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# The Conserved, Buried Aspartic Acid in Oxidized *Escherichia coli* Thioredoxin Has a p$K_a$ of 7.5. Its Titration Produces a Related Shift in Global Stability†

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ABSTRACT: Aspartic acid 26 in *Escherichia coli* thioredoxin is located at the bottom of a hydrophobic cavity, near the redox-active disulfide of the active site. Asp 26 is embedded in the protein except for part of the surface of one carboxyl oxygen. The high degree of evolutionary conversion of Asp 26 suggests that it plays a critical role in thioredoxin function. We have determined the p$K_a$ of Asp 26 by a novel electrophoretic method based on the relative electrophoretic mobilities of wild-type thioredoxin and of D26A thioredoxin (with Asp 26 replaced by alanine). The p$K_a$ of Asp 26 determined by this technique is 7.5, more than 3 units above the p$K_a$ of a solvated carboxyl side chain. The titration of Asp 26 is thermodynamically linked to the stability of thioredoxin. As expected if thioredoxin stability depends on the ionization state of Asp 26, $\Delta G°_{WT}$, the free energy of the cooperative denaturation reaction of wild-type thioredoxin by guanidine hydrochloride, varies with pH in a sigmoidal fashion in the vicinity of pH 7.5. Over the same pH range, the free energy for D26A folding, $\Delta G°_{D26A}$, is pH independent and D26A is highly stabilized compared to wild type. From the thermodynamic cycle describing the linkage of Asp 26 titration to thioredoxin stability, the difference in free energy between wild-type thioredoxin with protonated Asp 26 and wild-type thioredoxin with deprotonated Asp 26, $\Delta\Delta G°_{(COOH \rightarrow COO^-)}$, is calculated to be 4.9 kcal/mol. In good agreement with this value, we find that the difference in free energy between wild type and D26A denaturation, $\Delta\Delta G°_{(WT \rightarrow D26A)}$, is 4.6 kcal/mol at pH 8.5, where Asp 26 is mostly deprotonated. This indicates that the stabilization of D26A compared to wild type is primarily due to the electrostatic effects of removing the abnormally titrating aspartic acid and corroborates the p$K_a$ of 7.5 obtained for Asp 26 by the electrophoretic method. In the following paper [Langsetmo, K., Fuchs, J., Woodward, C., & Sharp, K. (1991) *Biochemistry* (following paper in this issue)], the role of Asp 26 titration in thioredoxin structure is further described. In that paper the excellent agreement of the experimental pH dependence of $\Delta G°_{WT}$ with a general expression for the linkage of protein titration groups and protein stability is reported.

*Escherichia coli* thioredoxin is a 108 amino acid, soluble protein that participates in diverse redox and regulatory reactions (Holmgren et al., 1975; Holmgren, 1985). It has an $\alpha/\beta$ structure (Katti et al., 1990; Eklund et al., 1984), of which the most prominent feature is the central five-stranded, twisted $\beta$-sheet (Figure 1). The only two cysteine residues in the molecule, Cys 32 and Cys 35, form the redox-active disulfide/dithiol in a reverse turn between the middle $\beta$-strand and the $\alpha$2-helix. Thioredoxin is purified in the oxidized form, with the active site cysteines in a disulfide bond. In the presence of reducing agents, the cysteines are in the dithiol form. Although reduced thioredoxin is much less stable than oxidized (Kelley et al., 1987; Langsetmo et al., 1989), reduction produces only small, local changes in the vicinity of the cysteines (Brown et al., 1987; Dyson et al., 1990).

*E. coli* thioredoxin has high sequence homology to other thioredoxins (Eklund et al., 1984; Gleason, 1986; Gleason & Holmgren, 1988). In addition to the active site sequence Trp-Cys-Gly-Pro-Cys, the completely conserved residues include two others of unusual conformation in the crystal structure. These are Pro 76, which is in a cis peptide bond, and Asp 26, which has a buried carboxyl group. We are

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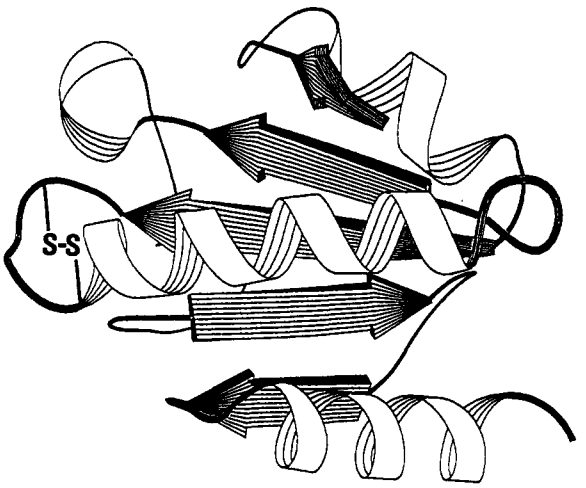



FIGURE 1: Oxidized *E. coli* thioredoxin. The Richardson-type drawing shows the five-stranded $\beta$-sheet and four helices. In this view, the active site disulfide is on the left side of the molecule. The drawing was generated from the high-resolution crystal structure (Katti et al., 1990) with the Protein Analysis Package of Callahan et al. (1990).

investigating the role of these residues in thioredoxin stability, structure, and activity. Previously we reported that thioredoxin has two folded forms that have different stability in urea denaturation and that we attribute to cis and trans isomers of the Pro 76 peptide bond (Langsetmo et al., 1989). Here we further examine the role of the buried carboxyl of Asp 26 in thioredoxin stability.

