Protein Science (1997), 6:657–665. Cambridge University Press. Printed in the USA.

Thermal denaturation of iso-1-cytochrome *c* variants: Comparison with solvent denaturation

LYNN M. HERRMANN[1] AND BRUCE E. BOWLER

Department of Chemistry and Biochemistry, University of Denver, Denver, Colorado 80208-2436

(RECEIVED August 20, 1996; ACCEPTED November 22, 1996)

Abstract

Thermal denaturation studies as a function of pH were carried out on wild-type iso-1-cytochrome *c* and three variants of this protein at the solvent-exposed position 73 of the sequence. By examining the enthalpy and T_m at various pH values, the heat capacity increment (ΔC_p), which is dominated by the degree of change in nonpolar hydration upon protein unfolding, was found for the wild type where lysine 73 is normally present and for three variants. For the Trp 73 variant, the ΔC_p value (1.15 ± 0.17 kcal/mol K) decreased slightly relative to wild-type iso-1-cytochrome *c* (1.40 ± 0.06 kcal/mol K), while for the Ile 73 (1.65 ± 0.07 kcal/mol K) and the Val 73 (1.50 ± 0.06 kcal/mol K) variants, ΔC_p increased slightly. In previous studies, the Trp 73, Ile 73, and Val 73 variants have been shown to have decreased *m*-values in guanidine hydrochloride denaturations relative to the wild-type protein (Herrmann L, Bowler BE, Dong A, Caughey WS. 1995. The effects of hydrophilic to hydrophobic surface mutations on the denatured state of iso-1-cytochrome *c*: Investigation of aliphatic residues. *Biochemistry 34*:3040-3047). Both the *m*-value and ΔC_p are related to the change in solvent exposure upon unfolding and other investigators have shown a correlation exists between these two parameters. However, for this subset of variants of iso-1-cytochrome *c*, a lack of correlation exists which implies that there may be basic differences between the guanidine hydrochloride and thermal denaturations of this protein. Spectroscopic data are consistent with different denatured states for thermal and guanidine hydrochloride unfolding. The different response of *m*-values and ΔC_p for these variants will be discussed in this context.

Keywords: circular dichroism; guanidine hydrochloride denaturation; heat capacity increment; iso-1-cytochrome *c*; *m*-values; protein stability; thermal denaturation

Understanding the factors that control the equilibrium between the native and denatured state of a protein is of great importance to elucidating the protein folding problem (Dill, 1990; Matthews, 1993; Shortle, 1995). Early work from Tanford's lab (Tanford, 1968) suggested that most denatured states approach a random coil, and therefore much research has focused on how native state interactions stabilize a folded protein (Privalov & Gill, 1988; Matthews, 1993). More recently it has become apparent that denatured states of proteins may be more complex than originally suspected (Dill & Shortle, 1991 ; Shortle, 1995). In previous work in our laboratory, guanidine hydrochloride (gdnHCl) denaturations were used to study a series of variants of the electron transfer protein, iso-1-cytochrome *c*. In these studies, a highly solvent-exposed site, lysine 73, was replaced with aromatic (Bowler et al., 1993) and aliphatic (Herrmann et al., 1995) amino acids. Many of the substitutions at site 73 led to decreases in the *m*-values for gdnHCl denaturation with respect to the wild-type (WT) protein where lysine is present (m^- variants, variants for which the *m*-value is >5% smaller than the WT value as defined by Green et al., 1992). The *m*-value from gdnHCl denaturation data is considered to be a measure of the difference in the solvent-exposed surface area between the native and denatured states (Schellman, 1978). The *m*-value is therefore believed to be a measure of the compactness of the denatured state (Dill & Shortle, 1991; Shortle, 1995). A highly significant negative correlation between the hydrophobicity of the amino acid at site 73 of iso-1-cytochrome *c* and $\Delta G_u^{\circ\ H_2O}$, the free energy of unfolding in the absence of denaturant (such a correlation is known as a reverse hydrophobic effect, see Pakula & Sauer, 1990), also suggested that a substantial portion of the changes in $\Delta G_u^{\circ\ H_2O}$ could be attributed to the denatured state. Such a correlation is also consistent with the predictions of heteropolymer

Reprint requests to: Bruce E. Bowler, Department of Chemistry and Biochemistry, University of Denver, 2050 E. Iliff Street, Denver, Colorado 80208-2436; e-mail: bbowler@du.edu.

[1] Present address: NIAID, National Institutes of Health, Rocky Mountain Laboratories, Hamilton, Montana 59840.

Abbreviations: gdnHCl, guanidine hydrochloride; $\Delta G_u^{\circ\ H_2O}$, free energy of unfolding in the absence of denaturant; *m*-value, the rate of change of ΔG_u° as a function of guanidine hydrochloride concentration; ΔC_p, heat capacity increment of protein unfolding; ΔASA, change in accessible surface area upon unfolding; WT, wild type; T_m, midpoint temperature of unfolding; ΔH_m, enthalpy of unfolding at T_m; ΔS_m, entropy of unfolding at T_m; $\Delta G_{D,T}$, free energy of unfolding at temperature, *T*; $\Delta H_{D,T}$, enthalpy of unfolding at temperature, *T*; $\Delta S_{D,T}$, entropy of unfolding at temperature, *T*.

theory for hydrophilic to hydrophobic surface mutations (Shortle et al., 1992).

By examining some of the most pronounced m^- mutants at position 73 using thermal denaturation methods, additional insight was sought into the important interactions governing the stability and structure of the denatured state of these mutants. Thermal denaturations can provide a direct means of obtaining valuable thermodynamic information about the relative effects of mutations on the energetics and structure of the denatured state versus the native state. An important parameter obtained from thermal denaturation studies is ΔC_p, the heat capacity increment, which is believed to result primarily from the increased degree of hydration of nonpolar groups caused by protein unfolding (Privalov, 1979). The heat capacity increment, like the m-value, appears to be proportional to the change in accessible surface area, ΔASA, upon unfolding (Privalov & Makhatadze, 1990, 1992; Gomez et al., 1995). A recent survey of protein unfolding data (Myers et al., 1995) shows strong correlations of ΔASA with m-values for both gdnHCl and urea denaturations and with ΔC_p for thermal denaturations. For proteins where ΔC_p and m-value data both exist, a good correlation between ΔC_p and the m-value was observed. We know of very few cases where m-values and ΔC_p values are known for variants of the same protein (Shortle et al., 1988, Betz & Pielak, 1992). It thus seemed important to pursue such data for our m^- mutants.

In this study, thermal denaturations monitored by circular dichroism were performed on Val 73, Ile 73, Trp 73, and WT iso-1-cytochromes *c*. The Val, Ile, and Trp variants at site 73 were the most pronounced m^- mutants from our previous studies (Bowler et al., 1993; Herrmann et al., 1995), and thus seemed the best choices for this study. Since ΔC_p measures the increase in hydration upon unfolding, if the thermally denatured state is more compact, as indicated for the gdnHCl denatured states of the m^- variants studied here, a decrease in ΔC_p should be expected for all mutants versus WT. Surprisingly, only small subtle differences in ΔC_p were detected in the variants with respect to WT. In addition there was no correlation between the ΔC_p and the m-value for the variants. The possible causes for this lack of correlation between ΔC_p and the m-value will be discussed.

