Trx (Figure 6). The buffers did, however, have a significant effect on the second step of DTNB reduction by these variants (Figures 6 and 7). Apparently, in the absence of an aspartate residue at position 26, the deprotonation of Cys35 limits the rate of the reduction of DTNB. This constraint is relaxed by an exogenous base. Of the three exogenous bases tested as a replacement for an aspartate side chain at position 26, imidazole was more effective than was acetate or Tris.

DISCUSSION

We propose that the reaction catalyzed by Trx proceeds by the mechanism shown in Figure 8. In the first step of this mechanism (step I), the thiolate form of Cys32 attacks a disulfide bond in a substrate to form a mixed disulfide. This nucleophilic attack requires Cys32 to be in the unprotonated thiolate form, which can be $>5 \times 10^{10}$-fold more reactive than the protonated thiol form (Bednar, 1990). At physiological pH, when Cys32 is unprotonated, Asp26 is protonated (Chivers et al., 1997b). In the mixed disulfide, however, the only pK_a of Asp26 is low, near 7.58 (LeMaster, 1996). So, in the second step of the mechanism (step II), the solvent-accessible carboxyl of Asp26 can readily lose a proton to solvent water. Now, the carboxylate of Asp26 is poised to remove a proton from the thiol of Cys35. In the final step of the mechanism (step III), the side chain of Asp26 abstracts a proton from the side chain of Cys35, and the thiolate attacks the mixed disulfide to form the reduced substrate and oxidized enzyme. This last step could occur in a concerted (as shown) or stepwise manner.

Two factors assist Cys35 in its nucleophilic attack on Cys32: the proximity of Cys35 to Cys32 (Figure 1) and the deprotonation of Cys35 to generate a thiolate nucleophile. Based on the data in Figures 3−7, we propose that the deprotonation of Cys35 in wild-type Trx is accomplished by Asp26. The results of the stopped-flow kinetics experi-

[2] The charge ($z = -3$) on Trx at pH 6.5 is estimated from its 15 anionic side chains (11 Asp + 4 Glu) and 12 cationic side chains (10 Lys + 1 Arg + 1 His). So, $n = 2z - 1 = -7$.

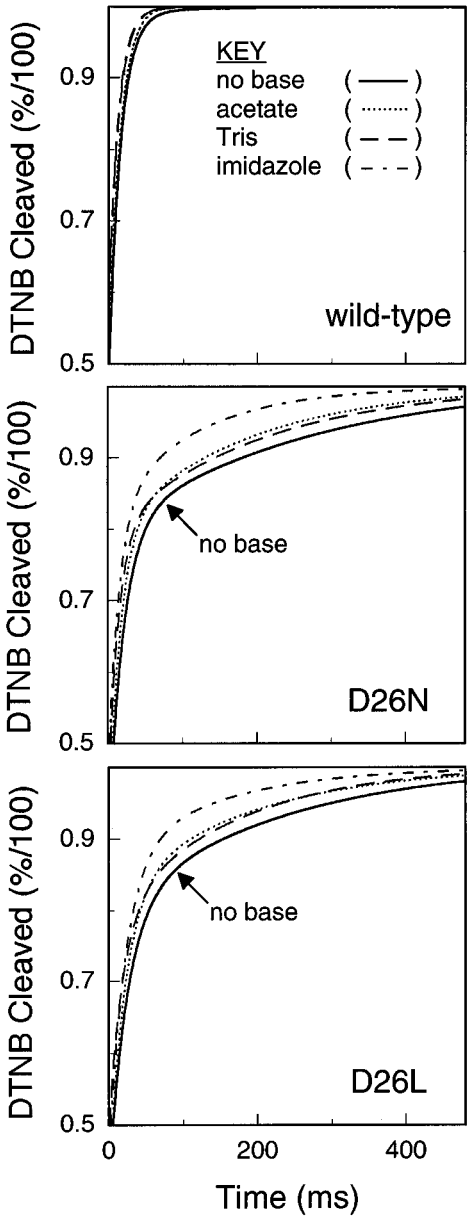


FIGURE 6: Effect of 0.10 M exogenous base on the time-course of the reduction of DTNB by wild-type and the D26N and D26L variants of thioredoxin. To highlight the second step in eq 3, the range of the ordinate is limited to 0.5 − 1.0. Assays were performed at 295 K in 0.01 M potassium phosphate buffer, pH 6.5, containing EDTA (1 mM). The ionic strength of each reaction was held constant at 0.20 M by the addition of potassium chloride.

