Articles

Potential Active-Site Base of Thioredoxin Reductase from *Escherichia coli*: Examination of Histidine^{245} and Aspartate^{139} by Site-Directed Mutagenesis†

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ABSTRACT: It has been proposed that an acid–base catalyst facilitates the reduction of thioredoxin by thioredoxin reductase from *Escherichia coli* [O'Donnell, M. E., & Williams, C. H., Jr. (1983) *J. Biol. Chem. 252*, 13795-13805]. The X-ray crystal structure reveals two groups which could potentially fulfill this role: His^{245} and Asp^{139}. Using site-directed mutagenesis, His^{245} was changed to asparagine (H245N) and alanine (H245A) and Asp^{139} was changed to glutamate (D139E), asparagine (D139N), and leucine (D139L). Steady-state kinetic analysis of the His^{245} mutants gave turnover numbers and K_m values similar to those of wild-type thioredoxin reductase. All three Asp^{139} mutants were altered in their overall kinetic properties: D139E had 38% of wild-type activity, D139N had 1.5%, and D139L had no measurable activity. Rate constants for the NADPH to 3-acetylpyridine adenine dinucleotide phosphate transhydrogenase activity were similar for all of the Asp^{139} and His^{245} mutants and wild-type thioredoxin reductase. Stopped-flow kinetic measurements of the reductase half-reaction of H245A and H245N gave rate constants that were up to 2-fold faster than those found for wild-type thioredoxin reductase, while all of the Asp^{139} mutants had rate constants comparable to those of wild-type. To further examine the causes of the low overall activity of D139N, the oxidative half-reaction was measured. The reoxidation of reduced D139N mixed with oxidized thioredoxin occurred at a very slow rate constant of 0.23 s^{-1}—about 1% that of wild-type enzyme. We suggest that Asp^{139} is the active-site acid catalyst which functions to protonate the thiolate anion of reduced thioredoxin. Thus, the reductive half-reaction is not affected in mutants of Asp^{139}; only the oxidative half-reaction is slowed, consistent with the proposed function of this residue as an acid–base catalyst.

Thioredoxin reductase from *Escherichia coli* catalyzes the transfer of electrons from NADPH to thioredoxin. Thioredoxin is a small (M_r = 12 000) monomeric protein containing one redox-active disulfide and having several metabolic roles (Moore et al., 1964; Russel & Model, 1986; Holmgren, 1989; Williams, 1992). Thioredoxin reductase is a member of a family of pyridine nucleotide–disulfide oxidoreductases which includes lipoamide dehydrogenase, glutathione reductase, and mercuric ion reductase (Williams, 1992). The amino acid sequence of thioredoxin reductase has been derived from the gene sequence (Russel & Model, 1986). The redox-active disulfide is composed of Cys^{135} and Cys^{138} (Thelander, 1970; Ronchi & Williams, 1972). Thioredoxin reductase is a dimer of identical subunits (M_r = 35 000), each having one redox-active disulfide and one FAD. The reaction sequence is initiated by binding of NADPH to oxidized thioredoxin reductase and passage of the electrons to the FAD. Electrons are equilibrated with the disulfide, and these three steps constitute the reductive half-reaction. The oxidative half-reaction involves dithiol–disulfide interchange between the nascent dithiol of thioredoxin reductase and the disulfide of thioredoxin.

Enzyme reduced with 1 equiv of NADH (to avoid complexes with NADPH and $NADP^+$) is an equilibrium mixture of four species: oxidized, 2-electron-reduced dithiol–FAD, 2-electron-reduced disulfide–$FADH_2$, and 4-electron-reduced forms (O'Donnell & Williams, 1983). The two 2-electron-reduced forms predominate and are present at approximately equal concentrations. This is a consequence of the fact that the disulfide/dithiol and FAD/$FADH_2$ redox potentials are separated by only 11 mV (O'Donnell & Williams, 1983).

It has been shown by several methods that Cys^{138} interacts more closely with the FAD than does Cys^{135} (Prongay et al., 1989; Prongay & Williams, 1990, 1992). It was proposed that Cys^{135} initiates dithiol–disulfide interchange with thioredoxin on the basis that, in lipoamide dehydrogenase and glutathione reductase, each thiol has a distinct role. Thus, Cys^{135} is referred to as the interchange thiol and Cys^{138} as the electron-transfer or flavin-interacting thiol. The X-ray crystal structure shows that Cys^{138} is indeed close to the flavin—3.0 Å from the C4a position of the isoalloxazine ring—whereas Cys^{135} is 4.4 Å from the C5a as shown in Figure 1 (Kuriyan et al., 1991; Waksman et al., 1994).

The dithiol–disulfide-interchange step is presumed to be initiated by attack of a thiol anion of Cys^{135} on the disulfide of thioredoxin. Deprotonation of Cys^{135} would be facilitated by a nearby base. This presumption is based on model reactions (Foss, 1961) and by analogy with the known mechanisms of lipoamide dehydrogenase and glutathione reductase (Mat-

