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Aspartic Acid 26 in Reduced *Escherichia coli* Thioredoxin Has a pK_a >9[†]

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ABSTRACT: Apparent pK_a values of active site residues Asp26, Cys32, and Cys35 in reduced thioredoxin have been characterized. Both wild-type thioredoxin and mutant D26A thioredoxin were selectively ^{13}C-enriched on cysteine β-carbons. In both proteins, the variation with pH of ^{1}HB1, ^{1}HB2, and ^{13}CB NMR chemical shifts has been measured. In wild-type reduced thioredoxin, for both cysteines, the pH *versus* chemical shift plots of HB1 protons can be fit to one titration with pK_a values of 7.0−7.1. In contrast, the HB2 protons and β-carbons give pH−chemical shift plots that clearly reflect more than one titration; fits to the data give apparent pK_a values of 7.0−7.3 and 9.5 for HB2 protons and 7.5−7.9 and 9.2−10.2 for CB carbons. In reduced D26A, all three probe chemical shifts have a pH dependence that is fit by one titration with pK_a of 7.4−7.9. The absence of a titration with pK_a >9 in D26A, taken together with cysteine thiol pK_a values of 7.1 and 7.9 determined by Raman spectroscopy [Li *et al.* (1993) *Biochemistry 32*, 5800−5808], indicates that the pK_a >9 in reduced thioredoxin is that of Asp26. This is highly significant in view of the previous observation that, in oxidized thioredoxin, Asp26 pK_a is 7.5 [Langsetmo *et al.* (1991) *Biochemistry 30*, 7603−7609]. The very high pK_a values of these carboxyls is consistent with their local environment in the three-dimensional structure; the Asp26 side chain in oxidized thioredoxin is almost but not completely buried, and in reduced thioredoxin it may be even more buried. The upward shift in pK_a of Asp26 in reduced thioredoxin accounts for the lower stability of reduced compared to oxidized thioredoxin and suggests that the function of this very highly conserved active site group is to regulate the redox potential of the enzyme through thermodynamic linkage to global stability. The higher pK_a of Asp26 in reduced thioredoxin also offers an explanation for the preference for reduced thioredoxin in phage maturation processes.

Escherichia coli thioredoxin, a soluble redox-active protein, is the archetype for the thioredoxin superfamily which includes thioredoxins, glutaredoxins, protein disulfide isomerases (Edman *et al.*, 1985), and the disulfide bond maturation factors (Bardwell *et al.*, 1991). Numerous functions are ascribed to thioredoxins, most of which involve its general protein disulfide−dithiol oxidoreductase activity. Thioredoxins reduce thioredoxin reductase and are hydrogen donors to ribonucleotide reductase and to bacterial and yeast sulfate reduction reactions. In eukaryotes, thioredoxins regulate photosynthetic enzymes (Wolosiuk *et al.*, 1977; Schurmann *et al.*, 1981) and are involved in activation of

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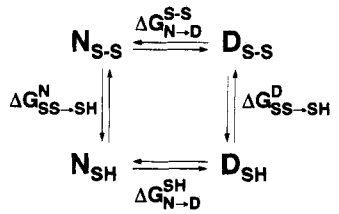
transcription factors NF-kB and AP-1 (Matthews *et al.*, 1992; Hayashi *et al.*, 1993; Schenk *et al.*, 1994). Recently, the primary sequence of adult T-cell leukemia derived factor was found to be identical to that of human thioredoxin (Tagaya *et al.*, 1989; Mitsui *et al.*, 1992; Bazzichi *et al.*, 1994).

In addition to oxidoreductase activities, thioredoxin is required in the replication cycle of several bacteriophages. The DNA polymerase of phage T7 requires prior complex formation with host thioredoxin (Modrich & Richardson, 1975a,b; Nordstrom *et al.*, 1981; Huber *et al.*, 1986). Coat assembly in the filamentous phages M13 and f1 also requires the presence of host thioredoxin (Russel & Model, 1985, 1986; Lim *et al.*, 1985). For the phage systems, reduced thioredoxin is preferred. Apparently the redox capability of thioredoxin is not necessary, since the mutant C32S,C35S replaces reduced thioredoxin in these systems, albeit with somewhat lower growth levels (Huber *et al.*, 1986).

E. coli thioredoxin is small (11 700 Da) and monomeric. A high-resolution crystal structure is reported for oxidized *E. coli* thioredoxin (Katti *et al.*, 1990) but not for reduced. The canonical thioredoxin fold has a central twisted β-sheet of four or five strands, packed on opposite sides by two α-helices, and an active site sequence motif -Cys-X-X-Cys-. The glutaredoxin fold is the same as thioredoxin, but with the deletion of a β-sheet strand and a helix from the N-terminus. In protein disulfide isomerases two of the four domains have high sequence similarity to thioredoxin (Edman *et al.*, 1985). DsbA crystal structure has the basic thioredoxin fold but also contains a large helical domain inserted between β-sheet strands three and four (Martin *et al.*, 1993). High-resolution NMR structures for both oxidized and reduced thioredoxin from *E. coli* (Jeng *et al.*, 1994) and humans (Qin *et al.*, 1994) are reported. In both, the small difference between oxidized and reduced in average structure or backbone dynamics involves increased local mobility of side chains in or near the dithiol active site in reduced thioredoxin.

In thioredoxin, protein folding stability is directly related to biological activity. While folding is an essential function of all proteins, in thioredoxin global stability (stability of the native state relative to the cooperatively unfolded state) plays a special role in determining redox potential. Oxidized (disulfide) and reduced (dithiol) forms of thioredoxin have significantly different stabilities, and this establishes a thermodynamic linkage between the global unfolding equilibria of oxidized and reduced and the disulfide−dithiol equilibria of folded and unfolded (Lin & Kim, 1989; Langsetmo *et al.*, 1991a; Sandberg *et al.*, 1991). The linkage is illustrated by a thermodynamic cycle between oxidized and reduced native thioredoxin (N_{S-S} and N_{SH}) and oxidized and reduced cooperatively unfolded thioredoxin (D_{S-S} and D_{SH}):



$\Delta E_F = \Delta E_U + \Delta\Delta G/(-nF)$, where ΔE_F is the redox potential of folded thioredoxin, ΔE_U is the redox potential of globally unfolded thioredoxin, n is the number of electrons transferred, F is Faraday's constant, and $\Delta\Delta G$ is $\Delta\Delta G°_{(ox\rightarrow red)}$, the difference in stability between oxidized and reduced thioredoxin ($\Delta G^{SS}_{(N\rightarrow D)} - \Delta G^{SH}_{(N\rightarrow D)}$). One outcome of the stability−redox linkage is that any additional process linked to stability also affects redox potential. In thioredoxin, two such linked processes have been described, Asp26 titration (Langsetmo *et al.*, 1991) and Ile75−Pro76 *cis*−*trans* isomerism (Langsetmo *et al.*, 1989). Folding stability mediates the modulation of thioredoxin redox potential by these processes. There are indications that global stability functions in regulating redox potential in glutaredoxin (Sandberg *et al.*, 1991) and in DsbA (Wunderlich *et al.*, 1993), which suggests that this phenomenon may be general for the thioredoxin superfamily.

