Thermodynamic Effects of Reduction of the Active-Site Disulfide of *Escherichia coli* Thioredoxin Explored by Differential Scanning Calorimetry†

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Received August 17, 1993; Revised Manuscript Received November 4, 1993®

ABSTRACT: Intramolecular disulfide bonds in protein molecules, whether present in the wild-type protein or engineered via site-directed mutagenesis, are capable of significantly increasing the stability. Establishing thermodynamic parameters associated with the redox formation of this linkage is often encumbered by other global structural changes within the protein molecule. The active site of *Escherichia coli* thioredoxin possesses a disulfide/dithiol in a short loop, oxidation/reduction of which is accompanied by little structural alteration of the protein. Data for the thermal denaturation of the reduced protein are presented, which on comparison to the data obtained for the oxidized form [Ladbury, J. E., Wynn, R., Hellinga, H. W., & Sturtevant, J. M. (1993) *Biochemistry 32,* 7526–7530] are used to establish thermodynamic parameters for the redox reaction in this molecule. Data for an isosteric double mutation in the active site of thioredoxin (Cys32Ser/Cys35Ser) are also presented. Although the wild-type and mutated proteins show a similar reduction in free energy compared to the oxidized form (-3.0 ± 0.4 and -3.1 ± 0.3 kcal mol^{-1}, respectively), the enthalpic and entropic contributions to this destabilization are different for the two proteins.

Covalent cross-linking provides an important means for the stabilization of proteins (Anfinsen & Scheraga, 1975) as has been demonstrated with, for example, ribonuclease T1 (Pace et al., 1988), T4 lysozyme (Perry & Wetzel, 1986; Matsumura et al., 1989a,b; Jacobson et al., 1992), λ-repressor (Sauer et al., 1986), and dihydrofolate reductase (Villafranca et al., 1987). Attributing thermodynamic parameters to the formation of a disulfide bond is thus of considerable interest with regard to the potential stabilizing effect of this reaction. However, in many cases where this has been attempted, either in wild-type or in engineered situations, complications in thermodynamic interpretation have evolved due to gross structural changes within the protein.

Thioredoxin possesses a dithiol/disulfide group, the oxidation/reduction of which results in a negligible, localized conformational change of the protein. The active site for the redox function of thioredoxin protrudes as part of a short 14-atom loop from the protein core. This site consists of a pair of cysteines separated by two residues (Cys32–Gly–Pro–Cys35). Immediately proximal to the active site is a flat hydrophobic surface which has been identified as the binding site for the gene 5 protein of phage T7 (Huber et al., 1986) and as being of importance in thioredoxin's role in filamentous phage assembly [Russel & Model, 1986; for reviews of the structure and function of thioredoxin, see Holmgren (1985, 1989)].

The similarity of the peptide chain structures of the two redox forms of the protein has been demonstrated by several spectroscopic techniques. Difference 2D-NMR spectroscopic studies of the oxidized versus the reduced form of the protein at pH 5.7 (Dyson et al., 1988) and pH 6.1 (Hiraoki et al., 1988) indicate changes limited to a small number of residues within the active site loop, in the central strand of the twisted β-sheet immediately preceding the active site, or comprising the hydrophobic surface described above.

Comparison between the crystal structure of the oxidized form [at pH 3.8 as a Cu^{2+} complex, determined to 1.68 Å by Katti et al. (1990)] and the NMR solution structure of reduced thioredoxin (at pH 5.7; Dyson et al., 1990) reveals local structural differences at the active site leading to an increase in the distance between the sulfur atoms of approximately 4.75 Å imposed by rotation of the side chain of Cys35 and a distortion of the Pro34 position. The N-terminal region has ill-defined solution structure and is significantly more mobile than the average structure (Stone et al., 1993), and variations observed when compared to the crystal structure may be artifacts of crystal packing and coordination of Cu^{2+} ions at Ser1, Asp2, and Asp10 (Dyson et al., 1990).

