Structural Determinants of the Catalytic Reactivity of the Buried Cysteine of *Escherichia coli* Thioredoxin†

David M. LeMaster*

Department of Biochemistry and National Magnetic Resonance Facility at Madison, University of Wisconsin−Madison, 420 Henry Mall, Madison, Wisconsin 53706

Received July 3, 1996⊗

ABSTRACT: The structurally homologous thioredoxins and thioltransferases/glutaredoxins possess a solvent-exposed cysteine sulfur which carries out a nucleophilic attack on the target disulfide as well as a structurally adjacent solvent inaccessible thiol. The mechanistic basis of the essentially exclusive redox reactivity of the thioredoxins in contrast to the thiol−disulfide exchange reactions characteristic of the thioltransferases lies in the relative reactivity of the buried cysteine. A stable analog of the mixed disulfide state of *Escherichia coli* thioredoxin is used to demonstrate a p*K* value of 11.1 for the solvent inaccessible Cys 35 thiol. NMR chemical shift pH titration analysis indicates a very low dielectric surrounding the Cys 35 sulfur providing a basis for both the elevated p*K* and the enhanced apparent nucleophilicity. The buried Asp 26 likely serves as the proton sink for the (de)protonation of Cys 35. Relevance to the reactivity of the mammalian protein isomerases is discussed.

Thioredoxins are ubiquitous enzymes which catalyze reduction of disulfides and oxidation of dithiols for a wide range of small molecule and protein substrates. In bacteria and yeast thioredoxin functions include hydrogen donation to ribonucleotide reduction (Laurent et al., 1964) and reduction of inorganic sulfate (Black et al., 1960). In eukaryotes thioredoxins regulate a host of photosynthetic enzymes (Wolosiuk et al., 1977), activate transcription factors NF-kB and AP-1 (Matthews et al., 1992; Schenk et al., 1994), and have been shown to be identical with the adult T-cell leukemia-derived factor (Tagaya et al., 1989). Much of the interest in thioredoxin biochemistry stems from the strong structural and functional homology shared with the mammalian protein disulfide isomerase (Edman et al., 1985) crucial in the maturation of secreted proteins.

Studies of the archetypical *Escherichia coli* thioredoxin have shown that these promiscuous redox catalysts exhibit ∼10000-fold enhanced kinetics for both the oxidation (Creighton, 1975; Holmgren, 1979a) and reduction (Holmgren, 1979b) reactions at neutral pH as compared to small molecule thiols and unstrained disulfides. Potentially competing with this redox reaction is a thiol−disulfide exchange reaction as illustrated in Figure 1. The long studied thioltransferase (Racker, 1955) has been shown (Gan & Wells, 1987) to be identical with the independently characterized glutaredoxin named for its enzymatic complementarity to thioredoxin (Holmgren, 1976). This protein shows a striking structural homology to thioredoxin although with little sequence homology (Eklund et al., 1984). Thioltransferase/glutaredoxin catalyzes both the thiol−disulfide exchange as well as the dithiol−disulfide redox reaction. Genetic mutation of the structurally buried active site cysteine only modestly reduces its thiol−disulfide exchange activity while eliminating the potential for the redox reaction (Yang & Wells, 1991; Bushweller et al., 1992). In marked contrast, *E. coli* thioredoxin shows no activity in the standard thioltransferase assay (Holmgren, 1978).

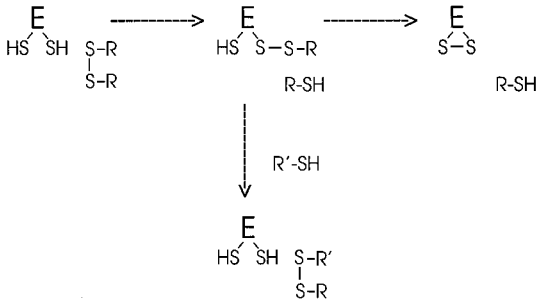

FIGURE 1: Catalysis of disulfide reduction and thiol−disulfide exchange. The top pathway illustrates the two-step disulfide reduction reaction involving an intramolecular displacement by the solvent inaccessible cysteine as is efficiently catalyzed by the thioredoxins. The branched reaction involves a displacement reaction with an exogenous thiol to form a mixed disulfide as is more efficiently catalyzed by the thioltransferase/glutaredoxins. The generic R and R′ are taken to symbolize cysteinyl groups of either small molecule (e.g., glutathione) or protein substrates.

This differential enzymatic behavior arises from the rate at which the solvent inaccessible thiolate attacks the mixed disulfide initially formed by the solvent accessible cysteine (Cys 35 and Cys 32, respectively, for *E. coli* thioredoxin). If this rate is rapid compared to attack by an exogenous thiolate, the thioltransferase activity is suppressed. The rate of this internal thiolate attack reflects how the protein structure modulates both the nucleophilicity of the Cys 35 thiolate and the stability of the linear trisulfur transition state formed in this S_N2 reaction.

