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Thermodynamics of unfolding for turkey ovomucoid third domain: Thermal and chemical denaturation

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Abstract

We have used thermal and chemical denaturation to characterize the thermodynamics of unfolding for turkey ovomucoid third domain (OMTKY3). Thermal denaturation was monitored spectroscopically at a number of wavelengths and data were subjected to van't Hoff analysis; at pH 2.0, the midpoint of denaturation (T_m) occurs at 58.6 ± 0.4 °C and the enthalpy of unfolding at this temperature (ΔH_m) is 40.8 ± 0.3 kcal/mol. When T_m was perturbed by varying pH and denaturant concentration, the resulting plots of ΔH_m versus T_m yield a mean value of 590 ± 120 cal/(mol·K) for the change in heat capacity upon unfolding (ΔC_p). A global fit of the same data to an equation that includes the temperature dependence for the enthalpy of unfolding yielded a value of 640 ± 110 cal/(mol·K). We also performed a variation of the linear extrapolation method described by Pace and Laurents, which is an independent method for determining ΔC_p (Pace, C.N. & Laurents, D., 1989, *Biochemistry 28*, 2520–2525). First, OMTKY3 was thermally denatured in the presence of a variety of denaturant concentrations. Linear extrapolations were then made from isothermal slices through the transition region of the denaturation curves. When extrapolated free energies of unfolding (ΔG_u) were plotted versus temperature, the resulting curve appeared linear; therefore, ΔC_p could not be determined. However, the data for ΔG_u versus denaturant concentration are linear over an extraordinarily wide range of concentrations. Moreover, extrapolated values of ΔG_u in urea are identical to values measured directly.

Keywords: heat capacity; linear extrapolation method; protein folding; van't Hoff analysis

One facet of "the protein folding problem" is understanding how proteins maintain their unique native conformations. While both the energetics and conformation of the native structure are clearly dictated by the primary amino acid sequence, the contributions of individual residues are at present ill-defined. Comprehensive studies of such contributions can most easily be addressed in small proteins, such as ovomucoid third domain (56 amino acid residues, MW = 6.1 kDa).

Our ultimate goal is to quantitatively describe the contribution of every amino acid residue to the global stability of ovomucoid third domain. Key information for these studies is knowledge of the free energy of unfolding (ΔG_u) over a wide range of experimental conditions. ΔG_u can be directly measured over only a narrow temperature range but, for a two-state process, can be predicted at any temperature from the modified Gibbs-Helmholtz equation:

$$\Delta G_u = \Delta H_m \cdot (1 - T/T_m) - \Delta C_p \cdot [(T_m - T) + T \cdot \ln(T/T_m)], \quad (1)$$

where T_m is the temperature at the midpoint of the transition and ΔH_m is the enthalpy of unfolding at this temperature. Both parameters are dependent upon experimental conditions but are often readily obtained from a single thermal denaturation experiment. ΔC_p is the difference in heat capacity between native and denatured protein and, to a first approximation, is independent of solution conditions and temperature between 20 and 80 °C (Privalov, 1979, 1990; Schellman & Hawkes, 1980; Pace & Laurents, 1989; Griko & Privalov, 1992). Therefore, once the value of ΔC_p has been determined, ΔH_m and T_m are sufficient for calculation of ΔG_u at any temperature T.

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Abbreviations: OMTKY3, turkey ovomucoid third domain; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GuHCl, guanidine hydrochloride; HPLC, high-performance liquid chromatography; T_m, temperature at the midpoint of unfolding; ΔH_m, enthalpy of unfolding at T_m; ΔS_m, entropy of unfolding at T_m; ΔG_u, Gibbs free energy of unfolding; ΔC_p, denaturational heat capacity change; K, equilibrium constant of protein unfolding; $\%R$, percent renaturation of protein after a denaturation experiment; OMCHI3, chicken ovomucoid third domain.

The only direct method for determining ΔC_p is calorimetry; however, this technique is not often readily available. Consequently, several spectroscopic approaches based upon van't Hoff analysis of denaturation have been developed as alternatives to calorimetry. Additionally, small proteins, such as turkey ovomucoid third domain (OMTKY3), have small values of ΔC_p (Alexander et al., 1992) that are difficult to measure directly due to fitting errors in the baselines of a single calorimetric scan.

Two spectroscopic methods have been employed in our study. Specifically, data from thermal denaturation experiments are used to construct plots of ΔH_m (van't Hoff) versus T_m; the slope of the resulting line is ΔC_p. These spectroscopic thermal denaturation experiments have also been analyzed by fitting nearly all data simultaneously to an equation that includes the temperature dependence for the enthalpy of unfolding. ΔC_p may also be determined by fitting plots of ΔG_u versus T to Equation 1. Data for such plots are generated by chemical denaturation and subsequent linear extrapolation of ΔG_u to zero denaturant concentration, as described by Pace and Laurents (1989). The small value of ΔC_p anticipated for ovomucoid third domain (Alexander et al., 1992) should provide a good test of the limits of these spectroscopic determinations of ΔC_p. In order to measure a small value, accurate data must be obtained over a wide temperature range.

Several characteristics of ovomucoid third domain make it attractive for studies of protein stability. Ovomucoid third domain is one of three domains in a serine proteinase inhibitor easily purified in large quantities from avian egg white. Protein sequences of 125 third domains have been determined (Laskowski et al., 1987, 1990), providing a large sequence database that facilitates extensive studies of protein stability. We have chosen to begin our investigations with the turkey variant (OMTKY3). OMTKY3 is highly soluble and stable over a wide range of pH and temperature (Ardelt & Laskowski, 1991), and its structure is well characterized both in the solid state and in solution (Read et al., 1983; Read & James, 1986; Robertson et al., 1988). Whole ovomucoid and its third domains have proven to be propitious for biophysical investigations (Waheed et al., 1977; Baig et al., 1978; Watanabe et al., 1981; Matsuda et al., 1981a,b; Griko & Privalov, 1984; Hildebrandt et al., 1988; Das et al., 1990, 1991; Ardelt & Laskowski, 1991). The present studies, while focused on determination of ΔC_p for OMTKY3, have also provided opportunities to test and develop methods for characterization of the thermodynamics of unfolding.

