Stability of Oxidized *Escherichia coli* Thioredoxin and Its Dependence on Protonation of the Aspartic Acid Residue in the 26 Position†

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Received December 15, 1992; Revised Manuscript Received April 28, 1993

ABSTRACT: The effects of pH in the range 6.0–8.0 on the thermodynamics of the reversible thermal unfolding of *Escherichia coli* thioredoxin in the oxidized state have been determined over a range of concentrations using differential scanning calorimetry. The thermal denaturation indicated an inverse temperature dependence on concentration. The data were shown to fit a model based on dimerization of both the native and denatured states of the protein. The degree of dimerization of both states was found to be pH dependent. The previously described importance of protonation of the anomalously titrating aspartic acid 26 residue [Langsetmo, K., Fuchs, J., & Woodward, C. (1991) *Biochemistry 30*, 7603–7609] was apparently verified by the agreement between the experimentally determined $\Delta\Delta G°_d$ and the calculated $\Delta\Delta G°_H$ in the pH range 7.0–8.0.

The interior of a globular protein is intriguing when one considers the contribution of individual residues to the overall stability. A high tolerance to structural variations is demonstrated by the ability of the protein to fold and retain activity after substitution of residues possessing charges where none previously existed (Sali *et al.*, 1988; Stites *et al.*, 1991; Dao-pin *et al.*, 1991; Hellinga *et al.*, 1992) or residues with bulky side groups into these precise well-packed regions (Lim & Sauer, 1991; Lu *et al.*, 1992; Wynn & Richards, 1993).

On the other hand, a single amino acid replacement can have dramatic effects on the free energy of denaturation (for example, Matsumura *et al.*, 1988; Shortle *et al.*, 1988, 1991; Bell *et al.*, 1992; Ladbury *et al.*, 1992). These studies emphasize the importance of each unit in these complex structures and how the balance between folded and unfolded forms can easily be upset (Kellis *et al.*, 1989; Lim & Sauer, 1991; Eriksson *et al.*, 1992). Attempts to explain these effects have been made by considering phenomena such as packing of side chains, hydrophobic/hydrophilic interactions, and charge effects (Matthes, 1987; Pakula & Sauer, 1989; Tidor & Karplus, 1991; Shirley *et al.*, 1992).

Escherichia coli thioredoxin is of interest with regard to the above in that it is capable of folding and retaining activity even with major changes in the main hydrophobic core (Hellinga *et al.*, 1992; Wynn & Richards, 1993) and yet it possesses an Asp residue in this region with an anomalous pK_a, protonation of which has a major effect on the protein's stability.

E. coli thioredoxin is a small (11.7-kDa) protein consisting of a five-stranded twisted β-sheet, four α-helices, and a 3_{10} helix. An important structural feature of this protein is the hydrophobic core which is believed to convey the observed high thermal stability [for reviews on the structure and function of this protein see Holmgren (1985, 1989)]. The Asp26 residue is highly conserved in thioredoxins and is thought to play a role in the control of the redox potential of the disulfide/dithiol active site (Trp-Cys32-Gly-Pro-Cys35) (Langsetmo *et al.*, 1991a).

The sigmoidal variation of the free energy of denaturation by guanidine hydrochloride, $\Delta G°_{WT}$, with pH shows that the stability of WT thioredoxin is linked to titration of Asp26. Electrophoretic mobility studies, based on comparison to the Asp26Ala mutant (Langsetmo *et al.*, 1991), have shown Asp26 to possess an anomalous pK_a of 7.5 (Asp usually has a pK_a of 3.9). Langsetmo *et al.* (1991a,b) calculated the difference in the free energy of protonation in the folded and unfolded states [$\Delta\Delta G_{(COOH-COO^-)}$] of Asp26 in wild-type thioredoxin as 4.9 kcal mol1^{-1}. This compared favorably with the difference in free energy between wild-type and D26A denaturation, $\Delta\Delta G°_{(WT-D26A)}$, at pH 8.5 (4.6 kcal mol^{-1}), where Asp26 is largely deprotonated. Langsetmo *et al.* (1991a) also showed that at pH 6.5 the Asp26 side chain was protonated since the net charge was equal to that of the D26A mutant as shown by equivalence of electrophoretic mobilities.