† This work was supported by National Institutes of Health Grant GM 38779. This study made use of the National Magnetic Resonance Facility at Madison, which is supported by NIH Grant RR02301 from the Biomedical Research Technology Program, National Center for Research Resources. Equipment in the facility was purchased with funds from the University of Wisconsin, the NFS Biological Instrumentation Program (DMB-8415048), NIH Biomedical Research Technology Program (RR02301), NIH Shared Instrumentation Program (RR02781), and the U.S. Department of Agriculture.

* For correspondence: phone, (608) 278-8539; fax, (608) 262-3453; e-mail, lemaster@nmrfam.wisc.edu.

⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1996.

S0006-2960(96)01607-8 CCC: $12.00

No direct measurement has been reported for the rate of attack of Cys 35 on the mixed disulfide. However, for the reverse process dithiothreitol reacts with oxidized *E. coli* thioredoxin with a rate constant of 1600 M^{-1} s^{-1} at pH 7.2 (Holmgren, 1979a). This contrasts with 10 M^{-1} s^{-1} for the reaction of dithiothreitol with neutral small molecule disulfides at pH 8.7 (Creighton, 1975). Correcting for the dithiothreitol thiolate concentration at pH 7, the disulfide of thioredoxin exhibits a 5000-fold enhanced reactivity at neutrality. The factors contributing to the stabilization of the linear trisulfur transition state will perforce equally affect the reactions in both directions.

Initial to the analysis of the enhanced Cys 35 apparent nucleophilicity is the determination of the cysteine p*K*. The NMR determination of the active site cysteine p*K* values has been significantly complicated by the chemical shift effects induced by not only the apparent interaction in the ionization of the cysteines but also that of the buried Asp 26 (Dyson et al., 1991; Jeng et al., 1995; Wilson et al., 1995). Asp 26 has been shown to have a p*K* of 7.5 in the oxidized form of the protein (Dyson et al., 1991; Langsetmo et al., 1991). However for the reduced protein the H^{β} resonances of Cys 32, Cys 35, and Asp 26 all show complex titration curves between pH 5.7 and 10.5. Although the corresponding ^{13}C titration behavior appears somewhat less problematic, an ongoing debate offers, on one hand, p*K* assignments for the reduced protein of 7.5 for Asp 26 and 9.5 for Cys 35 (Jeng et al., 1995; Jeng & Dyson, 1996) and, on the other hand, the reverse p*K* assignments for both *E. coli* (Wilson et al., 1995) and the homologous human thioredoxin (Qin et al., 1996) with each group accepting a Cys 32 p*K* value of ~7.0. Additional titration behavior has been noted for pH values above 10 which have been ascribed to denaturation effects (Jeng et al., 1995).

With regard to analysis of the enzymatic mechanism, it should be noted that the p*K* values of these three groups in the reduced state are only germane to the initial nucleophilic attack by Cys 32. The kinetically relevant p*K* for Cys 35 occurs in the mixed disulfide state. The catalytically germane p*K* for Asp 26 is presumably also in the mixed disulfide state given its probable role as a solvent inaccessible proton sink (vide infra).

Given the kinetic instability of the mixed disulfide state of *E. coli* thioredoxin, an analog system must be selected. We have chosen to analyze the derivative covalently modified at Cys 32 with iodoacetamide, making use of the previously noted marked differential reactivities of the two cysteine thiols (Kallis & Holmgren, 1980). The alternative C32S and C32A (Russel & Model, 1986) mutations were excluded as the alanine substitution would introduce a substantial void potentially rendering the C35 sulfur solvent exposed while the serine substitution introduces a strong hydrogen bond donor−acceptor adjacent to the C35 sulfur, likewise potentially strongly perturbing its ionization behavior. The similarity between sulfur and carbon in terms of both van der Waals radii and electronegativity has been commonly exploited in design of substrate analogs. In particular, alkyl sulfide derivatives of glutathione have been used to demonstrate specific substrate binding of thioltransferase/glutaredoxin (Hoog et al., 1982), while a methylene substitution for one of the sulfurs in the disulfide bridge of glutathione has been used to generate an inhibitor of glutathione reductase (Embrey et al., 1994).

NMR titration experiments demonstrate an Asp 26 p*K* of 7.58 for the alkylated derivative. On the other hand, the Cys 35 p*K* is markedly higher than earlier suggested and clearly directly coupled to the pH denaturation process. Evidence is presented for a low-dielectric environment surrounding the Cys 35 sulfur. These results are interpreted in terms of their relevance to both the deprotonation of Cys 35 and the nucleophilicity of the thiolate.

MATERIALS AND METHODS

For the natural abundance sample, the *E. coli* thioredoxin expression vector pDL59 in host *E. coli* MG1655 (Coli Genetic Stock Center) was used to produce the protein sample as described previously (LeMaster & Richards, 1988). For incorporation of [3-^{13}C]cysteine, the plasmid pDL59 was transformed into the cys^- auxotroph JM15 (Coli Genetic Stock Center). Alkylation with iodoacetamide was carried out in 0.1 M Tris and 2 mM EDTA (pH 7.0) following published procedures (Kallis & Holmgren, 1980). A ~0.2 mM solution of *E. coli* thioredoxin was incubated for 15 min at room temperature with a 4-fold excess of dithiothreitol. A concentrated solution of iodoacetamide was added to a final concentration of 2 mM and incubated for 2 min. The reaction was quenched with a large excess of dithiothreitol, and the mixture was dialyzed against 50 mM NH_4HCO_3 and lyophilized. Subsequent NMR analysis indicated the presence of only one species.

