The Role of the Buried Aspartate of Escherichia coli Thioredoxin in the Activation of the Mixed Disulfide Intermediate*

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David M. LeMaster*, Penelope A. Springer, and Clifford J. Unkefer
From the Chemical Science and Technology Group 4, Los Alamos National Laboratory, Los Alamos, New Mexico 87545

The structurally homologous protein disulfide isomerases and thioredoxins exhibit a 10^9 variation of redox equilibria. It is demonstrated that the kinetic distinction among these protein family members lies primarily in the rate of breakdown of the mixed disulfide intermediate. The conserved buried acid group serves as a proton transfer catalyst for the buried active site cysteine in the formation and breakdown of the mixed disulfide. The reduction rate of Escherichia coli thioredoxin by dithiothreitol is directly proportional to the fraction of Asp-26 in the protonated form over the pH range of 6–9. The kinetic role of Asp-26 is further probed via differential solvent kinetic isotope effect measurements versus a D26N variant. The differential solvent isotope effect of 0.6 is consistent with a direct proton donation to the thiolate leaving group (Cys-35) via an enforced general acid catalysis by trapping mechanism. Such a donation necessitates a structural rearrangement as these two buried side chains are separated by 6 Å in both the oxidized and reduced forms of the protein.

Despite substantial sequence variability, the mammalian and bacterial protein disulfide isomerases, as well as the ubiquitous thioredoxins, appear to share a common structural motif (1) and are generally assumed to utilize a common enzymatic mechanism to achieve their accelerated reactivities in both dithiol oxidation and disulfide reduction (Fig. 1). Although the conformation of the active sites are markedly similar (2, 3), there is a 10^9-fold variation in redox equilibria between Escherichia coli thioredoxin (E_0 = −260 mV (4)) and the E. coli protein disulfide isomerase protein DaA (E_0 = −0.1 mV (5)). Within the thioredoxin/protein disulfide isomerase family the active site disulfide (-CXXC-) sequence spans the top of the first turn of an a-helix (the a2 helix in E. coli thioredoxin) with the sulfur of the first cysteine exposed for reaction with exogenous thiols and disulfides while the second cysteine is buried within the protein interior. Much of the research into the mechanism has focused on the reactions of the solvent exposed thiol (Cys-32 in E. coli thioredoxin). Particularly noteworthy is the quantitative correlation between the redox potential and the pK of this solvent exposed thiol observed for a series of protein disulfide isomerase variants (6). This correlation is also proposed to extend to the E. coli thioredoxin (7).

Since K_{eq} equals (k_1/k_{−1})(k_{−2}/k_2) for Fig. 1, the large differences in redox equilibria between the protein disulfide isomerases and the thioredoxins must be manifested in the individual rate constants. E. coli thioredoxin exhibits a k_1 reaction rate of 120 s^{-1} s^{-1} with oxidized glutathione under the conditions reported for human PDI α domain in Fig. 1 (8). Given the similarity in the rate constants observed for this step, the major difference in kinetics exhibited by these two enzymes does not lie primarily in the reaction of the protein thiol with the target disulfide substrate. Hence, a fuller understanding of the factors determining the differential reactivities within the thioredoxin/protein disulfide isomerase family necessitates a more detailed analysis of the structural elements influencing the other three reaction steps.

Thioredoxins of all phyla as well as both the human and E. coli protein disulfide isomerases have a conserved buried acidic residue located behind the solvent inaccessible active site cysteine (1). In a recent analysis of the structural determinants of the catalytic reactivity of the buried Cys-35 of E. coli thioredoxin, we proposed that the buried Asp-26 serves as a proton transfer catalyst for the buried Cys-35 thiol in the formation and breakdown of the mixed disulfide intermediate (9). It should be noted that such a proton transfer necessitates a concomitant structural rearrangement. The present manuscript provides direct evidence for the proton transfer function of Asp-26 apparently via an “enforced general acid catalysis by trapping” (10) for the Cys-35 thiolate.

EXPERIMENTAL PROCEDURES

Preparation of the Wild Type and D26N E. coli Thioredoxins—DNA encoding wild type E. coli thioredoxin from the plasmid pDL59 (11) was amplified via the polymerase chain reaction (Amplitaq kit from Applied Biosystems Inc.) (12). The primers introduced into the PET 3a vector (Novagen, Inc.). Asn codon AAC was introduced at position 26 via site-directed mutagenesis (13). The D26N expression plasmid was transformed into E. coli strain BL21 (14). The ΔsrxA307 deletion was transduced into BL21 (14) by linkage to medE163 Tn10 from strain JH221 (kindly provided by M. Russel, Rockefeller University). Expression plasmid pDL59 in MG1655 (Coli Genetic Stock Center) was used to produce the wild type protein. Protein samples were purified as described previously (11).