The Asp26 in the oxidized form of thioredoxin is almost completely buried at the bottom of a deep hydrophobic cleft between the β1 sheet and the α2 helix near the active site, as shown by the 1.6-Å resolution crystal structure [obtained at pH 3.8 by Katti *et al.* (1989)]. By use of the algorithm of Lee and Richards (1971) it is apparent that in the static structure neither of the oxygen atoms of the Asp side chain is solvent accessible. Molecular dynamics simulations (Langsetmo *et al.*, 1990) have demonstrated that it is possible for one or both of the Asp26 side-chain oxygen atoms to interact with water via a transient channel extending to the surface.

The electrostatic potential of proximal residues is affected by the protonation state of the Asp26. Lys57 (not highly conserved in thioredoxins) is the only polar residue in this region that is electrostatically influenced. On the basis of electrostatic calculations and molecular modeling, this residue is thought to rotate into the hydrophobic cleft facilitating its interaction with the Asp26 carboxyl to form a salt bridge when it is charged and to move away when the Asp is neutral (Langsetmo *et al.*, 1991b).

On the basis of the anomalous pK_a of Asp26, in this work we apply thermal denaturation studies to investigate the effect of the protonation state of this residue on thermodynamic parameters describing the global stability of the protein in the pH range around this pK_a (pH 6.0–8.0). Thioredoxin has become an important system in understanding protein folding/unfolding reactions. This work is the first attempt to apply differential scanning calorimetry (DSC) to a detailed study

† This research was supported in part by Grant GM-04725 from the National Institutes of Health and Grant DMB-8810329 from the National Science Foundation.

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0006-2960/93/0432-7526$04.00/0

of the unfolding of this protein and represents a base for future work investigating the effects of its mutant forms.

MATERIALS AND METHODS

Materials. Wild-type thioredoxin was obtained by the method of LeMaster and Richards (1988). Stock solutions of up to 25 mg mL^{-1} were kept frozen in Tris buffer at pH 7.0 until required. Prior to use the protein was dialyzed extensively in 50 mM sodium phosphate buffer to the required pH. The concentration of protein was based on a molar extinction coefficient of 13.7 mM^{-1} cm^{-1} (Reutimann *et al.*, 1981).

Calorimetry. All data were derived from scans on the MC-2 calorimeter (MicroCal, Inc., Northampton, MA). In all experiments the reference cell was filled with the buffer used to dialyze protein samples. The instrumental baseline was determined prior to each protein scan with both cells filled with the aforementioned buffer. The calorimeter temperature scale was calibrated with hydrocarbon standards. Scans were made at 60 °C h^{-1} in all cases, except when heating-rate dependence was assessed, in which case a scan rate of 10 or 90 °C h^{-1} was used. The enthalpy of denaturation was found to be independent of the rate of heating confirming that the scan rate used in the experiments was sufficiently low to allow equilibrium to be attained at each point of the temperature scan.

Wild-type thioredoxin unfolding was found to be between 92% and 100% reversible in the pH range 6.0–8.0 if the sample was not heated more than 5 °C beyond $t_{1/2}$. This was considered a suitable test for reversibility because of the high temperatures required to denature the protein. Scans performed at pH values higher than 8.0 were shown to be less than 90% reversible.