The various pH samples were equilibrated by dialysis under argon against a buffer containing 100 mM NaCl, 20 mM NaH_2PO_4, 20 mM boric acid, and 0.2 mM EDTA in ^2H_2O (H_2O for the [3-^{13}C]cysteine-labeled sample). The pD values, adjusted sequentially from 5.7 to 10.4, are reported without isotope corrections. The ^1H NMR chemical shifts were obtained via DQF-COSY experiments (Rance et al., 1983) with a NOESY data set collected at pD 5.7 for assignment confirmation. The ^{13}C titration data was obtained via direct ^{13}C observation with a $^1H-^{13}C$ HMQC (Bax et al., 1983) reference spectrum used to demonstrate the ^1H to ^{13}C correlations of the cysteine resonances. ^1H NMR spectra were collected at 17.6 T, while ^{13}C NMR spectra were obtained at 11.7 T.

RESULTS

^1H and ^{13}C NMR pH Titration of Active Site Resonances. ^1H NMR resonance titrations from pD 5.7 to 10.4 were carried out on the Cys 32−carbamidomethyl *E. coli* thioredoxin via a series of two-dimensional homonuclear correlated spectra. The pD dependence of ^1H NMR resonances from the active site Cys 32, Cys 35, and Asp 26 is presented in Figure 2. With the exception of the Cys 35 H^{β} resonances all of the protons were independently fitted to a simple Henderson−Hasselbach titration yielding p*K* values of 7.58 (±0.02). The Cys 35 $H^{\beta 2}$ and $H^{\beta 3}$ resonance titrations were then fitted with this p*K* using data up to a pD of 8.2. Above this pD the Cys 35 $H^{\beta 2}$ and $H^{\beta 3}$ resonances coalesce and then subsequently exhibit a modest upfield migration.

Several aspects of the data of Figure 2 suggest that the pH 7.58 transition reflects the ionization of Asp 26. The resonances of Asp 26 H^{α}, $H^{\beta 2}$, and $H^{\beta 3}$ all shift upfield with increasing pH, and indeed the chemical shift dependence for each is markedly similar to that earlier reported for the

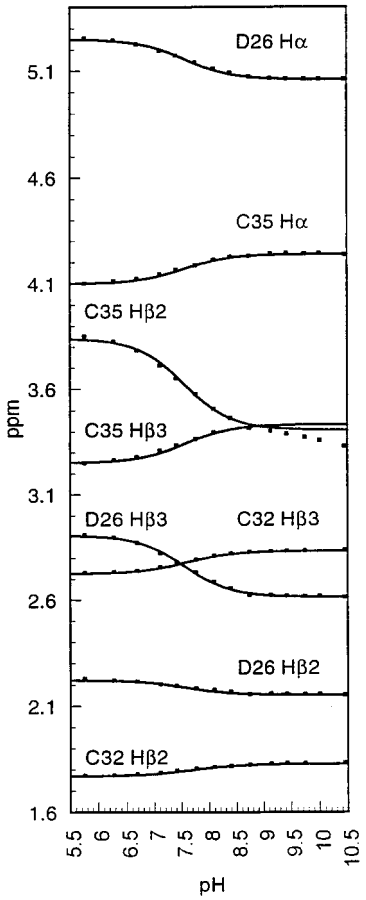

FIGURE 2: ^{1}H NMR chemical shift pH titration of the active site residues of Cys 32−carbamidomethyl *E. coli* thioredoxin. With the exception of the Cys 35 H$^\beta$ resonances, all titration data were independently fitted with a simple Henderson−Hasselbach dependence yielding an average p*K* value of 7.58 (±0.02). This p*K* value was then used to fit the Cys 35 H$^\beta$ data for pD values up to 8.2.