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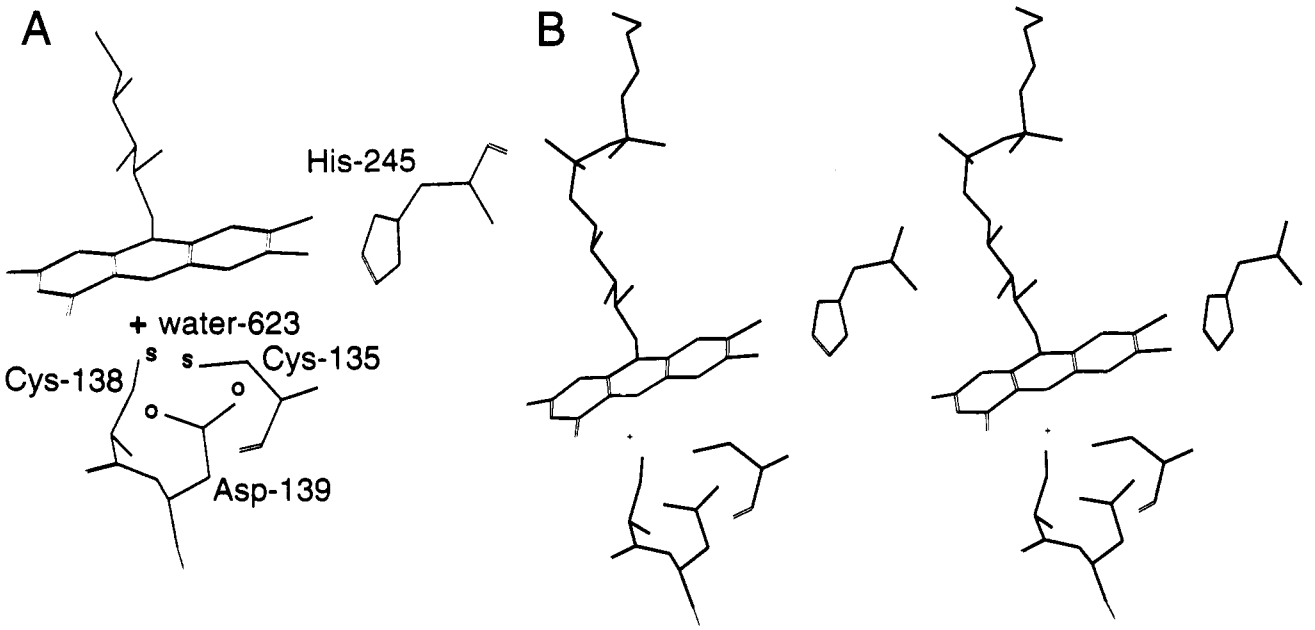

FIGURE 1: Structure of the active site of thioredoxin reductase. The view is of the *re* side of the isoalloxazine ring; N10 is closest, and N5 and the water are farthest away. (A) The sulfurs of Cys^{135} and Cys^{138} and the oxygens of Asp^{139} are designated by S and O, respectively. The sulfurs are in the foreground, and the oxygens are in the background. $Water^{623}$ is between N5 and the oxygens. His^{245} is located slightly above and to the right of the benzene ring of the FAD. (B) Stereoview of the same.

Table 1: Bacterial Strains, Phage, and Plasmids

E. coli strains	relevant phenotype	source or reference
HB101	*hsdS recA ara-14 supE lacY galK proA xyl-5 mtl-1 rpsL*	Boyer and Roulland-Dissoi (1969)
XL1-Blue	*recA1 endA1 gyrA96 thi hsdR17* (r_K^-, m_K^+) *supE44 relA1 lac*[*F' proAB lacI*^{q}*Z* M15 TN10(tet)]	Bullock et al. (1987)
A304	*trxB*$^-$ derivative of K38: HfrC *supII*$^+$ trxB::kan	Russel and Model (1986)
K1380	*trxB*$^-$ derivative of W3110: W3110 trxB15::kan zbj-1230::Tn10	M. Russel
W3110	*F*$^-$ *mcrA mcrB IN*(*rrnD-rrnE*)*1* λ^-	Hill and Harnish (1981)
plasmids		
pRBG156	high copy derivative of pBR322, Tp^r	Gayle et al. (1986)
pPMR14	clone of wild-type thioredoxin reductase	Russel and Model (1985)
pTrR13	H245N mutant of pPMR14	this study
pTrR27	H245A mutant of pPMR14	this study
pTrR1	wild-type TrR cloned into pRBG156	this study
pTrR50	D139L mutant of pTrR1	this study
pTrR53	D139N mutant of pTrR1	this study
pTrR57	D139E mutant of pTrR1	this study
phage		
f1R366	f1 phage-containing cloned TrR	Russel and Model (1985)

thews et al., 1977; Arscott et al., 1981). Indeed, the X-ray crystal structures of these enzymes have confirmed this hypothesis (Karplus & Schulz, 1987; Schierbeek et al., 1989) as have site-directed mutagenesis studies (Williams et al., 1989; Berry et al., 1989; Benen et al., 1992).

In the case of thioredoxin reductase, studies determining the pH dependence of the individual redox potentials of the flavin and disulfide centers suggested that an acid–base catalyst was located in the active site (O'Donnell & Williams, 1983). The X-ray crystal structure of thioredoxin reductase reveals two possible residues: His^{245}, which is located about 5 Å from the C8 methyl of the flavin ring, and Asp^{139}, which is near the thiols as seen in Figure 1 (Waksman et al., 1994). Asp^{139} is absolutely conserved in all sequences of thioredoxin reductase and thioredoxin-reductase-like proteins determined thus far, whereas His^{245} is found in only three other sequences (see Discussion). These two residues are the only potential acid–base catalysts in the region of the active site and therefore are good candidates for site-directed mutagenesis to examine their role in catalysis.