Results

Thermal denaturations of the WT and variant iso-1-cytochromes *c* as a function of pH were monitored by CD spectroscopy at 222 nm. The WT protein and all variant proteins in this study carry a mutation of cysteine 102 to serine to avoid the complication of disulfide dimerization during physical measurements (Bowler et al., 1993). Reversibility was tested by cooling the denatured protein in an ice bath and monitoring the recovery of ellipticity at 222 nm. In general reversibility was very good averaging 94%, 90%, 87%, and 82% for the Ile 73, Val 73, Trp 73, and WT data sets, respectively. The poorest reversibilities, 50–75%, were obtained at pH 3.00 and 3.25 for WT and at pH 3.00 for Trp 73. Control experiments showed that varying the rate of temperature increase from 0.5–2 °C/min did not affect the values of T_m or ΔH_m in any systematic way within experimental error. Our thermal denaturation data thus met the usual equilibrium and reversibility conditions required for thermodynamic analysis. Thermal denaturation data were analyzed by plotting ellipticity at 222 nm versus temperature and using a non-linear least-squares curve fitting program to obtain ΔH_m and T_m as described in Materials and methods. A typical data set with the best-fit curve to Equation 6 (Materials and methods) is shown in Figure 1. Multiple experiments were performed at each pH, allowing determination of the error in the measurement, since error from multiple experiments has been shown to significantly exceed error calculated directly from the non-linear least-squares fitting procedure (Cohen & Pielak, 1994). The means and standard deviations (or ranges) were calculated for ΔH_m and T_m at each pH and these values are shown for WT and variant proteins in Table 1. Ranges or standard deviations of the values of ΔH_m and T_m are generally about the same magnitude for different pHs and proteins, although the errors for the Trp variant are higher than for the other proteins.

The value of ΔH_m obtained at different pH values has been shown to depend only on T_m. In the pH range 3 to 5, buffer and protein ionization enthalpy effects appear to cancel each other (Pfeil & Privalov, 1976b, Potekhin & Pfeil, 1989). A plot of ΔH_m versus T_m thus can be made using Equation 8 (see Materials and methods) to obtain the heat capacity increment, ΔC_p, for protein unfolding. Such plots are shown for WT and each of the variants in Figure 2. The scatter in the ΔH_m versus T_m data is small for the WT, Ile 73, and Val 73 proteins; however, it is much larger for the Trp 73 data and this is reflected in the calculated error in ΔC_p. Within the error of the data, these plots are linear over the range of ΔH_m and T_m for which data were obtained. The ΔC_p values obtained for each protein are collected in Table 2.

Using the heat capacity increment (ΔC_p) obtained from the ΔH_m versus T_m plots, ΔH_D, ΔS_D, and ΔG_D can be calculated at a specific pH and temperature using Equations 9–11 (see Materials and methods) to allow direct stability comparisons of the WT and variant proteins. Calculations were carried out at a temperature of 298 K and a pH of 4.5 to allow ready comparison with other thermal denaturation data for iso-1-cytochrome *c* (Cohen & Pielak, 1994). The results are presented in Table 2.

We note that the value of $\Delta G_{D,298K}$ for WT(C102S) is lower than that of the closely related iso-1-cytochrome *c* WT(C102T) variant by 1.4 kcal/mol (Cohen & Pielak, 1994) indicating that threonine is more stabilizing at position 102 than serine. Compar-

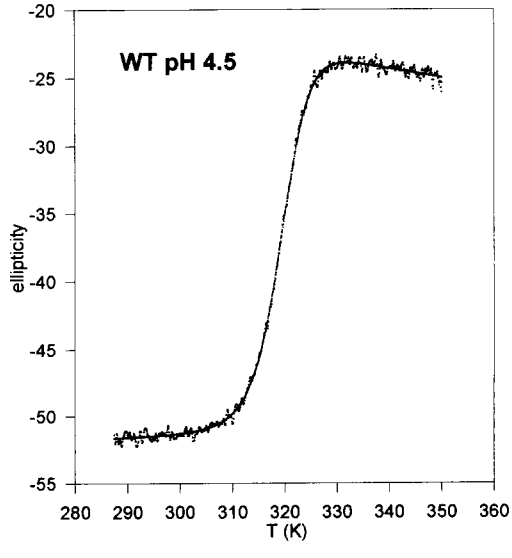

Fig. 1. Typical temperature-induced unfolding for iso-1-cytochrome *c*. Ellipticity is plotted versus temperature. The data shown are for the WT protein at pH 4.5. The solid curve is the result of a non-linear least-squares fit to Equation 6.

Table 1. *Enthalpy and midpoint temperature data for the thermal denaturation of WT and variant iso-1-cytochromes c as a function of pH*

	Wild Type			Trp 73	
pH	T_m (K)	ΔH_m (kcal/mol)	pH	T_m (K)	ΔH_m (kcal/mol)
3.00	294.5 ± 0.7[a]	39.6 ± 1.4[a]	3.00	295.8 ± 1.9[a]	43.5 ± 3.8[a]
3.25	295.6 ± 2.1[b]	42.0 ± 4.7[b]	3.25	295.8 ± 1.9[a]	49.8 ± 4.9[a]
3.50	303.7 ± 2.3[b]	54.2 ± 0.9[b]	3.50	300.5 ± 1.9[a]	50.5 ± 2.7[c]
3.75	307.9 ± 1.6[a]	62.3 ± 1.3[a]	3.75	302.2 ± 2.7[a]	58.6 ± 7.4[a]
4.00	313.7 ± 1.2[a]	69.8 ± 0.7[a]	4.00	303.4 ± 0.3[a]	67.8 ± 2.6[a]
4.25	316.1 ± 0.5[a]	73.7 ± 4.0[a]	4.25	304.5 ± 2.1[b]	68.5 ± 2.8[b]
4.50	318.0 ± 1.3[a]	73.4 ± 0.9[a]	4.50	309.1 ± 1.9[b]	64.2 ± 5.8[b]
4.75	321.6 ± 2.1[b]	79.6 ± 0.8[b]	4.75	312.0 ± 1.5[b]	71.4 ± 5.9[b]
5.00	325.7 ± 0.4[b]	81.9 ± 3.5[b]	5.00	320.6 ± 0.6[a]	78.0 ± 1.8[a]
			5.25	322.3 ± 0.7[a]	76.5 ± 3.1[a]
			5.50	320.4 ± 0.2[a]	79.3 ± 1.4[a]
	Val 73			Ile 73	
pH	T_m (K)	ΔH_m (kcal/mol)	pH	T_m (K)	ΔH_m (kcal/mol)
3.00	295.7 ± 1.8[a]	37.0 ± 5.6[a]	3.00	295.3 ± 1.1[a]	33.0 ± 5.1[a]
3.25	301.7 ± 1.2[a]	46.3 ± 1.5[a]	3.25	299.7 ± 1.1[a]	40.8 ± 1.1[a]
3.50	304.4 ± 0.7[a]	47.3 ± 0.7[a]	3.50	304.7 ± 0.8[a]	51.4 ± 1.0[a]
3.75	309.6 ± 0.9[a]	58.5 ± 0.7[c]	3.75	308.5 ± 1.7[a]	57.7 ± 1.2[a]
4.00	311.9 ± 0.2[a]	60.2 ± 2.1[a]	4.00	311.7 ± 0.3[a]	61.1 ± 0.6[c]
4.25	315.9 ± 0.3[a]	64.8 ± 0.8[a]	4.25	314.3 ± 0.9[a]	67.4 ± 2.2[a]
4.50	319.7 ± 0.6[b]	72.5 ± 1.8[b]	4.50	318.2 ± 0.5[a]	69.1 ± 1.7[a]
4.75	320.9 ± 0.2[a]	77.0 ± 1.5[a]	4.75	319.9 ± 0.1[a]	73.8 ± 4.7[a]
5.00	322.5 ± 0.8[b]	75.5 ± 1.4[b]	5.00	320.7 ± 0.8[a]	77.4 ± 0.7[a]