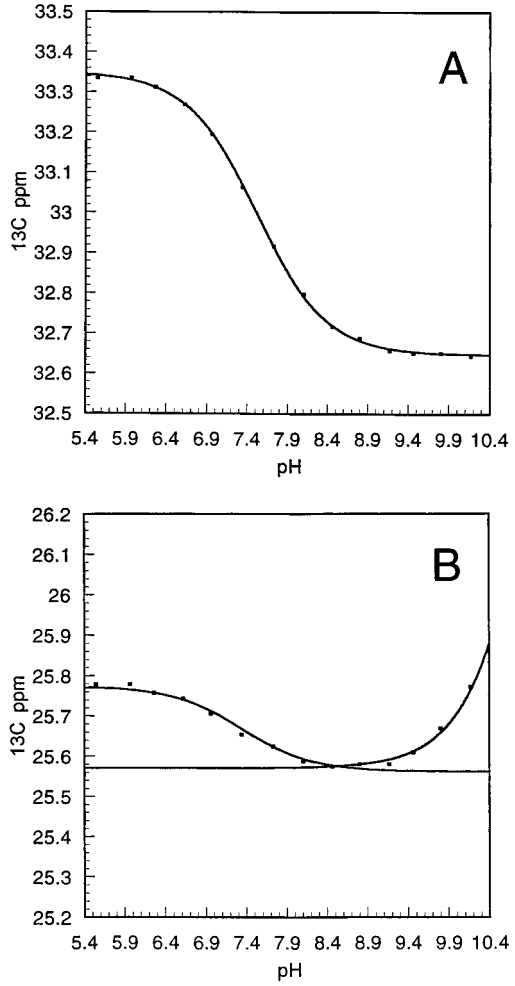

FIGURE 3: ^{13}C NMR chemical shift pH titration of the active site cysteine C$^\beta$ resonances of Cys 32−carbamidomethyl *E. coli* thioredoxin. Both the Cys 32 (panel A) and Cys 35 (panel B) titrations were fitted with p*K* values corresponding to that determined in Figure 2. In addition the high-pH titration behavior of Cys 35 was fitted assuming a 2.0 ppm amplitude for the complete transition.

oxidized protein (Dyson et al., 1991). In contrast the two H$^\beta$ resonances of Cys 35 shift in opposite directions, a behavior not expected for resonances inductively shifted by the ionization of the same side chain. Furthermore, unlike the other resonances illustrated in Figure 2, the Cys 35 H$^\beta$ resonances show an additional perturbation at high pH.

In order to clarify the interpretation of the titration behavior, spectra were collected on Cys 32−carbamidomethyl thioredoxin labeled with [3-^{13}C]cysteine. The pH dependence of the ^{13}C NMR chemical shifts is presented in Figure 3. Both Cys 35 C$^\beta$ as well as the blocked Cys 32 C$^\beta$ resonances undergo upfield shifts with a p*K* corresponding to that observed for the ^{1}H NMR resonances. In addition, at the higher pH values Cys 35 C$^\beta$ initiates a much larger downfield titration process consistent with deprotonation. Although the onset of protein denaturation precludes extension of the titration data, assuming a typical 2.0 ppm titration amplitude as is observed for the glutathione ionization (Jung et al., 1972), a Cys 35 p*K* of 11.1 can be estimated.

Structural interpretation of these results necessitates a molecular model. Given the solvent-exposed position of the nucleophilic Cys 32 thiol, it was anticipated that the structure of the Cys 32−carbamidomethyl thioredoxin would closely mimic that of the previously published reduced form (Jeng et al., 1994). It should be noted that the conformational differences between the oxidized and reduced states are known to be quite modest (Katti et al., 1990; Jeng et al., 1994). To demonstrate the similarity to the reduced structure, a comparison was made between the chemical shifts for all carbon-bound protons (excluding lysine protons beyond C$^\gamma$) of the Cys 32−carbamidomethyl thioredoxin at pD 5.7 and those for the reduced protein under similar conditions (Dyson et al., 1989). The overwhelming majority of the ^{1}H NMR resonances exhibit strikingly similar chemical shifts, occurring within 0.05 ppm of the reduced protein values. The few protons exhibiting larger differential shifts are all near Cys 32 S$^\gamma$, as given in Table 1. Note that alkylation induces a 9 ppm downfield shift for Cys 32 C$^\beta$. This qualitative structural assessment strongly indicates that the structure is faithfully preserved upon derivatization.

Table 1: All Carbon-Bound ^{1}H NMR Resonances of *E. coli* Thioredoxin Differing by Over 0.05 ppm for the Reduced and Cys 32−Carbamidomethyl (CAM) Forms at pH 5.7[a]

	CS			CS	
atom	red[b]	CAM	atom	red	CAM
Cys 32 H$^{\beta2}$	1.67	1.76	Cys 35 H$^{\beta3}$	3.79	3.85
Cys 32 H$^{\beta3}$	2.37	2.72	Ile 75 H$^{\gamma1}$ [c]	1.55	1.44
Pro 34 H$^\gamma$ [c]	2.28	2.22	Ile 75 H$^\delta$	0.41	0.34

[a] Excluding lysine resonances past C$^\gamma$. [b] Chemical shift values (Dyson et al., 1989). [c] Chirality not reported.

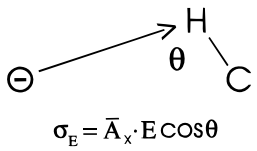

FIGURE 4: Simple bond polarization model for electric field-induced chemical shifts in a uniform field. The induced chemical shift σ^E is proportional to the component of the electric field directed along the H−C bond. The proportionality is given by A_x, the isotropic dipole-shielding polarizability (70 ppm/au) (Grayson & Raynes, 1995).

Electric Field Polarization Induced by Asp 26 Ionization. As illustrated in Figure 2, the Asp 26-induced shift of the Cys 35 $H^{\beta 2}$ resonance is 0.43 ppm even though the distance to the carboxylate group is 6.5 Å. Furthermore, the amplitudes of the titrations for the Cys 35 H^{α} and $H^{\beta 3}$ protons are also in excess of 0.13 ppm. These induced shifts arise either directly via a through-space electric field polarization at the ^1H nucleus or else indirectly via a pH dependent conformational transition. Several lines of evidence indicate that the electric field polarization is predominantly responsible. The practical relevance is that such large polarization effects imply minimal dielectric shielding between the Asp 26 carboxylate and the Cys 35 side chain.