In the high-resolution crystal structure of oxidized thioredoxin at pH 3.8 (Katti et al., 1990), the side-chain oxygen atoms of Asp 26 are embedded in a deep hydrophobic cavity near the active site disulfide. Asp 26 carboxyl oxygen atoms have zero static accessibility, as defined by Lee and Richards (1971), although part of one Asp 26 carboxyl oxygen atom lines the molecular surface (Richards, 1977; Connolly, 1983) of the cavity. Substitution of Asp 26 by alanine, to form the D26A[1] variant, greatly stabilizes thioredoxin (Langsetmo et al., 1990). This suggests that, in wild type (WT), the Asp 26 carboxyl group is situated in an unfavorable environment, and its p$K_a$ is strongly perturbed. The estimated stability difference between WT and D26A suggested that the p$K_a$ of Asp 26 is shifted up by more than 3 pH units from the value of fully solvated carboxyl groups (Langsetmo et al., 1990). To examine this further, we have determined the p$K_a$ of Asp 26 by a novel electrophoretic method and characterized more fully the stability difference between WT and D26A thioredoxin.

Our technique for determination of the Asp 26 acid dissociation constant involves analysis of the pH dependence of electrophoretic mobilities of wild type (WT) and D26A. The p$K_a$ of Asp 26 measured by this method is 7.5. If the destabilization of WT relative to D26A is entirely due to normalization of Asp 26 in denatured WT, then $\Delta\Delta G^\circ_{(WT \rightarrow D26A)}$, the difference in free energy between WT and D26A at high pH (where Asp 26 is fully deprotonated), should be 4.9 kcal/mol. This is in good agreement with the value of 4.6 kcal/mol we obtain for $\Delta\Delta G^\circ_{(WT \rightarrow D26A)}$ at pH 8.5. As expected if ionization of Asp 26 is important to its stability, WT thioredoxin unfolding is sensitive to pH in the vicinity of the Asp 26 p$K_a$, but D26A is not.

[1] D26A, thioredoxin with Asp 26 replaced by alanine; WT, wild-type thioredoxin; GuHCl, guanidine hydrochloride; CD, circular dichroism; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; TRIS, tris(hydroxymethyl)aminomethane; ACES, 2-[(carbamoylmethyl)-amino]ethanesulfonic acid.

In the following paper (Langsetmo et al., 1991), analysis of the pH dependence of $\Delta G^\circ$, and of $\Delta\Delta G^\circ_{(WT \rightarrow D26A)}$, is developed for the general case when stability depends on ionization of the mutated group. Application of the analysis to thioredoxin WT and D26A, using a p$K_a$ of 7.5 for Asp 26, gives an excellent fit to the experimental variation with pH of $\Delta G^\circ$ and $\Delta\Delta G^\circ_{(WT \rightarrow D26A)}$.

The functional implications for the perturbed p$K_a$ of Asp are significant. The complete evolutionary conversion of Asp 26, and its proximity to the redox-active disulfide and to the conserved *cis*-proline at position 76, are highly suggestive of a critical function of this residue in the catalytic mechanism of thioredoxin. This is further supported by the observation that in reduced, wild-type thioredoxin the p$K_a$ of Asp 26 changes to 7.0 (K.L., J.A.F., and C.W., unpublished results). A difference in p$K_a$ for Asp 26 in oxidized versus reduced thioredoxin means that there is a linked difference in the pH dependence of global stability between oxidized and reduced thioredoxin. Since redox potential is thermodynamically linked to the stability difference between oxidized and reduced (Lin & Kim, 1989), this results in a pH dependence of thioredoxin disulfide/dithiol equilibrium (redox potential) in the physiological pH range. We expect that the function of the buried Asp 26 lies in the thermodynamic linkage of Asp 26 titration, the stability of thioredoxin, and the redox potential of the active site cysteines.

## Materials and Methods

The *trx*A gene encoding thioredoxin has been cloned in a number of vectors, facilitating protein isolation and purification (Lim et al., 1985). High-level overexpression of wild-type and D26A thioredoxin and the protein purification protocol are described in Langsetmo et al. (1989, 1990). The mutation was confirmed by sequencing of the gene. Production of bona fide D26A was verified by quantitative amino acid analysis of purified protein. In isoelectric focusing experiments in this lab (V. Sandberg, unpublished results), purified WT and D26A both have isoelectric points within error of the previously reported value for WT, 4.5 (Holmgren, 1985).

Polyacrylamide gel electrophoresis was carried out with use of a Bio-Rad mini slab gel apparatus. Gels were cast with solutions of 15% acrylamide, 0.5% bisacrylamide, and 50 mM buffer adjusted to the specified pH with either HCl or NaOH. The pH of each gel is within 0.5 pH units of the buffer p$K_a$. Buffers were chosen with pH ranges that overlap, so that two buffers can be used for measurements at the same pH. Electrophoresis was performed at 20 V/cm, constant voltage, for 90 min. During electrophoresis, the tray buffer was pumped between the two reservoirs to maintain the ion distribution in the reservoir buffers. The pH was measured after electrophoresis with a flat surface combination electrode, with measurements taken in intervals throughout the entire surface of the gel. Gels that did not maintain a uniform pH throughout the gel were not included in the analysis.