[a]Range of two values.
[b]Standard deviation of three values.
[c]Error from non-linear least-squares fit was larger than the range, so it is reported.

ing WT to the other variants we find $\Delta G_{D,298K}$ for Trp 73 to have the lowest Gibbs free energy of denaturation. This was also the case for gdnHCl denaturation where Trp 73 was found to have the lowest Gibbs free energy without denaturant, $\Delta G_u^{\circ\ H_2O}$ (see Table 2). However, for Val 73 and Ile 73, $\Delta G_u^{\circ\ H_2O}$ values obtained by gdnHCl denaturations are more similar to the Trp 73 variant while $\Delta G_{D,298K}$ from thermal denaturations are closer in value to the WT.

Another way to probe for mutation-induced effects on the thermal unfolding of the WT versus the variant iso-1-cytochromes *c* is to monitor the number of protons transferred as a function of pH during denaturation. This can be done using equation 1:

$$\Delta\nu = (\Delta H_m/2.3RT_m^2) \times (\delta T_m/\delta\text{pH}) \quad (1)$$

where $\Delta\nu$ is the number of protons taken up upon denaturation (Privalov et al., 1969, 1986; Ptitsyn & Birshtein, 1969; Cohen & Pielak, 1994). To calculate $\delta T_m/\delta$pH, a second order polynomial was fit to a plot of T_m versus pH (Fig. 3A) and the derivative evaluated at the appropriate value of T_m. Equation 1 was then used to evaluate $\Delta\nu$ (Fig. 3B). Plots are shown only for WT, Ile 73, and Val 73, since the value of $\Delta\nu$ appears to be quite sensitive to $\delta T_m/\delta$pH, and the scatter in the data for Trp 73 may introduce significant error into the evaluation of this function. In general, our $\Delta\nu$ data are similar to data for the WT(C102T) protein (Cohen & Pielak, 1994), although our values of $\Delta\nu$ for WT(C102S) are in general slightly higher (0.2–0.5 more protons transferred). This difference is probably within the error of the method of calculating $\Delta\nu$. The differences between the WT and Val 73 and Ile 73 are likely within the error of the method as well. Therefore, it is not clear that any definite conclusion about differences in the thermal denaturation process for WT and variants can be drawn from analysis of proton transfer during thermal unfolding.

Given that the ΔC_p values and the previously obtained *m*-values were found not to correlate well, the CD spectra of gdnHCl-denatured iso-1-cytochrome *c* was compared to that of thermally denatured iso-1-cytochrome *c*. Previous FT-IR studies of the native states of these variants suggest that they have at most small (Ile 73) to no (Trp 73, Val 73) changes in secondary structure relative to the WT protein (Bowler et al., 1993; Herrmann et al., 1995). Similarly, analysis of the X-ray structure of iso-1-cytochrome *c* shows that lysine 73 is highly solvent-exposed (fractional solvent exposure of 0.85–0.90, see Bowler et al., 1993). Lysine 73 is also relatively isolated from other charged side chains (Bowler et al., 1993). Therefore, denatured state structural effects which could effect thermodynamic parameters are important to investigate. Wavelength scans of ellipticity from 210–250 nm were done at 25 °C, 70 °C, 3 M gdnHCl at 25 °C, and 3 M gdnHCl at 70 °C, all in 0.1 M acetate, pH 4.5. These data are shown in Figure 4 for the WT protein. Similar data were obtained for the Trp 73 variant (data not shown). The final ellipticity values for thermal versus gdnHCl

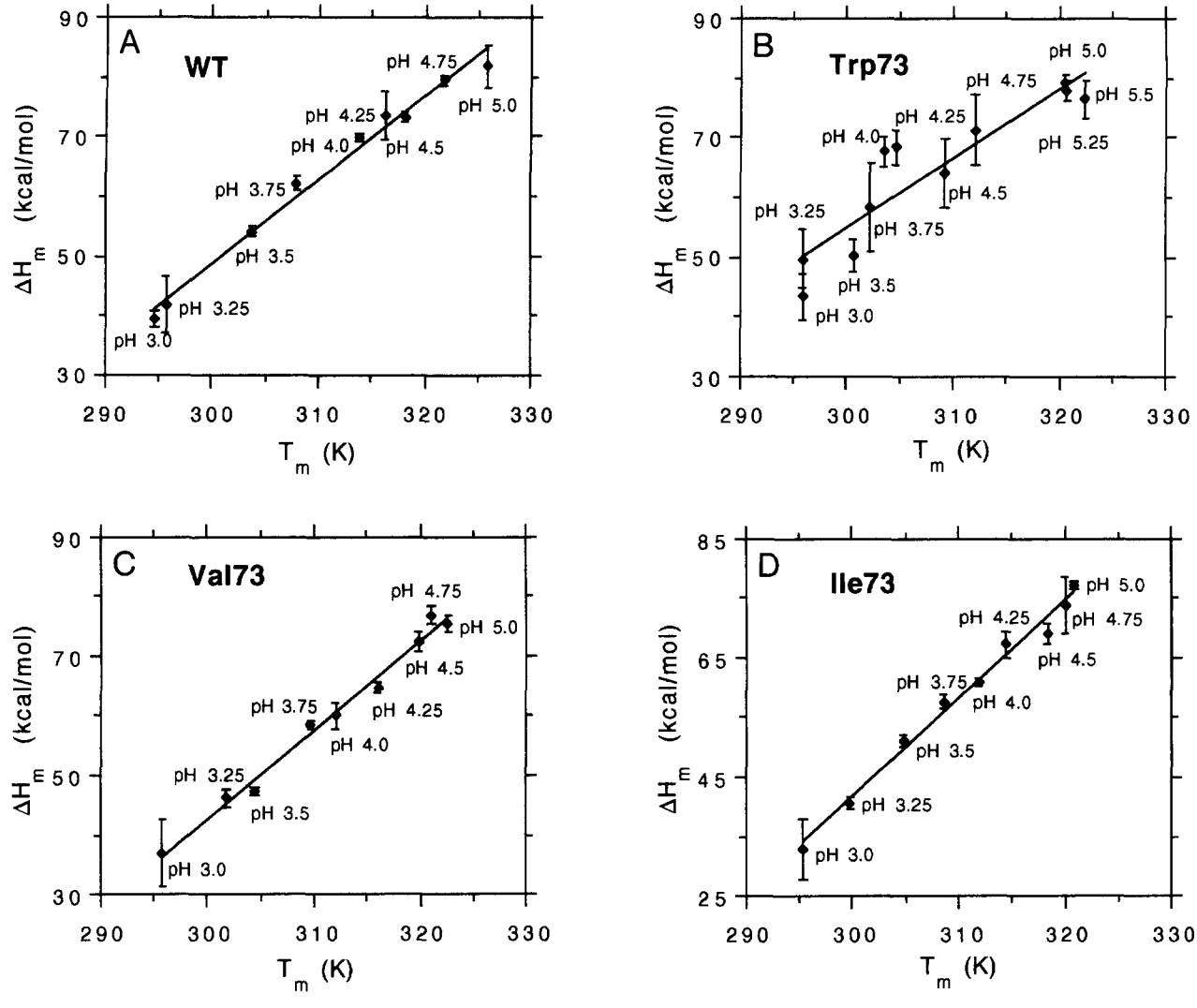

Fig. 2. Determination of ΔC_p. Plots of ΔH_m versus T_m are shown for the wild type protein (**A**) and for the Trp 73 (**B**), Val 73 (**C**), and Ile 73 (**D**) variants. Error bars are for one standard deviation or for the range of the data. The pH at which each data point was obtained is also indicated. The lines represent linear least-squares fits to the data points.