In this work, we focus on pK_a values of active site residues Cys32, Cys35, and Asp26. We find that, whereas in oxidized thioredoxin the Asp26 side-chain carboxyl has a pK_a more than 3 pH units above that expected for a free carboxyl (Langsetmo *et al.*, 1991a,b), in reduced thioredoxin its pK_a is even higher and is >9. A shift to higher pK_a of Asp26 when thioredoxin goes from oxidized to reduced has a number of intriguing functional implications. In addition, the high pK_a of Asp26 provides plausible explanations for several properties of thioredoxin that have been well described but heretofore unaccountable. These are the instability of reduced relative to oxidized thioredoxin and the preference for the reduced form in phage replication processes.

MATERIALS AND METHODS

Thioredoxin was expressed and purified as described in Langsetmo *et al.* (1989, 1990). The *trx*A or D26A mutant trx gene was expressed in a tac-kan *E. coli* vector and induced with IPTG (isopropyl β-D-thiogalactoside). A final Sephadex S-100 column (300 mL) equilibrated with 50 mM sodium phosphate, pH 7.0, was added to remove all Tris [tris(hydroxymethyl)aminomethane]. Thioredoxin enriched with ^{13}C on the β-carbons of active site Cys32 and Cys35 was expressed in cells auxotrophic for cysteine grown in the presence of exogenous cystine or cysteine containing ^{13}C-labeled β-carbons [purchased from Cambridge Isotope Laboratories; CLM-520 for L-cystine (99% 3,3′-$^{13}C_2$) and CLM-1968 for L-cysteine (99% 3-^{13}C)]. *E. coli* uptake of Cys is preferential for the oxidized form, cystine, prepared by bubbling filtered air through a cysteine solution at pH 8.3 until the cystine precipitates. The suspension was solubilized by acidification and filter sterilized; 0.0002% w/v cystine is sufficient to grow bacteria to maximum turbidity in minimal media (OD_{660} about 1.7−2.0) for WT.

The plasmid encoding thioredoxin was transfected into an *E. coli* strain (JF3593) which is auxotrophic for cystine (cysHIJ::Tn10) and also *trx*$^-$. JF3593 has an absolute requirement for cystine and will not grow on rich (LB) media without added cystine. Cells were grown in M97-enriched minimal media (McIntosh *et al.*, 1990) to minimize diversion of labeled cysteine into other biochemical pathways with resultant scrambling of the label, which occurs during growth of this strain on minimal media containing only essential amino acids and salts (Davis & Mingioli, 1950). The yield

is 75−100 mg of labeled WT thioredoxin/6 L of media. A slight increase in mass, within experimental error, is observed in labeled *vs* unlabeled protein by mass spectroscopy (11 671.84 ± 2.7 Da *vs* 11 674.0 ± 0.6 Da). Strains containing the expression system for D26A thioredoxin had a higher cystine requirement. The seed culture had 0.004% cystine; the main culture required 0.002% cystine in order to achieve the growth rates and cell densities of WT. Control of expression in the tac-kan system is not absolute; hence a small amount of thioredoxin is produced without induction. When WT thioredoxin is expressed, the plasmid-encoded protein complements the *trx*$^-$ defect. However, D26A is less active than WT (C. Hanson, J. Fuchs, and C. Woodward, unpublished results); therefore, the small amount expressed without induction does not complement the *trx*$^-$ defect, and cells grow more slowly. Yields of purified D26A are about one-third that of WT.

NMR samples were prepared by dissolving lyophilized thioredoxin (3−5 mM) in 50 mM sodium phosphate buffer in ^2H_2O, pH 7. To this was added 10 mM DTT (final concentration) in ^2H_2O to reduce the protein, as well as 1 mM 3-(trimethylsilyl)propionic acid (TSP) as an internal ^1H chemical shift standard in the 5 mm NMR tube. [3-^{13}C]-L-cysteine was used as an external ^{13}C reference. pH values are meter readings uncorrected for deuterium. NMR experiments were conducted on an AMX500 Bruker spectrometer, using modifications of the pulse sequences provided with the software. HMQC (heteronuclear multiple-quantum coherence) (Bax *et al.*, 1983) experiments were carried out at 298 K. Titrations were followed in 2D HMQC spectra of 256 scans and 72 t_1 increments. This was sufficient resolution to give well-shaped peaks and to enable us to complete a pH titration in 3 days, minimizing the change of air oxidation of the sample. Air oxidation in old samples is readily apparent in NMR spectra and was not observed in these experiments. Chemical shift assignments in both the ^1H and ^{13}C dimensions for WT thioredoxin peaks correlate well with those reported by Dyson *et al.* (1991), and no additional confirmation of their identity was carried out. ^1H and ^{13}C assignments of Cys32 and Cys35 in D26A are based on perfect $^1H-^{13}C$ correlation of their cross peaks in 2D HMQC spectra obtained over a range of pH values and upon assignments obtained using 2D $^1H-^{13}C$ HMQC-NOESY (nuclear Overhauser effect spectroscopy) and TOCSY (total correlated spectroscopy) in ^1H_2O. NOESY spectra were acquired using a mixing time of 150−175 ms, at pH 7 and 8. For Cys32 at pH 8, the HN chemical shift is 6.4 ppm, and HB chemical shifts are 2.33 and 1.78 ppm. Cys32 HBs show long-range NOEs to Ile75 HD at 0.38 ppm and Trp31 HE at 7.38 ppm and short-range NOEs to HN of the same residue. For Cys35 at pH 8, the HA chemical shift is 4.03 and HB chemical shifts are 3.48 and 2.88 ppm. A long-range NOE is observed between Cys35 HBs and Trp28 HE at 7.05 ppm. At pH 7, Cys35 HBs show NOEs to the HN of the same residue at 7.88 ppm. These chemical shift assignments and NOEs are similar to those of WT, suggesting no major conformational change in the mutant. Although the cross peak of Cys35 HB2 either falls under a large DTT peak or comigrates with Cys32 HB1 for some of the titration, the variation of chemical shift with pH can clearly be followed over the entire range, pH 6−10. The structure of D26A has not been determined, but its circular dichroism and NMR spectra are very similar to those of WT. Assignments have been made of active site residues using $^1H-^{13}C$ HMQC TOCSY and NOESY experiments, and $^1H-^{15}N$ sequential assignments have been made for ^{15}N-labeled D26A (E. Barbar, N. Wilson, and C. Woodward, unpublished results).