The simplest useful model for explaining electric field-induced chemical shifts is that of bond polarization in a uniform field (Buckingham, 1960). As illustrated in Figure 4 the chemical shift term σ^E is proportional to the projection of the electric field on the C−H vector. For Cys 35 $H^{\beta 2}$ the C−H vector is nearly parallel to and directed away from the vector from the Asp 26 carboxylate (Jeng et al., 1994). This predicts an upfield shift as the negative charge of the carboxylate induces electron density at the Cys 35 C^{β} to shift toward the $H^{\beta 2}$ nucleus. In contrast both H^{α} and $H^{\beta 3}$ have C−H vectors directed toward the Asp group although at a more oblique angle ($\theta = 136°$ and 120°, respectively), and they exhibit a correspondingly smaller downfield shift. Note that we have independently verified the previously reported (Chandrasekhar et al., 1994) chiral assignments of the cysteine H^{β} resonances via stereoselective deuteration (Homer et al., 1993).

As a further demonstration of the significance of the electric field contribution to the observed pH dependent shifts, analysis was carried out on the titration behavior of 27 methine and methylene protons within 8 Å of the Asp 26 carboxylate which exhibited well-resolved COSY cross-peaks throughout the titration. Excluding four protons for which the angle between the H−C and H−carboxylate vectors were within 5° of perpendicular, all but two protons exhibited pH-induced chemical shifts in the direction consistent with the simple bond polarization model. Such a correlation could not be expected if the differential shifts arose from an ionization-induced conformational transition. Furthermore, the methyl resonances within this 8 Å region generally shift less than 0.015 ppm. Such an insensitivity is to be expected from the bond polarization model due to the averaging arising from the methyl rotation, while a conformational transition generally would not give rise to such a clear difference between the methyl and methine + methylene resonances.

A bond polarization model for the pH dependence of the chemical shifts provides an explanation for the somewhat surprising observation that the pH-induced upfield shift change for Cys 32 C^{β} is substantially larger than that of Cys 35 C^{β} (Figure 3) despite being over 3 Å farther away from the Asp 26 carboxyl. As seen in Figure 2, both Cys 32 H^{β} resonances exhibit downfield shifts suggestive of polarization of electron density toward the C^{β} nucleus, while the Cys 35 H^{β} resonances migrate in opposite directions suggestive of a mixed polarization effect at the C^{β} nucleus.

Under appropriate conditions the simple bond polarization model can provide a reasonable upper bound for the effective dielectric constant as sensed by a given ^1H nucleus. Advances in derivative Hartree−Fock theory (Dykstra & Jasien, 1984) have lead to reliable calculation of the shielding polarizability tensors relating the electric field strength to the induced chemical shifts (Auspurger & Dykstra, 1995). These calculations have been used to estimate minimum effective dielectric constant of ∼2 for protein interiors based on the population spread in observed chemical shifts (Augspurger et al., 1992). Using the simple bond polarization model combined with a linear electric field coefficient along the C−H bond of 70 ppm/au (Grayson & Raynes, 1995), an effective dielectric constant is obtained for both Cys 35 $H^{\beta 2}$ and Cys 35 $H^{\beta 3}$ of 2.0 due to a −1.0 charge located at a distance corresponding to midway between the carboxylate oxygens of Asp 26.

This dielectric calculation strongly suggests a marked absence of reorientable dipoles in the region between the Asp 26 carboxyl and the Cys 35 sulfur. This low-dielectric environment provides a ready explanation for the substantially elevated thiol p*K*. It also serves to rationalize the presumably elevated nucleophilicity of the Cys 35 thiolate implied by the rapid breakdown of mixed disulfide intermediate.

DISCUSSION

Small molecule thiol studies have demonstrated a clear correlation between thiolate nucleophilicity and the thiol p*K* in the cases for which the p*K* shift is caused by the inductive effects of electronic-withdrawing or -donating constituents. However, the Bronsted exponential coefficient for this correlation is only 0.5 (Szajewski & Whitesides, 1980), yielding a 16-fold increase in the intrinsic nucleophilicity of a thiolate having a p*K* shifted 2.4 pH units above the reference value (glutathione p*K* is 8.7; Reuben & Bruice, 1976).

Polar coordinating interactions are well known to be a dominant determinant of nucleophilicity (Reichardt, 1988). In the case of the thiolate−disulfide reaction a switch from aqueous to the polar aprotic solvent dimethyl sulfoxide results in a 10^4-fold increase in rate (Singh & Whitesides, 1990). In the solution structure of the reduced *E. coli* thioredoxin (Jeng et al., 1994), only two polar groups exhibit both the distance and dipole orientation potentially suitable to interact strongly with the Cys 35 sulfur. The sole short range negative dipole interaction is with the Cys 32 carbonyl oxygen (distance to Cys 35 S^{γ} is 3.3 Å). The second short distance is between Ala 29 H^N and the Cys 35 S^{γ}, having a separation of 2.8 Å in the reduced state. Although fairly short, this is 0.3−0.4 Å beyond the optimal hydrogen bond separation found in high-resolution X-ray structures (Gregoret et al., 1991). This comparative lack of interactions to stabilize the Cys 35 thiolate would be expected to give rise to both an elevated p*K* value and a substantially enhanced nucleophilicity.