Table 2. *Thermodynamic parameters for unfolding of WT and variant iso-1-cytochromes c*

	WT	Trp 73	Val 73	Ile 73
$\Delta G_{D,298K}$[a] (kcal/mol)	3.7 ± 0.2	2.1 ± 0.4	3.8 ± 0.1	3.3 ± 0.1
$\Delta H_{D,298K}$[a] (kcal/mol)	45.4 ± 1.5	51.4 ± 6.9	40.0 ± 2.2	35.8 ± 2.2
$\Delta S_{D,298K}$[a] (kcal/mol K)	0.140 ± 0.005	0.17 ± 0.02	0.121 ± 0.007	0.109 ± 0.007
ΔC_p (kcal/mol K)	1.40 ± 0.06	1.15 ± 0.17	1.50 ± 0.06	1.65 ± 0.07
$\Delta G_u^{o\ H_2O}$ (298 K)[b] (kcal/mol)	5.66 ± 0.41[c]	4.23 ± 0.35[c]	4.28 ± 0.30[d]	4.47 ± 0.12[d]
m (298 K)[b] (kcal/mol M)	4.87 ± 0.32[c]	4.16 ± 0.34[c]	4.01 ± 0.24[d]	4.29 ± 0.12[d]

[a] All values are for pH 4.5 data.
[b] All data acquired at pH 7.5 in 20 mM Tris-HCl, 40 mM NaCl.
[c] Data from Bowler et al. (1993).
[d] Data from Herrmann et al. (1995).

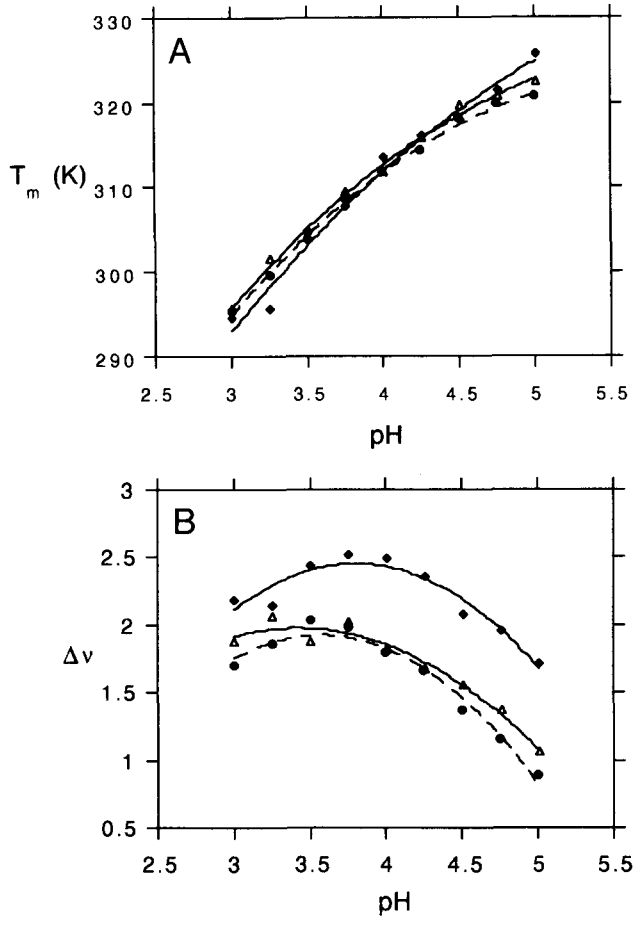

Fig. 3. Determination of the number of protons involved in the thermal unfolding equilibria of WT and variant iso-1-cytochromes *c* as a function of pH. **A:** T_m versus pH data for the WT protein (solid diamonds) and the Ile 73 (solid circles) and Val 73 (open triangles) variants. The curves (solid for WT and Val 73; dashed for Ile 73) represent the fits of each data set to a second order polynomial. **B:** Number of protons transferred from the native state to the denatured state upon unfolding, $\Delta\nu$, versus pH, for the WT protein (solid diamonds) and the Ile 73 (solid circles) and Val 73 (open triangles) variants. The curves (solid for WT and Val 73; dashed for Ile 73) represent the fits of each data set to a second order polynomial and have no theoretical significance.

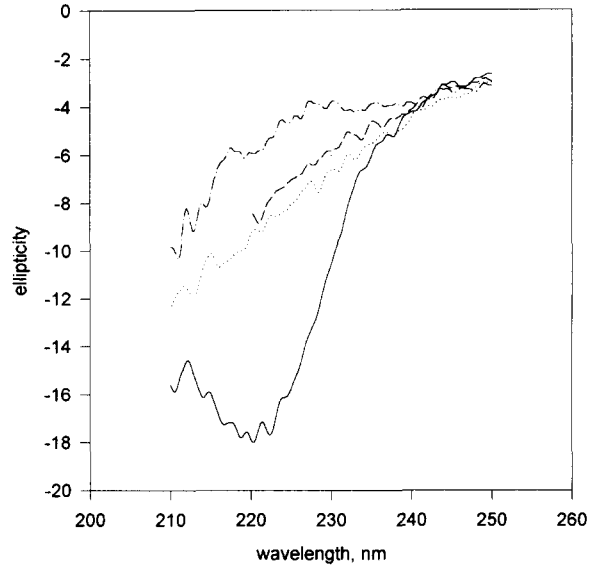

Fig. 4. CD spectra of native and denatured WT iso-1-cytochrome *c*. Spectra are shown of the protein in 3 M gdnHCl at 25 °C (– · – · –), in 3 M gdnHCl at 70 °C (– – – –), at 70 °C with buffer only (·····) and at 25 °C with buffer only (———). The buffer in all spectra is 100 mM acetate at pH 4.5.

denaturation are significantly different. Interestingly, heating the gdnHCl-denatured sample to 70 °C results in a CD spectrum that approximates the intensity and shape of the thermally-denatured sample.

Since ellipticity is the difference in the absorbance of left versus right circularly polarized light, spurious changes in ellipticity unrelated to structural features of the denatured state may occur if the extinction coefficient of the absorbance band being monitored changes significantly as a function of environment (temperature, [gdnHCl]). To correct for this possibility uv/vis absorbance spectra were obtained in the wavelength range of 210–300 nm at the same concentrations used for CD spectra (data not shown). The spectra show that temperature conditions do not affect the absorbance spectrum greatly near 222 nm. However, there are absorbance differences between a thermally-denatured protein and a gdnHCl denatured protein, indicating that these environments do affect the extinction coefficients at 222 nm somewhat. To assess these differences quantitatively, percent differences were calculated between these denatured states with respect to absorbance and ellipticity (Table 3). As can be seen from Table 3, the percent change in absorbance completely compensates the percent difference in ellipticity in the 70 °C versus the 3 M gdnHCl at 70 °C case, indicating the slight changes observed in ellipticity shown in Figure 4 are not significant from a structural standpoint. Thus, these two denaturing conditions are essentially equivalent. However, this is not the case with the other two comparisons in Table 3. Both of these indicate that the percent difference in absorbance cannot fully compensate for changes in ellipticity; thus, the differences in Figure 3 between 3 M gdnHCl at 25 °C and buffer at 70 °C and between 3 M gdnHCl at 25 °C and 3 M gdnHCl at 70 °C are real. Similar CD spectra for gdnHCl-denatured versus thermally-denatured protein and for gdnHCl-denatured protein at high temperature have been observed for the C102T variant of iso-1-cytochrome *c* (Betz & Pielak, 1992).