Titration data were fit using the program NonLin of Robert Bernstein and D. Wayne Bolen, which is based on the nonlinear least squares analysis of Johnson and Frasier (1985). Each data set was fit to one pK_a value; in the cases that satisfactory fits were not obtained with one pK_a, fits to two pK_a values were carried out. Several starting pK_a values were used to verify convergence to the same values. If input chemical shifts are first normalized to a scale of 0−1, this often resulted in swifter convergence; the resultant numbers are then used as starting values in analysis of the actual input chemical shifts to get the final apparent pK_a value(s).

RESULTS

WT Thioredoxin. E. coli thioredoxin contains two cysteine residues, Cys32 and Cys35, at the active site. These were ^{13}C-enriched at the β-carbon by expression of the protein in appropriate auxotrophs grown in the presence of ^{13}C-labeled cystine. Figure 1 shows 2D HMQC spectra of reduced, ^{13}C-labeled WT thioredoxin at several pH values. Hydrogens attached to ^{13}C are selectively detected. Limited scrambling of ^{13}C label is apparent; some ^{13}C is incorporated into other amino acids, presumably by metabolism of the labeled cystine added during growth. Thioredoxin samples contain dithiothreitol (DTT) to ensure that they remain reduced; prominent DDT peaks are indicated in Figure 1. Assignments of Cys32 and Cys35 HB1 and HB2 resonances are unambiguous; throughout the titration they are correlated with the same ^{13}C cross peaks which at pH 6 have the ^{13}C chemical shifts assigned to Cys32 CB and Cys35 CB by Dyson *et al.* (1991), and their ^1H chemical shifts at pH 6 are the same as those assigned to Cys32 and Cys35 HB1 and HB2 protons by Dyson *et al.* (1991).

Figure 2 shows the pH dependence of the chemical shifts of cysteine CB protons, obtained from 2D HMQC spectra. Cys32 HB1 and Cys35 HB1 data fit an expression for a single titration with pK_a values of 7.1 and 7.0, respectively. However, neither Cys35 HB2 nor Cys32 HB2 data can be fit to a single titration; both data sets are better fit to an expression for two titrations, with pK_a values of 7.3 and 9.5 for Cys32 HB2 and 7.0 and 9.5 for Cys35 HB2. More extensive experimental baselines below and above the titration cannot be obtained because thioredoxin precipitates at pH <6 and denatures at pH >10. Although the absence of extended baselines increases the uncertainty in pK_a values derived from fits to the data, it is clear that the two HB protons on the same carbon of each cysteine residue reflect different titration behavior. Chemical shifts of the HB2 protons are influenced not only by titration of a group with pK_a around 7 but also by a second group titrating at much higher pH. Further, from the same 2D HMQC spectra used to obtain the ^1H data in Figure 2A,B, cysteine ^{13}CB chemical shifts are also obtained as a function of pH (Figure 2C). Similar to WT cysteine HB2 protons, the chemical shifts of both ^{13}CB atoms are affected by two titrations. The pK_a

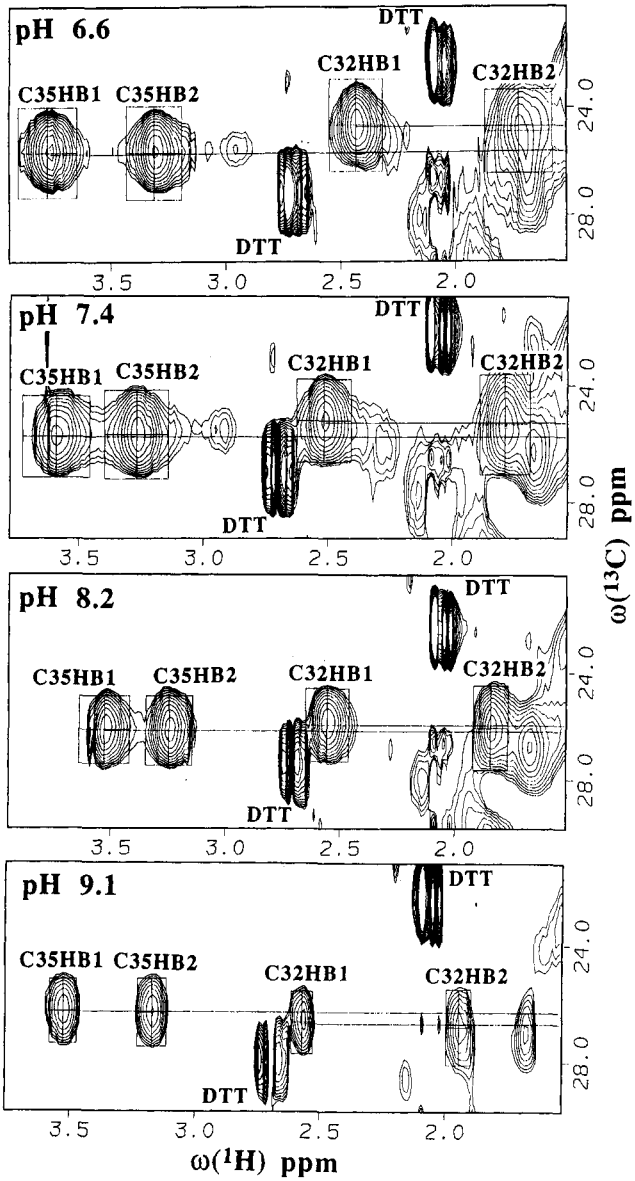


FIGURE 1: $^{1}H-^{13}C$ HMQC spectra of reduced wild-type thioredoxin at several pH values. Cysteine peaks are boxed and labeled; DTT peaks are indicated. Samples are 4 mM protein, 50 mM sodium phosphate buffer, 100% $^{2}H_2O$, and 10 mM DTT at 298 K.

values derived from fits to the data are 7.5 and 9.2 for Cys32 and 7.9 and 10.2 for Cys35. Apparent pK_a values derived from fits to the titration data of HB1, HB2, and CB of cysteines in WT thioredoxin are summarized in Table 1.

It has been reported for some time that one or both cysteine residues in reduced thioredoxin have perturbed pK_a values in the range 6.7−8 (Kallis & Holmgren, 1980; Dyson *et al.*, 1991). Our Raman experiments indicate that both cysteines have depressed pK_a values in the range 7.1−7.9 (Li *et al.*, 1993). Taking the Raman experiments together with the D26A data presented below, we conclude that the group titrating with pK_a >9 is the carboxyl of Asp26 and the groups titrating with pK_a in the range 7−7.9 are the cysteine thiols.