Fluorescence Stopped Flow Experiments—Fluorescence stopped flow experiments utilized an OLIS RSM spectrophotometer (On Line Instrument Systems, Inc.). The average of at least six mixing experiments were determined for each set of reagent conditions. The pH value of the mixing effluent are reported in all cases. For fluorescence experiments 40 μM oxidized protein in 0.2 M HEPES buffer was mixed 1:1 with dithiothreitol in 0.5 mM EDTA, disodium salt. Constant ionic strength for the HEPES buffer solution with a [Na^+] of 0.2 M was achieved by varying the proportion of 2 M NaOH and 2 M NaCl added to a solution of HEPES in free acid form. The dithiothreitol concentrations used ranged from 5 to 320 mM depending on the pH of the protein solution. An excitation wavelength of 285 nm and emission signals above 325 nm were used.

RESULTS

Kinetics of Reduction of Wild Type and D26N E. coli Thioredoxin by Dithiothreitol—Due to the kinetic instability of the mixed disulfide intermediate, the rate constant k_{−2} for reduc-

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‡ To whom correspondence should be addressed. Tel.: 505-667-6686; Fax: 505-667-0110; E-mail: lemaster@lanl.gov.

† The abbreviation used is: PDI, protein disulfide isomerase.

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tion of E. coli thioredoxin with glutathione cannot be obtained via the experimental approach used to obtain the individual rate constants of the human PDI α domain (8). The rate for reduction by excess glutathione is as follows (15).

\[
\text{Rate} = k \cdot \frac{[\text{glutathione}]}{[\text{enzyme}]} \cdot \left( k_2 + k_{-2}[\text{glutathione}] \right) \]  

(Eq. 1)

For experimentally accessible glutathione concentrations the rate is proportional to \([\text{glutathione}]^2\), indicating that \(k_2 \gg k_{-2}[\text{glutathione}]\). Under these conditions observed rate constant \((k_{\text{app}})\) equals \(k \cdot k_{-2}/k_{-2}^2\). Since the mixed disulfide intermediate was not trapped, the individual rate constants could not be deconvoluted.

Direct determination of \(k_{-2}\) can be obtained via the reduction of E. coli thioredoxin with dithiothreitol. In this case the breakdown of the mixed disulfide intermediate involves a rapid intramolecular cycling of the dithiothreitol. The rate for reduction by excess dithiothreitol is shown as follows.

\[
\text{Rate} = k \cdot \frac{[\text{dithiothreitol}]}{[\text{enzyme}]} \cdot \frac{1}{k_{-2}} \]  

(Eq. 2)

The \(\Delta E^\circ\) for the reduction of E. coli thioredoxin by dithiothreitol is 50 mV (via NAD(P)/H couple) (4, 16). Hence \(K_{d_{2-}} = (k_k/k_{-2}^2)k_{k2}/k_{-2} = 1/46\). As is evident from Equation 2, \(k_{\text{app}}\) provides a lower bound estimate of \(k_{-2}\). Given the value of 120 m\(^{-1}\) s\(^{-1}\) for \(k_2\) and 400 m\(^{-1}\) s\(^{-1}\) for \(k_{-2}\) (see below), \((k_2/k_{-2})\) for \(k_{\text{app}}\) is 1/14. Equation 2 indicates that \(k_{\text{app}}\) is at most a 7% underestimate of \(k_{-2}\).

Implicit in this analysis is the assumption that the intrinsic reactivities for the initial step of attack (\(k_2\) and \(k_{-2}\)) are similar for both glutathione and dithiothreitol. Mercaptoethanol and glutathione reduce the protein disulfide isomerases at similar rates (15). Likewise reduced protein disulfide isomerase reacts with oxidized glutathione only 4-fold faster than it does with the disulfide form of mercaptoethanol (17). These results are consistent with model redox kinetics measurements demonstrating that both the thiols and the disulfide bonds of glutathione are a factor of two more reactive than the thiol and disulfide forms of mercaptoethanol and dithiothreitol at neutral pH (16, 18). Selective enzymatic binding of substrate would distort this simple reactivity comparison. The thioredoxin/protein disulfide isomerase enzymes show no signs of substrate saturation up to several millimolar for both glutathione (19) and dithiothreitol (20), suggesting minimal binding interactions. In the present study the linear dependence of the dithiothreitol reaction was found to extend to above 100 mm.