MATERIALS AND METHODS

Enzymes and Reagents. All restriction endonucleases and T4 DNA ligase were obtained from Gibco-BRL, Boehringer Mannheim, or New England Biolabs. Reagents and enzymes used for the mutagenesis procedure were included in the Amersham mutagenesis kit. Dideoxy sequencing was performed by using the Sequenase version 2 system (U.S. Biochemical Corp.) according to the manufacturer's instructions with deoxyadenosine 5'-[α-^{35}S]thiotriphosphate (Amersham). Some automated sequencing reactions were also performed in the University of Michigan Biomedical Research Core Facility. Glucose-6-phosphate, RNase A, trimethoprim, NADPH, $APyADP^+$,[1] DTNB, and baker's yeast glucose-6-phosphate dehydrogenase were purchased from Sigma.

DNA and Bacterial Strains. Bacterial strains, bacteriophage, and plasmids are shown in Table 1. Plasmid pPMR14 (Russel & Model, 1985), which was originally designed for

[1] Abbreviations: APyADPH and $APyADP^+$, reduced and oxidized forms of 3-acetylpyridine adenine dinucleotide phosphate, respectively.

Table 2: Oligonucleotides Used for Mutagenesis[a]

mutation	oligonucleotide	new restriction enzyme site
wild-type	5′-AGTATTCGGGCTGAGACCGATAGCAACAAACAG-3′	
H245A	5′-AGTATTCGGGCTGGCACCGATAGCAACAA-3′	(*Nla*IV)
H245N	5′-ATTCGGGCTGATACCGATCGCAACAAACAG-3′	(*Pvu*I)
wild-type	5′-AACACGTTGGACGCTGCCAAAGAAGAT-3′	
D139L	5′-CGTTGGACGGATCCAAAGAAG-3′	(*Sty*I)
D139N	5′-AACACGTTGTACGTTGCCAAAGA-3′	(*Nsp*HI)
D139E	5′-ACGTTGTACACTTCCAAAGAAGAT-3′	(*Afl*III)

[a] The underlined bases were mutated from wild-type thioredoxin reductase.

expression of wild-type *trxB* gene, was used to construct derivatives that expressed the mutant thioredoxin reductases H245A and H245N. In order to obtain higher yields of wild-type and subsequent mutants of thioredoxin reductase, the entire 1.7-kb *Eco*RI fragment of f1R366 replicative form DNA, containing the *trxB* gene, was cloned into *Eco*RI-cleaved pRBG156. The resulting high copy number plasmid pTrR1 was used to express wild-type thioredoxin reductase. It was also used in cassette replacement mutagenesis to express mutants D139L, D139N, and D139E. Strain A304 (Russel & Model, 1986) or strain K1380 served as the host for expression of the mutated thioredoxin reductases. *E. coli* W3110 was used to express wild-type thioredoxin reductase from plasmid pTrR1, and strain XL1-Blue was used as the recipient of the mutagenesis reaction products.

Plasmid DNA and bacteriophage replicative form DNA were isolated by using standard alkaline lysis methods (Sambrook et al., 1989). The double-stranded circular forms of DNA were further purified by treatment with 50 μg/mL RNase A at 37 °C for 30 min followed by chromatography on a 1- × 3-cm Superose 12 column (Pharmacia) equilibrated with 0.1 M NaCl, 10 mM EDTA, pH 8, as the buffer. Production of single-stranded DNA from bacteriophage f1R366 and its mutated derivatives were performed according to standard methods (Sambrook et al., 1989).

Oligonucleotide-Directed Mutagenesis. The oligonucleotides that were used for mutagenesis reactions are shown in Table 2. The introduction of the new restriction sites allowed for simplified screening of potential mutants.

Mutagenesis reactions were carried out by using the phosphorothioate method (Sayers et al., 1988) using the enzymes and reagents supplied in the Amersham mutagenesis kit, version 2.0. Single-stranded f1R366 DNA served as the template. Double-stranded circular DNA resulting from the mutagenesis reactions was transformed into *E. coli* XL1-Blue to obtain phage plaques. Individual plaques were picked, and replicative form DNA was isolated from each by the miniprep method (Sambrook et al., 1989). The resulting DNA was digested with the appropriate restriction enzyme to screen for the presence of the desired mutation. Replicative form DNA from individual isolates which contained the newly created mutations was purified and used to construct plasmids expressing the altered enzymes.

Construction of Plasmids Expressing Mutated Thioredoxin Reductases. Replicative form DNA of phage carrying the mutations for H245A and H245N was digested with *Pvu*II and *Spl*I, and the resulting 152-bp fragment was purified from a 1% agarose gel by using the NA45 ion-exchange membrane (S&S) according to the manufacturer's instructions. The same steps were performed on plasmid pPMR14, except that the 152-bp wild-type fragment was discarded and the large 6.7-kbp fragment was recovered from the gel. The two recovered fragments were mixed, ligated, and transformed into *E. coli* HB101. Plasmid DNA was isolated from several ampicillin-resistant colonies and screened for the presence of the mutated fragment with the appropriate restriction enzymes. One of each plasmid carrying the potential mutated fragment was then sequenced across the *Pvu*I–*Spl*I sites to verify the presence of the desired mutations and the proper insertion of the mutant cassette. The resulting plasmids pTrR27 (H245A) and pTrR13 (H245N) were used for subsequent growth and purification of the mutated thioredoxin reductases.

The same strategy was used for expression of D139L, D139N, D139E, except that restriction enzymes *Ksp*I and *Rsr*II were used to excise the 289-bp mutated cassette from the replicative form phage DNA and plasmid pTrR1 was used as the recipient. The resulting constructs pTrR50 (D139L), pTrR53 (D139N), and pTrR57 (D139E) were then sequenced through the entire *Ksp*I–*Rsr*II region to verify the new mutation.