Data Analysis. The DSC scans were fit using the program DIAS, which accommodates a two-state model with incomplete dissociation/association to a dimer in both the native and denatured states of the protein:

$$^1/_2N_2 \overset{K_N}{\rightleftharpoons} N \overset{K}{\rightleftharpoons} D \overset{K_D}{\rightleftharpoons} {}^1/_2D_2 \qquad (1)$$

where N and D are the native and denatured forms of the protein, respectively, and K_N and K_D are the corresponding equilibrium constants for the dimerization reactions. The curve-fitting program is based on

$$K = \frac{1 - (1 + 8\alpha K_D{}^2[N]_0)^{1/2}}{1 - (1 + 8\,(1-\alpha)K_N{}^2[N]_0)^{1/2}} \frac{K_N{}^2}{K_D{}^2} \qquad (2)$$

where K is the equilibrium constant for the denaturation reaction, α is the fraction of protein denatured, which at $t_{1/2}$ is equal to 0.5, and $[N]_0$ is the initial concentration of native protein. For a full discussion of this equation see Sturtevant (1987) and Tanaka *et al.* (1993).

The DIAS program fits the data for given dimerization constants with three adjustable parameters: $t_{1/2}$, the temperature where K is equal to 1, Δh_{cal}, the calorimetric enthalpy expressed in calories per gram, and β, the ratio of ΔH_{vH} and Δh_{cal}, where ΔH_{vH} is the van's Hoff enthalpy expressed in kilocalories per mole. It is assumed that both K_N and K_D do not vary significantly over the temperature range of the denaturation reaction (N to D) and thus that no enthalpy changes accompany the dimerization reactions. This leads to a concentration dependence of $t_{1/2}$ as described by Sturtevant (1987).

For each pH the data points for each scan were best-fit based on the criterion of standard deviation of the excess heat capacity. Fits were attempted with fixed values of K_N and K_D until the optimal values were obtained. The values of K_N and K_D over the range of concentrations for a given pH were constant.

RESULTS AND DISCUSSION

The data obtained from 43 scans of oxidized wild-type thioredoxin in the pH range 6.0–8.0 are shown in Table I.

The object of DSC curve-fitting procedures is to find the simplest thermodynamic and physical model that describes the experimental observation. In the analysis detailed in this paper we have adopted the model described above on the basis of the fact that there is an inverse dependence of $t_{1/2}$ on concentration for unfolding of thioredoxin at a given pH (see Figure 1). This indicates that the denatured state of the protein attains a higher degree of association than the native state. Other, more complex models have been considered, but the introduction of additional adjustable parameters does not improve our understanding of this system.

An increase in the dimerization constants of both the native and denatured forms with decreasing pH is observed, as shown in Table II. Thus, both the native and denatured forms of the protein show a tendency to dimerize with decreasing pH. The native form of the protein has been clearly demonstrated to form dimers at pH values below its isoelectric point at pH 4.5 (Holmgren, 1985) which are likely to be the same as those observed in the crystal structure. Langsetmo *et al.* (1991a) found precipitation of the protein at pH values less than pH 6.0 (presumably due to oligomerization). Ultracentrifugation studies (Laurent *et al.*, 1964) performed over a range of pH values (pH 2.0, 4.5, and 7.0) give molecular weight values in the range 12 300–12 600, 5%–10% higher than the molecular weight of 11 700. This is indicative of an associative event in this pH range. Attempts to determine accurately the degree of dimerization by ultracentrifugation studies at the pH values described by this work are likely to be thwarted by the relatively low percentages of the dimeric form in the concentration ranges considered. The solvent-exposed residue His6 on thioredoxin has a pK_a in the pH range studied in this work and may well influence the pH-dependent dimerization observed.

The magnitudes of K_N and K_D are reduced to an extent that experimentally they are indistinguishable above pH 7.0; nonetheless, the temperature–concentration dependence indicates that there is association of the denatured state to a higher degree than the native state. The dependence of $t_{1/2}$ on concentration at a give pH is a function of the ratio of K_N to K_D rather than of their absolute values. Thus, based on the data shown in Figure 1, the ratio of K_N to K_D for the values at pH 7.5 and above have to be in the range of those at the lower pHs.