Results

Our thermodynamic characterization of OMTKY3 stability relies upon van't Hoff analysis of denaturation, which is predicated upon three assumptions. First, the unfolding reaction must be two-state with no stable intermediates. Second, ΔC_p is independent of temperature (Privalov, 1979, 1990; Schellman & Hawkes, 1980; Pace & Laurents, 1989; Griko & Privalov, 1992). Finally, the effect of ΔC_p on the enthalpy of unfolding is assumed to be negligible over the transition region of an unfolding curve, allowing the determination of ΔH_m from the slope of the plot of ln K versus $1/T$. We have performed several experiments in order to test assumptions 1 and 3 and to optimize the reaction conditions.

Thermal denaturation

OMTKY3 was heated at a variety of rates during thermal denaturation in order to identify conditions that permitted equilibration of the unfolding reaction while maximizing the reversibility of unfolding. Data were fit to the equation

$$y_{obs} = \frac{(y_n + m_n T) + (y_d + m_d T)\{\exp[\Delta H_m/R \cdot (1/T_m - 1/T)]\}}{\{1 + \exp[\Delta H_m/R \cdot (1/T_m - 1/T)]\}} \quad (2)$$

(see Materials and methods for derivation). In Equation 2, y_{obs} is an experimental observable, T is the temperature in Kelvin, R is the gas constant, and ΔH_m and T_m are as defined previously. The parameters y_n and y_d refer to the y-intercepts of the native and denatured baselines, respectively, while m_n and m_d are the slopes of the baselines. The results of these experiments are summarized in Table 1. Values of T_m and ΔH_m converged at scan rates of 0.5 °C/min and 0.25 °C/min, while lower reversibility was obtained with the slower rate. Therefore, a heating rate of 0.5 °C/min was used for all subsequent experiments. Reversibility was optimal when the sample was cooled as rapidly as possible. Reported confidence limits in these and all subsequent experiments are errors for the fit; experimental errors are similar to fitting errors (Table 1).

Two types of experiments were performed to test the assumption that denaturation of OMTKY3 is a two-state process. First, a series of CD spectra were collected at temperatures in the thermal transition zone (Fig. 1). All spectra except that for 90 °C have isodichroic points at 229 nm and 284 nm, which is consistent with a two-state process. During the extended time (several hours) at 90 °C, OMTKY3 undergoes irreversible denaturation, causing the deviation from these points.

A second test of the two-state unfolding hypothesis is thermal denaturation monitored with different probes. To be consistent with this hypothesis, the resulting curves must be coincident when expressed as apparent fraction native versus temperature (Kim & Baldwin, 1982). We have monitored thermal denaturation of OMTKY3 with CD at 222 nm, which reports on secondary structure, and

Table 1. *Optimization of heating rate*[a]

Rate (°C/min)	ΔH_m (kcal/mol)	T_m (°C)	% Reversibility
10	36.3 (1.3)	68.5 (0.5)	~100
5	39.2 (1.3)	66.8 (0.3)	~100
2	41.2 (1.1)	64.5 (0.3)	98.1
	42.7 (1.4)	64.6 (0.3)[b]	99.5
1	41.8 (1.1)	63.9 (0.3)	98.1
	41.0 (1.2)	64.0 (0.2)	96.2[c]
	41.8 (1.2)	64.1 (0.2)	98.4[d]
0.5	42.8 (1.8)	63.2 (0.3)	94.5
	42.9 (1.3)	63.3 (0.2)	96.5[d]
0.25	42.9 (1.2)	63.4 (0.2)	92.1[d]

[a] Samples contained 20–30 μM OMTKY3, 100 mM KCl, pH 2.0. Unless otherwise indicated, the cooling rate is equal to the heating rate. Denaturation was monitored with CD at 222 nm. Numbers in parentheses indicate confidence intervals at one standard deviation. Experimental error in T_m is 0.4 °C.

[b] A second thermal denaturation of this sample yielded statistically identical results.

[c] This sample remained at the maximum temperature (95 °C) for 10 min longer than other samples.

[d] The cooling rate was 100 °C/min.

290 nm, which is a measure of tertiary structure (Adler et al., 1973). We have also monitored denaturation at 238 nm, which probably reports changes in the environment around Tyr 31 (see Kinemage 1; Swint & Robertson, unpubl. results). Figure 2 contains raw data as well as curves representing the best fit to Equation 2. The data points in Figure 2B and C are mostly obscured by the fitted curve; the quality of the data is typical of all the thermal denaturation experiments in our studies. The normalized curves are coincident, demonstrating that unfolding is probably a two-state process (Fig. 2D).

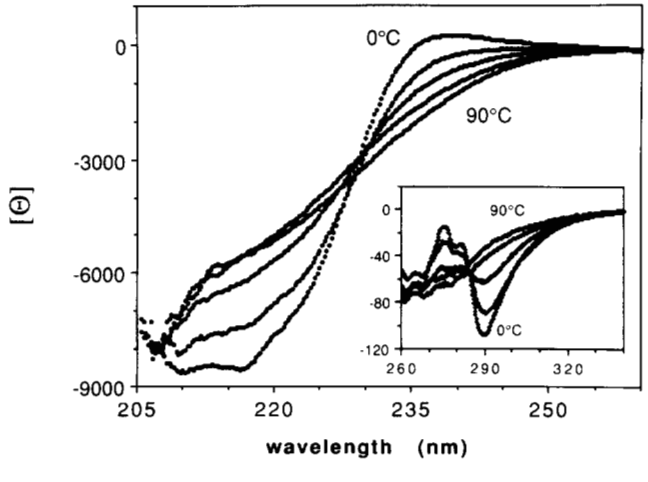

Fig. 1. CD spectra of OMTKY3. Samples contained 0.1 mg/mL and 2 mg/mL (inset) OMTKY3, pH 2.0, and spectra were taken at 0, 50, 60, 70, and 90 °C. The units of $[\theta]$ are deg/(cm^2·dmol residue).