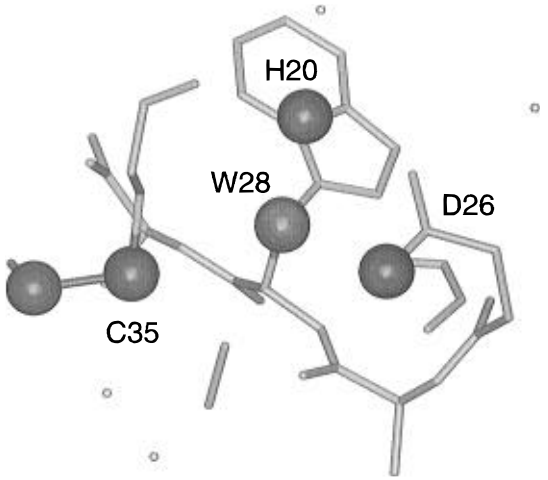

FIGURE 5: Region of oxidized *E. coli* thioredoxin involving Cys 35 S^{γ} and Asp 26 carboxyl. All heavy atoms within 6 Å of Trp 28 C^{β} are displayed. The isolated circles correspond to atoms for which no covalently attached heavy atoms lie within this sphere. Trp 28 C^{β} lies ~2.5 Å below the plane defined by Cys 35 S^{γ}, Asp 26 $O^{\delta 2}$, and the crystallographic water (W403). With a modest backward displacement of Trp 28 C^{β}, the internal cavity lying above this atom could readily accommodate either a water molecule or Cys 35 S^{γ} upon a χ_1 rotation of this residue. Either rearrangement provides a mechanism for proton transfer between Cys 35 S^{γ} and Asp 26.

The reaction rate of the buried Cys 35 must also reflect the energetics of the transition state. In our analysis of crystallographic refinement of the oxidized form of protein (Katti et al., 1990), two arguments were offered for the potential structural stabilization of the transition state. Following the general arguments of Hol (Hol, 1985), it was pointed out that, since the sulfurs of the disulfide span the top of the N-terminal end of the α_2 helix, the diffuse positive helix dipole field could serve to stabilize the linear trisulfur transition state. In the X-ray analysis a direct hydrogen bond between Cys 35 H^N and Cys 32 S^{γ} was pointed out. It was noted that this specific interaction would be expected to enhance the susceptibility of Cys 32 S^{γ} to attack. Such electrophilic interactions are known to stabilize the central sulfur of the linear trisulfur transition state (Pappas, 1977). Unfortunately, the crystallographic analysis has been widely misinterpreted as arguing for a hydrogen bond between Cys 35 H^N and Cys 32 S^{γ} in the reduced state, apparently reflecting a confusion over the distinct electrophilic and nucleophilic functions of Cys 32 during the different stages of the redox process. Indeed recent determination of the markedly increased amide exchange rate of Cys 35 H^N in the reduced form argues strongly against maintenance of a Cys 35 hydrogen bond (Jeng & Dyson, 1995).

The obvious major remaining question with regards to the reactivity of the buried Cys 35 is the mechanism by which the thiol proton is removed to form the reactive thiolate and conversely reprotonated upon reduction of the Cys 32−Cys 35 disulfide. In both the reduced and oxidized structures, no suitable basic group is located within 5 Å of the S^{γ}. The buried position of the Cys 35 sulfur would require substantial protein heavy atom displacement in the active site in order to render it directly accessible to acid/base groups of the substrate molecule as has been recently proposed (Jeng et al., 1995).

An alternate mechanism of proton transfer is illustrated in Figure 5. This figure includes all heavy atoms within 6 Å of the Trp 28 C^{β} which in this view is at the center of a triangle formed by Cys 35 S^{γ}, Asp 26 $O^{\delta 2}$, and a crystallographic water (W403). Trp 28 C^{β} lies ~2.5 Å below the plane defined by these three atoms. The side chains of Pro 76 and Ile 38 enclose the top of this cavity at a distance of ~7 Å above Trp 28 C^{β}. There is a significant packing defect lying between Cys 35 S^{γ} and the Asp 26 carboxylate. Indeed a 1.4 Å radius probe (i.e., size of a water molecule) can be positioned midway between Trp 28 C^{β} and the Ile 38 side chain without creating van der Waals overlap with the protein atoms. If the Cys 35 χ_1 angle is rotated from gauche$^-$ to trans, the S^{γ} is brought to within approximate van der Waals contact with the $O^{\delta 2}$ of Asp 26. Only the C^{β} of Trp 28 lies appreciably closer than van der Waals distance to the S^{γ} in this orientation. Alternatively, a modest displacement of Trp 28 C^{β} might also allow migration of a water molecule into a position spanning between the Cys 35 S^{γ} and the Asp 26 carboxylate. Note that the low apparent dielectric constant is consistent with the packing defect lying between Cys 35 and Asp 26 and that it suggests that on average water occupancy in this cavity is low. With regard to the plausibility of a local conformational rearrangement of Trp 28, ^{13}C NMR relaxation analysis of the oxidized form of thioredoxin (LeMaster & Kushlan, 1996) indicates that both main chain and side chain positions throughout the active site loop extending from Phe 27 to Lys 36 undergo a conformational transition in the millisecond range consistent with the reported 1600 M^{-1} s^{-1} rate of reduction at pH 7.2 (Holmgren, 1979a). Use of Asp 26 as a solvent inaccessible proton sink provides a function for its p*K* being elevated to the physiological pH as well as an explanation for the absolute conservation of an acid group at this site for all thioredoxins and mammalian protein disulfide isomerases (Eklund et al., 1991) (note Anabaena 7120 thioredoxin is reported to have tyrosine at this site; Alam et al., 1989).