The structural information gleaned from circular dichroism (CD) (Reutimann et al., 1981; Brown et al., 1987) and optical rotary dispersion (ORD) (Stryer et al., 1967) spectra shows essentially no change between the oxidized and reduced states of the protein. No detectable changes between the structures of the two redox forms of thioredoxin were observed for the backbone amide vibrations by Raman spectroscopy (Li et al., 1993).

Mutations proximal to the active site of thioredoxin serve as further illustrations of the isolation of this region from effects on the global structure. The Pro34Ser replacement produces no discernible effect on the stability of the oxidized form of the protein (Gleason, 1992). The mutation Lys36Glu leads to positional changes limited to residues in the vicinity of the active site as shown by X-ray crystallography (Nikkola et al., 1993).

† 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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® Abstract published in *Advance ACS Abstracts,* January 1, 1994.

0006-2960/94/0433-3688$04.50/0

Despite the limited structural changes associated with going from one oxidation state to the other, there are clearly some significant changes to the behavior of the protein. On reduction of the active site, the mean transition temperature of thermal denaturation has been reported as dropping by 10–12 °C (Holmgren, 1985; Kelley et al., 1987; Hiraoki et al., 1988). Studies by guanidine hydrochloride and urea denaturation showed that cleavage of the disulfide bonds lowers the stability of native thioredoxin by about 2.4 kcal mol^{-1} at neutral pH (Kelley et al., 1987). Lin and Kim (1989, 1991) via dithiol–disulfide equilibrium studies estimate the difference in stability between the two forms of the protein to be 3.6 kcal mol^{-1} at pH 8.7. Attempts to crystallize reduced thioredoxin [thioredoxin-$(SH)_2$] or to reduce oxidized thioredoxin (thioredoxin-S_2) crystals have been unsuccessful. Also, the gene 5 protein of T7 binds to reduced thioredoxin [and a Cys32Ser/Cys35Ser mutant (Huber et al., 1986)] rather than to the oxidized form.

The structures of both the oxidized and reduced thioredoxin have been well characterized. The consistency of the structure between these forms facilitates isolation of the effects of the redox reaction. This work represents the first attempt to use differential scanning calorimetry (DSC) to elucidate the thermodynamic parameters associated with formation of a covalent cross-link in thioredoxin. The data obtained from thermal denaturation of the reduced form of thioredoxin are compared to those for the oxidized protein (Ladbury et al., 1993) in the pH range 6.0–8.0 to enable quantitation of the free energy and the enthalpy and entropy of disulfide bond formation. Further exploration of this reaction is also made via a mutagenic form of thioredoxin where the active-site cysteine residues have been substituted by serines (Cys32Ser/Cys35Ser) which precludes disulfide bond formation.

MATERIALS AND METHODS

Materials. Wild-type thioredoxin was obtained from an overexpression construct and purified as described by Hellinga et al. (1991). The Cys32Ser/Cys35Ser mutant thioredoxin was produced using the protocol described by Hellinga et al. (1992).

Protein solutions were kept frozen in Tris buffer at pH 7.0 at concentrations of up to 25 mg·mL^{-1}. Prior to use, the protein was dialyzed extensively in 50 mM sodium phosphate buffer to the required pH. In the case of the reduced protein, the buffer solutions additionally contained 0.5–2 mM dithiothreitol (DTT). For experiments with the reduced proteins, precautions were taken to limit exposure of solutions to atmospheric oxygen. In these experiments, solutions were deaerated and saturated with nitrogen gas. The concentration of protein was based on a molar extinction coefficient of 13.7 mM^{-1}·cm^{-1} (Reutimann et al., 1981).

Calorimetry. All scans were obtained using the MC-2 calorimeter (MicroCal Inc., Northampton, MA). Experimental details have been described elsewhere (Ladbury et al., 1993).

In all cases, the reversibility of unfolding was assessed by heating the protein to between 5 and 10 °C beyond $t_{1/2}$ and rescanning after rapid cooling. Within the pH ranges herein reported, the unfolding reactions of both the wild-type and the mutant forms of thioredoxin were found to be between 90% and 100% reversible.