We have also examined the concentration dependence for the thermal denaturation of OMTKY3. As can be seen in Figure 3, ΔH_m and T_m are invariant over a range of protein concentrations spanning approximately 2.5 orders of magnitude. The constant value of T_m indicates that the protein is behaving as a monomer (Sturtevant, 1987) and is consistent with the results of sedimentation equilibrium studies in which OMTKY3 at pH 2.0 behaves as a monomer over a similar range of protein concentrations (Jeffrey C. Hansen, pers. comm.). All errors in the fits of T_m are less than the 0.4 °C experimental error of the temperature probe. ΔH_m appears to be larger in the thermal transitions monitored at 222 nm (Fig. 3), which is observed in subsequent experiments as well, but the difference is not statistically significant at two standard deviations. The weighted means of ΔH_m and T_m for OMTKY3 at pH 2.0, no buffer, are 40.8 ± 0.3 kcal/mol and 58.6 ± 0.4 °C. Renaturation in these experiments is usually greater than 90%. OMTKY3 thus appears to undergo a two-state thermal denaturation that is independent of protein concentration.

Determination of ΔC_p

Two complementary spectroscopic approaches have been taken in order to determine ΔC_p for OMTKY3. ΔC_p may be determined from the slope of plots of ΔH_m versus T_m or from the second derivative of plots of ΔG_u versus T (Equation 1).

Perturbation of T_m with pH

The CD signal at 238 nm was chosen for monitoring the thermal denaturation of OMTKY3 as a function of pH. While the magnitude of the signal change at 238 nm is smaller than that observed at 222 nm, the scatter in the data is much lower at 238 nm. The pH of the samples ranged from 1.5 to 3.0; above pH 3.0, the thermal transition was incomplete even at 90 °C. The pH of the samples did not change significantly (less than 0.1 pH units) over the experimental temperature range. The results of the fits to Equation 2 are summarized in Table 2. The slope of ΔH_m versus T_m, as determined by weighted linear regression, yields a ΔC_p of 870 ± 170 cal/(mol·K) (Fig. 4, ▲). The weighting factor for individual data points was $1/(\sigma_{\Delta H_m})^2$, which we presume also includes errors in T_m.

Because much of our future work will involve NMR studies of OMTKY3 dissolved in D_2O, we have examined the effects of D_2O on the thermodynamics of unfolding and found no significant change in ΔC_p, 780 ± 250 cal/(mol·K) (Table 2; Fig. 4, △). Interestingly, ΔH_m of OMTKY3 is increased in D_2O when compared to a sample in H_2O at the same temperature. Also, at any given pH, stability appears to increase in the presence of D_2O. Similar behavior has been observed for a number of other

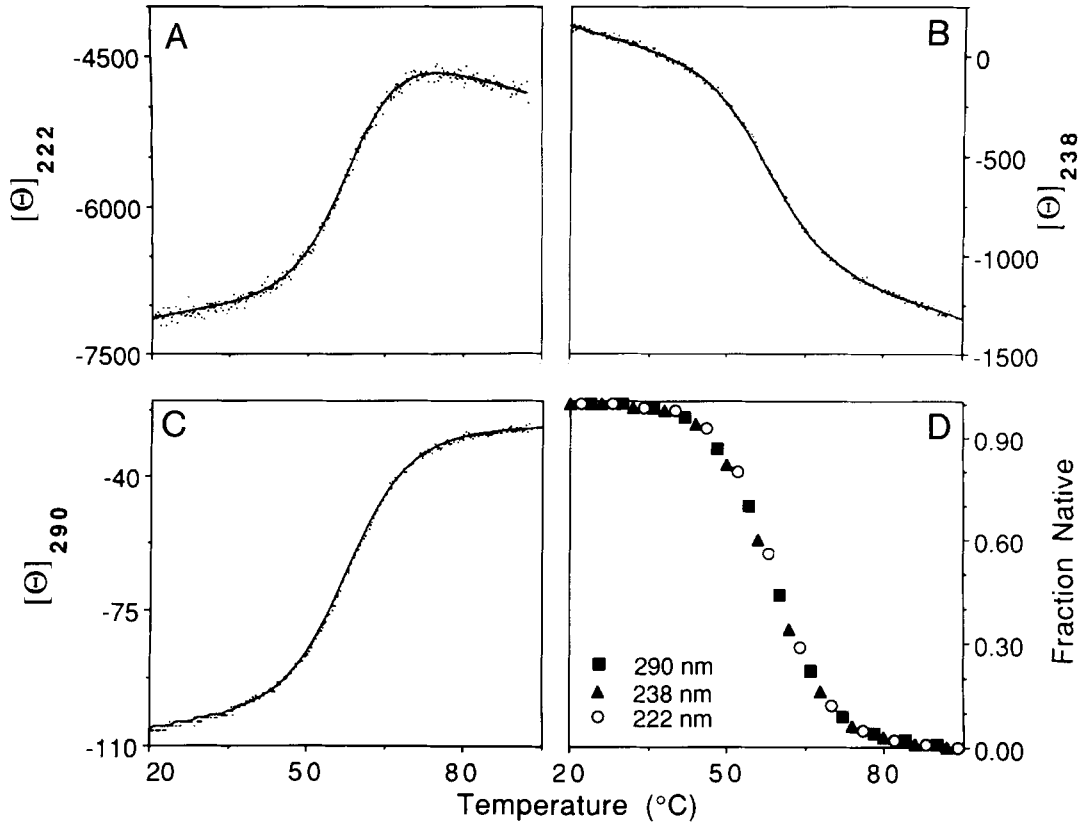

Fig. 2. Thermal denaturation of OMTKY3. Thermal denaturation was monitored with CD at **(A)** 222, **(B)** 238, and **(C)** 290 nm. Sample concentrations were 0.1, 0.3, and 8.0 mg/mL, respectively, pH 2.0. Samples contained no buffer or salt beyond that used to adjust the pH. The units of $[\theta]$ are deg/(cm^2·dmol residue). Solid lines represent the best fits to Equation 5; many data points are obscured by these lines. **D:** Fraction native versus temperature curves were generated from the fits in A–C.

proteins (Hermans & Scheraga, 1959; Lemm & Wenzel, 1981; Kenneth P. Murphy, pers. comm.). The difference in T_m values may also be due, in part, to isotope effects on the measured pH values (Glasoe & Long, 1960; Appel & Yang, 1965).