After staining with Coomassie blue, the gel was digitized with use of AppleScan to drive an optical scanner interfaced with a Macintosh II computer. The wet, stained slab was placed in a self-sealing plastic bag for scanning. The mobilities of the bands were measured interactively on the digitized image of the gel on the screen with use of utilities of the program AppleScan. The mobility is measured reproducibly to 0.1 mm. To minimize the influence of slight variations in current from one side of the gel to the other, mobilities, taken as the distance from the top of the gel, were determined on WT and D26A bands in adjacent lanes. Results from 8–16 adjacent pairs of WT and D26A bands (2 pairs per gel) were averaged to obtain

the values reported at each pH for the ratio of mobility WT:mobility D26A.

Circular dichroism (CD) spectra were recorded with use of a modified Jasco J41C spectropolarimeter with a jacketed 1-mm path length cell maintained at 25 °C. Protein stock solutions were prepared in 50 mM succinate (pH 6.0, pH 6.5), TES (pH 7.0, pH 7.5), and TRIS (pH 8.0, 8.5, and 9.0). Samples with varying concentrations of GuHCl were prepared by dilution of stock solutions to a final protein concentration of 0.5 mg/mL. Protein concentration was determined by absorbance at 280 nm, with use of the wild-type molar extinction coefficient of 13 700 (Reutimann et al., 1981). The samples were equilibrated for 15 min before spectra from 270 to 215 nm were recorded. The data, mean residue weight ellipticity, $[\Theta]_{MRW}$, versus [GuHCl], were analyzed with use of the program NonLin. Values of $\Delta G°$ in the absence of denaturant are obtained by nonlinear least-squares fit of $[\Theta]_{MRW}$ at each GuHCl concentration to the equation

$$[\Theta]_{MRW} = f_F(b_f - b_u) + b_u \quad (1)$$

where the fraction folded, $f_F$, equals $K_{eq}/(1 + K_{eq})$ and $b_f$ and $b_u$ are the values of the folded and unfolded base lines, respectively, at that GuHCl concentration. The base lines include the parameters $i_f$ and $i_u$, the base-line values in the absence of GuHCl, and $s_f$ and $s_u$, which are the [GuHCl]-dependent slopes of the base lines. Then, $b_f = i_f + s_f$[GuHCl] and $b_u = i_u + s_u$[GuHCl]. $\Delta G°_{[GuHCl]}$ is the free energy value at a specified GuHCl concentration; $K_{eq} = \exp(\Delta G°_{[GuHCl]}/RT)$; $\Delta G°_{[GuHCl]} = m$[GuHCl] + $\Delta G°$, where $m$ is [GuHCl] dependence of the free energy, and $\Delta G°$ is the free energy in the absence of denaturant (Pace, 1986). The base-line parameters $b_f$ and $b_u$ refer to the [GuHCl] dependence of $[\Theta]_{MRW}$ of the native and the unfolded states, i.e., the [GuHCl] dependence of the $[\Theta]_{MRW}$ above and below the transition region.

The fitting program NonLin of Robert Brenstein and D. Wayne Bolen was used to fit the data for ratio of electrophoretic mobility versus pH, and the data for ellipticity versus [GuHCl]. [NonLin is based on the nonlinear least-squares analysis method of Johnson and Frasier (1985), and may be obtained from Brenstein and Bolen at P.O. Box 160, Carbondale, IL 62903.]

Electrostatic potentials of thioredoxin atoms with protonated Asp 26 and with deprotonated Asp 26 were calculated with use of the program DelPhi [reviewed in Sharp and Honig (1990)] and are further described in the following paper (Langsetmo et al., 1991).

## Results

*p$K_a$ of the Asp 26 Carboxyl Group.* We have determined the p$K_a$ of Asp 26 by comparison of the electrophoretic mobilities of WT thioredoxin and D26A as a function of pH. The method is based on the expectation that changes in net charge due to titration of Asp 26 will be present in WT but not in the alanine-substituted variant. At pH's where Asp 26 in WT is fully deprotonated, WT and D26A will differ by one charge, and this should be reflected in their electrophoretic mobilities. In contrast, at pH's where Asp 26 in WT is protonated, WT and D26A should have similar electrophoretic mobilities. At intermediate pH's, where Asp 26 is titrating, the differences in electrophoretic mobilities of WT and D26A should be proportional to the ratio of protonated to unprotonated Asp 26.

The electrophoretic behavior of WT and D26A over the range 6–9 is shown in Figure 2. At pH 8.5, compared to D26A, WT has a higher mobility toward the anode (greater net negative charge). This mobility difference corresponds

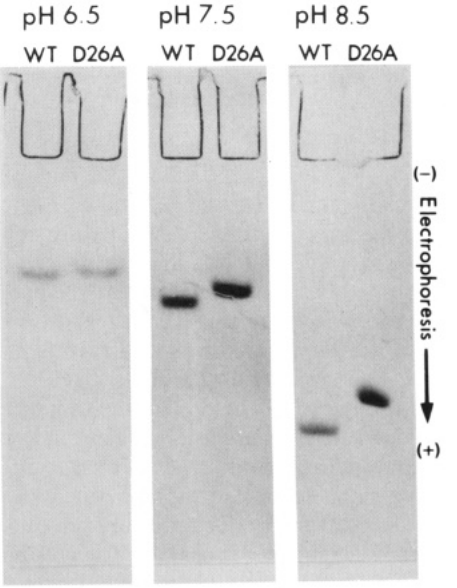



FIGURE 2: Native gel electrophoresis of wild-type and D26A thioredoxin. Three representative gels at pH 6.5, 7.5, and 8.5 are shown. Each well was loaded with 3 μg of protein, and electrophoresis is toward the anode. The gels are cast in 50 mM buffer, with use of ACES at pH 6.5, TES at 7.5, and TRIS at pH 8.5.