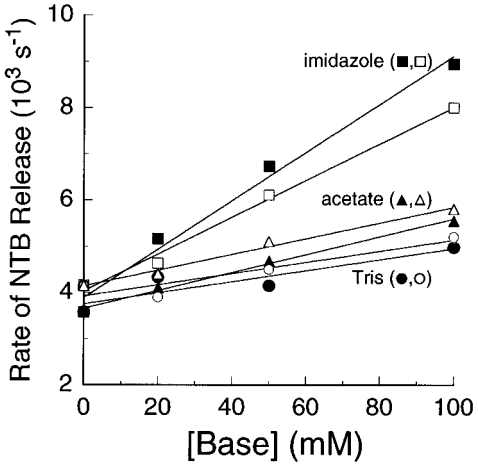


FIGURE 7: Effect of the concentration of exogenous base on the rate constant for the second step in the reduction of DTNB by the D26N (closed symbols) and D26L (open symbols) variants of thioredoxins. Assays were performed at 295 K in 0.010 M potassium phosphate buffer, pH 6.5, containing EDTA (1 mM). The ionic strength of each reaction was held constant at 0.20 M by the addition of potassium chloride. Data points are the mean values (±10%) for six experiments. The lines are linear least-squares fits and have the following slopes for the D26N and D26L variants, respectively: $k_{imidazole}$, 5×10^4 and 4×10^4 M^{-1} s^{-1}; $k_{acetate}$, 2×10^4 and 2×10^4 M^{-1} s^{-1}; k_{Tris}, 1×10^4 and 1×10^4 $M^{-1}s^{-1}$.

ments (Figure 4) demonstrate directly that the presence of Asp26 accelerates cleavage of the enzyme−substrate mixed disulfide. The D26N and D26L enzymes can recruit an exogenous base to compensate for the absence of the aspartate residue (Figures 6 and 7). Imidazole, acetate, and Tris can assist the deprotonation of Cys35 in the variants.

The first step of DTNB reduction by Trx is not affected by the presence of an aspartate residue at position 26 (Figure 4). This result suggests that Cys32 has a similar pK_a in the wild-type, D26N, and D26L enzymes. The same result was obtained by NMR spectroscopy (Chivers et al., 1997b). In the NMR experiments, however, a second microscopic pK_a of 9.2 was detected for Cys32 in wild-type Trx. This microscopic pK_a is not apparent in the results of the stopped-flow experiments. Detection of microscopic pK_a's by monitoring the rate of a chemical reaction requires that the chemical reaction occur more quickly than does proton exchange by the nucleophile. Apparently, the first step of DTNB reduction by Trx is too fast to allow for detection of the microscopic pK_a's of Cys32.

Water or buffer molecules have access to Cys35 in the mixed disulfide between Cys32 and NTB. Imidazole, acetate, and Tris can apparently deprotonate Cys35 either directly or via an intervening water molecule. This result is consistent with the ability of small molecules to form covalent bonds with Cys35 (Kallis & Holmgren, 1980). Although buffer catalysis is evident (Figures 6 and 7), the data do not obey the Brønsted relationship (Bender et al., 1984; Jencks, 1987), suggesting that steric constraints could limit access to Cys35. This interpretation is supported by the absence of a dependence on ionic strength (Figure 5), which indicates that Cys35 is at least somewhat buried in the Trx variants.

Formation of a mixed disulfide between Trx (Cys32) and a large substrate, such as TR (Cys135), appears to limit the access of small molecules to the active site. The presence of 0.10 M Tris accelerated the cleavage of the Trx_{SH}^{S-NTB} mixed disulfide in D26N Trx and D26L Trx (Figure 6), making the variants' cleavage rate close to that of wild-type Trx. All of the TR assays were performed in a solution containing 0.10 M Tris. The cleavage of the Trx_{SH}^{S-TR} mixed disulfide is significantly slower in the D26N and D26L enzymes than in wild-type Trx (Figure 3). The contribution of Asp26 to catalysis is therefore greater in reactions with large substrates.