Perturbation of T_m with combinations of denaturant and pH

Initial attempts to chemically denature OMTKY3 were complicated by its extraordinary resistance to chemical denaturation. Native OMTKY3 is stable in 7 M GuHCl, pH 2.0, at temperatures below 10 °C. We have taken advantage of this stability by using GuHCl and urea in conjunction with pH as perturbants of T_m.

Previously described controls were repeated for samples containing GuHCl. The heating and cooling rates chosen for experiments performed in the absence of chemical denaturant were found to be valid in the presence of 6 M GuHCl. When OMTKY3 is subjected to thermal denaturation in the presence of 6 M GuHCl, a single isodichroic point is observed at 232 nm; this red shift from the previous point at 229 nm is expected from known solvent effects on absorption spectra (Donovan, 1969). The near-UV isodichroic point disappears in the presence of GuHCl. Thermal transitions in 3, 6, and 7 M GuHCl monitored at 222, 238, and 290 nm are coincident (Table 3). Thermal denaturation of OMTKY3 thus appears to be a two-state process in the presence of GuHCl.

Thermal denaturation of OMTKY3 in 5.9 M GuHCl, with pH varied from 1.5 to 4.8, was monitored with CD at 238 nm. Data were fit to Equation 2 (Table 4). Reversibility in these experiments is typically between 70 and 85%. Weighted linear regression of a plot of ΔH_m versus T_m yields an apparent ΔC_p of 600 ± 110 cal/(mol·K) (Fig. 4, ○). Data from thermal denaturation experiments performed in various concentrations of GuHCl at pH 2 also fall on this line (Table 3).

Thermal denaturation was performed in the presence of 1–7 M GuHCl and monitored with CD at 222, 238, and 290 nm (Table 3). Weighted linear regression of these data yields a value of 670 ± 70 cal/(mol·K) for ΔC_p. An examination of the residuals reveals a systematic deviation in the data obtained at 222 nm (Fig. 4, □), which appear to fall on an independent line when compared to the data obtained at 238 and 290 nm (Fig. 4, ●). This trend is the

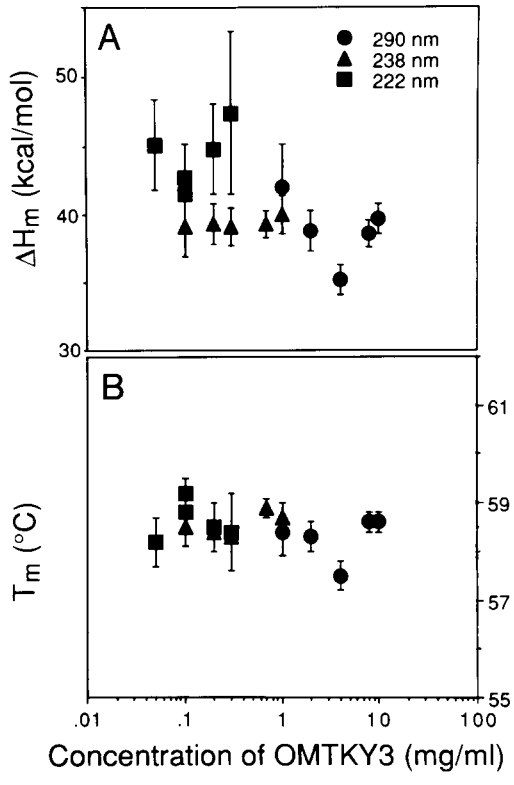

Fig. 3. ΔH_m **(A)** and T_m **(B)** versus concentration of OMTKY3. Thermal denaturation was performed in the absence of buffer or added salt at pH 2.0 and monitored with CD at the indicated wavelength. Error bars represent confidence limits at one standard deviation.

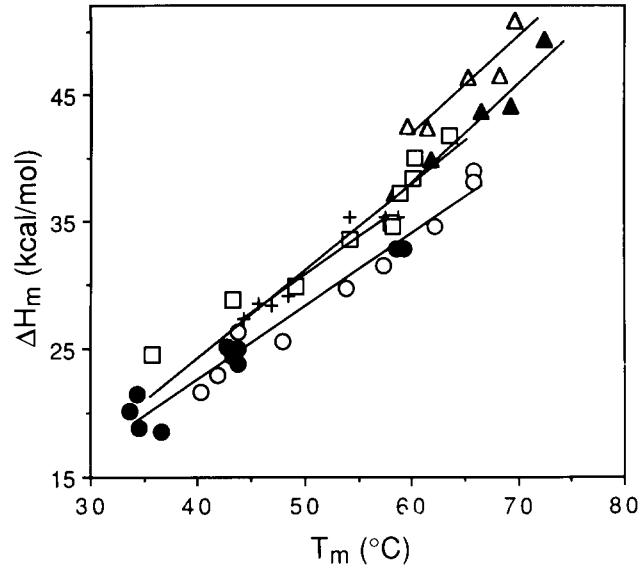

Fig. 4. Determination of ΔC_p for OMTKY3. Thermal denaturation was performed under a variety of conditions in order to obtain data for plots of ΔH_m versus T_m. Weighted linear regression was used to fit each line; ΔC_p for each was determined from the slope. Experimental specifics are found in the respective tables. ▲, Varied pH in H_2O, ΔC_p = 870 ± 170 cal/(mol·K). △, Varied pH* in D_2O, ΔC_p = 780 ± 250 cal/(mol·K). ○, Varied pH in 5.9 M GuHCl, ΔC_p = 600 ± 110 cal/(mol·K). ●, Varied concentrations of GuHCl; thermal denaturation monitored with CD at 238 and 290 nm, ΔC_p = 580 ± 30 cal/(mol·K). □, Varied concentrations of GuHCl; thermal denaturation monitored with CD at 222 nm, ΔC_p = 760 ± 160 cal/(mol·K). +, Varied concentrations of urea, ΔC_p = 610 ± 120 cal/(mol·K).

same as that previously noted in the test of the two-state unfolding assumption. Although intriguing, the present experimental methods are not sufficiently sensitive to determine whether this trend is significant. Values of ΔC_p from both data at 222 nm alone, 760 ± 160 cal/(mol·K) (Fig. 4, □), and at 238 and 290 nm, 580 ± 30 cal/(mol·K) (Fig. 4, ●), are not significantly different from any other calculated values.