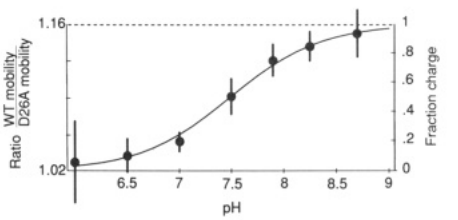



FIGURE 3: Ratio of wild-type mobility:D26A mobility versus pH. Mobility is the distance from the top of the gel to the bands of wild type and D26A in adjacent lanes at each pH. Data points are the average of 8–16 determinations; vertical bars give the standard deviation. The dotted line represents the ratio for a one-charge difference taken from an internal standard. The right vertical axis is a normalization of the mobility ratio to a one charge-difference. The curve is a nonlinear least-squares fit of eq 2, with p$K_a$ 7.5.

to a one-charge difference, which can be calibrated from the change in electrophoretic mobility of thioredoxin due to a single specific deamidation (first brought to our attention by M. Santoro and D. W. Bolen, personal communication). As the pH is lowered toward their p$I$ (=4.5), the mobilities of WT and D26A are decreased and the difference between their relative mobility is also decreased. At pH 6.5 the mobilities are essentially the same (Figure 2), indicating no difference in net charge; that is, at pH 6.5 Asp 26 is essentially in the –COOH form. At pH 7.5, the difference in electrophoretic mobilities between WT and D26A is intermediate between no difference in net charge and a one-charge difference.

The change in mobiliity of WT relative to D26A may be expressed as the ratio of the distance between the top of the gel and the WT band to the distance between the top of the gel and the D26A band. Since measurements are taken for adjacent bands in the same gel, the distance from the top of the gel to the ion front is the same for both bands. Ratios of WT:D26A mobilities, as a function of pH, are shown in Figure 3. Data at pH < 6 cannot be obtained since thioredoxin undergoes reversible precipitation in the vicinity of its p$I$. Each data point represents the average of 8–16 measurements obtained from 4–8 gels; vertical bars give the standard deviation. The solid curve gives a fit of the data to a single titration with p$K_a$ = 7.5 ± 0.15. A three-parameter nonlinear least-squares

fit of the data to the equation

$$R(\text{WT:D26A}) = \frac{b_L + (b_H \times 10^{(pH-pK_a)})}{1 + 10^{(pH-pKa)}} \quad (2)$$

was carried out with use of the program NonLin of Brenstein and Bolen. $R$(WT:D26A) is the ratio of the mobilities of WT to D26A, $b_L$ is the low-pH base line, and $b_H$ is the high-pH base line. In this algorithm, the base lines are not fixed but are allowed to vary with the fit. The best fit gives values of 1.02 for the low-pH base line and 1.16 for the high-pH base line. If these base-line values are taken as 0 and 1, respectively, then the data can be expressed as the fraction of a charge difference between WT and D26A, as given on the right vertical axis of Figure 3. The dotted line shows the ratio of mobilities of deamidated to unmodified D26A at each pH; this provides an internal standard for a one-charge difference at all pH's and demonstrates that the differences in mobilities of WT and D26A over this pH range are not due to a nonspecific effect of changes in net charge. The possibility of any contribution from sulfhydryls due to partial reduction of the protein(s) was eliminated by quantitative Ellman's assay of oxidized samples before electrophoresis.

*Development of the Method.* To obtain reproducible data like that shown in Figures 2 and 3, several aspects of the electrophoretic technique must be standardized. In particular, care must be taken to ensure that the pH is maintained throughout the gel. To demonstrate this, the pH of the gel was measured directly with a flat surface combination pH electrode at numerous points throughout the slab immediately after electrophoresis and before staining. Typically, either the gels were very uniform in pH or they changed spuriously across the gel, with pH varying from 3 to 11. To maintain uniform pH gels, we found that several precautions are necessary. First, buffers should have p$K_a$ values within 0.5 pH units of the desired pH. Second, during electrophoresis, the buffer is recirculated between upper and lower reservoirs using a peristaltic pump. Buffer recirculation is necessary to maintain a uniform ionic composition in the tray buffers, which are rather small volumes (100 mL/each tray) in the mini gel apparatus used here. The tube-pinching action of the pump prevents electrical current from being drawn through the pump tubing. Buffer recirculation is essential for pH uniformity with most buffers (e.g., ACES) but not with others (e.g., TRIS). Buffer recirculation may be unnecessary if the electrophoresis apparatus has larger reservoirs. Third, constant voltage (20 V/cm) rather than constant current is used to minimize effects of ionic strength differences between buffers used at different pH's. The buffers examined are listed in Table I. Only buffers that maintain pH to less than ±0.01 pH units across the entire gel surface were used for analysis. Finally, the possibility of effects from specific interactions of buffer ions with the protein was excluded by choosing buffers with overlapping ranges. Gels were cast with different buffers, but at the same pH, and the ratio of WT:D26A mobilities compared. Thioredoxin does not exhibit any difference in the ratio attributable to a specific buffer effect.