Data Analysis. The DSC scans were fit to a two-state model with incomplete dissociation/association to a dimer in both the native and denatured states of the protein as previously described (Sturtevant, 1987; Tanaka et al., 1993; Ladbury et al., 1993).

Table 1: Data from DSC Scans of Wild-Type Reduced and C32S/C35S Mutagenic Forms of Thioredoxin[a]

concn (μM)	concn (mg/mL)	$t_{1/2}$ (°C)	% dimer N	% dimer D	ΔH_{cal} (kcal/mol)	β/MW	ΔC_p [kcal/(mol·K)]	SD (%)
Reduced Wild-Type Thioredoxin								
(A) pH 6.00 ($K_N = 1.2 \times 10^3$ M^{-1}; $K_D = 2.5 \times 10^7$ M^{-1})								
409	4.79	72.87	38.2	99.3	58.38	1.33	2.34	1.88
291	3.41	74.16	32.5	99.2	66.57	1.12	1.73	2.03
196	2.29	74.99	26.2	99.0	78.39	0.92	1.71	2.15
(B) pH 7.00 ($K_N = 1.0 \times 10^2$ M^{-1}; $K_D = 1.0 \times 10^4$ M^{-1})								
428	5.01	72.41	7.3	71.2	75.70	1.07	2.42	1.34
324	3.79	73.36	5.8	67.7	67.60	1.25	1.53	0.53
308	3.60	73.09	5.5	67.0	75.91	1.12	2.49	0.58
239	2.80	73.69	4.4	63.5	77.92	1.10	2.04	0.93
164	1.92	74.17	3.1	58.0	83.40	1.01	1.86	1.54
C32S/C35S Mutagenic Form of Thioredoxin								
(A) pH 6.00 ($K_N = 1.2 \times 10^3$ M^{-1}; $K_D = 1.2 \times 10^5$ M^{-1})								
565	6.61	76.48	43.8	91.9	47.27	1.66	1.09	0.86
440	5.15	76.84	39.5	90.8	51.95	1.44	1.36	1.28
392	4.58	77.25	37.5	90.3	50.78	1.53	0.92	0.65
313	3.67	77.67	33.7	89.2	51.71	1.47	0.90	0.60
(B) pH 7.00 ($K_N = 4.0 \times 10^2$ M^{-1}; $K_D = 6.4 \times 10^3$ M^{-1})								
1013	11.86	73.15	34.6	75.8	79.21	1.04	2.28	0.67
706	8.26	73.75	28.7	71.8	91.26	0.89	2.34	0.58
423	4.95	74.44	21.1	65.3	93.72	0.86	2.26	1.05
382	4.47	74.71	19.7	63.9	89.62	0.91	2.14	0.74
285	3.34	75.28	16.1	59.6	83.77	1.05	1.20	0.83
255	2.98	74.65	14.8	57.9	95.82	0.82	3.25	1.53
161	1.88	75.48	10.4	50.5	91.26	1.00	2.53	1.03
(C) pH 8.00 ($K_N = 4.0 \times 10^2$ M^{-1}; $K_D = 3.6 \times 10^3$ M^{-1})								
500	5.86	71.71	23.4	59.4	83.80	0.97	2.48	0.30
370	4.33	72.10	19.3	54.7	87.63	0.94	2.70	0.41
327	3.83	72.38	17.7	52.7	81.08	1.05	2.14	0.39
241	2.82	72.53	14.2	47.6	84.83	0.99	2.43	0.77
188	2.20	72.71	11.7	43.4	86.81	0.99	2.40	1.59

[a] The values of K_N and K_D are converted to the units M^{-1} to enable comparison to reported data. From eq 1 in Ladbury et al. (1993), the values of K_N and K_D have the units M$^{-1/2}$. The values reported here, therfore, correspond to the equation $2N \rightleftharpoons N_2$ and $2D \rightleftharpoons D_2$, respectively. The percentage dimer is given in the native, N, and denatured, D, states.