OMTKY3 was also thermally denatured in the presence of 1–8 M urea (Table 5), and an apparent ΔC_p of 610 ± 120 cal/(mol·K) was obtained (Fig. 4, +). Percent reversibility at 6, 7, and 8 M urea is excellent, but denaturation in lower concentrations is essentially irreversible. However, when the 7 M sample was subjected to a second thermal denaturation, the thermodynamic parameters were statistically identical to the first denaturation experiment even though the second denaturation was essentially irreversible. This observation suggests that the irreversible denaturation occurs after the unfolding process is complete, and that the thermodynamic data obtained at lower urea concentrations are valid. Also, the value of ΔC_p obtained is in good agreement with those from other experiments.

Table 2. *Perturbation of T_m with pH*[a]

Solvent	pH	ΔH_m (kcal/mol)	T_m (°C)	% Reversibility
H_2O	1.5	37.1 (1.7)	58.3	94
	2.0	39.9 (1.4)	62.0	83
	2.5	43.8 (1.1)	66.7	76
	2.8	44.2 (1.5)	69.4	71
	3.0	49.5 (1.8)	72.5	79
D_2O	*1.4[b]	42.5 (1.5)	59.6	97
	*2.0	42.4 (1.4)	61.5	96
	*2.4	46.3 (1.3)	65.5	95
	*2.6	46.6 (1.4)	68.2	90
	*2.8	50.9 (1.8)	69.8	90

[a] Experiments were performed in 10 mM each potassium phosphate and potassium acetate and monitored with CD at 238 nm. OMTKY3 concentration was 0.3 mg/mL. Numbers in parentheses represent confidence intervals at one standard deviation. Error in T_m was less than the experimental error of 0.4 °C.

[b] Asterisk indicates apparent pH, without correction for isotope effects, of an experiment performed in D_2O.

Global analysis of ΔC_p

The weighted average of the six ΔC_p values determined by van't Hoff analysis is 590 ± 25 cal/(mol·K). Clearly, the values of both the mean ΔC_p and its confidence lim-

Table 3. *Thermal denaturation of OMTKY3 in GuHCl*[a]

[GuHCl] (M)	λ (nm)	[OMTKY3] (mg/mL)	ΔH_m (kcal/mol)	T_m (°C)	% Reversibility
1.0	222	0.1	41.9 (1.9)	63.6 (0.4)	77
1.1	238	0.3	37.3 (1.3)	66.0 (0.2)	82
	290	2.5	–	–	0[b]
2.0	222	0.1	40.0 (2.0)	60.3 (0.4)	58
2.25	222	0.1	38.5 (2.5)	60.2 (0.6)	73
2.5	222	0.1	37.3 (2.5)	58.9 (0.6)	42
2.75	222	0.1	34.9 (2.0)	58.1 (0.6)	54
3.0	222	0.1	34.6 (1.7)	58.2 (0.6)	89
	290	1.5	32.9 (1.7)	58.5 (0.5)	96
3.1	238	0.3	32.9 (1.6)	59.1 (0.4)	89
	290	2.5	–	–	0[b]
4.0	222	0.1	33.5 (1.7)	54.2 (0.4)	89
5.0	222	0.1	29.9 (1.7)	49.2 (0.6)	86
6.0	222	0.1	28.8 (2.0)	43.3 (0.7)	82
	238	0.3	23.8 (1.7)	43.7 (0.9)	91
	238[c]	0.3	25.2 (2.7)	42.8 (1.3)	78
	238[d]	0.3	25.0 (2.0)	43.8 (1.0)	91
	290	2.5	24.5 (0.9)	43.3 (0.4)	88
7.0	222	0.1	24.6 (2.3)	35.7 (1.0)	81
	238	0.3	18.9 (1.8)	34.7 (2.5)	83
	238	1.0	18.6 (1.5)	36.7 (1.4)	82
	290	10.0	21.5 (0.6)	34.3 (0.5)	91
7.1	290	2.1	20.1 (1.2)	33.8 (0.9)	*97

[a] Samples contained 0.1 M KCl, pH 2.0, and denaturation was monitored with CD at the indicated wavelength. Numbers in parentheses represent confidence intervals at one standard deviation. The experimental error in T_m is 0.4 °C. Percent renaturation was calculated between native and denatured baselines at 0 °C, except (*), where it was calculated at 5 °C.
[b] Protein precipitated during experiment.
[c] Heating rate was 1 °C/min.
[d] Heating rate was 0.25 °C/min.

Table 4. *Perturbation of T_m with pH in 5.9 M GuHCl*[a]

pH	ΔH_m (kcal/mol)	T_m (°C)	% Reversibility
1.5	22.9 (1.7)	42.1 (1.0)	76
	21.7 (1.6)[b]	40.3 (1.0)	100
1.9	26.3 (2.1)	43.8 (0.9)	70
2.4	25.6 (1.6)	48.0 (0.7)	81
2.9	29.8 (1.8)	51.8 (0.6)	79
3.5	31.5 (1.3)	57.5 (0.4)	86
4.0	34.6 (1.3)	62.3 (0.3)	85
4.8	39.0 (1.4)	66.0 (0.3)	82
	38.1 (1.5)[b]	66.0 (0.4)	84

[a] Samples contained 10 mM each potassium phosphate and potassium acetate, 5.9 M GuHCl, and 0.3 mg/mL OMTKY3. Thermal denaturation was monitored with CD at 238 nm. Numbers in parentheses represent confidence intervals at one standard deviation. Experimental error in T_m is 0.4 °C.
[b] Second thermal denaturation performed on the same sample.

its are skewed by the value of 580 ± 30 cal/(mol·K) (Fig. 4). This mean value does fall within one confidence interval of four experimental determinations and is within two confidence intervals of the remaining two, so we consider it a good estimation. The confidence limits for most of the determinations of ΔC_p were roughly 20%, so we feel that more reasonable confidence limits for the mean value are of this magnitude, or ± 120 cal/(mol·K).