D26A Thioredoxin. As for WT thioredoxin, the D26A mutant was expressed in cells grown on minimal media supplemented with cystine containing ^{13}C-label on the β-carbons. As with WT thioredoxin, 2D HMQC spectra taken at incremental pH values from pH 6 to pH 10 have two pairs of ^{13}C-correlated ^{1}H cross peaks that move with

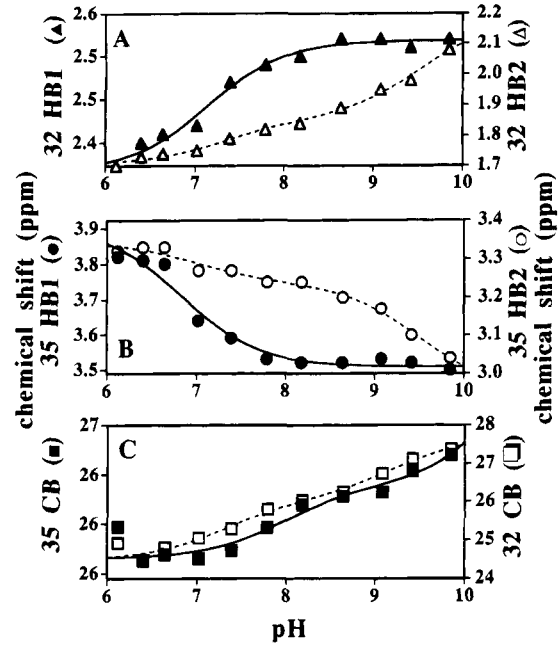


FIGURE 2: Variation of chemical shift with pH for cysteine $^{1}HB1$, $^{1}HB2$, and ^{13}CB in reduced wild-type thioredoxin. (A) Cys32 HB1 (▲) is fit to one titration (solid line). Cys32 HB2 (△) is fit to two titrations (dashed line). (B) Cys35 HB1 (●) is fit to one titration (solid line). Cys35 HB2 (○) is fit to two titrations (dashed line). (C) Cys32 CB (□) and Cys35 CB (■) are each fit to two titrations (solid line for C32; dashed line for C35). pK_a values of fitted curves are given in Table 1. All fits were obtained using the program NonLin, as described in Materials and Methods.

Table 1: Apparent pK_a Values Derived from Fits to Cysteine ^{1}HB and ^{13}CB Chemical Shift *vs* pH for Wild-Type and D26A Thioredoxin

	WT trx	D26A trx
Cys32 HB1	7.1	7.5
Cys32 HB2	7.3, 9.5	7.7
Cys35 HB1	7.0	7.4
Cys35 HB2	7.0, 9.5	7.8
Cys32 CB	7.5, 9.2	7.8
Cys35 CB	7.9, 10.2	7.9

pH (Figure 3). The pH dependence of cysteine HB1 and HB2 chemical shifts of D26A (Figure 4A,B) is strikingly different from that of WT thioredoxin (Figure 2). For D26A, the data for all four HB protons can be fit to one titration. Apparent pK_a values for Cys32 HB1 and Cys35 HB1 are 7.5 and 7.4, respectively. Cys32 and Cys35 HB2 titrations are better fit to pK_a values of 7.7 and 7.8. Further, ^{13}CB chemical shifts of cysteines in D26A have a pH dependence consistent with a single titration, rather than two (Figure 4C and Table 1). A titrating group with pK_a >9 clearly does not affect the chemical shifts of any ^{13}C-correlated ^{1}H cross peak nor the ^{13}CB chemical shifts in D26A. Since Asp26 is replaced by alanine in D26A, these data indicate that the higher pK_a influencing the HB2 protons and CB carbon in WT is Asp26 side-chain carboxyl. The titrations observed for D26A are assigned to the cysteine thiols. These are more similar to each other in D26A than in WT; both are around 7.5. Raman data support the conclusion from NMR that pK_a values of Cys32 and Cys35 are more nearly equal to each other in D26A than in WT (S. Vohnik, C. Hanson, R. Tuma, C. Woodward, and G. Thomas, unpublished results).

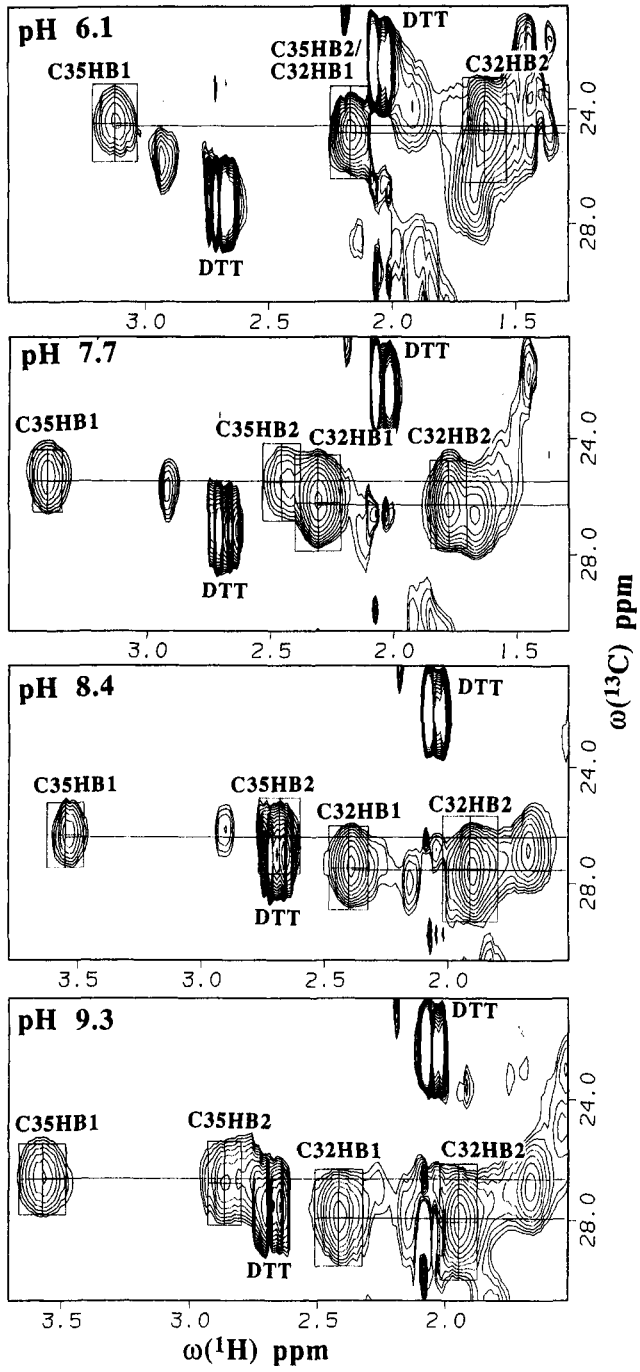


FIGURE 3: $^{1}H-^{13}C$ HMQC spectra of reduced D26A thioredoxin at several pH values. Cysteine peaks are boxed and labeled; DTT peaks are indicated.