Table 3. *Comparisons in the percent difference for different solution conditions in absorbance and ellipticity at 222 nm for WT iso-1-cytochrome c*

Solution conditions compared[a]	Difference in absorbance at 222 nm (%)	Difference in ellipticity at 222 nm (%)
25 °C 3 M gdnHCl vs. 70 °C 3 M gdnHCl	8	31
25 °C 3 M gdnHCl vs. 70°C	21	38
70 °C 3 M gdnHCl vs. 70 °C	14	11

[a]All data were acquired at pH 4.5 in 0.1 M acetate.

Discussion

An initial point to clarify since we are monitoring thermal denaturations down to reasonably low pH is that we are not crossing over into a region where a molten globule state of cytochrome *c* might be stabilized (Potekhin & Pfeil, 1989; Kuroda et al., 1992; Fink et al., 1994). We do not expect molten globule states to be involved at either end point of our low pH denaturations as these states appear to be primarily driven by anion binding and the concentration of anion should be lowest (<10 mM) in our low pH acetate buffers. Heating is also known to disrupt molten globule states.

The main assumption in our derivation of ΔC_p is that ΔH_m is dependent upon T_m but not pH. This assumption has been experimentally tested through the observation that the ionization enthalpies of acetate buffers and of protein unfolding compensate each other almost precisely (Pfeil & Privalov, 1976b). It has also been observed that ΔC_p values obtained by the pH variation method and by direct calorimetric measurements are indistinguishable within experimental error (Potekhin & Pfeil, 1989). In the case of cytochrome *c*, His 26 and His 18 have abnormally low pK_a values, <3.5 and <3.0, respectively (Cohen & Hayes, 1974), although the pK_a of His 18 will be abnormally low in the denatured state as well due to heme iron ligation (Babul & Stellwagen, 1971; Muthukrishnan & Nall, 1991). Thus, it remains likely that up to one histidine may be protonated upon unfolding of cytochrome *c* in the pH range 3–5. Its protonation enthalpy would be too large to be compensated by the acetate buffer ionization enthalpy (Privalov et al., 1989). However, it has been demonstrated for myoglobin unfolding that correction for the ionization enthalpy of histidine has an insignificant effect on the ΔC_p obtained from a plot of ΔH versus T_m (Privalov et al., 1986). Therefore, by examining the change in enthalpy with respect to the change in T_m under differing conditions of pH, ΔC_p can be estimated reliably. For the WT(C102S) iso-1-cytochrome *c*, the ΔC_p value obtained in these studies is within error the same as the value of 1.37 kcal/mol K obtained for the closely related WT(C102T) iso-1-cytochrome *c* (Cohen & Pielak, 1994).

The heat capacity increment (ΔC_p), which to a first approximation is temperature independent (Privalov et al., 1989), is proportional to the change in solvent-accessible surface area upon unfolding, ΔASA (Brandts & Hunt, 1967; Privalov, 1979; Privalov & Makhatadze, 1990, 1992; Gomez et al., 1995). Since ΔC_p is a measure of ΔASA as is the *m*-value for solvent denaturations (Shortle et al., 1988; Myers et al., 1995), one might expect that for the iso-1-cytochrome *c* variants studied here (see Table 2) the ΔC_p values from our thermal denaturation experiments should correlate with *m*-values obtained from gdnHCl denaturations. Such a correlation was reported for a series of staphylococcal nuclease variants (Shortle et al., 1988). For all the m^- mutants examined here, Val 73, Ile 73, and Trp 73, the ΔC_p did not change greatly with respect to the WT, the largest change being for the Trp 73 variant. In general, there was no strong effect on ΔC_p. In addition, there was no direct correlation of ΔC_p with *m*-values.

Interpretation of a lack of correlation between the *m*-value and ΔC_p may come from a consideration of whether denatured states produced under different denaturing conditions are the same. Work by Tanford and coworkers suggested that thermally denatured proteins have more residual structure than gdnHCl-denatured proteins (Tanford, 1968; Greene & Pace, 1974). Cooperative loss of structure from thermally-denatured lysozyme, chymotrypsin and RNase A upon addition of gdnHCl provides convincing evidence that thermally denatured states contain residual structure (Aune et al., 1967). Furthermore, the ΔC_p for thermal denaturation of lysozyme was found to be less than for gdnHCl denaturation (Tanford & Aune, 1970). However, later calorimetric work indicated that ΔC_p for thermally-denatured and gdnHCl-denatured lysozyme were identical (Pfeil & Privalov, 1976a). It has also been demonstrated recently that there can be differences between $\Delta G_u^{\circ\ H_2O}$ values obtained from urea versus gdnHCl denaturations for the same protein. Thus, even within the realm of chemical denaturations, structural differences in the end points of the unfolding equilibrium may exist, some of which may be attributable to the denatured state (Greene & Pace, 1974; Pace et al., 1990; Dekoster et al., 1993). Studies with helical coiled coils have demonstrated that a gdnHCl denaturation may mask electrostatic interactions leading to denaturation reflecting mainly hydrophobic interactions whereas urea denaturations reflect both hydrophobic and electrostatic interactions (Monera et al., 1994). Thus, the end point of denaturation may be distinct much like an increase in ionic strength leads to the more compact molten globule state under acid denaturation conditions for many proteins (Fink et al., 1994). For barnase there is evidence of more differences between gdnHCl-denatured and thermally-denatured states than between acid-denatured and thermally-denatured states (Oliveberg et al., 1994). Certainly, for cytochrome *c*, differences have been observed for the degree of compactness of the gdnHCl-denatured versus the urea-denatured state of this protein (Tsong, 1974, 1975).

The differences in CD spectra we show in Figure 4 are consistent with different structural end points for thermally- versus gdnHCl-denatured iso-1-cytochrome *c*. The question remains as to the nature of this structural difference. It is initially tempting to suggest that more residual structure remains in the thermally-denatured state, due to the greater magnitude of the negative ellipticity. However, some β-turn structures are known to give positive contributions to ellipticity in the 220 nm region (Rose et al., 1985). Thus, heating could be causing loss of such residual β-turn structure and leading to an increase in negative ellipticity at 220 nm. Similarly, an extended helix structure, commonly found in polyproline homopolymers, has been found to be stabilized by the presence of urea and gdnHCl in both peptide homopolymers and proteins (Tiffany & Krimm, 1972, 1973; Woody, 1992). This extended helix structure gives a positive contribution to ellipticity at 220 nm as well. Loss of denaturant binding to the protein at higher temperature destabilizes the extended helix structure resulting in a decrease in positive ellipticity at 220 nm as temperature is increased (Tiffany & Krimm, 1972, 1973). A recent study of the denatured states of a number of proteins in which CD effects similar to those in Figure 4 were observed (Privalov et al., 1989), as well as a calorimetric study (Makhatadze & Privalov, 1992), came to the same general conclusion. GdnHCl-denatured proteins at room temperature are not complete random coils, but adopt a denaturant stabilized extended helix structure. Only heat-denatured proteins or gdnHCl-denatured proteins at high temperature approach a random coil.