Reduction of E. coli thioredoxin is accompanied by an increase of tryptophan fluorescence which can be used to monitor the reaction (20). When the D26N mutant is reduced by dithiothreitol for a series of pH values from 6 to 9, the apparent rate constant is linearly dependent on [OH\(^{-}\)] as illustrated in the log-log plot of Fig. 2A. This is the standard behavior observed in model thiol-disulfide reactions indicating the limiting diffusion kinetics of the reactive thiolate. In marked contrast, the wild type enzyme is substantially more reactive at the lower pH values, while its reactivity becomes nearly independent of [OH\(^{-}\)] above pH 8. The nature of this kinetic distinction is clarified by normalizing the rate constants to the concentration of the reactive dithiothreitol thiolate species (Fig. 2B). The enhanced reactivity of the wild type enzyme clearly follows a simple proton titration behavior with a pK value of 7.23 similar to that previously reported for Asp-26 in the oxidized protein (21, 22). In this form of the protein there is no other ionizable group in the active site region which could account for this behavior.

At pH 7.4 the apparent rate constant for reduction of E. coli thioredoxin by dithiothreitol (400 m\(^{-1}\) s\(^{-1}\)) agrees quite well with the \(k_{-2}\) reported for reduction of the human protein disulfide isomerase α domain reaction with glutathione at pH 7.4 are included (17).

Experiments analogous to those of Fig. 2 were repeated using a 0.1 m potassium phosphate buffer over the same pH range. These experiments yielded quite similar results suggesting the absence of a significant specific ion effect. Linearity was verified at each pH using four different dithiothreitol concentrations. For both buffer systems the reactivity of the wild type enzyme converged to a value near that of the mutant around pH 9. Likewise reproducible is the modest increase in reactivity of D26N mutant at lower pH (Fig. 2B), which may reflect the decreasing negative charge of this acidic protein and its effects on diffusion of the dithiothreitol thiolate.

These data strongly suggest the presence of two distinct mechanisms for the wild type enzyme. At pH values below 9 the reactivity of E. coli thioredoxin is proportionate to the fraction of protonated form of Asp-26, while at higher pH values a mechanism not involving protein ionization becomes dominant. The convergence of the reactivities of both the wild type and D26N enzymes at high pH argues for a common enzymatic catalysis mechanism, which for the D26N enzyme involves no net protein ionization over the pH range examined. Although combined acid and base catalyzed reactions are quite common in enzymology, these generally give rise to the familiar bell shaped pH profile reflecting the pH ranges for which either the acid or based catalyzed steps are rate-limiting. In the present case the reaction rate is proportionate to both [DTT\(^{-}\)] and [Asp-26\(^{-}\)] over at least a 3 pH unit range, indicating that both species contribute directly to the rate-limiting process.

**Solvent Kinetic Isotope Effects on the Reduction of Thioredoxin by Dithiothreitol**—Kinetic isotope measurements provide one of the most sensitive experimental monitors of proton transfer mechanisms (23, 24). Unfortunately, enzyme reactions rarely allow for the systematic variation of both substrates and catalytic groups often necessary to obtain unambiguous mechanistic interpretations. Hence interpretation often draws heavily upon analogy to small molecule studies. The differential solvent kinetic isotope effect for the wild type and D26N enzymes ([\(k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}}\)]\(\text{WT}\)\(/k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}}\)\(\text{D26N}\)) were determined based on the expectation that the wild type mechanism differs from the mutant reaction by introduction of an additional Asp26-dependent step. Hence the differential solvent kinetic isotope effect is anticipated to reflect only the isotope selectivity of this Asp26-dependent step. This differential analysis also offers the practical benefit of increased precision as inaccuracies in the matching of the H\(_2\)O and D\(_2\)O buffers cancel out.

The ratio of the solvent kinetic isotope effects for the wild type versus D26N mutant as a function of pH are given in Fig. 3. From pH 6 to pH 9 the kinetic isotope effect for the Asp26-dependent step is constant at 0.6. These values can not arise from a simple solvent isotope dependent pK shift of the aspartate as they are constant across the range of that pK. The differential kinetic isotope measurements clearly demonstrate a qualitative difference in mechanism between the wild type
Mechanism of Asp-26-dependent Proton Transfer—Any satisfactory model for the mechanism of the wild type enzyme must account for both the proportionality of the rate to [DTT]−[Asp-26−] and the pH-insensitive differential solvent kinetic isotope effect. In principle, protonation of Asp-26 could function indirectly via an activating conformational transition, which is energetically inaccessible to either the carboxamide or carboxylate forms of the enzymes. In this case the kinetic isotope effect would reflect changes in hydrogen bond strengths and hydration interactions in H2O versus D2O. Although as discussed in more detail below, the separation of the Asp-26 and Cys-35 side chains appear to necessitate some conformational rearrangement, the role of Asp-26 as a general acid in a direct proton transfer appears to offer a more plausible explanation of the isotope effects.

SN2 reactions are notoriously insensitive to solvent isotope effects. The reason is apparent from consideration of Fig. 4A. The model thiolate-disulfide reaction is initiated by diffusion of a thiolate ion to the active site followed by the attack on the disulfide. The leaving thiolate ion diffuses away to react with water. As no proton transfer occurs during the rate-limiting step of disulfide attack, a small solvent isotope effect (−1.2) is commonly observed reflecting the lower diffusion rates resulting from the higher viscosity of the D2O solution (25).