*pH Dependence of Thioredoxin Stability.* The unfolding of wild-type and D26A thioredoxin by GuHCl at various pH's is shown in Figure 4. The WT transition is pH dependent; the GuHCl concentration at the midpoint shifts from 2.1 M at pH 9 to 2.6 M at pH 6. In contrast, the D26A unfolding transition is unaffected by pH over the range 7–8.5 (rightmost curve, Figure 4). Protein stability in the absence of denaturant may be estimated from the GuHCl unfolding data in Figure 4 (Pace, 1986). Values of $\Delta G°$ in the absence of GuHCl, and

Table I: Buffers Tested for Suitability in Electrophoresis Experiments[a]

| buffer | p$K_a$ | gel pH uniform | pH range |
|---|---|---|---|
| acetate | 4.75 | – | |
| citrate | 5.40 | + | 5.4–5.9 |
| succinate | 5.57 | + | 5.5–5.6 |
| MES | 6.15 | + | 6.1–6.5 |
| BIS-TRIS | 6.50 | – | |
| ADA | 6.60 | + | 6.5–7.0 |
| BIS-TRIS–propane | 6.80 | – | |
| PIPES | 6.80 | + | 6.8–6.9 |
| ACES | 6.90 | + | 6.8–7.3 |
| TES | 7.50 | + | 7.3–8.0 |
| HEPES | 7.55 | – | |
| TRIS | 8.30 | + | 7.7–8.7 |
| CHES | 9.50 | – | |
| CAPS | 10.40 | + | 9.9 |

[a] Buffers than did not maintain a uniform pH throughout the gels after electrophoresis were not used in further experiments. The last column gives the range of pH values over which each buffer was used. Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; BIS-TRIS, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; ADA, *N*-(2-acetamido)iminodiacetic acid; BIS-TRIS–propane, 1,3-bis[[tris-(hydroxymethyl)methyl]amino]propane; PIPES, piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid)]; ACES, 2-[(carbamoylmethyl)amino]ethanesulfonic acid; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TRIS, tris(hydroxymethyl)aminomethane; CHES, 2-(cyclohexylamino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)-propanesulfonic acid.

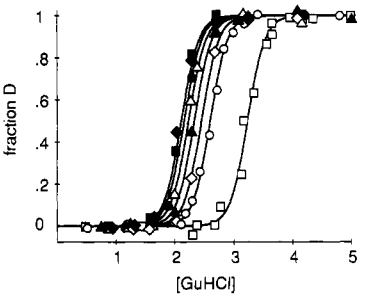



FIGURE 4: Guanidine hydrochloride unfolding of D26A and wild-type thioredoxin. Guanidine hydrochloride unfolding is measured by circular dichroism for D26A at pH 7–8.5 (□). For wild type, unfolding was measured at pH 6 (○), pH 6.5 (◇), pH 7 (▲), pH 7.5 (△), pH 8, (●), pH 8.5 (■), and pH 9 (◆). The fraction denatured as a function of guanidine hydrochloride concentration was determined from the percent ellipticity change relative to the ellipticity difference between fully native and fully denatured thioredoxin at 225 nm. The curves are a best fit of the data to a two-state unfolding transition, as described in the Materials and Methods section. Unfolding is measured in 50 mM buffers at pH 6.5 in ACES, at pH 7.0 and 7.5 in TES, and at pH 8.0 and 8.5 in TRIS.

of $[\text{GuHCl}]_m$, midpoint guanidine hydrochloride concentrations, are given in Table II along with values of $\Delta\Delta G°_{(\text{WT}\rightarrow\text{D26A})}$ for the difference in free energies between WT and D26A at pH 7–8.5. The GuHCl denaturation parameters are obtained from a nonlinear least-squares fit of CD ellipticity to $\Delta G°$, the base lines, and $m$, the variation of $\Delta G°$ with GuHCl concentration, as described in the Methods and Materials section. At pH 8.5, where Asp 26 in WT is presumed to be mostly deprotonated, $\Delta\Delta G°_{(\text{WT}\rightarrow\text{D26A})}$ is 4.6 kcal/mol.

Other studies of WT thioredoxin folding/unfolding have been at pH 7. Our $\Delta G°$ value of 9.5 kcal/mol at pH 7 (Table II) is in good agreement with other values reported for pH 7, namely, 8.7 kcal/mol (Kelley & Richards 1987), 9.0 kcal/mol (Kelley et al., 1987), and 9.4 kcal/mol (M. Santoro and D. W. Bolen, unpublished results).