RESULTS AND DISCUSSION

DSC scans for wild-type and mutant (Cys32Ser/Cys35Ser) forms of thioredoxin were performed in the pH range 6.0–8.0. The data derived from these scans are shown in Table 1.

As previously explained (Ladbury et al., 1993), the object of the curve-fitting procedures used for DSC scans is to provide the simplest thermodynamic and physical model that best describes the experimental observation. We have fit the data to a model accounting for a reversible dimerization of both the native and denatured protein. The model herein used is based on the fact that there is an inverse dependence of $t_{1/2}$ on concentration for both thioredoxin-$(SH)_2$ and mutant Cys32Ser/Cys35Ser at all the pH values studied, hence giving evidence for increased association of the denatured form of these proteins. Adoption of more complex models involving higher oligomeric forms has been attempted, but these, by their nature, involve additional parameters which, although slightly improving the data fit, give a number of best-fit options.

Further support for this model is that there is clear evidence for dimeric and possible higher oligomeric forms of thioredoxin. Thioredoxin-S_2 aggregates to form dimers below the isoelectric point (Holmgren, 1985). Dimerization of the oxidized protein was observed between pH 6.5 and 8.0 (Ladbury et al., 1993). Reversible oligomerization at low pH has been demonstrated by ultracentrifugation studies for thioredoxin-$(SH)_2$ (Laurents

et al., 1964). A potential site for the association of thioredoxin is the N-terminus since the pK_a of the surface residue His6 is 5.9 (Hiraoki et al., 1988) instead of the usual 6.8, suggesting that the residue is removed from solvent.

Table 1 shows that the dimerization constants are inversely dependent on pH in both the native and denatured forms, as was observed for the oxidized form of thioredoxin (Ladbury et al., 1993), and may be extrapolated down to lower pH values where the protein exists as a dimer, as described above. These data are consistent with the reported higher propensity for association of the reduced native protein (Holmgren, 1985). At pH values around 4.5, NMR studies indicate considerable differences in the conformation of oxidized and reduced thioredoxin (Holmgren & Roberts, 1976; Hiraoki et al., 1988) which may be due to differing degrees of dimerization/oligomerization. For the reduced protein, the increased dimerization of the denatured state has been attributed to intermolecular disulfide bond formation causing line broadening in NMR studies of the denatured protein (Hiraoki et al., 1988). The reversibility of the transitions observed in this work and the similar values of K_D with the Cys32Ser/Cys35Ser form, which cannot form a covalent linkage, preclude this conclusion. It is not clear based on the structural consistency of the two redox forms of thioredoxin why there are different degrees of dimerization between the two forms.

The ratio of K_N to K_D has an effect on the dependence of $t_{1/2}$ on concentration and hence on the stability of the protein. The value of this ratio for oxidized thioredoxin (Ladbury et al., 1993) is lower than those for both the reduced and mutant thioredoxins; however, we do not observe any pronounced change in the dependence described. To account for this lack of observed dependence, several possible conclusions can be inferred: (i) the differences in the ratios observed are not sufficient to lead to significant changes; (ii) small errors in the low values of K_N and K_D for the oxidized protein would lead to large changes in the ratio; thus, the differences in these ratios may be minimal if the errors have been underestimated; and (iii) if there was enthalpy associated with the dimerization event, this would have an effect on the concentration dependence of $t_{1/2}$. We were unable to satisfactorily fit DSC scans for this third event since the additional parameters invoked gave rise to a number of possible "best-fit" options and hence confusing interpretation. For the thermodynamic analysis discussed henceforth, we assume that the effect of the differences in the ratio of K_N to K_D has a negligible effect on the comparative stability of the various forms of thioredoxin.