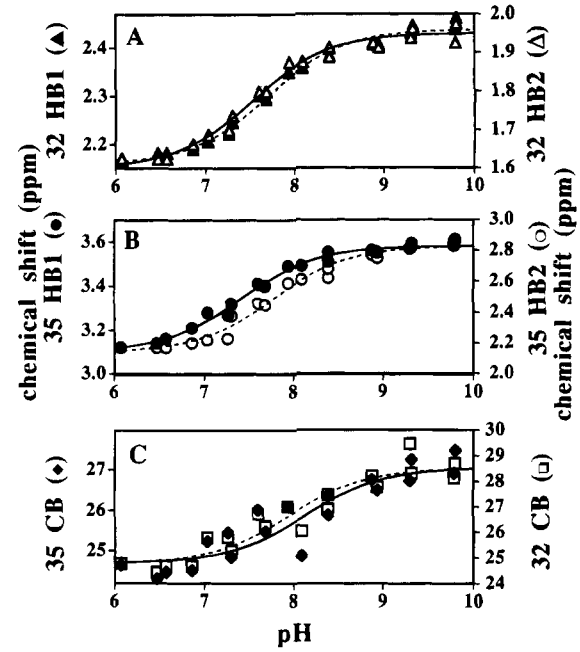


FIGURE 4: Variation of chemical shift with pH for cysteine $^{1}HB1$, $^{1}HB2$, and ^{13}CB in reduced D26A thioredoxin. (A) Cys32 HB1 (▲) and Cys32 HB2 (△) are each fit to one titration (solid line for Cys32 HB1; dashed line for Cys32 HB2). (B) Cys35 HB1 (●) and Cys35 HB2 (○) are each fit to one titration (solid line for Cys35 HB1; dashed line for Cys35 HB2). (C) Cys32 CB (□) and Cys35 CB (◆) are each fit to one titration (solid line for C32; dashed line for C35). pK_a values of fitted curves are given in Table 1. All fits were obtained using the program NonLin, as described in Materials and Methods.

DISCUSSION

Asp26 Side-Chain Carboxyl in Reduced Thioredoxin Has pK_a >9. Chemical shifts of hydrogens bound to cysteine β-carbons are sensitive to the ionization state of the thiol group covalently attached to the same carbon, as well as to the ionization state of other groups distant in sequences but close three dimensionally. For each cysteine in reduced WT thioredoxin, chemical shifts of the two gem-coupled hydrogens on the same β-carbon vary differently with pH. While the chemical shift *vs* pH curves of both HB protons reflect the titration of the attached thiol, one HB also reports titration of a second group. The pH−chemical shift curves of HB1 protons fit a single titration with pK_a values of about 7. In contrast, pH−chemical shift curves of both HB2 protons fit two titrations, with pK_a values of about 7 and 9.5. Likewise, the ^{13}C chemical shifts of the CB carbons in both WT thioredoxin cysteines report two titrations, with apparent pK_a values of 7.5−7.9 and 9.2−10.2. For all of these, we assign the pK_a values between 7−8 to the respective cysteine thiol titrations and the pK_a values >9 to Asp26 carboxyl titration. These assignments are consistent with the pK_a values of the cysteine thiols (7.1 and 7.9) measured for reduced thioredoxin by Raman spectroscopy (Li *et al.*, 1993) and are confirmed by pH−chemical shift data for D26A. In reduced D26A, both HB protons and the CB to which they are bound give pH−chemical shift curves that fit one titration with apparent pK_a values in the range 7.4−7.9 (Figure 4 and Table 1). Since D26A does not have a carboxyl at position 26, these titrations are assigned to the respective thiols. A titration with pK_a >9 observed in WT is absent in D26A, as predicted by its assignment to Asp26.

The observation that in reduced WT thioredoxin Asp26 titration affects chemical shifts of cysteine CB, and one HB but not the other, is consistent with differences in distance of the carboxyl to the two HB protons of each cysteine in the NMR structures of reduced thioredoxin (Jeng *et al.*, 1994). Further, Dyson *et al.* (1991) report two titrations for most protons in the region of the active site of reduced thioredoxin. They also observe a similar behavior with respect to the pH dependence of chemical shifts of gem-coupled cysteine HB protons; one is fit to a single titration, while the other apparently reflects at least two titrations.

Table 2: Comparison of Thioredoxin Active Site pK_a Values

trx	Cys32	Cys35	C32/C35	Asp26	method	reference
E. coli WT (ox)	n/a	n/a	n/a	7.5	electrophoresis	Langsetmo *et al.* (1991)
E. coli WT (ox)	n/a	n/a	n/a	7.5	NMR	Dyson *et al.* (1991)
E. coli WT (red)	7.4	7.9		9.5	NMR	this work
E. coli WT (red)			7.1, 7.9		Raman	Li *et al.* (1993)
E. coli WT (red)			7.1−7.4, 8.4	7.1−7.4	NMR	Dyson *et al.* (1991)
E. coli WT (red)			6.4		fluorescence	Reutiman *et al.* (1981)
E. coli WT (red)	6.7	>8.5			alkylation	Kallis & Holmgren (1980)
E. coli D26A (red)	7.8	7.9		n/a	NMR	this work
E. coli C32S,C35S	n/a	n/a		8.3	NMR	Dyson *et al.* (1994)
DsbA (red)	6.7 (C30)	9.5 (C33)			alkylation	Nelson & Creighton (1994)
human (red)	6.3	7.5−8.6		<2 or >10.6	NMR	Forman-Kay *et al.* (1992)

A highly significant result of these experiments is that the pK_a of Asp26, already highly perturbed in oxidized thioredoxin, rises even further when the protein is reduced, to a value >9. Active site cysteine thiol pK_a values have been estimated by several groups for oxidized and reduced *E. coli* and human thioredoxins and for DsbA (Table 2). Asp26 pK_a is 7.5 in oxidized WT *E. coli* thioredoxin (Langsetmo *et al.*, 1991a; Dyson *et al.*, 1991). In reduced WT *E. coli* thioredoxin, Holmgren and associates (Kallis & Holmgren, 1980) determined that at least one cysteine thiol pK_a is perturbed downward to around 6.7, and their proposal that the lowered pK_a of one or both cysteine thiols functions to provide a thiolate at physiological pH is widely accepted. Li *et al.* (1993) determined the cysteine thiol pK_a values to be approximately 7.1 and 7.9 from the pH dependence of the intensity of SH bands in Raman spectra, which has the advantage of direct observation of thiol sulfur ionization state. As discussed below, there is reason to have more confidence in ^{13}C-derived pK_a values (Oda *et al.*, 1994), and in this regard it is notable that our ^{13}C-derived values are consistent with the values from Raman spectroscopy. Dyson *et al.* (1991) observed three apparent pK_a values in ^{1}H chemical shift *vs* pH curves of reduced *E. coli* thioredoxin. The authors discussed several possible assignments of the three pK_a values, two of which were in the range 7.1−7.4 and one at 8.4; Cys32 was assigned to the lower pK_a, but it was considered unlikely that the titration with pK_a around 8.4 could be that of Asp26. For the thioredoxin mutant C32S,C35S, Dyson *et al.* (1994) observed an apparent pK_a of 8.4 for Asp26 and suggested that local changes due to replacement of cysteines with serines were responsible for the rise in Asp26 pK_a. Our data suggest rather that serine replacements in C32S,C35S affect the environment in a way that lowers Asp26 pK_a from >9 to 8.4. In reduced human thioredoxin, no titration of Asp26 is detected, and it was concluded that it must either titrate at very low or very high pH, or if it titrates, there is no effect on chemical shift of observable protons (Forman-Kay *et al.*, 1992).