In 2.2 M GuHCl, WT thioredoxin is about half-unfolded at pH 7.5, where Asp 26 is about half-titrated. In the presence of 2.2 M GuHCl, and at pH's near the p$K_a$, the fraction

Table II: Guanidine Hydrochloride Denaturation Parameters for *E. coli* Thioredoxin WT and D26A in the pH Range of Asp 26 Titration[a]

| | WT | | D26A | | $\Delta G°_{(D26A)} - \Delta G°_{(WT)}$ |
|---|---|---|---|---|---|
| pH | $[GuHCl]_m$ (M) | $\Delta G°$ (kcal/mol) | $[GuHCl]_m$ (M) | $\Delta G°_{D26A}$ (kcal/mol) | $\Delta\Delta G°_{(WT\rightarrow D26A)}$ (kcal/mol) |
| 9.0 | 2.1 | 8.6 | | | |
| 8.5 | 2.1 | 8.6 | 3.2 | 13.2 | 4.6 |
| 8.0 | 2.2 | 8.8 | 3.2 | 13.2 | 4.4 |
| 7.5 | 2.2 | 9.0 | 3.2 | 13.2 | 4.2 |
| 7.0 | 2.3 | 9.5 | 3.2 | 13.2 | 3.7 |
| 6.5 | 2.4 | 10.0 | | | |
| 6.0 | 2.6 | 10.6 | | | |

[a] Free energies, $\Delta G°$, and guanidine hydrochloride concentrations at the unfolding midpoint, $[GuHCl]_m$, are derived from data in Figure 4 as described in the Methods and Materials section. $\Delta\Delta G°_{(WT\rightarrow D26A)}$ is the difference in free energies between WT and D26A at the specified pH.

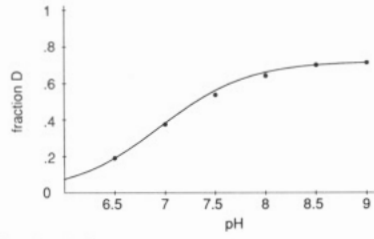



FIGURE 5: Thioredoxin fraction denatured versus pH in 2.2 M GuHCl. The fraction denatured was determined from the percent ellipticity change relative to the ellipticity difference between fully native and fully denatured thioredoxin at 225 nm. The curve is calculated from the thermodynamic linkage of the stability of thioredoxin and p$K_a$ shift of Asp 26 [see Langsetmo et al., (1991)].

denatured, $f_D$, gives a sensitive measure of the pH dependence of overall stability. This is shown by the data points in Figure 5. As discussed below, it may often be more convenient to measure $f_D$ in intermediate concentrations of denaturant than to determine the full-denaturation curves shown in Figure 4.

## Discussion

We have developed a simple, inexpensive method for determination of the p$K_a$ of a protein ionizable group by coelectrophoresis of WT protein and its "mutant" that has the ionizable group replaced by a neutral side chain. This technique is expected to be generally applicable to soluble proteins. The electrophoretic analysis is based on two reasonable assumptions. First, for ionizable groups other than the one mutated, p$K_a$ is the same in both WT and mutant. Second, any differences in hydrodynamic radius, or other frictional properties, between WT and mutant protein are not pH dependent.

Application of the electrophoresis method to Asp 26 in thioredoxin gives a p$K_a$ of 7.5 for the carboxyl side chain. This is 3.6 pH units above the p$K_a$ for fully solvated carboxyl side chains. The structural basis for the abnormally high p$K_a$ arises from the local environment of the carboxyl group in folded thioredoxin. The high-resolution crystal structure is solved at pH 3.8 (Katti et al., 1990), where Asp 26 is fully protonated. A cross-section of thioredoxin showing the side chains of D26, C32, C35, P76, and K57 is given in Figure 6, from an orientation similar to that in the Richardson-type drawing in Figure 1. The Asp 26 carboxyl group is near the center of the molecule, located at the bottom of a deep cleft, as shown in Figure 6, which renders the molecular, or Connolly, surface in dots.

One of the Asp 26 carboxyl oxygens (OD2) is completely buried, and some of the other (OD1) forms part of the molecular surface at the bottom of the cavity. One side of the cleft is formed primarily by Trp 28 side-chain atoms. This suggests that the function of Trp 28 is to provide a hydrophobic environment for the Asp 26 cleft. In homologous thioredoxins, Trp 28 is highly, but not completely, conserved (Gleason & Holmgren, 1988). Other atoms contributing to the molecular surface of the cleft are the amino group of Lys 57, the methyl group of Ala 39, and several backbone atoms of Cys 35. The rest of the active site disulfide is essentially buried, except for a small portion of Cys 32 (SG and CB) that is exposed to the other surface of the molecule shown below the active site in Figure 6. The highly conserved Pro 76, in a cis peptide bond, is also associated with the active site. Pro 76 is buried except for a small exposure of the backbone N to the outer surface close to Cys 32 CB. The Pro 76 side-chain atoms BC and CD are in van der Waals contact with Cys 35 SG.

Electron densities assigned to two water molecules are observed in the cleft (not shown in Figure 6), one within H-bonding distance of Asp 26 OD2. Directly to the right of the Asp 26 carboxyl group in Figure 6 is a cavity containing a crystallographic water (not shown); a second cavity by Pro 76 is large enough for a water, but no electron density is observed crystallographically within the cavity (Katti et al., 1990). Although we do not yet have a crystal structure of thioredoxin with Asp 26 in the $-COO^-$ form, it seems probable