At pH 7.0 for thioredoxin-$(SH)_2$, the mean ratio β/MW ($=\Delta H_{vH}/\Delta H_{cal}$) is 1.11 ± 0.08 (Table 1) whereas for the (Cys32Ser/Cys35Ser) mutagenic form this value is 0.94 ± 0.08 at pH 7.0 and 0.99 ± 0.04 at pH 8.0. As discussed by Sturtevant (1987), when this ratio is unity a two-state thermal denaturation transition can be inferred, whereas values greater than unity are representative of greater intermolecular cooperativity and values less than unity indicate the formation of intermediate states. Since the amino acid substitution is made in a portion of the protein in which chemical changes have little effect on the global structure, we may expect a similar unfolding behavior of these two sytems. We can assume, therefore, that the deviations of the ratio β/MW from unity are not significant at these pH values.

At pH 6.0 for both the reduced and the mutant forms of the protein, the ratio of β/MW appears to deviate more obviously from unity, as was observed for the oxidized protein. This is likely to be a reflection of the reversible aggregation that has been observed for both reduced and oxidized thioredoxin as the pH approaches the pI (pH 4.5) as described above. Increased intermolecular cooperativity was indicated by observation of precipitation in titrations of thioredoxin performed at 1 mM concentration at 25 °C in the pH range 5.5–3.5 (Hiraoki et al., 1988).

Plotting ΔH_{cal} against $t_{1/2}$ for the data at pH 7.0 for the reduced form and at pH 7.0 and 8.0 for the mutated form of thioredoxin indicates positive changes in heat capacity, ΔC_p, but large standard deviations in the attempted linear fits. The mean value of ΔC_p for both forms of thioredoxin obtained from the individual scans in this pH range is 2.26 (±0.44) kcal mol^{-1} K^{-1}. This compares with 2.7 (±0.7) kcal mol^{-1} K^{-1} obtained from the plot of ΔH_{cal} against $t_{1/2}$, 1.88 (±0.62) kcal mol^{-1} K^{-1} obtained from the individual scans (Ladbury et al., 1993), and 1.66 (±0.05) kcal mol^{-1} K^{-1} from the work of Santoro and Bolen (1992) for the oxidized form of thioredoxin. The similarity of the values obtained for reduced and oxidized forms of thioredoxin adds further support to the conclusion that the redox reaction produces little structural alteration in the protein. For example, if a large change in the exposed hydrophobic surface area occurred, this would lead to a significant change in ΔC_p.

If structural integrity between the various forms of thioredoxin can be assumed (except in the binding site region), then 57 individual values of ΔC_p have been obtained by DSC (i.e., 17 herein analyzed at pH 7.0 and 8.0 and 40 from the oxidized protein between pH 6.5 and 8.0; Ladbury et al., 1993). The mean value of ΔC_p from these is 1.93 (±0.67) kcal mol^{-1} K^{-1}. Values of ΔC_p obtained from individual DSC scans are not considered to be as accurate as those obtained from the plot of ΔH_{cal} against $t_{1/2}$, since they are more prone to error from the somewhat arbitrary positioning of pre- and posttransition base lines. However, due to the limited temperature range of $t_{1/2}$ in the plots of ΔH_{cal} against $t_{1/2}$, the mean of the 57 scans may give the best value available for this quantity from our data.

The data at pH 6.0 for both the reduced and mutant thioredoxins show clear deviation from the trend in plots of ΔH_{cal} against $t_{1/2}$ (as observed for the oxidized form of thioredoxin; Ladbury et al., 1993). As previously discussed, it is not clear why this is apparent; however, reversible oligomerization events that have been observed at low pH may persist to a significant extent at this pH. Thus, in addition to an equilibrium involving the formation of the dimeric state described in this work, a further equilibrium to a higher oligomeric state could be hypothesized. Enthalpy associated with the formation of this higher oligomeric state could lead to deviation from linearity in the plots of ΔH_{cal} against $t_{1/2}$.