At a given pH, the ratio β/MW ($= \Delta H_{vH}/\Delta H_{cal}$) varies from 1.04 ± 0.05 at pH 6.5 to 1.49 ± 0.07 at pH 8.0 (Table I). For a strictly two-state process this ratio equals unity (Sturtevant, 1987). It is unclear why this ratio is not constant over the pH range studied. A ratio greater than unity corresponds to greater intermolecular cooperativity of the protein than is accounted for by the curve-fitting model. To bring the value of β down, to give a ratio of unity, requires increasing the values of K_N and K_D; however, in all cases where this was attempted the fit of the data was found to suffer, as shown by an increased standard deviation. It should be noted that changing the value of β does not significantly affect the values of $t_{1/2}$ or ΔH_{cal}. To demonstrate this, the data at pH 7.0 (Table I) were fit using K_N = 400 M^{-1} and K_D = 1600 M^{-1}. This resulted in the individual values of both

Table I: Data for DSC Scans of Wild-Type Thioredoxin (Oxidized) in the pH Range 6.50–8.00

concn			% dimer[a]					
(μM)	(mg/mL)	$t_{1/2}$ (°C)	N	D	ΔH_{cal} (kcal/mol)	β/mw	ΔC_p [kcal/(mol·K)]	SD (%)
				pH 6.00 (K_N = 400 M^{-1}; K_D = 1600 M^{-1})[b]				
297	3.47	86.85	16.5	37.3	76.4	1.21	1.54	0.46
197	2.30	87.82	12.2	30.5	81.3	1.18	0.71	0.70
98	1.15	88.44	6.8	20.0	83.4	1.22	0.98	0.60
				pH 6.50 (K_N = 225 M^{-1}; K_D = 625 M^{-1})				
758	8.87	84.12	21.2	37.3	97.81	1.00	1.53	0.67
293	3.43	85.56	10.5	22.2	100.50	1.09	1.82	0.65
211	2.47	85.94	8.0	17.8	103.66	1.06	1.65	0.71
160	1.88	86.14	6.3	14.6	102.49	1.08	1.86	0.86
66	0.78	86.43	2.8	7.0	106.12	1.00	1.76	1.38
				pH 6.75 (K_N = 225 M^{-1}; K_D = 400 M^{-1})				
413	4.83	84.63	13.8	20.8	100.15	1.11	1.43	0.57
257	3.01	85.24	9.5	14.9	103.55	1.09	1.26	0.61
212	2.48	85.54	8.1	12.9	102.61	1.09	1.20	0.39
189	2.22	85.33	7.3	11.6	94.54	1.19	1.45	0.87
				pH 7.00 (K_N = 100 M^{-1}; K_D = 400 M^{-1})				
577	6.75	84.75	9.5	25.6	79.68	1.29	1.22	1.34
576	6.74	84.78	9.4	25.5	81.78	1.26	1.31	0.51
318	3.72	85.41	5.7	17.4	87.40	1.25	1.46	0.79
290	3.39	85.46	5.2	16.3	84.47	1.25	1.69	0.59
227	2.65	85.42	4.2	13.6	95.47	1.16	1.04	0.31
227	2.65	85.18	4.2	13.6	98.40	1.11	1.29	0.42
193	2.26	85.80	3.6	12.0	91.45	1.11	0.82	1.17
189	2.21	85.82	3.5	11.5	85.13	1.32	1.18	0.45
98	1.14	86.01	1.9	6.8	96.53	1.14	1.30	1.50
				pH 7.25 (K_N = 100 M^{-1}; K_D = 100 M^{-1})				
1043	12.20	81.95	15.1	15.1	91.96	1.28	1.73	0.91
738	8.63	82.50	11.5	11.5	90.68	1.33	1.77	1.27
456	5.33	83.40	7.8	7.8	96.29	1.30	1.18	0.52
343	4.00	83.74	6.1	6.1	90.68	1.29	1.18	1.33
161	1.88	84.66	3.0	3.0	95.12	1.26	1.01	1.26
103	1.20	85.05	2.0	2.0	96.76	1.22	0.95	1.07
				pH 7.50 ($K_N \leq$ 5 M^{-1}; $K_D \leq$ 5 M^{-1})				
498	5.83	82.11			85.41	1.39	1.81	1.28
369	4.31	82.73			92.66	1.32	2.57	0.76
297	3.48	83.40			96.76	1.29	1.79	0.47
217	2.54	83.48			97.34	1.24	1.90	1.25
				pH 7.75 ($K_N \leq$ 5 M^{-1}; $K_D \leq$ 5 M^{-1})				
1146	13.41	80.49			78.04	1.80	2.39	0.74
414	4.85	82.17			93.25	1.39	2.59	1.92
348	4.08	82.08			84.71	1.42	2.64	1.37
183	2.15	83.16			80.15	1.60	3.45	1.28
175	2.04	83.37			86.11	1.43	1.43	1.40
129	1.51	83.97			89.27	1.40	1.55	0.94
121	1.42	84.51			83.30	1.44	1.78	1.29
84	0.99	83.71			87.63	1.42	3.23	1.25
80	0.94	83.90			91.26	1.31	2.28	1.03
				pH 8.00 ($K_N \leq$ 5 M^{-1}; $K_D \leq$ 5 M^{-1})				
501	5.86	81.17			82.95	1.54	2.73	1.07
312	3.65	82.00			81.90	1.55	2.69	1.08
57	0.67	83.30			85.88	1.39	3.84	1.58