Purification of Mutant Thioredoxin Reductases. E. coli strain A304 transformed with either pTrR13 (H245N) or pTrR27 (H245A) was grown in SOB medium (Sambrook et al., 1989) containing 100 μg/mL ampicillin. *E. coli* K1380 transformed with pTrR50 (D139L), pTrR53 (D139N), or pTrR57 (D139E) was grown in Mueller–Hinton Broth (Difco) supplemented after autoclaving with 100 mL/L of a solution of 0.17 M KH_2PO_4, 0.72 M K_2HPO_4, 10 mL/L 20% (w/v) glucose, and 50 μg/mL trimethoprim. Cells from cultures grown to saturation were harvested, resuspended in 1–2 volumes of 0.1 M Na/K phosphate, 0.3 mM EDTA, pH 7.6, and disrupted by sonication. Streptomycin sulfate was added to a final concentration of 2% (w/v) and the mixture centrifuged at 40000*g* for 90 min. Ammonium sulfate was added to 30% and then 80% saturation. The 30–80% fraction was dialyzed in 10 mM Na/K phosphate, 0.3 mM EDTA, pH 7.6, and loaded onto a 1- × 12-cm 2′,5′-ADP$^+$ agarose column. After the column was washed exhaustively, the enzyme was eluted with stepwise increases of NaCl in 100 mM increments. The major peak from the column, eluting at 500 mM NaCl, was ≥95% pure thioredoxin reductase as judged by SDS polyacrylamide gel electrophoresis and by spectroscopic analysis. Enzyme concentration was determined by using an extinction coefficient of 11 300 M^{-1} at 456 nm for the enzyme-bound FAD (Williams et al., 1967).

Kinetic Analysis. Steady-state kinetics were examined using the previously described coupled assay which measures the reduction of DTNB (Prongay et al., 1989) while varying the concentrations of the substrates, thioredoxin, and NADPH. The concentrations of the mutated thioredoxin reductases in the assays were 6 nM for H245A and H245N and 25 nM for D139E.

For mutant enzymes D139N, D139L, and D139E, assays for thioredoxin reduction, NADPH oxidase, and NADPH–APyADP$^+$ transhydrogenase activities were run at fixed concentrations of the appropriate substrates. Thioredoxin reduction and NADPH oxidase activities were monitored by the disappearance of NADPH at 340 nm, while NADPH–

APyADP+ transhydrogenase activity was measured by the increase in APyADPH absorbance at 390 nm. The kinetic parameters for both wild-type and mutated enzymes were determined by using a rectangular hyperbolic fit to the data.

Rapid reaction kinetics were measured with a stopped-flow apparatus, having a light path of 2 cm, interfaced with a Tracor Northern diode array spectrophotometer. Enzyme and thioredoxin solutions were made anaerobic by placing them in a glass vessel which was repeatedly evacuated and flushed with oxygen-scrubbed nitrogen. NADPH solutions were made anaerobic by bubbling the oxygen-scrubbed nitrogen through the solution for 20 min prior to introduction into the stopped-flow instrument.

For measurement of the oxidative half-reaction for D139N in the stopped-flow spectrophotometer, the enzyme was first reduced by using the xanthine/xanthine oxidase system (Massey, 1990) in a mixture that contained 39.5 μM D139N, 525 μM xanthine, 75 nM xanthine oxidase, and 100 units/mL catalase in Na/K phosphate buffer, pH 7.6. Enzyme reduction was monitored spectrally and allowed to proceed at room temperature.

RESULTS

Spectral Analysis. The absorbance of oxidized, wild-type, and mutated enzymes was virtually identical (data not shown). The peaks for H245A, H245N, D139L, D139N, and D139E were located at 380 and 456 nm and were the same as for wild-type enzyme. The shoulder in the 480-nm region, indicative of the hydrophobic environment of the flavin (Harbury, 1959), is unchanged in the mutant enzymes. Apparently, neither His^{245} nor Asp^{139} are not close enough to noticeably perturb the absorbance properties of the flavin.

The fluorescence excitation of several mutants was examined at pH 7.6, 12 °C, and measured at 456 nm: H245A, H245N, and D139L had 95%, 85%, and 99%, respectively, of the fluorescence excitation of wild-type thioredoxin reductase, again indicating that His^{245} and Asp^{139} are not close enough to purturb the flavin fluorophore. The fluorescence excitation at 456 nm (emission wavelength 550 nm) was measured as a function of pH and resulted in pK_a values of 7.01 for H245N, 7.30 for H245A, and 7.53 for D139L (Figure 2). Wild-type enzyme has a pK of 7.03 (O'Donnell & Williams, 1983), and the replacement of His^{245} or Asp^{139} with less polar residues results in a small increase in this pK_a value.

Steady-State Kinetic Analysis. The catalytic activities of H245A and H245N were reduced significantly compared with that of wild-type enzyme (Table 3). Lineweaver–Burk plots of the initial velocities of the reaction gave parallel lines, as expected for a ping-pong mechanism for thioredoxin reductase. There is noticeable substrate inhibition by NADPH for H245A, as shown in the secondary plot of the intercepts of the linear portion of the Lineweaver–Burk plots (Figure 3). Michaelis–Menten plots using nonlinear least-squares fitting (and neglecting the points where substrate inhibition was obvious for H245A) provided the kinetic parameters shown in Table 3. The K_m values are similar to those seen with wild-type thioredoxin reductase. These data demonstrate that His^{245} does not have a major role in catalytic turnover.