The free energy, $\Delta G°$, for the thermal denaturation of the reduced and the Cys32Ser/Cys35Ser forms of thioredoxin for 300 μM protein solutions at pH 7.0 has been calculated from the equation below as previously described (Kitamura & Sturtevant, 1989; Connelly et al., 1991):

$$\Delta G°(\text{at } T_{1/2O}) = \Delta H_R[(T_{1/2R} - T_{1/2O})/T_{1/2R}] - \Delta C_p[T_{1/2R} - T_{1/2O} + T_{1/2O} \ln(T_{1/2O}/T_{1/2R})]$$

where $T_{1/2}$ ($=t_{1/2} + 273.15$ K) is the temperature where the concentrations of native and denatured protein are equal for reduced (or mutant), R, or for oxidized, O, thioredoxin. For both the reduced and mutant forms of thioredoxin, $T_{1/2}$ at the above conditions was obtained from the linear relationship between $1/T_{1/2}$ and the natural logarithm of concentration ($t_{1/2}$ = 73.20 and 74.85 °C, respectively) and ΔH_R from the linear relationship between the calorimetric enthalpy, ΔH_{cal}, and concentration (75.85 and 91.11 kcal mol^{-1}, respectively).

Table 2: Thermodynamic Data for the Thermal Unfolding of Reduced and Mutant (C32S/C35S) Thioredoxin[a]

protein	$\Delta t_{1/2}$ (°C)	$\Delta\Delta G°$ (kcal mol^{-1})	$\Delta\Delta H$ (kcal mol^{-1})	$T\Delta\Delta S°$ (kcal mol^{-1})
reduced	−12.1 ± 0.1	−3.0 ± 0.4	10.3 ± 4.2	13.3 ± 4.2
C32S/C35S	−10.5 ± 0.2	−3.1 ± 0.3	22.5 ± 4.2	25.6 ± 4.2

[a] pH 7.00; 300 μM protein; 85.32 °C (the $t_{1/2}$ of oxidized thioredoxin).

The ΔC_p used for the calculation of $\Delta G°$ was 1.93 (±0.67) kcal mol^{-1} K^{-1} obtained as described above. The change in entropy on denaturation, $\Delta S°$, at $T_{1/2R}$ is equal to ($\Delta G°$(at $T_{1/2R}$) − ΔH_R)/$T_{1/2R}$. The thermodynamic values obtained are compared in Table 2 to data for the oxidized protein under the same conditions where $t_{1/2}$ is 85.32 °C [derived from the data of Ladbury et al. (1993)]. This comparison to thioredoxin-S_2 avoids an extrapolation involving ΔC_p over a large temperature range. We previously used a ΔC_p of 2.7(±0.7) kcal mol^{-1} K^{-1} for calculation of the free energy of denaturation of thioredoxin-S_2; however, since these data covered such a limited temperature range, recalculation using the value 1.93 (±0.67) kcal mol^{-1} K^{-1} has a negligible effect.

The effect on $T_{1/2}$ of reducing the disulfide bond in the oxidized protein is a reduction of 12.1 °C. This is comparable to that $\Delta T_{1/2}$ reported previously (Holmgren, 1985; Kelley et al., 1987; Hiraoki et al., 1988). The difference in $T_{1/2}$ for the reduced and mutant forms of thioredoxin in this work is 1.5 °C. The significance of this will be discussed below.

Both the reduced and the Cys32Ser/Cys35Ser forms of the protein are destabilized compared to oxidized wild type with $\Delta\Delta G°$ values of −3.0 and −3.1 kcal mol^{-1}, respectively. This destabilization for disulfide bond reduction in thioredoxin can be compared to −3.5 (±0.3) and −3.1 (±0.6) kcal mol^{-1} obtained by Lin and Kim (1989) from studies of equilibria between thioredoxin and glutathione and from CD spectroscopy, respectively. Further comparisons can be drawn between the observed free energies for reduction of a single disulfide bond in ribonuclease T1 (cross-linking residues 2 to 10, −3.4 kcal mol^{-1} at 12.5 °C; Pace et al., 1988), T4 lysozyme (cross-linking residues 1 to 15, −2.3 kcal mol^{-1} at 55.4 °C; Johnson et al., 1978), immunoglobulin light chain (−4.0 to −4.7 kcal mol^{-1} at 25.0 °C; Goto et al., 1987), and ribonuclease A (cross-linking residues 7 to 41, −4.9 kcal mol^{-1} at 40.0 °C; Lin et al., 1984). The close agreement of the $\Delta G°$ herein obtained with that obtained for reduction of the disulfide bond between residues 2 and 10 in ribonuclease T1 (Pace et al., 1988) is worth emphasizing since, as with the active site of thioredoxin, this disulfide bond is partially solvent-accessible and the protein folds and retains most of its enzyme activity when this bond is reduced.