An inherent difficulty in determination of pK_a values from the pH dependence of NMR chemical shifts is that the signal reflects a summed effect of all titrating groups; the change in chemical shift with pH is a function of the change with pH of the total local electrostatic environment at that site [and of any other pH-dependent contribution(s) to chemical shift]. Therefore, the apparent pK_a derived from fits to the pH−chemical shift curves may not be the actual pK_a of a given titrating group(s). In oxidized thioredoxin, in which the only titrating active site group is Asp26, Dyson *et al.* (1991) observe a range of pK_a values from 6.9 to 8.2 for different reporter groups, with an average around 7.5. For reduced thioredoxin, this range is even wider (Dyson *et al.*, 1991). Similarly, we observe variation in apparent pK_a values from cysteine ^{1}HB and ^{13}CB chemical shifts. Since the titrating sulfur is chemically bonded to the ^{13}C reporting group, the ^{13}CB titrations may give the most reliable values of reduced thioredoxin cysteine thiol pK_a (Oda *et al.*, 1994).

Structural Implications of a High pK_a for Asp26 in Reduced Thioredoxin. Although it may at first appear highly anomalous for an aspartic acid to have pK_a >9, an abnormally raised pK_a is expected for a carboxyl buried in a hydrophobic environment. The elevated pK_a is primarily the result of a large desolvation penalty associated with charging the group in a medium of low dielectric constant. In the crystal structure of oxidized thioredoxin, the Asp26 side chain is located at the bottom of a deep crevice; one carboxyl oxygen is completely buried, while the other is slightly exposed to solvent and hydrogen bonded to a crevice water. Electrostatic calculations on oxidized thioredoxin predict a pK_a for Asp26 of ≥10 (Langsetmo *et al.*, 1991b; Dyson *et al.*, 1994).

The fact that Asp26 pK_a is as low as 7.5 in oxidized thioredoxin suggests that the dynamic accessibility to water is greater than indicated in the average (crystal) structure, since small changes in buriedness of the Asp26 side chain can have large effects on the local effective dielectric constant and therefore on the acid dissociation equilibrium. In Langsetmo *et al.* (1991b) it was suggested that Lys57 may play a role in lowering Asp26 pK_a from the expected value of ≥10; however, replacement of Lys57 with glycine has no effect on Asp26 pK_a in oxidized thioredoxin (C. Hanson, J. Fuchs, and C. Woodward, unpublished results). In the NMR structure of reduced thioredoxin, when surface area is computed by the method of Lee and Richards (1971) or of Connolly (1983), there is no apparent access to solvent for the Asp26 side chain, and the crevice observed in oxidized thioredoxin is not present. It is feasible that the higher pK_a of Asp26 in reduced thioredoxin is due to a decrease in average accessibility of Asp26 carboxyl to water and an associated decrease in local effective dielectric constant favoring protonation even at very high pH.

Difference in Stability between Oxidized and Reduced Thioredoxin. Oxidized thioredoxin is significantly more stable than reduced; the difference in stability between oxidized and reduced, $\Delta\Delta G^\circ_{(ox\rightarrow red)}$, is reported to be 2.0 kcal/mol at pH 7 (Kelley *et al.*, 1987) and 3.5 kcal/mol at pH 8.7 (Langsetmo *et al.*, 1989). Although this stability difference has been recognized for a long time, no satisfactory explanation has been published. There are no obvious differences

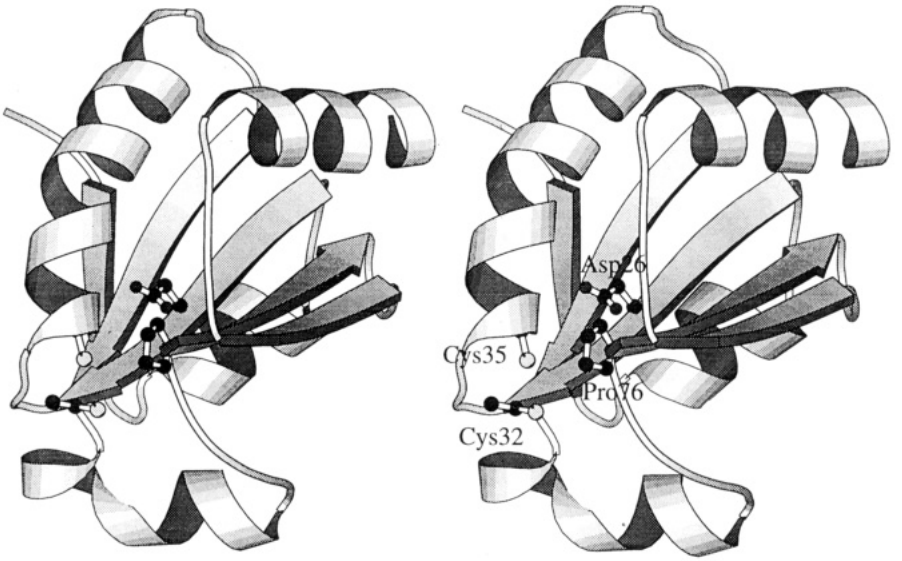


FIGURE 5: Stereoview of the average reduced structure determined by NMR (Jeng *et al.*, 1994). Side chains of active site residues Asp26, Cys32, Cys35, and Pro76 are shown. The figure was prepared using the program MolScript (Kraulis, 1991).

between the NMR structures of oxidized and reduced thioredoxin which can be invoked as a basis for the stability difference (Jeng *et al.*, 1994). However, the high pK_a of Asp26 in reduced thioredoxin provides an explanation.