The data from the thermal denaturations were also fit simultaneously to Equation 3 (at bottom of page; see Materials and Methods for derivation). Unique values of the curve parameters y_n, m_n, y_d, m_d, ΔH_m, and T_m were determined for each thermal denaturation experiment within the larger set, whereas the value of ΔC_p was common to all experiments. This approach has the additional advantage of including the temperature dependence for the enthalpy of unfolding and only relies upon the assumptions of two-state denaturation and the temperature independence of ΔC_p. Several data sets are necessary for convergence of ΔC_p.

In most cases, values of y_n, m_n, y_d, m_d, ΔH_m, and T_m from global fits are statistically identical to those obtained from individual fits, although the confidence limits are up to 2.5 times larger. In the "super global fit" of 42 thermal denaturation experiments, the maximum possible due to computer limitations, confidence limits on the parameters are up to five times as large as those for individual fits. In cases where the values of T_m determined by individual fits are low, the global fits skewed both T_m and ΔH_m to even lower values. This phenomenon probably results from both uncertainties in the data, due to short native baselines and similar slopes for the baseline and transition regions, and bias in the fitting procedure toward denaturation experiments with larger values of T_m and ΔH_m.

Global fits of ΔC_p converged on real (nonzero) values that agree with values derived from van't Hoff analysis and have reasonable confidence intervals. A value of 910 ± 210 cal/(mol·K) was determined for the experiments in which T_m was perturbed with pH. The global fit of changing pH* in D_2O did not converge to a reasonable value of ΔC_p, probably due to the limited temperature range of T_m from these experiments. Denaturation in which pH was changed in the presence of 5.9 M GuHCl yields a value of 610 ± 110 cal/(mol·K). For experiments in which the concentration of GuHCl was changed with thermal denaturation monitored at 222 nm, the value of ΔC_p obtained by a global fit is 630 ± 140 cal/(mol·K),

$$y_{obs} = \frac{(y_n + m_nT) + (y_d + m_dT)\cdot\exp\{(\Delta C_p\cdot((T_m/T - 1) + \ln(T/T_m))] - [\Delta H_m\cdot(T_m/T - 1)])/R\}}{1 + \exp\{([\Delta C_p\cdot((T_m/T - 1) + \ln(T/T_m))] - [\Delta H_m\cdot(T_m/T - 1)])/R\}} \quad (3)$$

Table 5. *Thermal denaturation of OMTKY3 in urea*[a]

Urea (M)	ΔH_m (kcal/mol)	T_m (°C)	% Reversibility
1	35.3 (1.7)	58.8 (0.4)	*[c]
2	35.4 (1.4)	57.5 (0.4)	*
3	35.4 (1.4)	54.1 (0.3)	*
6	29.2 (1.4)	48.6 (0.4)	92[d]
7	28.6 (1.9)	45.5 (0.7)	~100
	28.4 (2.0)[b]	47.0 (0.7)	*
8	27.4 (2.4)	44.1 (0.9)	~100

[a] Samples contained 0.3 mg/mL OMTKY3, pH 2.0. Thermal denaturation was monitored with CD at 238 nm. Numbers in parentheses represent confidence intervals at one standard deviation. Experimental error in T_m is 0.4 °C.

[b] Repeated thermal denaturation performed on same sample.

[c] Asterisk indicates almost 100% irreversibility. See text for discussion.

[d] Percent renaturation in 6 M urea was determined by denaturing the same sample a second time.

while the denaturation monitored at 238 and 290 nm yields a value of 560 ± 50 cal/(mol·K). From thermal denaturation experiments in the presence of changing urea concentrations, a value of 860 ± 170 cal/(mol·K) was obtained. The "super global fit" from 42 of the thermal denaturation experiments was also performed and yielded a value of 640 ± 110 cal/(mol·K).

ΔC_p determined by linear extrapolation in the presence of denaturants

We also attempted to measure ΔC_p for OMTKY3 using the approach developed by Pace and Laurents (1989). This approach, which involves measuring ΔG_u at a variety of temperatures using the linear extrapolation method, proved impractical in its original form because of the high stability of OMTKY3 toward chemical denaturation. We instead employed an approach in which thermal denaturation experiments are performed in the presence of varying concentrations of denaturant; this provides the same type of information, free energies of unfolding as a function of temperature and denaturant concentration, after transposition of the data.

OMTKY3 in 0.1 M KCl, pH 2.0, was subjected to thermal denaturation in the presence of 0–7 M GuHCl and monitored with CD at 222 nm. The data were fit to Equation 2 and results are presented in Table 3. The parameters in Table 3 were used to generate curves in which the fraction of native OMTKY3 is plotted as a function of temperature. Isothermal slices were then taken through these curves at temperatures where there were at least four data points having fraction native values between 0.95 and 0.05. After conversion of the fraction native to a free energy of unfolding (ΔG_u), the data were replotted as ΔG_u versus the concentration of denaturant to obtain the

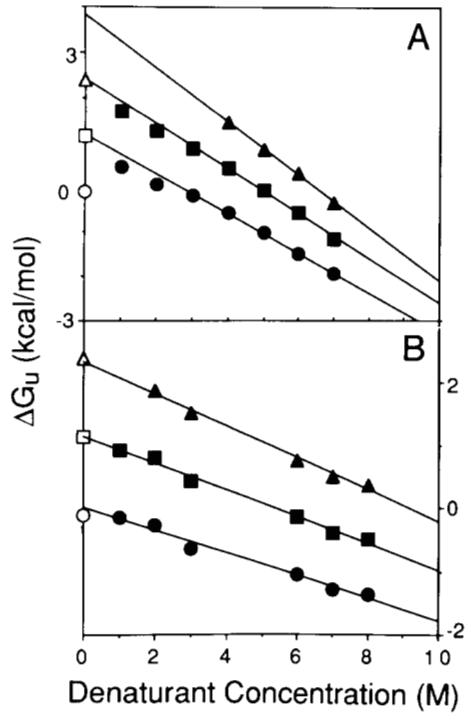

Fig. 5. Linear extrapolation of ΔG_u to zero denaturant concentration at 40 °C (▲), 50 °C (■), and 60 °C (●). Filled symbols represent ΔG_u calculated in the presence of denaturant. Open symbols are values of ΔG_u obtained from thermal denaturation of OMTKY3, pH 2.0, in the absence of any salt, buffer, or denaturant. **A:** Samples contained 1–7 M GuHCl, 0.1 mg/mL OMTKY3, 0.1 M KCl, pH 2.0. Thermal denaturation was monitored with CD at 222 nm. The extrapolated values of ΔG_u are 3.8, 2.4, and 1.2 kcal/mol at 40, 50, and 60 °C, respectively. The directly measured values at these temperatures are 2.4, 1.1, and −0.1 kcal/mol. **B:** Samples contained 1–8 M urea, 0.3 mg/mL OMTKY3, pH 2.0. Thermal denaturation was monitored with CD at 238 nm. Linear regression was used to fit all data; extrapolated values at the three temperatures are 2.3, 1.2, and 0.0 kcal/mol.

extrapolated free energy of unfolding at zero denaturant concentration (Fig. 5A). The error in calculated and extrapolated values of ΔG_u is approximately ±5%. The plots of ΔG_u as a function of GuHCl cover an extraordinary range of denaturant concentrations.