In the context of an unconstrained thermally-denatured state that may approach a random coil, it is relatively straightforward to explain the slight increases in ΔC_p observed for the Ile 73 and Val 73 variants. Both of these amino acids are beta-branched and such amino acids are expected to lead to slight expansion of a random coil denatured state (Tanford, 1968; Miller & Goebel, 1968). Expansion of the denatured state of a protein to a more

extended structure is expected to expose more buried hydrophobic surface area (Creamer et al., 1995). Using the parameterization of Freire and coworkers (Gomez et al., 1995), the increase in ΔC_p observed for the Ile 73 variant corresponds to an increase in exposed hydrophobic surface area of 560 ± 290 Å^2. This number is a lower limit that assumes that no additional exposure of polar surface area occurs.

The less random denatured state which occurs under gdnHCl denaturing conditions, apparently leads to exposure of less hydrophobic surface area for the same set of variants. The exact nature of this effect is unclear at this point. Previous work from our laboratory discussed in the introduction is consistent with a hydrophobic effect acting on the denatured state (Bowler et al., 1993; Herrmann et al., 1995) for these hydrophobic replacements at a highly surface-exposed site.

Conclusion

The main observation in this work is that ΔC_p values obtained using thermal denaturation methods and *m*-values obtained by gdnHCl denaturation methods for a set of iso-1-cytochrome *c* variants do not correlate well. Interpretation of CD data for gdnHCl-denatured versus thermally-denatured states, based on data from the literature (Privalov et al., 1989; Woody, 1992), indicates that the denatured state is more random under heat denaturation conditions. This increased randomness in the heat-denatured state appears to be adequate to cause different responses to mutation under gdnHCl-denaturation versus thermal-denaturation conditions. Experiments are in progress to further characterize this novel result.

Materials and methods

Iso-1-cytochrome c preparation

Site-directed mutagenesis, other molecular biology techniques to produce yeast cells maintaining a plasmid which encodes iso-1-cytochrome *c*, and procedures for isolation of iso-1-cytochrome *c* have been described previously (Bowler et al., 1993; Herrmann et al., 1995, 1996). Iso-1-cytochrome *c* (~3 mg/mL) was taken from a −70 °C frozen stock or a recently isolated refrigerated stock and purified using a Pharmacia HPLC and a Waters SP HR-8 cation exchange column as described previously (Bowler et al., 1994). After elution, the protein was concentrated to 2–3 mg/mL and transferred into 50 mM sodium phosphate, 1 mM EDTA using Amicon centriprep-10 (10,000 MW cut off) ultrafiltration devices. About 0.5 mg was oxidized and loaded onto a refrigerated (4 °C) G-25 Sephadex column equilibrated with 0.1 M sodium acetate pH 4.5. Acetate buffers are used in thermal denaturation studies because they have been found to effectively compensate for the ionization enthalpy of protein unfolding in the pH range 3–5 (Pfeil & Privalov, 1976b). The 0.1 M sodium acetate buffers were prepared by mixing a 0.1 M sodium acetate solution with a 0.1 M acetic acid solution to achieve the desired pH. It was found that adjusting a buffer that was 0.1 M in sodium acetate with concentrated acetic acid (17.4 M) and using this for circular dichroism studies caused significant spectral noise due to the strong absorbance of the carboxylic group of acetic acid in the far UV (210–250 nm) region. After elution from the G-25 resin, Amicon Centricon-3 (3,000 MW cut off) ultrafiltration units were used to place the protein in the desired pH buffer using 0.1 M acetate buffer adjusted for pH as stated above. A minimum of two dilutions and reconcentrations with the new buffer were carried out (overall 400× dilution of the original buffer). Control experiments indicate that this procedure adjusted the pH to within 0.05 of the desired pH for all but the lowest pHs (3.0, 3.25 and 3.5) which were within 0.1 pH units of the desired pH. The concentration of oxidized iso-1-cytochrome *c* was measured using a Zeiss PM6 spectrophotometer at the visible absorbance wavelengths: 550 nm, 541.75 nm, 526.5 nm, and 339 nm as described previously (Bowler et al., 1993).

Thermal denaturations

All thermal denaturation experiments were carried out at an iso-1-cytochrome *c* concentration of 30 μM in a jacketed 0.1 cm path length cell. A JASCO 500-C spectropolarimeter was used to measure the ellipticity at 222 nm as a function of temperature. The temperature was incremented at a rate of 1 °C/min for 55 min using a computer-interfaced Fisher Iso-temp 9110 circulating water bath running DTC10-PC software (Polyscience). The temperature of the sample was monitored directly using a Cole Palmer iron constantan thermocouple, type J, connected to a voltmeter and this in turn connected to a Linear recorder/plotter. The thermocouple calibration was checked in an ice water bath (0 °C) before each run. The spectropolarimeter was interfaced to a computer in order to record data as a function of time. Over 800 data points were collected during each thermal denaturation experiment. At the end of each experiment, the sample cell was taken out of the spectropolarimeter and placed into an ice bath for approximately 5 min (until the temperature was below the starting temperature) to check reversibility. The two openings to the sample cell were sealed by placing a Teflon stopper in one and an engineered thermocouple holder/stopper in the other. No detectable sample evaporation occurred during the course of thermal denaturation experiments under these conditions. Heating rates of 0.5 °/min and 2 °/min were also used to check that enthalpy values did not depend on heating rate.

The starting and ending set temperatures depended on the pH of the protein solution. If the pH was 3.0, 3.25, or 3.5, the starting temperature was 2–5 °C, and the ending temperature was 57–60 °C, if the pH was 3.75, 4.0, or 4.25, the starting temperature was 10–15 °C and the ending temperature was 65–70 °C, and if the pH was 4.5, 4.75, or 5.0, the starting temperature was 15–20 °C, and the ending temperature was 70–75 °C. These nine pH values were used for the wild-type and the Val 73, Ile 73, and Trp 73 variants. For Trp 73, two more pHs, 5.25 and 5.5, were included to obtain a more accurate estimate of ΔC_p.

Circular dichroism of thermally vs. guanidine hydrochloride denatured iso-1-cytochrome c

Wavelength scans from 210-250 nm were taken of the WT and the Trp 73 variant at a concentration of 15 μM using the computer-interfaced JASCO 500-C spectropolarimeter. CD spectra for both proteins were obtained at 25 °C, 70 °C, 3 M gdnHCl at 25 °C, and 3 M gdnHCl at 70 °C. For the 3 M gdnHCl at 70 °C sample, 220 nm was the lowest wavelength for the scan because of background noise problems at shorter wavelengths. The sample cell was the same as previously described above, and the buffer conditions were 0.1 M acetate buffer, pH 4.5.

Data analysis

The data from both the time and wavelength scans were smoothed three times using the CD interface software, PROCDAT (Landis Instrument, Oakland, NJ). The data were transferred to a Packard Bell pentium computer and fit using a non-linear least-squares routine adapted for use with Sigma Plot 4.0. All data points were included in the fit. The non-linear fitting program was provided by Gary Pielak and David Cohen at the University of North Carolina (Cohen & Pielak, 1994). This non-linear fitting method provided a better fit for the data than did the linear-extrapolation method, which introduced some subjectivity in assigning baselines, especially when fitting the lower pH data where the unfolding transition is broader.