The activity of D139E was also measured in the coupled assay and exhibited 38% of the activity of wild-type thioredoxin reductase. The K_m for thioredoxin was markedly higher than for wild-type enzyme; an explanation for this is not obvious. D139N and D139L had activities that were so low that it was not practical to use the coupled assay system because of interference from the low basal levels of NADPH oxidase.

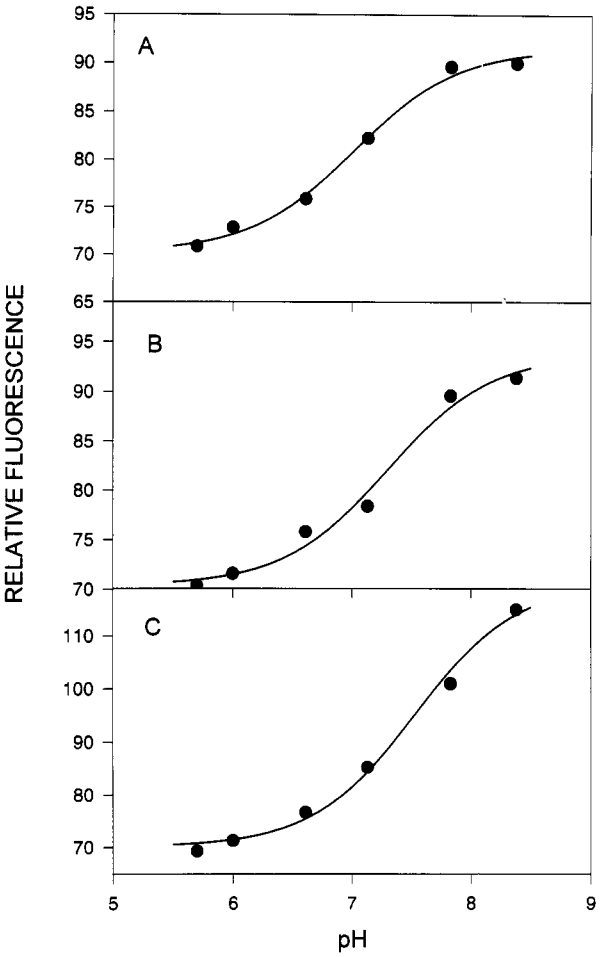

FIGURE 2: Effect of pH on fluorescence of thioredoxin reductase mutants: H245A (panel A), H245N (panel B), and D139L (panel C). Fluoresence excitation values (emission wavelength 550 nm) at 12 °C are on an arbitrary, relative scale, but the fluorescence of the mutants has been compared to that of wild-type enzyme at pH 7.6 (see the text). The solid lines are theoretical fits to the pK_a values given in the text.

Table 3: Steady-State Results for Wild-Type Thioredoxin Reductase and Thioredoxin Reductase Mutants[a]

	turnover[b] number	K_m(NADPH)	turnover[b] number	K_m(thioredoxin)
WT[c]	2000	1.2		2.8
H245A	1000 ± 60	2.0 ± 2.1	685 ± 14	0.67 ± 0.06
H245N	355 ± 27	0.45 ± 0.16	357 ± 8	0.35 ± 0.04
D139E	600 ± 50	5.1 ± 0.9	940 ± 70	29.0 ± 4.7

[a] Assay conditions are described in Prongay et al. (1989). [b] Turnover numbers have units of mol of substrate min^{-1} mol of FAD^{-1}, and K_m values are in μM. [c] Williams (1976).

Therefore, D139N and D139L turnover was assayed at fixed NADPH concentrations and at varying thioredoxin concentrations, yielding the results in Table 4. All mutants of Asp^{139} were, on the other hand, relatively unchanged with respect to their reaction with NADPH: the NADPH to APyADP+ transhydrogenase activities (Table 4) and the NADPH oxidase activities (data not shown) were similar to those seen in wild-type enzyme. The Asp^{139} mutants were, however, clearly altered in their reaction with thioredoxin. D139E had a V_{max} less than half that of wild-type thioredoxin reductase. When measured in a noncoupled assay, D139N had extremely low thioredoxin reduction activity while D139L had no detectable level of activity.

Rapid Reaction Kinetics. Reduction of thioredoxin reductase by NADPH occurs in three distinct phases when

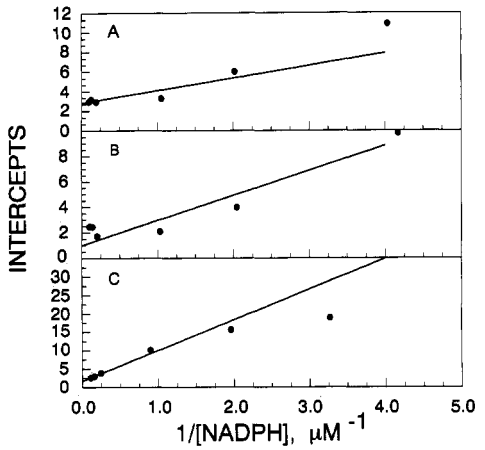

FIGURE 3: Lineweaver–Burk secondary plots for thioredoxin reductase mutants: (A) H245N, (B) H245A, and (C) D139E. The solid lines are theoretical fits of the kinetic constants shown in Table 3.

Table 4: Turnover Numbers for Wild-Type and Asp[139] Mutants

	thioredoxin reduction[a]	transhydrogenase[b]
WT	1511 ± 143	452 ± 74
D139L	0.0	385 ± 24
D139N	24.5 ± 2.3	448 ± 60
D139E	561 ± 20	360 ± 62

[a] Thioredoxin reduction measured at 10 μM NADPH at varying thioredoxin concentrations by monitoring A_{340}. [b] Transhydrogenase activity measured at 80 μM NADPH and varying concentrations of APyADP+.