Although mechanistically distinct the redox vs thiol−disulfide exchange pathways illustrated in Figure 1 are often not easily experimentally deconvoluted, particularly in regards to the reactions catalyzed by the protein disulfide isomerase. Both direct redox transfer and catalysis of glutathione−substrate protein mixed disulfide followed by intramolecular displacement yield the same products. On one hand, stoichiometric amounts of the mammalian protein disulfide isomerase have been shown to catalyze both the oxidative and rearrangement phases in the standard assay of oxidative renaturation of reduced ribonuclease A (Lyles & Gilbert, 1991). Furthermore, mutation of the buried cysteine to serine at both active sites not only markedly reduces the activity in shortening the oxidative lag phase for ribonuclease renaturation, but it also results in a 2−8-fold reduction in the disulfide-unscrambling activity (Walker et al., 1996). On the other hand, mutation of the buried cysteines of either the yeast (LaMantia & Lennarz, 1993) or rat (Laboissiere et al., 1995) protein disulfide isomerase still yields complementation of the lethal deletion of the wild type yeast isomerase indicating that the unscrambling activity is sufficient for viability. As mutation of the buried cysteines leads to accumulation of high levels of the mixed disulfide form of the isomerase, it has been proposed that the buried cysteine may serve as an escape mechanism for breaking down unproductive intermediates (Walker et al., 1996).

Both scientific and commercial considerations have stimulated an interest in genetically engineering *E. coli* thioredoxin

so as to mimic the activity of the protein disulfide isomerase. To date the most successful efforts appear to arise from mutations which function in part by raising the redox potential closer to that observed for the isomerase (Lundstrom et al., 1992). The research described herein indicates that mutations modulating the reactivity of the buried cysteine may also serve to enhance the disulfide isomerase activity of thioredoxin variants.

ACKNOWLEDGMENT

H. J. Dyson is thanked for making the coordinates of the solution structure of *E. coli* thioredoxin previous to their becoming available through the Brookhaven Data Bank.