[a] The percentage dimer is given in the native, N, and denatured, D, states. In the pH range 7.5–8.0, the percentage dimer is too low to be accurately determined. [b] The values of K_N and K_D are converted to the units M^{-1} to enable comparison to reported data. The values of K_N and K_D obtained using eq 1 have the units $M^{-1/2}$. The values presented here, therefore, correspond to the equations $2N \rightleftharpoons N_2$ and $2D \rightleftharpoons D_2$, respectively.

$t_{1/2}$ and ΔH_{cal} changing by approximately 1%. The mean value of β/MW was reduced from 1.21 to 0.97; however, the mean standard deviation of the fit to the DSC scans was increased from 0.8% to 1.4%, which is a significant deviation.

A plot of ΔH_{cal} against $t_{1/2}$ for the data shown in Table I appears to deviate from a linear relationship (Figure 2). However, on closer inspection the nonlinearity becomes obvious only on inclusion of data obtained at pH 6.0. The scatter of the data points in Figure 2 is typical of plots obtained in this way. We do not understand why this variation of enthalpy occurs from precise curve-fitting procedures; however, enthalpy values (like the experimentally determined values for ΔC_p) are highly dependent on the discretionary positioning of pre- and posttransition baselines.

Why do we observe lower enthalpies for the denaturation reaction at pH 6.0? The enthalpy of a reaction is dependent on both the initial and final states of the system. In the denaturation of a protein the reduction of enthalpy could result from (i) the incomplete folding of the initial state (e.g., a molten globular state) or (ii) the incomplete unfolding of the final state (e.g., the existence of residual structure). With regard to (i), reversible acid unfolding of thioredoxin at pH 4.8 (pD 5.2, since measurements were made in D_2O) has been observed by NMR (Holmgren & Roberts, 1976). Thus, at pH 6.0 some degree of unfolding may persist. With regard to (ii), the presence of residual structure resulting from association of the protein in oxidized WT thioredoxin has been observed by Fourier transform infrared spectroscopy