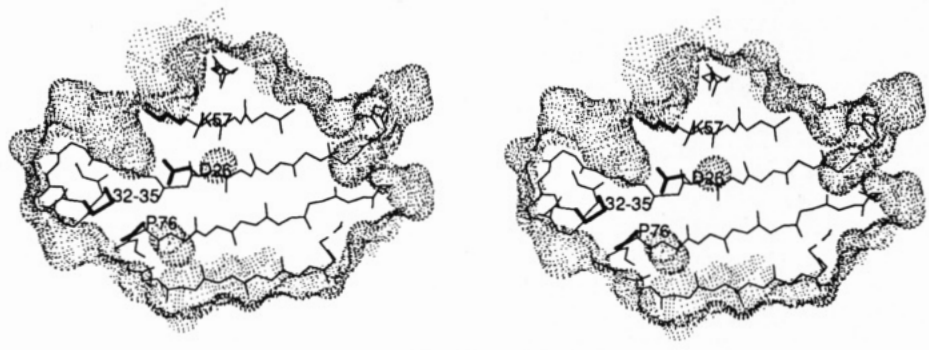



FIGURE 6: The structure of *E. coli* thioredoxin viewed in cross-section through the hydrophobic cleft. The orientation is the same as in Figure 1. In bold lines are the side-chain atoms of Asp 26, Lys 57, Pro 76, Cys 32, and Cys 35, which form the active site disulfide. The carboxyl group of Asp 26 is located at the bottom of the cleft. The molecular surface of the protein is represented by dots (Connolly, 1983). Two internal cavities are observed close to Asp 26 and Pro 76. The stereo drawing has the right eye image on the left side, and vice versa.

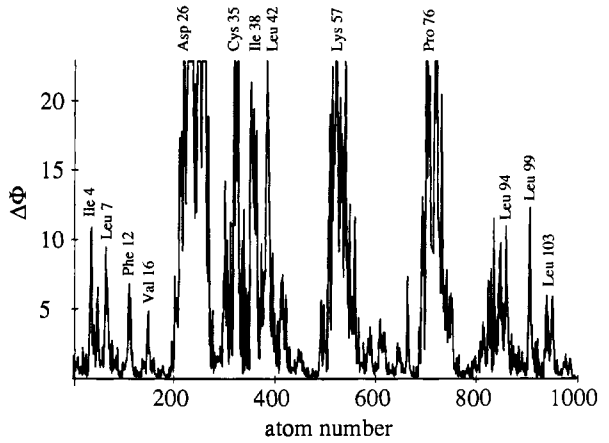
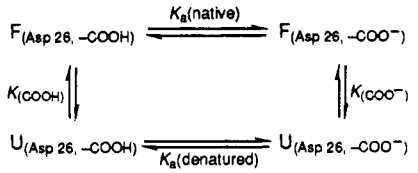



FIGURE 7: Thioredoxin atoms whose electrostatic potentials are affected by the charging of Asp 26. At each atom the electrostatic potential, Φ, is calculated for thioredoxin with Asp 26 in the –COOH form and with Asp 26 in the $-COO^-$ form. The difference between the two, $-COO^-$ ΔΦ, in units of $kT$, is plotted against atom number. The atom numbers correspond to numbering in the crystal coordinate list. The residues containing the clusters of atoms with large ΔΦ are indicated above the plot.

Scheme I




that its dynamic structure includes small rapid flutuations that would allow some contact of solvent water and/or hydrated protons with the carboxyl group.

The protein moieties in the microenvironment of the Asp 26 carboxyl group are highly apolar. This is illustrated by Figure 7, which shows atoms affected by ionization of the Asp 26 side chain. Other than Lys 57 and the active site cysteines, the difference in electrostatic potential resulting from the charging of Asp 26 is significant only for apolar residues. The anomalous $pK_a$ of Asp 26 reflects the energetic penalty for deprotonating the carboxyl in this hydrophobic milieu (Langsetmo et al., 1991). On the basis of electrostatic calculations combined with molecular modeling, it appears highly likely that the side chain of Lys 57 rotates into the cleft to facilitate its interaction with the Asp 26 carboxyl when it is charged and then moves back out of the cleft when the Asp 26 carboxyl is neutral, as described in the following paper (Langsetmo et al., 1991).

Thermodynamic linkage of protein stability with titration of a group with shifted $pK_a$ is expected (Sali et al., 1988). A raised $pK_a$ for a carboxyl group reflects the energetic cost of burying the charge, and this in turn destabilizes the protein. The linkage of Asp 26 carboxyl titration with stability is shown by the thermodynamic cycle given in Scheme I where $K_a$(native) and $K_a$(denatured) are the acid dissociation constants for Asp 26 in the native and denatured forms of the protein ($K_a = 10^{-pKa}$). $F_{(Asp26\text{-}COOH)}$, $F_{(Asp26,\text{-}COO^-)}$, $U_{(Asp26,\text{-}COOH)}$, and $U_{(Asp26,\text{-}COO^-)}$ are the folded and unfolded states of protonated and deprotonated Asp 26. $K_{(COOH)}$ and $K_{(COO^-)}$ are the equilibrium constants for cooperative denaturation of thioredoxin with protonated and deprotonated Asp 26. In the thermodynamic cycle, $K_a$(native)/$K_a$(denatured) = $K_{(COOH)}/K_{(COO^-)}$. If $pK_a$(native) > $pK_a$(denatured), then $K_{(COOH)} < K_{(COO^-)}$, and $F_{(Asp26,\text{-}COO^-)}$ is less stable than $F_{(Asp26,\text{-}COOH)}$. That is, titration should destabilize the protein while substitution of the abnormally titrating group by a nonionizable group should stabilize the protein. Both of these expectations are observed for thioredoxin (Figure 4 and Table II).