A mechanism consistent with the differential kinetic isotope effect can be derived by noting that in Fig. 4A, for the case of the E. coli thioredoxin reaction the leaving Cys-35 thiolate is not free to diffuse away from the Cys-32 sulfur. Indeed the cysteine sulfurs remain in van der Waals contact in the reduced state (26). Furthermore, the initially released Cys-35 thiolate is removed from contact with the aqueous phase. If the protonation of the thiolate is appreciably slower than the typical diffusion limited reaction, it must compete with the reverse Cys-35 thiolate attack on the mixed disulfide linkage. Such kinetics offer a formal analogy to the thiolate reaction with aldehydes (Fig. 4B). In their analysis of the thiolate reaction with acetaldehyde, Gilbert and Jenkins state "enforced general acid catalysis by trapping must appear whenever the rate constant for the breakdown of T− [hemithioacetal anion] to reactants, k−1, becomes larger than the rate constant for conversion of T− to TH by proton abstraction from water, kH/" (10). Using dilute weak acid catalysts they observed up to a 100-fold increase in trapping efficiency.

For E. coli thioredoxin it is proposed that the requisite kinetics are achieved by the decrease in the rate of reaction with water, kH, due to the solvent inaccessibility. Note that for the D26N mutant both the water catalyzed quenching of the thiolate and the reversal of the mixed disulfide formation would be pH-independent, consistent with the simple kinetic behavior of Fig. 2. These internal return kinetics would merely lead to a decrease in the observed rate constant. In this model, the Asp-26 carboxyl of the wild type protein serves to quench the Cys-35 thiolate more rapidly thus markedly decreasing the amount of internal return. As the pK of Cys-35 is over 11 (9), at physiological pH the quenching of the thiolate is expected to reduce the reactivity by 108. Asp-26 must, of course, catalyze the reverse reaction with equal efficiency. Thus in the equilibrated mixed disulfide state, the Asp-26 carboxylate will serve to remove the Cys-35 thiol proton facilitating the attack on the mixed disulfide linkage. Support for this model comes from the solvent kinetic isotope effect observed in the Gilbert and Jenkins study on the thiolate reaction with acetaldehyde (10). The magnitude of the isotope effect depends of the proton source. For catalysis by weak general acids versus water, kH/D2O was observed to be −1.0 and 1.8, respectively. The ratio of these solvent kinetic isotope effects corresponds closely to the value of 0.6 observed for the wild type and D26N enzymes.
The variation in branching ratios can be estimated from equilibrium stability measurements for the reaction of mercaptoethanol with E. coli thioredoxin. Using high concentrations of mercaptoethanol, equilibrium values of $K_1 = k_1/k_{-1} = 1.95$ and $K_2 = k_2/k_{-2} = 2.13$ M were obtained (31). Given the similar redox potentials of the glutathione and mercaptoethanol redox couples, similar values for the glutathione mixed disulfide are anticipated. When combined with the $k_1$ and $k_{-2}$ values for E. coli thioredoxin discussed herein and compared with the rate constants for the human PDI $\alpha$ domain summarized in Fig. 1, one concludes that the attack of the buried cysteine on the mixed disulfide intermediate ($k_{-2}$) is $\approx$ 50-fold faster for E. coli thioredoxin (i.e. $\approx 1000$ s$^{-1}$) than for the PDI $\alpha$ domain. Conversely, the attack of exogenous glutathione on the mixed disulfide ($k_{-1}$) is predicted to be $\approx 1000$-fold faster for the PDI $\alpha$ domain than for E. coli thioredoxin (i.e. $\approx 60$ M$^{-1}$ s$^{-1}$). The internal consistency of these estimates is independently verified by the predicted $k_{app}$ for glutathione reduction of E. coli thioredoxin $k_1/k_2k_{-2} < 25$ M$^{-1}$ s$^{-1}$ in reasonable agreement with the reported value of $\approx 10$ M$^{-1}$ s$^{-1}$ (8).

The most readily apparent sequence difference between the protein disulfide isomerases and the thioredoxins is the substitution of histidine for proline in the active site (i.e. -CGHC for the human PDI $\alpha$ domain versus -CGPC for E. coli thioredoxin). The present kinetic analysis combined with the evolutionary conservation of these residues suggests that the active site histidine likely serves to facilitate the attack of an exogenous thiol on the protein disulfide isomerase mixed disulfide intermediate. Similarly, the thioredoxin Pro-34 may enhance the internal attack of Cys-35 on the mixed disulfide via its effect on the helical twist of the active site sequence (32).