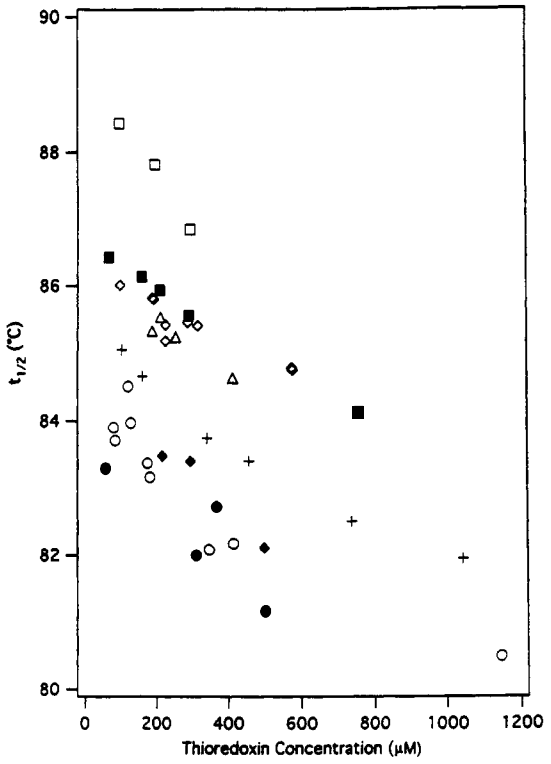

FIGURE 1: Plot of $t_{1/2}$ against concentration of oxidized WT thioredoxin indicating the effect of pH. (□) pH 6.00; (■) pH 6.50; (△) pH 6.75; (◇) pH 7.00; (+) pH 7.25; (◆) pH 7.50; (○) pH 7.75; (●) pH 8.00.

Table II: Variation of the Association Equilibrium Constants, K_N and K_D, with pH for Wild-Type Thioredoxin (Oxidized), as Fit Using DIAS

pH	K_N (M^{-1})	K_D (M^{-1})
6.00	400 ± 80	1600 ± 400
6.50	225 ● 60	625 ● 100
6.75	225 ± 60	400 ● 80
7.00	100 ± 35	400 ● 80
7.25	100 ● 35	100 ± 35
7.50	<5 ± 4	<5 ± 4
7.75	<5 ± 4	<5 ± 4
8.00	<5 ± 4	<5 ± 4

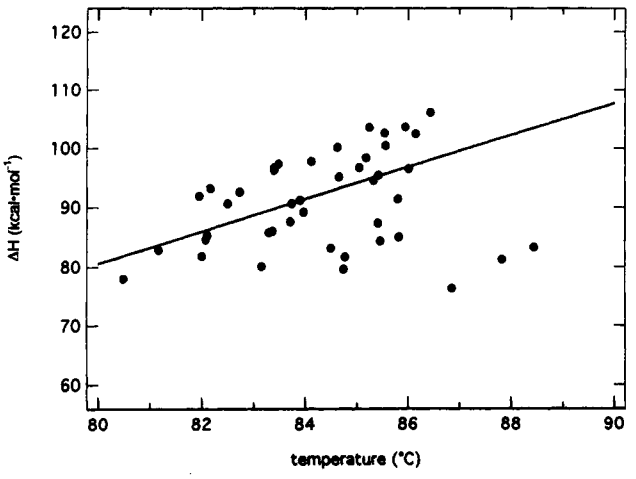

FIGURE 2: Plot of ΔH_{cal} against $t_{1/2}$ for 43 scans on oxidized WT thioredoxin. The solid line is the linear least-squares fit for the data in Table I excluding the values at pH 6.00. Slope 2.71 ± 0.68 kcal·mol^{-1}·K^{-1}; intercept −136.04 ± 57.23 kcal·mol^{-1}.