If the stabilization of D26A is due primarily to removal of the $-COO^-$ form of Asp 26, then the expected $\Delta\Delta G°_{(WT\rightarrow D26A)}$ is approximately equal to the difference between the free energies of $F_{(Asp26,\text{-}COOH)}$ and $F_{(Asp26,\text{-}COO^-)}$; i.e., $\Delta G°_{(COOH)} - \Delta G°_{(COO^-)} = (-RT \ln K_{(COOH)}) - (-RT \ln K_{(COO^-)})$. As a consequence of the cycle in Scheme I

$$\Delta G°_{(COOH)} - \Delta G°_{(COO^-)} = 2.303RT\Delta pK_a \qquad (3)$$

where $\Delta pK_a = pK_a$(native) – $pK_a$(denatured). From eq 3, $\Delta G°_{(COOH)} - \Delta G°_{(COO^-)} = 4.9$ kcal/mol at 25 °C, and with use of a $pK_a$(denatured) of 3.9 (Dawson et al., 1969), which makes $\Delta pK_a = 3.6$. In good agreement with this, at pH 8.5, the experimental $\Delta\Delta G°_{(WT\rightarrow D26A)}$ is 4.6 kcal/mol (Table II).

The pH dependence of WT thioredoxin stability in the vicinity of Asp 26 $pK_a$ is shown in Figure 4. From the full-denaturation curves for WT the overall free energy, $\Delta G°_{WT}$, is determined at each pH (Table II). However, the same information is obtained from a simpler measurement. This is the pH dependence of the fraction denatured, $f_D$, in GuHCl at a concentration near the unfolding midpoint when pH ≅ $pK_a$. For thioredoxin at pH 7.5, $[GuHCl]_m = 2.2$ M (Table II). From the WT curves in Figure 4, it can be seen that, in 2.2 M GuHCl, $f_D$ should vary sigmoidally with pH in the range pH 6–9. This is demonstrated in Figure 5. The data points are the fraction denatured obtained from CD ellipticity at 220 nm (Pace, 1986). The curve in Figure 5 is calculated from the equation $f_D = K_{WT}/(1 + K_{WT})$, where $K_{WT}$ is the overall equilibrium constant (related to $\Delta G°_{WT}$ in Table II) at a given pH. The variation of $K_{WT}$ with pH is described in the following paper (Langsetmo et al., 1991). Thus, data of the type in Figure 5 can be used as a diagnostic indicator of thermodynamic linkage between protein stability and titration of a group with abnormal $pK_a$.

## Added in Proof

After the present work had been completed, an NMR study of thioredoxin titrations by Dyson et al. (1991) came to our attention. Those authors also report a titration with $pK_a$ = 7.5 in oxidized thioredoxin and 7.0 in reduced thioredoxin, which they assign to Asp 26.

**Registry No.** Asp, 56-84-8.

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# Linkage of Thioredoxin Stability to Titration of Ionizable Groups with Perturbed p$K_a$[†]

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ABSTRACT: The highly conserved, buried, Asp 26 in *Escherichia coli* thioredoxin has a p$K_a$ = 7.5, and its titration is associated with a sizable destabilization of the protein [Langsetmo, K., Fuchs, J., & Woodward, C. (1991) *Biochemistry* (preceding paper in this issue)]. A fit of the experimental pH dependence of thioredoxin stability to a theoretical expression for the pH/stability relation in proteins agrees closely with a p$K_a$ value of 7.5 for Asp 26. The agreement between the experimental and theoretical changes in protein stability due to substitution of Asp 26 by alanine is also good. The local structure in the vicinity of Asp 26 in the low-pH crystal structure (with uncharged Asp 26) is hydrophobic, indicating that the aspartate would be highly destabilized. In theoretical calculations, the desolvation penalty for deprotonating Asp 26 in this environment is similar to the total protein folding energy. As a consequence, the Asp 26 p$K_a$ would be much greater than 7.5, and/or the protein might not fold. This suggests that a compensating process partially stabilizes the Asp 26 carboxyl group when it is charged. A simple model for this is proposed, whereby the Lys 57 side chain rotates to form a salt bridge with Asp 26 when it is deprotonated.

Thioredoxin contains a buried aspartic acid at residue 26, with a p$K_a$ of 7.5 (Langsetmo et al., 1991). Deprotonation of Asp 26 significantly destabilizes the protein. Conversely, replacement of Asp 26 with alanine enhances the stability of the "mutant" D26A[1] relative to WT (Langsetmo et al., 1990). The evolutionary conservation of Asp 26 in various species of thioredoxin (Gleason & Holmgren, 1988) implies an important biological role for its destabilizing effect. Its function may be to exert a critical influence on thioredoxin catalytic activity by pH-dependent modulation of the redox potential of the active site disulfide bond.

Expressions have been developed for the pH dependence of the stability of a protein when stability is linked to titration of an ionizable group with perturbed p$K_a$ (Yang et al., 1991). We show here that the pH dependence of $\Delta G°$ for thioredoxin unfolding in the vicinity of Asp 26 p$K_a$, pH 6-9, is in excellent agreement with the predictions of this analysis. Likewise, the

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[1] D26A, thioredoxin with Asp 26 replaced by alanine; WT, wild-type thioredoxin.

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