Curiously, Asp26 is not conserved in all glutaredoxins (Figure 2), which catalyze the reduction of oxidized glutathione. A mixed disulfide with this small substrate may not exclude access of water or buffer molecules to the buried cysteine residue (Bushweller et al., 1994). Alternatively, one of the two carboxylate groups in glutathione itself could act as a base during substrate-assisted catalysis (Carter & Wells, 1987; Widersten et al., 1996). If catalysis by glutaredoxin

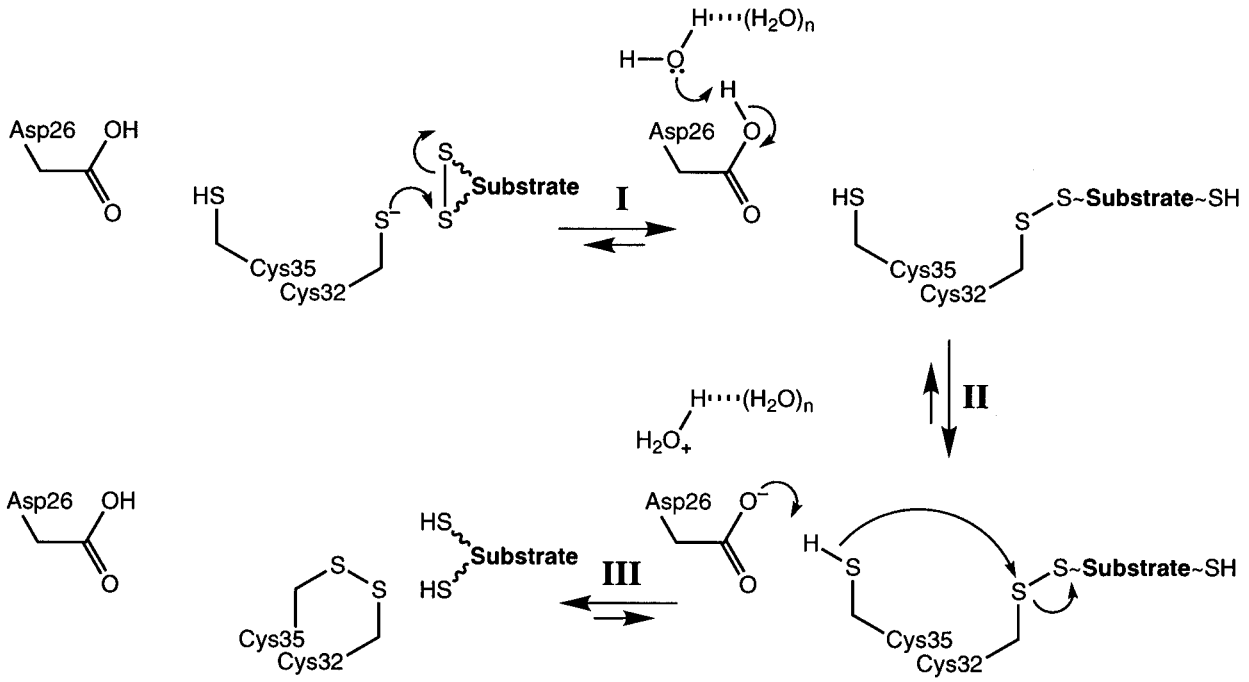


FIGURE 8: Proposed mechanism for catalysis by thioredoxin. Step III could occur in a stepwise manner via an intermediate having an Asp26 carboxyl and a Cys35 thiolate. Other thiol:disulfide oxidoreductases could recruit the Asp/Glu and Cys residues listed in Figure 2 to effect an analogous mechanism.

proceeded by either of these two mechanisms, then deprotonation of the buried cysteine residue would not require the assistance of an enzymic base.

The pK_a values of the side chain of Asp26 is 7.5 in oxidized Trx and 7.5 or 9.2 (depending on the protonation state of Cys32) in reduced Trx (Chivers et al., 1997b). Thus, at physiological pH, Asp26 will not always be in the correct protonation state for catalysis. When Trx is acting as a reductant, Asp26 must be deprotonated to act as a base once the enzyme−substrate mixed disulfide forms. At pH 7.0, however, Asp26 will be protonated in most mixed disulfide molecules because it has a pK_a near 7.58 (LeMaster, 1996). In these molecules, Asp26 must somehow lose its proton. A reasonable mechanism for this process involves proton abstraction by bulk water, which can approach Asp26 from the side of the enzyme molecule distal from the active site. Interestingly, three or four water molecules are observed in proximity to Asp26 in the crystalline structures of thioredoxins from both *E. coli* and Anabena (Katti et al., 1990; Saarinen et al., 1995).