(FTIR) to 95 °C at pH 6.5 (J.E.L., M. Jackson, and H. Mantsch, unpublished data). This feature is also observed by circular dichroism spectroscopy (Chen *et al.*, 1991) and FTIR

Table III: Changes in $T_{1/2}$ and ΔG for a 300 μM Solution of Wild-Type Thioredoxin (Oxidized) in the pH Range 6.50–8.00 Compared to the Values at pH 7.0 ($t_{1/2}$ = 85.32 °C)

pH	$\Delta T_{1/2}$ (K)	$\Delta\Delta G°_d$ (kcal mol^{-1})	$\Delta\Delta G°_H$ (kcal mol^{-1})
6.50	−0.01 ± 0.22	0.00 ± 0.11	0.7
6.75	−0.67 ● 0.26	−0.17 ± 0.12	0.3
7.00	0.00 ± 0.26	0	0
7.25	−1.25 ± 0.37	−0.36 ± 0.14	−0.3
7.50	−2.20 ± 0.32	−0.57 ± 0.13	−0.5
7.75	−2.87 ± 0.48	−0.74 ± 0.16	−0.7
8.00	−3.52 ± 0.34	−0.90 ● 0.14	−0.8

(J.E.L., M. Jackson, and H. Mantsch, unpublished data) in staphylococcal nuclease, another protein with a large β-sheet structural element. If there were a net enthalpic reduction from the presence of this residual β-sheet resulting from association in the denatured state, this would be expected to increase with increased $t_{1/2}$ (*i.e.*, with increased concentration of dimer) giving rise to a negative effect on ΔC_p. This is not observed, as shown by the linear least-squares fit (the solid line) in Figure 2 (which is derived neglecting the data at pH 6.0). However, the fact that the lower enthalpies at pH 6.0 do not appear to be the result of the dimerization event addressed by this work does not preclude the occurrence of reversible association through a different mechanism at this pH.

If we assume that the enthalpic contribution of the dimerization is negligible, the value of ΔC_p from the slope of the points in Figure 2 is 2.7 kcal mol^{-1} K^{-1} (excluding data obtained at pH 6.0 as discussed above). There is a large uncertainty associated with this value [standard deviation (σ) = 0.7]. The ΔC_p obtained as the mean of the ΔC_p values from the mean of individual scans at each pH (excluding pH 6.0) is 1.88 ± 0.62 kcal mol^{-1} K^{-1}. This is closer to that obtained by Santoro and Bolen (1992) of 1.66 ± 0.05 kcal mol^{-1} K^{-1} from thermal denaturation of thioredoxin in the presence of NaCl and/or GdnHCl in 0.01 M phosphate buffer at pH 7.0. Values of ΔC_p obtained from individual scans are not considered to be as accurate as those obtained from the plot of ΔH against $t_{1/2}$ [as mentioned above and in Kitamura *et al.* (1989)].

The free energy of unfolding of thioredoxin can be calculated as described by Connelly *et al.* (1991). The $T_{1/2}$ (= $t_{1/2}$ + 273.15 K) at a given concentration is calculated from the van't Hoff plot of the natural logarithm of concentration against $1/T_{1/2}$. The ΔG values at each pH were calculated at 300 μM concentration and compared to the value at pH 7.0 ($t_{1/2}$ = 85.32 °C). Due to the large standard deviation attributed to measurement of ΔC_p it was not considered prudent to extrapolate values to 25 °C. The calculated free energies are shown in Table III, where

$$\Delta\Delta G°_d = \Delta G°_{d(pH=x)} - \Delta G°_{d(pH=7.0)} \quad (3)$$

where x is any of the pH values studied in this work. Also shown in Table III are the changes in $T_{1/2}$ at each pH compared to pH 7.0, where

$$\Delta T_{1/2} = T_{1/2(pH=x)} - T_{1/2(pH=7.0)} \quad (4)$$

The propagated error in the $\Delta G°_d$ values quoted is dependent on assuming that where a term tends to zero the error also tends to zero. Since $\Delta G°_{d(pH=7.0)}$ is equal to zero, by definition, in calculating $\Delta\Delta G°_d$ an error on $\Delta G°_{d(pH=7.0)}$ has been estimated on the basis of the average error in $\Delta G°_{d(pH=x)}$. The error quoted in $\Delta T_{1/2}$ is the propagated error based on the standard deviation of an interpolated point on the van't Hoff plot mentioned above. A general trend of reduced stability accompanying the increase in pH, and resulting deprotonation