Theoretical treatment has indicated that the entropic contribution to the free energy for the reduction reaction is linearly related to the logarithm of the number of residues in the loop formed by the disulfide cross-link (Schellman, 1955; Flory, 1956; Poland & Scheraga, 1965; Pace et al., 1988). On the basis of the equation derived by Pace and co-workers, the entropic contribution to the change in free energy on reduction of the disulfide bond in thioredoxin, with the cysteine residues separated by just two residues, should be 4.17 kcal mol^{-1}. However, it should be noted that the largest deviations from the theoretical values observed by Pace et al. occurred with smaller loop sizes.

The values of $\Delta\Delta H$ and $\Delta\Delta S°$ obtained by extrapolation to 85.32 °C (358.47 K) are shown in Table 2. These values involve the large error associated with ΔC_p, 1.93 (±0.67) kcal mol^{-1} K^{-1}, and the uncertainties in ΔH_{cal} of approximately ±4 kcal mol^{-1}. The large increases observed in both values are consistent with the overall destabilizing effect of increasing the entropy (and hence stabilization) of the denatured state of the protein on removal of the disulfide bond in the active site.

In view of the fact that the only difference between the reduced and the mutant forms of thioredoxin is the substitution of two sulfurs by two oxygens in the active site, the large differences between the values of $\Delta\Delta H$ and $\Delta\Delta S°$ for the two forms are surprising. It has, in addition, been observed that the binding constant for the mutated protein to T7 gene 5 protein is less than that for the reduced wild-type form (Huber et al., 1986). These changes in $\Delta\Delta H$ and $\Delta\Delta S°$ could be the result of structural differences in both the native and denatured forms of the protein; however, if a random-coil denatured form is assumed in both cases, no significant energetic differences in the denatured forms of the mutant and reduced protein should be expected. Thus, the search for the source of this effect is limited to the native state. Possible explanations could be the following: (i) The positioning of the hydroxyl groups in the Cys32Ser/Cys35Ser mutated protein may enable hydrogen bonding to main chain amide groups or between the serines (Li et al., 1993) to occur (hydrogen bonding involving –OH groups is stronger than that with –SH groups; for example, the boiling point of methanol is 65 °C whereas that for methane thiol is 6 °C). Intramolecular interactions would result in increased entropic and enthalpic contributions on denaturation of the protein. (ii) The pK_a of Cys32 is about 7.0, well below that for a solvated cysteine residue (Kallis & Holmgren, 1980; Dyson et al., 1991; Li et al., 1993). The interaction for a partially ionized side chain in the reduced form that is absent from the double serine mutant may have a significant enthalpy and entropy contribution to free energy. Since no structural data exist for the Cys32Ser/Cys35Ser form of the protein, it is difficult to explain the differences in enthalpic and entropic contributions to the free energy for these two highly similar molecules. This is yet another example of how, despite the relative simplicity of the mutation, our ability to predict full thermodynamic characterization for the molecule is extremely limited (Sturtevant, 1993).

The redox status of the active site of thioredoxin clearly has a significant effect on the stability of the protein. We have demonstrated that this is derived, at least in part, from the entropic contributions to the denatured forms of the protein in the respective forms.

ACKNOWLEDGMENT

We gratefully acknowledge Prof. F. W. Richards for his continued support of this work. We also thank Dr. J. R. Livingstone for advice on ultracentrifugation studies.

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