Replacing Asp26 has some analogy to replacing the C-terminal cysteine of a CXXC motif. If Cys35 is not deprotonated, then a mixed disulfide between Trx and substrate cannot undergo intramolecular attack by the enzyme. In CXXS-containing enzymes, such as wild-type Eug1p (Tachibana & Stevens, 1992), CLHS/CIHS PDI (LaMantia & Lennarz, 1993), CGHS PDI (Laboissière et al., 1995; Walker et al., 1996), and CGPS Trx (Chivers et al., 1996), the mixed disulfide cannot be cleaved by the enzyme but instead must rely on either a substrate or a solution thiol for scission. Apparently, D26N Trx and D26L Trx do not rely completely on a nonenzymic thiol for cleavage of a mixed disulfide. Still, replacing Asp26 of Trx (like replacing the analogous Asp/Glu of other thiol:disulfide oxidoreductases; Figure 2) is liable to make its mixed disulfides less labile. This stability (which is kinetic, not thermodynamic) increases the probability that the mixed disulfide will be cleaved by a nonenzymic thiol. Replacing the active-site Asp/Glu residue could therefore enhance the ability of thiol:disulfide oxidoreductases to catalyze the isomerization (relative to the reduction) of disulfide bonds in protein substrates (Laboissière et al., 1995; Walker et al., 1996; Chivers et al., 1998; Walker & Gilbert, 1997).[3]

The kinetic stability of mixed disulfides in thiol:disulfide oxidoreductases lacking an Asp/Glu residue has another consequence. Because the mixed disulfides of such variants are less reactive, reaching redox equilibrium between a variant and substrate takes longer and could in theory take much longer. It is therefore a formal possibility that an observed $E^{\circ\prime}$ could be a *kinetic* reduction potential rather than a thermodynamic one. But because different concentrations of Trx yielded similar values of $E^{\circ\prime}$ in our assays using TR, the values of $E^{\circ\prime}$ derived from the data in Figure 3 are thermodynamic.

The mechanism in Figure 8 differs from other mechanisms proposed recently for Trx. Dyson, Holmgren, and co-workers have proposed that Cys35 is deprotonated by a substrate thiolate (Jeng et al., 1995; Dyson et al., 1997). According to their mechanism, the rate of mixed disulfide cleavage would not rely on an enzymic base. We have observed otherwise (Figures 4, 6, and 7). Woodward, Fuchs, and co-workers have suggested that the role of Asp26 is to "regulate" the value of $E^{\circ\prime}$ (Wilson et al., 1995). Because Asp26 titrates near pH 7 (Chivers et al., 1997b), it necessarily has an effect on $E^{\circ\prime}$ (Chivers et al., 1997a). We observe that the $E^{\circ\prime}$ of the Cys32−Cys35 disulfide bond does indeed increase in variants lacking Asp26, but only slightly. Most recently, Glockshuber and co-workers made an analogous mutation in DsbA, replacing Glu24 with a glutamine residue (Jacobi et al., 1997). They did not consider a role for Glu24

[3] In the analogy of Walker et al. (1996) and Walker and Gilbert (1997), replacing the Asp/Glu residue slows the intramolecular clock of the CXXC motif.

of DsbA in general acid/base catalysis, proposing instead that the role of this residue is to direct the folding pathway of DsbA. In contrast, we have demonstrated that Asp26 has a direct role in catalysis, as depicted in Figure 8.

CONCLUSIONS

The role of the common Asp/Glu residue in catalysis by dithiol:disulfide oxidoreductases has been examined in Trx, the best characterized enzyme in this family. Replacing this residue, which is Asp26 in Trx, with an asparagine or leucine decreases the rate of both Trx reduction and Trx oxidation by TR. The oxidation and reduction reaction of Trx occur in two steps, via an enzyme − substrate mixed disulfide. The rate of mixed disulfide formation from reduced Trx does not rely on Asp26. But, the breakdown of the mixed disulfide is slower in the Trx variants. The rate of this step is increased by added buffers, which apparently act in lieu of the sidechain of Asp26. These data indicate that Asp26 of Trx acts as an acid/base in the oxidation/reduction of a substrate. The analogous Asp/Glu residue in the active sites of protein disulfide isomerase, DsbA, and other thiol:disulfide oxidoreductases is likely to have a similar role. Finally, the significance of our findings extends beyond these enzymes. To the best of our knowledge, we have herein provided the first evidence for general acid/base catalysis in a thiol:disulfide interchange reaction.

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