The plots of ΔG_u versus denaturant concentration are linear over the range of 1–7 M GuHCl. However, we have reason to believe that the small deviations below 2.75 M GuHCl, though not statistically significant, may be real. Santoro and Bolen (1992) observed a similar phenomenon when thioredoxin was thermally denatured in the presence of low GuHCl concentrations, which they attributed to changing ionic strength. Increasing ionic strength has a noticeable stabilizing effect on OMTKY3 (data not shown). We attempted to control for ionic strength effects on OMTKY3 in two ways. Several early experiments were performed in the presence of 0.1 M KCl (Tables 1, 3), but this salt concentration is not high enough to fully compensate for molar changes in GuHCl. We next attempted

to perform thermal denaturation experiments in 3 M isoionic solutions of GuHCl and KCl, but OMTKY3 precipitated during the experiments. Because the presence of KCl has deleterious effects on the solubility of OMTKY3 and the reversibility of unfolding, KCl was left out of subsequent samples.

Free energies resulting from extrapolation to zero denaturant were replotted as ΔG_u versus temperature. Both the extrapolated ΔG_u values (Fig. 5A) and T_m (>66 °C) are larger than the directly measured values in the absence of both denaturant and salt (Fig. 5A; T_m of 58.4 °C). This difference is probably due to the high ionic strength of GuHCl solutions. In fact, values obtained from thermal denaturation in 0.1 M KCl alone approach the GuHCl extrapolated values: ΔG_u is 1.7 kcal/mol at 50 °C, and T_m is 63.4 °C. However, we reasoned that ΔC_p is unlikely to be affected significantly by the salt effect exerted by GuHCl (Privalov, 1979, 1990; Griko & Privalov, 1992) and determinations of ΔC_p from these data are likely to be valid. Ideally, a plot of ΔG_u versus temperature results in a parabolic curve whose second derivative is ΔC_p (Pace & Laurents, 1989). The data for OMTKY3, however, fall on an apparently linear region of the stability curve. We were thus unable to determine a ΔC_p value with this approach.

A similar result was obtained when OMTKY3 was thermally denatured in the presence of 1–8 M urea (Table 5). Extrapolations were linear over the ranges of 1–8 M urea and 29–77 °C (Fig. 5B). Unfortunately, the plot of ΔG_u versus temperature was also linear. However, both the extrapolated ΔG_u values (Fig. 5B) as well as the T_m (approximately 60 °C) are statistically identical to values of ΔG_u and T_m obtained by thermal denaturation in the absence of denaturant and salt.

Discussion

Both van't Hoff analysis and global fits of the spectroscopic thermal denaturation experiments provide reasonable and statistically indistinguishable estimates of ΔC_p, 590 ± 120 and 640 ± 110 cal/(mol·K), respectively. Therefore, the assumption that ΔC_p has a negligible effect on ΔH_m over the temperature range of unfolding, allowing its determination from the slope of the plot of ln K versus $1/T$, is valid for OMTKY3. For the sake of simplicity, the remainder of the discussion will employ the values of ΔC_p obtained by this analysis.

We can now predict ΔG_u for OMTKY3 with Equation 1, using the mean ΔC_p of 590 ± 120 cal/(mol·K) and ΔH_m and T_m from a single thermal denaturation experiment in the desired solution conditions. The spread of the six ΔC_p determinations used to calculate the mean is only 300 cal/(mol·K), which compares favorably to the precision of spectroscopic and calorimetric determinations of ΔC_p for other proteins (Privalov et al., 1986; Pace & Laurents, 1989). Because ΔC_p for OMTKY3 is small, ΔG_u versus T will appear linear over the temperature range in which most future experiments will be performed, and stability curves generated from different values of ΔC_p are coincident within the error of our measurements. Practically speaking, all six measured values of ΔC_p are therefore equivalent. The stability curves generated by our extreme values of ΔC_p, 870 and 580 cal/(mol·K), differ by less than 0.5 kcal/mol at temperatures above 25 °C for OMTKY3 at pH 2.0. One origin of the spread may be from the dependence of ΔC_p on solution conditions or temperature, but such an effect would have to be larger than 300 cal/(mol·K), or roughly twice the confidence interval of most ΔC_p determinations, in order to be statistically distinguishable. Therefore, within the error of the measurements, ΔC_p for OMTKY3 is the same in both the presence and absence of chemical denaturants.

The value of ΔC_p we have obtained, 590 ± 120 cal/(mol·K), is in reasonable agreement with previous calorimetric studies of the closely related chicken ovomucoid third domain (OMCHI3) (Griko & Privalov, 1984). OMTKY3 differs from OMCHI3 by only three solvent exposed amino acid residues (Laskowski et al., 1987). Griko and Privalov (1984) determined that ΔC_p is 2.2 ± 0.5 kcal/(mol·K) for intact chicken ovomucoid. Since third domain is one of three homologous domains, we assume that one domain contributes approximately one-third of the total ΔC_p, or 730 cal/(mol·K). We have also calculated values of ΔC_p that range from 910 to 515 cal/(mol·K) from the enthalpy data for OMCHI3; these bracket our range of ΔC_p values for OMTKY3.