The linkage of Asp26 titration to protein global stability is discussed in Langsetmo *et al.* (1991a,b). There we considered the pH dependence of the destabilizing effect on oxidized thioredoxin from the perturbed Asp26 pK_a of 7.5; at physiological pH the anomalous pK_a of Asp26 destabilizes oxidized thioredoxin by about 4.2 kcal/mol. Reduction of thioredoxin further perturbs Asp26 pK_a to >9 and thereby further destabilizes the protein. Along with theAsp26 pK_a, other contributions to the difference in stability between oxidized and reduced thioredoxin that must also be taken into account include the thiol pK_a values, the disulfide cross-link, and Ile75−Pro76 peptide isomerism. Both cysteine thiols in reduced thioredoxin have pK_a values that are perturbed downward (Li *et al.*, 1993, and Table 1), which *stabilizes* reduced relative to oxidized. The perturbation upward of Asp26 pK_a in reduced thioredoxin provides a destabilizing process that compensates the thiol pK_a effects on $\Delta\Delta G^\circ_{(ox\rightarrow red)}$. Other contributions to $\Delta\Delta G^\circ_{(ox\rightarrow red)}$ are expected from chain entropy effects of the disulfide cross-link (Sandberg *et al.*, 1991) and from the increased population of the *trans* isomer of the Ile75−Pro76 peptide bond (Langsetmo *et al.*, 1989), both of which destabilize reduced relative to oxidized.

As described in Langsetmo *et al.* (1991a,b), one can calculate the variation with pH of the equilibrium constant (and related ΔG°) for cooperative unfolding due to titration of any number of groups with perturbed pK_a. If this is done for both WT and a mutant in which a perturbed group is replaced with a neutral group, then the pH dependence of $\Delta\Delta G^\circ_{(WT\rightarrow mut)}$ due only to loss of the titration of the replaced group may be calculated. Similarly, by carrying out the calculation for oxidized thioredoxin with Asp26 pK_a of 7.5, and for reduced thioredoxin with Asp26 pK_a of 9.5, Cys32 pK_a of 7.4, and Cys35 pK_a of 7.9, one can estimate $\Delta\Delta G^\circ_{(ox\rightarrow red)}$ as a function of pH, if only these groups contribute to the stability difference. This calculation indicates that perturbed pK_a values of groups 26, 32, and 35 collectively destabilize reduced relative to oxidized thioredoxin by 0.2 kcal/mol at pH 7 and 1.1 kcal/mol at pH 8.7. To this must be added contributions to stabilization of oxidized from chain entropy effects of the disulfide bond, roughly estimated to be about 2 kcal/mol (Sandberg *et al.*, 1991; Pace *et al.*, 1988) and assumed to be pH independent, and from a higher population of 75−76 *trans* isomer (Langsetmo *et al.*, 1989), for which no reliable estimate is presently available. Adding 2 kcal/mol to the summed effects of 26, 32, and 35 titrations gives values of $\Delta\Delta G^\circ_{(ox\rightarrow red)}$ of 2.2 kcal/mol at pH 7 and 3.1 kcal/mol at pH 8.7, which are similar to the experimental values. In summary, the pK_a values of 26, 32, and 35 in reduced thioredoxin determined in this study account for the pH dependence of the stability difference between oxidized and reduced and give values of $\Delta\Delta G^\circ_{(ox\rightarrow red)}$ that are reasonable in light of other processes known to destabilize reduced relative to oxidized thioredoxin.

Functional Implications of a High pK_a for Asp26 in Reduced Thioredoxin. In thioredoxin, the stability difference between oxidized and reduced links global stability to the disulfide−dithiol equilibrium described by the redox potential. Therefore, additional processes linked to global stability are also linked to redox potential. We have identified two of these, Asp26 titration (Langsetmo et al., 1991a) and Ile75−Pro76 cis−trans isomerism (Langsetmo et al., 1989). Asp26 is conserved in all thioredoxins except Anabaena (Eklund et al., 1991), and Pro76 is absolutely conserved in thioredoxins. Figure 5 shows the relative locations of the active site cysteines to Asp26 and Pro76 in reduced thioredoxin.

The role of Asp26 is, we propose, to confer a destabilizing influence on reduced thioredoxin relative to oxidized thioredoxin. As discussed above, a mechanistic requirement for a thiolate ion at physiological pH explains the function of a low pK_a for one or both cysteines (Kallis & Holmgren, 1980) but results in stabilization of reduced relative to oxidized thioredoxin, which would make thioredoxin a less effective reductant. Asp26 compensates with a destabilization penalty of about 4 kcal/mol for the oxidized form and about 7 kcal/

mol for the reduced from [using eq 3 of Langsetmo *et al.* (1991a)]. The perturbation of Asp26 pK_a to 7.5 in oxidized thioredoxin provides for sensitive pH modulation in the physiological pH range of the stability difference between oxidized and reduced thioredoxin and of the linked redox potential. It seems clear that the function of Asp26 is not primarily to provide an electrostatic environment that depresses the cysteine thiol pK_a. With a pK_a >9, Asp26 in reduced thioredoxin would be neutral at physiological pH. While the absence of Asp26 in D26A is associated with some change in active site thiol pK_a values (Table 1), the change is not large. This is in agreement with Raman studies which indicate that, like reduced WT (Li *et al.*, 1993), reduced D26A also has depressed values of cysteine pK_a (S. Vohnik, C. Hanson, R. Tuma, C. Woodward, and G. Thomas, unpublished results).

In contrast to the redox−stability linkage relationship in thioredoxin, reduced DsbA is more stable than oxidized DsbA by 5.4 kcal/mol (Wunderlich *et al.*, 1993). As Wunderlich *et al.* point out, this is significant because, while thioredoxin is a strong reductant, with a redox potential of −0.23 to −0.26 V (Holmgren, 1968; Berglund & Sjoberg, 1970; Gleason, 1992), DsbA is a strong oxidant with a redox potential of −0.089 V (Wunderlich *et al.*, 1993). This suggests that the stability difference between oxidized and reduced forms determines whether a thioredoxin-like protein will be a reductant or an oxidant and that processes which modulate global stability similarly modulate redox potential. Reduced thioredoxin tends to return to the more stable oxidized state and does so when reducing other proteins; oxidized DsbA tends to return to the more stable reduced state and does so when oxidizing other proteins. DsbA has a glutamic residue at the same three-dimensional position as Asp26 in thioredoxin, but its pK_a has not yet been reported.

In this regard it is significant that another protein in the thioredoxin superfamily, glutaredoxin A, which has isoleucine at the same three-dimensional position as Asp26 in thioredoxin, does not have a stability difference between oxidized and reduced, but glutaredoxin N, with an additional five amino acids at the N-terminus, is more stable in the oxidized form, suggesting a functional difference between the two species (Sandberg *et al.*, 1991).