REFERENCES

Alam, J., Curtis, S. E., Gleason, F. K., Gerami-Nejad, M., & Fuchs, J. A. (1989) *J. Bacteriol. 171*, 162−171.
Auspurger, J. D., & Dykstra, C. E. (1995) *Annu. Rep. NMR Spectrosc. 30*, 1−36.
Augspurger, J., Pearson, J. G., Oldfield, E., Dykstra, C. E., Park, K. D., & Schwartz, D. (1992) *J. Magn. Reson. 100*, 342−357.
Bax, A., Griffey, R. H., & Hawkins, B. L. (1983) *J. Magn. Reson. 55*, 301−315.
Black, S., Harte, E. M., Hudson, B., & Wartofsky, L. (1960) *J. Biol. Chem. 235*, 2910−2916.
Buckingham, A. D. (1960) *Can. J. Chem. 38*, 300−307.
Bushweller, J. H., Aslund, F., Wuthrich, K., & Holmgren, A. (1992) *Biochemistry 31*, 9288−9293.
Chandrasekhar, K., Campbell, A. P., Jeng, M. F., Holmgren, A., & Dyson, H. J. (1994) *J. Biomol. NMR 4*, 411−432.
Creighton, T. E. (1975) *J. Mol. Biol. 96*, 767−776.
Dykstra, C. E., & Jasien, P. G. (1984) *Chem Phys. Lett. 109*, 388−393.
Dyson, H. J., Holmgren, A., & Wright, P. E. (1989) *Biochemistry 28*, 7074−7087.
Dyson, H. J., Tennant, L. L., & Holmgren, A. (1991) *Biochemistry 30*, 4262−4268.
Edman, J. C., Ellis, L., Blacher, R. W., Roth, R. A., & Rotter, W. J. (1985) *Nature 317*, 267−270.
Eklund, H., Cambillau, C., Sjoberg, B. M., Holmgren, A., Jornvall, H., Hoog, J. O., & Branden, C. I. (1984) *EMBO J. 3*, 1410−1417.
Eklund, H., Gleason, F. K., & Holmgren, A. (1991) *Proteins 11*, 13−28.
Embrey, K. J., Mehta, A., Carrington, S. J., Jaohari, R., McKie, J. H., & Douglas, K. T. (1994) *Eur. J. Biochem. 221*, 793−799.
Gan, Z. R., & Wells, W. W. (1987) *J. Biol. Chem. 262*, 6699−6703.
Grayson, M., & Raynes, W. T. (1995) *Magn. Reson. Chem. 33*, 138−143.
Gregoret, L. M., Rader, S. D., Fletterick, R. J., & Cohen, F. E. (1991) *Proteins 9*, 99−107.
Hol, W. G. J. (1985) *Prog. Biophys. Mol. Biol. 45*, 149−195.
Holmgren, A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2275−2279.
Holmgren, A. (1978) *J. Biol. Chem. 253*, 7424−7430.
Holmgren, A. (1979a) *J. Biol. Chem. 254*, 9113−9119.
Holmgren, A. (1979b) *J. Biol. Chem. 254*, 9627−9632.
Homer, R. J., Kim, M. S., & LeMaster, D. M. (1993) *Anal. Biochem. 215*, 211−215.
Hoog, J. O., Holmgren, A., Silva, C. D., Douglas, K. T., & Seddon, A. P. (1982) *FEBS Lett. 138*, 59−61.
Jeng, M.-F., & Dyson, H. J. (1995) *Biochemistry 34*, 611−619.
Jeng, M. F., & Dyson, H. J. (1996) *Biochemistry 35*, 1−6.
Jeng, M.-F., Campbell, A. P., Begley, T., Holmgren, A., Case, D. A., Wright, P. E., & Dyson, H. J. (1994) *Structure 2*, 853−868.
Jeng, M. F., Holmgren, A., & Dyson, H. J. (1995) *Biochemistry 34*, 10101−10105.
Jung, G., Breitmaier, E., & Voelter, W. (1972) *Eur. J. Biochem. 24*, 438−445.
Kallis, G. B., & Holmgren, A. (1980) *J. Biol. Chem. 255*, 10261−10265.
Katti, S., LeMaster, D. M., & Eklund, H. (1990) *J, Mol. Biol. 212*, 167−184.
Laboissiere, M. C., Sturley, S. L., & Raines, R. T. (1995) *J. Biol. Chem. 270*, 28006−28009.
LaMantia, M. L., & Lennarz, W. J. (1993) *Cell 74*, 899−908.
Langsetmo, K., Fuchs, J. A., & Woodward, C. (1991) *Biochemistry 30*, 7603−7609.
Laurent, T. C., Moore, E. C., & Reichard, P. (1964) *J. Biol. Chem. 239*, 3436−3444.
LeMaster, D. M., & Richards, F. M. (1988) *Biochemistry 27*, 142−150.
LeMaster, D. M., & Kushlan, D. M. (1996) *J. Am. Chem. Soc. 118*, 9255−9264.
Lundstrom, J., Krause, G., & Holmgren, A. (1992) *J. Biol. Chem. 267*, 9047−9052.
Lyles, M. M., & Gilbert, H. F. (1991) *Biochemistry 30*, 619−625.
Matthews, J., Wakasuki, N., Virelizier, J., Yodoi, J., & Hays, R. (1992) *Nucleic Acids Res. 20*, 3821−3830.
Pappas, J. A. (1977) *J. Am. Chem. Soc. 99*, 2926−2930.
Qin, J., Clore, G. M., & Gronenborn, A. M. (1996) *Biochemistry 35*, 7−13.
Racker, E. (1955) *J. Biol. Chem. 217*, 867−874.
Rance, M., Sorensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R., & Wuthrich, K. (1983) *Biochem. Biophys. Res. Commun. 117*, 479−485.
Reichardt, C. (1988) *Solvents and Solvent Effects in Organic Chemistry*, VCH Verlagsgesellschaft mbH, Weinheim.
Reuben, D. M. E., & Bruice, T. C. (1976) *J. Am. Chem. Soc. 98*, 114−121.
Russel, M., & Model, P. (1986) *J. Biol. Chem. 261*, 14997−15005.
Schenk, H., Klein, M., Erdbrugger, W., Droge. W., & Schulze-Ostoff, K. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1672−1676.
Singh, R., & Whitesides, G. M. (1990) *J. Am. Chem. Soc. 112*, 6304−6309.
Szajewski, R. P., & Whitesides, G. M. (1980) *J. Am. Chem. Soc. 102*, 2011−2026.
Tagaya, Y., Maeda, Y., Mitsui, A., Kondo, N., Matsui, M., Hamuro, J., Brown, N., Arai, K.-I., Yokota, T., Wakasugi, H., & Yodoi, J. (1989) *EMBO J. 8*, 757−764.
Walker, K. W., Lyles, M. M., & Gilbert, H. F. (1996) *Biochemistry 35*, 1972−1980.
Wilson, N. A., Barbar, E., Fuchs, J. A., & Woodward, C. (1995) *Biochemistry 34*, 8931−8939.
Wolosiuk, R., Buchanan, B., & Crawford, N. (1977) *FEBS Lett. 81*, 253−258.
Yang, Y., & Wells, W. W. (1991) *J. Biol. Chem. 266*, 12766−12771.

BI961607O