Table 5: Rate Constants for Reductive Half-Reaction

	k_2 (s^{-1})	k_3 (s^{-1})
WT[a]	55	9
H245A	102.5 ± 2.6	6.4 ± 0.3
H245N	85.7 ± 0.5	3.5 ± 0.3
D139L	44.0 ± 1.0	3.6 ± 0.3
D139N	39.9 ± 1.1	14.8 ± 1.7
D139E	49.8 ± 0.5	7.0 ± 0.5

[a] Williams et al. (1991).

observed in the stopped-flow spectrophotometer at 456 nm, 1 °C (Massey et al., 1970; Williams et al., 1992). The first phase, which is completed in the 3-ms dead time of the instrument, is seen as a slight drop in absorbance at 456 nm and an increase at 570 nm, due to the formation of an NADPH–FAD charge-transfer complex. The second phase is the reduction of the flavin and is detected as a rapid decrease in absorbance at 456 nm. H245A and H245N had faster rate constants for this phase than wild-type thioredoxin reductase, while D139E, D139N, and D139L had similar rate constants (Table 5, Figure 4). The third phase, presumed to represent the NADP+ dissociation rate (Massey et al., 1970; Williams et al., 1992), is similar and rate-limiting for all of the mutants as is true for wild-type enzyme.

To further investigate the lower overall activities of Asp[139] mutants, the oxidative half-reaction of D139N was measured in the stopped-flow spectrophotometer. The rates of reoxidation measured at 456 nm were only 1% of those previously reported for wild-type thioredoxin reductase as shown in Figure 4 (Navarro et al., 1991). Thus, mutation of Asp[139] specifically affects the reduction of thioredoxin by reduced thioredoxin reductase. D139N resembled wild-type enzyme in that the reoxidation proceeded in a single phase and the rate constants were independent of thioredoxin concentration.

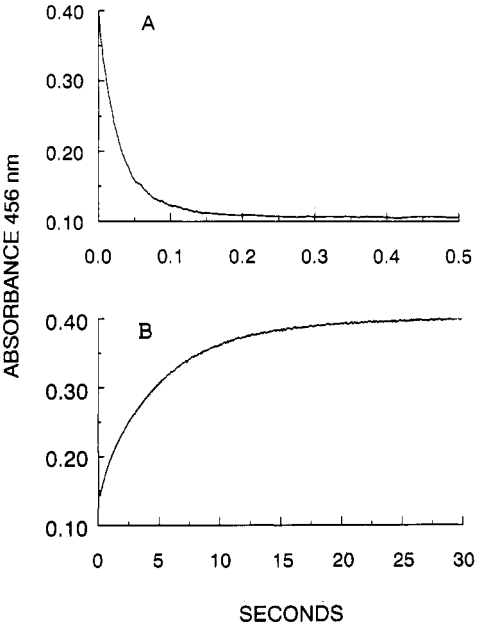

FIGURE 4: Kinetics of reductive and oxidative half-reactions of D139N: (A) reduction of 10.4 μM D139N by 20.7 μM NADPH at 1 °C and (B) oxidation of 18.9 μM reduced D139N by 97.5 μM thioredoxin at 25 °C.

DISCUSSION

Lipoamide dehydrogenase and glutathione reductase utilize the imidazole side chain of a histidine residue as a base or acid catalyst, respectively, of dithiol–disulfide interchange between the active-site disulfide–dithiol and the substrate (Williams, 1992). Site-directed mutagenesis of His[444] of *E. coli* lipoamide dehydrogenase to a glutamine results in the loss of more than 99% of the activity (Williams et al., 1989; Benen et al., 1992). Likewise, conversion of His[439] to an alanine in *E. coli* glutathione reductase brings about a similar loss in activity (Deonarian et al., 1989). In both altered enzymes, the half-reaction involving dithiol–disulfide interchange is slowed but the pyridine nucleotide half-reaction, which includes electron exchange between dithiol/disulfide and FAD/FADH−, is also affected (Williams et al., 1989; Benen et al., 1992).[2] It was postulated that the acid–base catalyst functions in the electron exchange by stabilizing one or the other thiol as a thiolate, in agreement with mechanisms proposed earlier (Arscott et al., 1981; Wong et al., 1988; Leichus & Blanchard, 1992). Interpretation of data on the pH dependence of the redox potential of thioredoxin reductase assumed that this enzyme would also use an acid–base catalyst (O'Donnell & Williams, 1983).

Examination of the three-dimensional structure of thioredoxin reductase shows that His[245] is the only histidine residue in the active site and thus represents a logical candidate for being the acid–base catalyst (Kuriyan et al., 1991). However, preliminary experiments with His[245] mutants showed that they had significant activity. We therefore looked for other residues which could function similarly near the active-site disulfide. The most likely candidate was Asp[139], the OD1 of which is located 6.3 Å from Cys[135] and the OD2 is 5.1 Å from Cys[138], the electron-transfer thiol (Figure 1). There is a water molecule between Asp[139](OD2) and the N5 position (3.2 and 2.9 Å, respectively) of the isoalloxazine ring which could

[2] P. Rietveld, L. D. Arscott, C. H. Williams, Jr., submitted for the Eleventh International Symposium on Flavins and Flavoproteins, Nagoya, Japan, July 1993.

facilitate proton transfer. It should be noted that Asp^{139} is absolutely conserved in the eight sequences of thioredoxin reductase and thioredoxin-reductase-like proteins, such as alkyl hydroperoxide reductase, determined thus far. His^{245}, on the other hand, is present at the homologous position in only three of the eight sequences (Mathieu et al., 1992; Tartaglia et al., 1990; Aharonowitz et al., 1993; Xu et al., 1991; Schräder & Andreesen, 1992; Niimura et al., 1993; Cohen et al., Gene Bank acession number X76119). The goals of the present study were to replace His^{245} with an asparagine residue (which is not titratable but has a polarity similar to that of imidazole) and alanine (which is not titratable and apolar), to replace Asp^{139} with glutamate (which alters the position of the carboxylate), asparagine, and leucine, and to examine the effects of these replacements on catalysis.