A Higher pK_a in Reduced Thioredoxin May Explain the Preference for the Reduced Form in Phage Replication Systems. A preference for the reduced form has been shown in the functioning of the bacteriophage replication cycle (Russel & Model, 1986). However, the redox activity of thioredoxin is not required for this function; mutants in which active site cysteines are replaced also support phage growth although at a somewhat lower level (Russel & Model, 1986). The primary differences detected by NMR between oxidized and reduced thioredoxins is an increased internal mobility of active site residues in reduced, and these do not obviously explain the phage preference for reduced thioredoxin (Jeng *et al.*, 1994). A raised pK_a of Asp26 in reduced thioredoxin offers a possible explanation for the preference. Functioning of thioredoxin in phage replication presumably involves specific binding of a factor critical to phage maturation. This binding may be inhibited by the presence of a partial negative charge at Asp26 at physiological pH and facilitated by the neutralization of this charge in reduced thioredoxin due to the raised pK_a, thus accounting for the preference for the reduced form. Widespread changes in local electrostatic field throughout the thioredoxin molecule are expected when Asp26 is neutralized [*e.g.*, Figure 4 in Langsetmo *et al.* (1990)].

REFERENCES

Bardwell, J. C. A., McGovern, K., & Beckwith, J. (1991) *Cell 67*, 581−589.

Bax, A., Griffey, R., & Hawkins, B. (1983) *J. Magn. Reson. 55*, 301.

Bazzichi, A., Incaprera, M., & Garzelli, C. (1994) *Int. J. Oncol. 5*, 41−46.

Berglund, O., & Sjoberg, M.-B. (1970) *J. Biol. Chem. 245*, 6030−6035.

Connolly, M. L. (1983) *Science 221*, 709−713.

Davis, B., & Mingioli, A. (1950) *J. Bacteriol. 60*, 17.

Dyson, H. J., Tennant, L., & Holmgren, A. (1991) *Biochemistry 30*, 4262−4268.

Dyson, H. J., Jeng, M.-F., Model, P., & Holmgren, A. (1994) *FEBS Lett. 339*, 11−17.

Edman, J., Ellis, L., Blacher, R., Roth, R., & Rutter, W. (1985) *Nature 334*, 267−270.

Eklund, H., Gleason, F., & Holmgren, A. (1991) *Proteins: Struct., Funct., Genet. 11*, 13−28.

Forman-Kay, J., Clore, G., & Gronenborn, A. (1992) *Biochemistry 31*, 3442−3452.

Gleason, F. K. (1992) *Protein Sci. 1*, 609−616.

Hayashi, T., Ueno, Y., & Okamoto, T. (1993) *J. Biol. Chem. 268*, 11380−11388.

Holmgren, A. (1968) *Eur. J. Biochem. 6*, 475−485.

Huber, H., Russel, M., Model, P., & Richardson, C. (1986) *J. Biol. Chem. 261*, 15006−15012.

Jeng, M.-F., Campbell, A., Begley, T., Holmgren, A., Case, D., Wright, P., & Dyson, H. J. (1994) *Structure 2*, 853−868.

Johnson, M., & Frasier, S. (1985) *Methods Enzymol. 117*, 301−342.

Kallis, G.-B., & Holmgren, A. (1980) *J. Biol. Chem. 255*, 10261−10265.

Katti, S., LeMaster, D., & Eklund, H. (1990) *J. Mol. Biol. 212*, 167−184.

Kelley, R., Shalongo, W., Jagannadham, M., & Stellwagen, E. (1987) *Biochemistry 26*, 1406−1411.

Kraulis, P. (1991) *J. Appl. Crystallogr. 24*, 946−950.

Langsetmo, K., Fuchs, J. A., & Woodward, C. (1989) *Biochemistry 28*, 3211−3220.

Langsetmo, K., Sung, Y.-C., Fuchs, J. A., & Woodward, C. (1990) in *Current Research in Protein Chemistry: Techniques, Structure & Function* (Villafranca, J., Ed.) pp 449−456, Academic Press, New York.

Langsetmo, K., Fuchs, J. A., & Woodward, C. (1991a) *Biochemistry 30*, 7603−7609.

Langsetmo, K., Fuchs, J. A., Woodward, C., & Sharp, K. A. (1991b) *Biochemistry 30*, 7609−7614.

Lee, B., & Richards, F. M. (1971) *J. Mol. Biol. 55*, 379−400.

Li, H., Hanson, C., Fuchs, J. A., Woodward, C., & Thomas, G. J., Jr. (1993) *Biochemistry 32*, 5800−5808.

Lim, C.-J., Geraghty, D., Fuchs, J. A. (1985) *J. Bacteriol. 163*, 311−316.

Lin, T.-Y., & Kim, P. S. (1989) *Biochemistry 28*, 5282−5287.

Martin, J., Bardwell, J. C. A., & Kuriyan, J. (1993) *Nature 365*, 464−468.

Matthews, J., Wakasuki, N., Virelizier, J., Yodoi, J., & Hay, R. (1992) *Nucleic Acids Res. 20*, 3821−3830.

McIntosh, L., Wand, A., Lowry, D., Redfield, A., & Dahlquist, F. (1990) *Biochemistry 29*, 6341−6362.

Mitsui, A., Hirakawa, T., & Yodoi, J. (1992) *Biochem. Biophys. Res. Commun. 186*, 1220−1226.

Modrich, P., & Richardson, C. (1975a) *J. Biol. Chem. 250*, 5508−5514.

Modrich, P., & Richardson, C. (1975b) *J. Biol. Chem. 250*, 5515−5522.

Nelson, J., & Creighton, T. E. (1994) *Biochemistry 33*, 5974−5983.

Nordstrom, B., Randahl, H., Salby, I., & Holmgren, A. (1981) *J. Biol. Chem. 256*, 3112−3117.

Oda, Y., Yamazaki, T., Nagayama, K., Kanaya, S., & Nakamura, H. (1994) *Biochemistry 33*, 5275−5284.
Pace, C. N., Grimsley, G., Thomson, J., & Barnett, B. (1988) *J. Biol. Chem. 263*, 11820−11825.
Qin, J., Clore, G., & Gronenborn, A. (1994) *Structure 2*, 503−522.
Reutiman, H., Straub, B., Luisi, P., & Holmgren, A. (1981) *J. Biol. Chem. 256*, 6796−6803.
Russel, M., & Model, P. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 29−33.
Russel, M., & Model, P. (1986) *J. Biol. Chem. 261*, 14997−15005.
Russel, M., & Holmgren, A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 990−994.
Russel, M., Model, P., & Holmgren, A. (1990) *J. Bacteriol. 172*, 1923−1929.
Sandberg, V. A., Kren, B., Fuchs, J. A., & Woodward, C. (1991) *Biochemistry 30*, 5475−5484.
Schenk, H., Klein, M., Erdbrugger, W., Droge, W., & Schulze-Ostoff, K. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1672−1676.
Schurmann, P., Maeda, K., & Tsugita, A. (1981) *Eur. J. Biochem. 116*, 37−45.
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.
Wolosiuk, R., Buchanan, B., & Crawford, N. (1977) *FEBS Lett. 81*, 253−258.
Wunderlich, M., Jaenicke, R., & Glockshuber, R. (1993) *J. Mol. Biol. 233*, 559−566.

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