of Asp26, is observed in the $\Delta\Delta G°_d$ and $\Delta T_{1/2}$ data at pH values above pH 7.0. Below pH 7.0 the stability of the protein shows some variation, being destabilized by 0.17 kcal mol^{-1} at pH 6.75 and showing no net change at pH 6.5.

If we assume that the value of pK_a for the Asp26 residue calculated by Langsetmo *et al.* (1991a) is correct, we can predict the stability of thioredoxin associated with the protonation of this residue. The net contribution to free energy of an anomalously titrating group will vary depending on the net difference in the pK_a values in the native and unfolded conformations and the pH of the solution (Tanford, 1970; Thomson, 1990). To assess the contribution to free energy of the protonation of anomalously titrating residues compared to that at pH 7.0 ($\Delta\Delta G°_H$), the following equation is used [derived from Tanford (1970)]:

$$\Delta\Delta G°_H = -RT \ln \left[\frac{1 + (K_u/[H^+])}{1 + (K_F/[H^+])}\right]_{pH=x} + RT \ln \left[\frac{1 + (K_u/[H^+])}{1 + (K_F/[H^+])}\right]_{pH=7} \quad (5)$$

where K_F and K_U are the acid dissociation constants in the folded and unfolded states of the protein, respectively (obtained from the respective pK_a values, *i.e.*, 7.5 and 3.9) and [H$^+$] is the hydrogen ion concentration.

$\Delta\Delta G°_H$ was calculated for each pH and is shown in Table III. There appears to be good agreement between $\Delta\Delta G°_d$ and $\Delta\Delta G°_H$ above pH 7.0. This indicates that above this pH the destabilization due to deprotonating the Asp26 residue (or effectively adding a charge to the hydrophobic core of the protein) accounts for the reduction of free energy in this pH region. At pH 6.5 and 6.75 the agreement is less satisfactory.

As previously mentioned the Lys57 residue is the only charged residue in the hydrophobic cleft whose electrostatic potential is affected by the presence of charge on Asp26. Langsetmo *et al.* (1991b) concluded that this residue exhibits an abnormal pK_a value depending on the distance between the Asp26 oxygen atom on the δ carbon and the Lys57 nitrogen atom on the ϵ carbon. The value of $\Delta\Delta G°_H$ for Lys57 can be calculated from eq 5 on the basis of the anomalous pK_a of 10.25 (Langsetmo *et al.*, 1991b). Adding the contribution of this residue to the $\Delta\Delta G°_H$ values calculated in Table III has no effect, since they are of the order of 1 cal mol^{-1}, which is outside the error inherent in these values.

From the data detailed here, there appear to be two major influences on the stability of thioredoxin. The first is the deprotonation of the Asp26 residue, which introduces a charge into the protein hydrophobic core. As the pH is increased this becomes an increasingly destabilizing influence. The second is the presence of dimer. Since the concentration of dimer in the denatured state is always greater than that in the native form, decreasing the amount of dimer present will have a stabilizing effect. Thus, as the pH and the degree of dimerization are reduced, increased stability will result. The two influences outlined above tend to oppose each other. At pH 6.5–6.75 there is less correlation between $\Delta\Delta G°_d$ and $\Delta\Delta G°_H$. $\Delta\Delta G°_d$ does not vary significantly in this pH range from that at pH 7.0. Numerous factors play a role in the stability of thioredoxin in the region of pH 6.5–7.0; however, the opposition of the two influences discussed may be important.

ACKNOWLEDGMENT

We thank Drs. J. A. Thomson, N. Kishore, and A. Tamura for helpful discussions.

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