The evidence presented here clearly demonstrates that His^{245} is not a viable active-site base. Steady-state analysis showed that H245A and H245N exhibited significant activity compared to wild-type enzyme. There was, however, detectable substrate inhibition by NADPH when H245A was analyzed, which indicates that interaction between the enzyme and the pyridine nucleotide may have been altered in this mutated enzyme.

Since His^{245} is not the active-site acid–base, other experiments were performed in order to examine any potential role in catalysis. Anaerobic titrations with dithionite showed that the electron-uptake properties of H245A and H245N were virtually identical to that of wild-type thioredoxin reductase (data not shown). Although these results do not directly address whether the redox potentials of the flavin and disulfide centers have changed, the separation between them has not appeared to change noticeably. Stopped-flow studies of the reductive half-reaction revealed that both mutated enzymes had faster rates of reduction than wild-type thioredoxin reductase, which may indicate that a slight drop in the redox potential of the flavin has affected the kinetics.

O'Donnell and Williams (1983) noted that there was a titratable residue with a pK_a of 7.03, observed by measuring the pH dependence of the fluorescence of oxidized enzyme. This residue was distinct from the active-site acid–base catalyst (O'Donnell & Williams, 1983). Our results disclose that His^{245} is not this second residue since the pK_a values detected by changes in fluorescence were raised only slightly in H245N and H245A.

In all of the experimental results above, there is an overall pattern that H245N has properties closer to wild-type enzyme than does H245A. In steady-state analysis, H245N shows no substrate inhibition with NADPH while H245A has pronounced inhibition at high NADPH concentrations. Wild-type thioredoxin reductase exhibits no detectable inhibition by NADPH. The pK_a values detected by the pH dependence of the enzyme fluorescence are identical for wild-type and H245N and are shifted up by 0.27 pH unit in H245A. In stopped-flow studies, H245A exhibited rate constants of enzyme reduction nearly twice that of wild-type enzyme while H245N had intermediate rate constants. Such findings would be expected, since the conversion of a histidine to an alanine is a greater perturbation in polarity than changing a histidine to an asparagine.

While thioredoxin reductase shares many properties with other members of the flavoprotein reductase family, there are some significant differences as well. In thioredoxin reductase, all of the chemical reactions occur on the *re* face of the flavin, whereas in glutathione reductase and lipoamide dehydrogenase, pyridine nucleotide binding and the disulfide reactions are on *re* and *si* sides of the flavin rings, respectively. In addition, the active-site histidine of glutathione reductase and lipoamide dehydrogenase is contributed by the opposite subunit of the dimeric enzyme (Karplus & Schulz, 1987; Schierbeek el at., 1989), while in thioredoxin reductase, Asp^{139} is on the same polypeptide chain as are the rest of the active-site residues. From the data presented here, it is clear that alterations of Asp^{139} profoundly affect the catalytic properties specifically in the oxidative half-reaction of thioredoxin with the reduced enzyme. Therefore, it seems likely that Asp^{139} functions as the acid catalyst for the dithiol–disulfide-interchange reaction between thioredoxin and reduced thioredoxin reductase. If Asp^{139} is the immediate proton donor to thioredoxin as the mixed disulfide forms, this would be analogous to the major function of the acid–base catalyst in glutathione reductase, namely, protonation of the first dissociating glutathione, inhibiting the reverse reaction (Wong et al., 1988). Since the alteration of Asp^{139} has a minimal affect on the reductive half-reaction, it does not appear to play an important role in electron transfer between reduced flavin and the active-site disulfide. This is in marked contrast to the situation for lipoamide dehydrogenase and glutathione reductase where modification of the acid–base catalyst slows electron transfer between the flavin and the disulfide/dithiol markedly (Williams et al., 1989; Benen et al., 1992).[2]

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REFERENCES

Aharonowitz, Y., Av-Gay, Y., Schreiber, R., & Cohen, G. (1993) *J. Bacteriol. 175*, 623–629.

Arscott, D. L., Thorpe, C., & Williams, C. H., Jr. (1981) *Biochemistry 20*, 1513–1520.

Benen, J., van Berkel, W., Dieteren, N., Arscott, D., Williams, C., Jr., Veeger, C., & de Kok, A. (1992) *Eur. J. Biochem. 207*, 487–497.

Berry, A., Scrutton, N. S., & Perham, R. N. (1989) *Biochemistry 28*, 1264–1269.

Boyer, H. W., & Roulland-Dussoi, D. (1969) *J. Mol. Biol. 41*, 459–477.

Bullock, W. O., Fernandez, J. M., & Stuart, J. M. (1987) *Biotechniques 5*, 876–879.

Deonarain, M. P., Berry, A., Scrutton, N. S., & Perham, R. N. (1989) *Biochemistry 28*, 9602–9607.