By assuming a two state reversible equilibrium process between the native and denatured states, the fraction of denatured protein at temperature T, $f_{D,T}$ is given by the following equation:

$$f_{D,T} = (\Theta_{222,T} - \Theta_{N,T})/(\Theta_{D,T} - \Theta_{N,T}) \quad (2)$$

where $\Theta_{222,T}$ is the measured ellipticity at temperature T at 222 nm and $\Theta_{N,T}$ and $\Theta_{D,T}$ are the native and denatured state ellipticities at that temperature, respectively. $\Theta_{N,T}$ and $\Theta_{D,T}$ are defined in Equations 3 and 4, where m_N, b_N

$$\Theta_{N,T} = m_N T + b_N \quad (3)$$

$$\Theta_{D,T} = m_D T + b_D \quad (4)$$

and m_D, b_D are the slopes and intercepts of the native and denatured state baselines, respectively. The equilibrium constant for denaturation, $K_{D,T}$, is given by Equation 5:

$$K_{D,T} = [D]/[N] = f_{D,T}/(1 - f_{D,T}). \quad (5)$$

The dependence of $\Theta_{222,T}$ on temperature is obtained by substituting Equation 2 into Equation 5 and solving for $\Theta_{222,T}$ to yield Equation 6:

$$\Theta_{222,T} = [\Theta_{N,T} + (\Theta_{D,T})K_{D,T}]/[1 + K_{D,T}]. \quad (6)$$

The temperature dependence of $K_{D,T}$ is given by Equations 7a and 7b. For the purposes of curve fitting ellipticity versus temperature data to Equation 6, the standard expression for the temperature dependence of $\Delta G_{D,T}$ (Becktel & Schellman, 1987) is used as given in Equation 7b:

$$K_{D,T} = \exp -\{\Delta G_{D,T}/RT\} \quad (7a)$$

$$= \exp -\{(\Delta H_m(1 - T/T_m) - \Delta C_p[(T_m - T) + T\ln(T/T_m)])/RT\}. \quad (7b)$$

In Equation 7b, ΔH_m is the van't Hoff enthalpy of denaturation at T_m (the temperature where $K_{D,T} = 1$), R is the gas constant, T is temperature in degrees Kelvin, and ΔC_p is the heat capacity change associated with protein unfolding. Two or three thermal unfolding experiments were carried out at each pH. The ΔH_m and T_m values obtained from the non-linear least-squares fit to Equation 6 were averaged, and a standard deviation (SD) or range was calculated as appropriate.

Values of ΔC_p for each iso-1-cytochrome c variant were determined by linear least-squares fits of ΔH_m versus T_m using data obtained at different pHs and the equation:

$$\Delta H_m = \Delta C_p T_m + \Delta H^\circ_m. \quad (8)$$

The enthalpy of denaturation at temperature T, $\Delta H_{D,T}$, was calculated using the equation:

$$\Delta H_{D,T} = \Delta H_m + \Delta C_p(T - T_m). \quad (9)$$

The entropy of denaturation at temperature T, $\Delta S_{D,T}$, was calculated using Equation 10 where ΔS_m equals $\Delta H_m/T_m$:

$$\Delta S_{D,T} = \Delta S_m + \Delta C_p \ln(T/T_m). \quad (10)$$

The free energy of denaturation at temperature T was calculated using the equation:

$$\Delta G_{D,T} = -RT\ln(K_{D,T}) = \Delta H_m(1 - T/T_m) - \Delta C_p[(T_m - T) + T\ln(T/T_m)]. \quad (11)$$

Error analysis

The error in ΔC_p was calculated from the scatter of data points about the least-squares line using standard methods (Taylor, 1982). To calculate the error involved in $\Delta G_{D,T}$, a standard propagation of error (Taylor, 1982) was used according to the following equation:

$$\sigma_{F(x,y,z)} = \{[(\delta F/\delta x)\sigma_x]^2 + [(\delta F/\delta y)\sigma_y]^2 + [(\delta F/\delta z)\sigma_z]^2\}^{1/2} \quad (12)$$

where the errors from three independent variables, namely, ΔH_m, T_m, and ΔC_p, are represented by x, y, and z, and σ is the standard deviation for each of the values at a specific pH and temperature. Therefore, the error in $\Delta G_{D,T}$ is given by

$$\sigma_{\Delta G_{D,T}} = \{[(1 - T/T_m)\sigma_{\Delta H_m}]^2 + [(T - T_m - T\ln(T/T_m))\sigma_{\Delta C_p}]^2 + [(\Delta C_p T/T_m + \Delta H_m T/T_m^2 - \Delta C_p)\sigma_{T_m}]^2\}^{1/2}. \quad (13)$$

The errors for $\Delta S_{D,T}$ and $\Delta H_{D,T}$ were calculated using Equation 12 by assuming two independent variables instead of three. Since ΔS_m and ΔH_m are considered independent variables dependent upon T_m, T_m is not considered an independent variable in Equations 9 and 10. Thus, the errors involved in $\Delta S_{D,T}$ and $\Delta H_{D,T}$ are reliant upon $\sigma_{\Delta S_m}, \sigma_{\Delta C_p}$ and $\sigma_{\Delta H_m}, \sigma_{\Delta C_p}$, respectively.

Acknowledgments

This work was supported by NSF grant # MCB 93004751 (B.E.B.) We thank David Cohen and Gary Pielak for providing software for fitting thermal denaturation data.

References

Aune KC, Salahuddin A, Zarlengo MH, Tanford C. 1967. Evidence for structure in acid- and heat-denatured proteins. *J Biol Chem 242*:4486–4489.

Babul J, Stellwagen E. 1971. The existence of heme-protein coordinate-covalent bonds in denaturing solvents. *Biopolymers 10*:2359–2361.

Becktel WT, Schellman JA. 1987. Protein stability curves. *Biopolymers 26*:1859–1877.

Betz SF, Pielak GJ. 1992. Introduction of a disulfide bond into cytochrome *c* stabilizes a compact denatured state. *Biochemistry 31*:12337–12344.

Bowler BE, Dong A, Caughey WS. 1994. Characterization of the guanidine-HCl denatured state of iso-1-cytochrome *c* by infrared spectroscopy. *Biochemistry 33*:2402–2408.

Bowler BE, May K, Zaragoza T, York P, Dong A, Caughey WS. 1993. Destabilizing effects of replacing a surface lysine of cytochrome *c* with aromatic amino acids: Implications for the denatured state. *Biochemistry 32*:183–190.

Brandts JF, Hunt L. 1967. The thermodynamics of protein denaturation. III. The denaturation of ribonuclease in water and in aqueous urea and aqueous ethanol mixtures. *J Am Chem Soc 89*:4826–4838.

Cohen DS, Pielak GJ. 1994. Stability of yeast iso-1-ferricytochrome *c* as a function of pH and temperature. *Protein Sci 3*:1253–1260.

Cohen JS, Hayes MB. 1974. Nuclear magnetic resonance titration curves of histidine ring protons. *J Biol Chem 249*:5472–5477.

Creamer TP, Srinivasan R, Rose GD. 1995. Modeling unfolded states of peptides and proteins. *Biochemistry 34*:16245–16250.