Foss, O. (1961) in *Organic Sulfur Compounds* (Karasch, N., Ed.) Vol. 1, pp 83–96, Pergamon Press, New York.

Gayle, R. B., Vermersch, P. S., & Bennett, G. N. (1986) *Gene 41*, 281–288.

Harbury, H. A., LaNoue, K. F., Loach, P. A., & Amick, R. M. (1959) *Proc. Natl. Acad. Sci. U.S.A. 45*, 1708.

Hill, C. W., & Harnish, B. W. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7069–7072.

Holmgren, A. (1989) *J. Biol. Chem. 264*, 13963–13966.

Karplus, P. A., & Schulz, G. E. (1987) *J. Mol. Biol. 95*, 701–729.

Kuriyan, J., Krishna, T. S. R., Wong, L., Guenther, B., Pahler, A., Williams, C. H., Jr., & Model, P. (1991) *Nature 352*, 172–174.

Leichus, B. N., & Blanchard, J. S. (1992) *Biochemistry 31*, 3065–3072.

Massey, V. (1990) in *Flavins and Flavoproteins* (Curti, B., Zanetti, G., & Ronchi, S., Eds.) pp 59–66, Walter de Gruyter and Co., Berlin.

Massey, V., Matthews, R. G., Foust, G. P., Howell, L. G., Williams, C. H., Jr., Zanetti, G., & Ronchi, S. (1970) in *Pyridine Nucleotide-Department Dehydrogenases* (Sund, H., Ed.) pp 393–409, Springer-Verlag, Berlin.

Mathieu, I., Meyer, J., & Moulis, J.-M. (1992) *Biochem. J. 285*, 255–262.

Matthews, R. G., Ballou, D. P., Thorpe, C., & Williams, C. H., Jr. (1977) *J. Biol. Chem. 252*, 3199–3207.

Moore, E. C., Reichard, P., & Thelander, L. (1964) *J. Biol. Chem. 239*, 3445–3452.

Navarro, J. A., Gleason, F. K., Cusanovich, M. A., Fuchs, J. A., Meyer, T. E., & Tollin, G. (1991) *Biochemistry 30*, 2192–2195.

Niimura, Y., Ohnishi, K., Yarita, Y., Hidaka, M., Masaki, H., Uchimura, T., Suzuki, H., & Kozaki, M. (1993) *J. Bacteriol. 175*, 7945–7950.

O'Donnell, M., & Williams, C. H., Jr. (1983) *J. Biol. Chem. 258*, 13795–13805.

O'Donnell, M. E., & Williams, C. H., Jr. (1984) *J. Biol. Chem. 259*, 2243–2251.

Prongay, A. J., & Williams, C. H., Jr. (1990) *J. Biol. Chem. 265*, 18968–18975.

Prongay, A. J., & Williams, C. H., Jr. (1992) *J. Biol. Chem. 266*, 25181–25188.

Prongay, A. J., Engelke, D. R., & Williams, C. H., Jr. (1989) *J. Biol. Chem. 264*, 2656–2664.

Ronchi, S., & Williams, C. H., Jr. (1972) *J. Biol. Chem. 247*, 2083–2086.

Russel, M., & Model, P. (1985) *J. Bacteriol. 163*, 238–242.

Russel, M., & Model, P. (1986) *J. Biol. Chem. 261*, 14997–15005.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) in *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sayers, J. R., Schmidt, W., & Eckstein, F. (1988) *Nucleic Acids Res. 16*, 791–802.

Schierbeek, A. J., Swarte, M. B. A., Dijkstra, B. W., Vriend, G., Read, R. J., Hol, W. G. J., Drenth, J., & Betzel, C. (1989) *J. Mol. Biol. 206*, 365–379.

Schräder, T., & Andreesen, J. R. (1992) *Eur. J. Biochem. 206*, 79–85.

Tartaglia, L. A., Storz, G., Brodsky, M. H., Lai, A., & Ames, B. N. (1990) *J. Biol. Chem. 265*, 10535–10540.

Thelander, L. (1970) *J. Biol. Chem. 245*, 6026–6029.

Waksman, G., Krishna, T. S. R., Williams, C. H., Jr., & Kuriyan, J. (1994) *J. Mol. Biol.* (in press).

Williams, C. H., Jr. (1976) in *The Enzymes* (Boyer, P. D., Ed.) Vol. 13, pp 89–173, Academic Press, New York.

Williams, C. H., Jr. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., Ed.) Vol. 3, pp 121–209, CRC Press, Boca Raton, FL.

Williams, C. H., Jr., Zanetti, G., Arscott, L. D., & McAllister, J. K. (1967) *J. Biol. Chem. 242*, 5226–5231.

Williams, C. H., Jr., Allison, N., Russel, G. C., Prongay, A. J., Arscott, L. D., Datta, S., Sahlman, L., & Guest, J. R. (1989) *Ann. N. Y. Acad. Sci. 573*, 55–65.

Williams, C. H., Jr., Prongay, A. J., Lennon, B. W., & Kuriyan, J. (1991) in *Flavins and Flavoproteins* (Curti, B., Zanetti, G., & Ronchi, S., Eds.) pp 497–504, Walter de Gruyter and Co., Berlin.

Wong, K. K., Vanoni, M. A., & Blanchard, J. S. (1988) *Biochemistry 27*, 7091–7096.

Xu, X., Koyama, N., Cui, M., Yamagishi, A., Nosoh, Y., & Oshima, T. (1991) *J. Biochem. 109*, 678–683.