Dekoster GT, Robertson AD, Stock AM, Stock JB. 1993. Urea and guanidine-HCl yield different unfolding free energies for cheY: Which denaturant provides the most reliable free energy values? In: Angeletti RH, ed. *Techniques in protein chemistry IV*. New York: Academic Press. pp 533–540.

Dill KA. 1990. Dominant forces in protein folding. *Biochemistry 29*:7133–7155.

Dill KA, Shortle D. 1991. Denatured states of proteins. *Annu Rev Biochem 60*:795–825.

Fink AL, Calciano LJ, Goto Y, Kuiotsu T, Palleros DR. 1994. Classification of acid denaturation of proteins: Intermediates and unfolded states. *Biochemistry 33*:12504–12511.

Gomez J, Hilser VJ, Freire E. 1995. The heat capacity of proteins. *Proteins: Struct Funct Genet 22*:404–412.

Green SM, Meeker AK, Shortle D. 1992. Contributions of the polar, uncharged amino acids to the stability of staphylococcal nuclease: Evidence for mutational effects on the free energy of the denatured state. *Biochemistry 31*:5717–5727.

Greene RF, Pace CN. 1974. Urea and guanidine hydrochloride denaturation of ribonuclease, lysozyme, α-chymotrypsin and β-lactoglobulin. *J Biol Chem 249*:5388–5393.

Herrmann L, Bowler BE, Dong A, Caughey WS. 1995. The effects of hydrophilic to hydrophobic surface mutations on the denatured state of iso-1-cytochrome *c*: Investigation of aliphatic residues. *Biochemistry 34*:3040–3047.

Herrmann LM, Flatt P, Bowler BE. 1996. Site-directed replacement of the invariant lysine 73 of *saccharomyces cerevisiae* iso-1-cytochrome *c* with all ribosomally encoded amino acids. *Inorg Chim Acta 242*:97–103.

Kuroda Y, Kidokoro S, Wada A. 1992. Thermodynamic characterization of cytochrome *c* at low pH: Observation of the molten globule state of the cold denaturation process. *J Mol Biol 223*:1139–1153.

Makhatadze GI, Privalov PL. 1992. Protein interactions with urea and guanidinium chloride: A calorimetric study. *J Mol Biol 226*:491–505.

Matthews BW. 1993. Structural and genetic analysis of protein stability. *Ann Rev Biochem 62*:139–160.

Miller WG, Goebel CV. 1968. Dimensions of protein random coils. *Biochemistry 7*:3925–3935.

Monera OD, Kay CM, Hodges RS. 1994. Protein denaturation with guanidine hydrochloride or urea provides a different estimate of stability depending on the contributions of electrostatic interactions. *Protein Sci 3*:1984–1991.

Muthukrishnan K, Nall BT. 1991. Effective concentrations of amino acid side chains in an unfolded protein. *Biochemistry 30*:4706–4710.

Myers JK, Pace CN, Scholtz JM. 1995. Denaturant *m* values and heat capacity changes: Relation to changes in accessible surface areas of protein unfolding. *Protein Sci 4*:2138–2148.

Oliveberg M, Viulleumier S, Fersht AR. 1994. Thermodynamic study of acid denaturation of barnase and its dependence on ionic strength: Evidence for residual electrostatic interactions in the acid/thermally denatured state. *Biochemistry 34*:8826–8832.

Pace CN, Laurents DV, Thomson JA. 1990. pH dependence of the urea and guanidine hydrochloride denaturation of ribonuclease A and ribonuclease T1. *Biochemistry 29*:2564–2572.

Pakula AA, Sauer RT. 1990. Reverse hydrophobic effects relieved by amino acid substitutions at a protein surface. *Nature 244*:363–364.

Pfeil W, Privalov PL. 1976a. Thermodynamic investigations of proteins. II. Calorimetric study of lysozyme denaturation by guanidine hydrochloride. *Biophys Chem 4*:33–40.

Pfeil W, Privalov PL. 1976b. Thermodynamic investigation of proteins. III. Thermodynamic description of lysozyme. *Biophys Chem 4*:41–50.

Potekhin S, Pfeil W. 1989. Microcalorimetric studies of conformational transitions of ferricytochrome *c* in acidic solution. *Biophys Chem 34*:55–62.

Privalov PL. 1979. Stability of proteins: Small globular proteins. *Adv Protein Chem 33*:167–241.

Privalov PL, Gill SJ. 1988. Stability of protein structure and hydrophobic interaction. *Adv Protein Chem 39*:191–234.

Privalov PL, Griko YV, Venyaminov SY, Kutyshenko VP. 1986. Cold denaturation of myoglobin. *J Mol Biol 190*:487–498.

Privalov PL, Makhatadze GI. 1990. Heat capacity of proteins II. Partial molar heat capacity of the unfolded polypeptide chain of proteins: Protein unfolding effects. *J Mol Biol 213*:385–391.

Privalov PL, Makhatadze GI. 1992. Contribution of hydration and non-covalent interactions to the heat capacity effect on protein unfolding. *J Mol Biol 224*:715–723.

Privalov PL, Ptitsyn OB, Birshtein TM. 1969. Determination of stability of the DNA double helix in an aqueous medium. *Biopolymers 8*:559–571.

Privalov PL, Tiktopolo EI, Venyaminov SY, Griko YV, Makhatadze GI, Khechinashvili NN. 1989. Heat capacity and conformation of proteins in the denatured state. *J Mol Biol 205*:737–750.

Ptitsyn OB, Birshtein TM. 1969. Method of determining the relative stability of different conformational states of biological macromolecules. *Biopolymers 7*:435–445.

Rose GD, Gierasch LM, Smith JA. 1985. Turns in peptides and proteins. *Adv Prot Chem 37*:1–109.

Schellman JA. 1978. Solvent denaturation. *Biopolymers 17*:1305–1322.

Shortle D. 1995. Staphylococcal nuclease: A showcase of *m*-value effects. *Adv Protein Chem 46*:217–247.

Shortle D, Meeker AK, Freire E. 1988. Stability mutants of staphylococcal nuclease: Large compensating enthalpy-entropy changes for the reversible denaturation reaction. *Biochemistry 27*:4761–4768.

Shortle D, Chan HS, Dill KA. 1992. Modeling the effects of mutations on the denatured states of proteins. *Protein Sci 1*:210–215.

Tanford C. 1968. Protein denaturation. *Adv Prot Chem 23*:121–282.

Tanford C, Aune KC. 1970. Thermodynamics of the denaturation of lysozyme by guanidine hydrochloride. III. Dependence on temperature. *Biochemistry 9*:206–211.

Taylor JR. 1982. *An introduction to error analysis*. Mill Valley, California: University Science Books.

Tiffany LM, Krimm S. 1972. Effect of temperature on the circular dichroism spectra of polypeptides in the extended state *Biopolymers 11*:2309–2316.

Tiffany LM, Krimm S. 1973. Extended conformations of polypeptides and proteins in urea and guanidine hydrochloride. *Biopolymers 12*:575–587.

Tsong TY. 1974. The trp-59 fluorescence of ferricytochrome *c* as a sensitive measure of the over-all protein conformation. *J Biol Chem 249*:1988–1990.

Tsong TY. 1975. An acid induced conformational transition of denatured cytochrome *c* in urea and guanidine hydrochloride solutions. *Biochemistry 14*:1542–1547.

Woody RW. 1992. Circular dichroism and conformation of unordered polypeptides. *Adv Biophys Chem 2*:37–79.