Several models have been developed in order to explain the structural features contributing to the difference in heat capacity between native and denatured proteins. ΔC_p has been correlated with changes, resulting from denaturation, in the solvent-accessible polar (ΔA_p) and nonpolar (ΔA_{np}) surface areas of the protein (Livingstone et al., 1991; Murphy & Gill, 1991; Spolar et al., 1992). We have chosen to use the simplest model, that of Murphy and Gill, which is based upon amino acid composition. They modeled ΔC_p with the dissolution of crystalline cyclic dipeptides in water, derived relationships between various protein moieties and ΔC_p, and accurately predicted ΔC_p of four well-characterized proteins. Applying this model to OMTKY3, we calculated a value of 649 cal/(mol·K) for ΔC_p, which is well within the range of our experimental determinations.

OMTKY3 is similar in size to bovine pancreatic trypsin inhibitor (BPTI), which has 58 amino acids and three disulfide bonds, and to tendamistat, with 74 amino acids and two disulfide bonds. Values of ΔC_p for both BPTI, approximately 400 cal/(mol·K) at 87 °C, and tendamistat, 690 cal/(mol·K), are also similar to that of OMTKY3 (Renner et al., 1992; Kim et al., 1993). The small values of ΔC_p for these proteins result in broad stability curves and high values of T_m similar to those of

other small globular proteins, the IgG-binding domains B1 and B2 of streptococcal protein G, studied by Alexander et al. (1992). They also compared ΔC_p per residue for 10 small, well-characterized proteins that yielded an average value of 14 ± 2 cal/(mol·K) per residue (Alexander et al., 1992). The low value for OMTKY3, 11 ± 2 cal/(mol·K) per residue, may be due to the three disulfide bonds in OMTKY3 (Kinemage 1) and a concomitant reduction in ΔA_{np} and ΔC_p (Doig & Williams, 1991).

Privalov and Khechinashvili (1974) have noted that when the enthalpy of unfolding is reported per mole of amino acid residue, the values for globular proteins converge to a common value at a temperature near 100 °C. Murphy and Gill (1991) have made further calculations, using their own data from solid model compounds in conjunction with the data of Privalov and Khechinashvili, to arrive at an average value of 1.35 ± 0.11 kcal/(mol residue) at 100.5 °C. Extrapolations for OMTKY3 using the values of ΔC_p determined in this study (Fig. 4) generated a range of values from 1.03 to 1.31 kcal/(mol residue), which is again in reasonable agreement with values for other globular proteins. The specific entropy of unfolding converges to 4.3 ± 0.1 cal/(mol residue·K) at about 112 °C (Baldwin, 1986; Murphy et al., 1990). For OMTKY3, the extrapolated value of ΔS at 112 °C is comparable, 3.7 cal/(mol residue·K) with an estimated uncertainty of 1 cal/(mol residue·K).

The pH dependence of OMTKY3 stability is also consistent with our expectations. As described by Wyman (1964), the linkage relationship between changes in pH and protein stability is

$$d \ln K/d \ln a_H = n_{H,D} - n_{H,N} = \Delta n_H, \quad (4)$$

where Δn_H is the difference in the number of proton binding sites between native and denatured protein. For OMTKY3, Δn_H is 1 or less, which is consistent with the pH range (1.5–3.0) of our experimental conditions; most sites on the protein are protonated in this range. Two amino acids in OMTKY3, Asp 27 and the carboxy-terminal Cys 56, have pK_a values less than 3 (Ortiz-Polo, 1985; Robertson, 1988; Schaller & Robertson, unpubl. results), which are probably responsible for this result (Kinemage 1).

OMTKY3 does appear unusual in its extreme resistance to chemical denaturation. In an attempt to understand this behavior, we employed the analysis of Tanford (1970), who extended Equation 4 to a more general form that describes protein interactions with chemical denaturants:

$$d \ln K/d \ln a_X = n_{X,D} - n_{X,N} = \Delta n_X. \quad (5)$$

The parameter Δn_X has been recently defined by Timasheff (1992) to be "the contribution of newly exposed groups to the total thermodynamic interaction of the protein with solvent components both at exchangeable and nonexchangeable sites." By this definition, Δn_X is descriptive of interactions between OMTKY3 and chemical denaturants regardless of whether denaturation is caused by specific binding events (Schellman, 1976) or is due to more general changes in solvent properties (Alonso & Dill, 1991).

The activity coefficient for GuHCl is calculated with the following equation (Aune & Tanford, 1969; Pace & Vanderburg, 1979):

$$a_{GuHCl} = 0.6761 \cdot M - 0.1468 \cdot M^2 + 0.02475 \cdot M^3 + 0.001318 \cdot M^4. \quad (6)$$

The plus sign in the last term of Equation 6 as reported in Pace and Vanderburg (1979) is correct; in the paper by Pace (1986), this sign was inadvertently changed to a minus. We have derived an equation for urea activity from the data of Stokes (1967) for solutions at 40 °C:

$$a_{urea} = -7.79 \cdot 10^{-2} + 0.990 \cdot M. \quad (7)$$

For OMTKY3, the slopes of ln K versus ln a_{GuHCl} or ln a_{urea} are temperature dependent. This effect may be the result of the temperature dependence of the activity coefficient, a_X (Makhatadze & Privalov, 1992). However, the highest values of Δn_{GuHCl} and Δn_{urea} are 2 and 2.5, respectively, which is consistent with our observation that OMTKY3 is not easily unfolded by chemical denaturants.

We were somewhat surprised when ΔC_p values calculated from experiments in which samples contained different denaturant concentrations were equivalent to other determinations. Perturbation of T_m with chemical denaturant has been suggested by Santoro and Bolen (1992), but they have not compared the resulting ΔC_p with values determined by more conventional methods. Both calorimetric experiments and theoretical predictions indicate that ΔC_p in the presence of increasing denaturant concentration is expected to be higher than in its absence (Pfiel & Privalov, 1976; Makhatadze & Privalov, 1992). The apparent increase in ΔC_p is a consequence of both the sign of denaturant association enthalpy and its proportionality to denaturant concentration (see Fig. 23 of Privalov [1979]). We therefore expected that perturbing the T_m with chemical denaturant would yield a value of ΔC_p larger than that determined in the absence of denaturant. In fact, ΔC_p for OMTKY3 measured in the presence of varied denaturant concentration was statistically identical to the value determined in its absence.

The simplest explanation for this phenomenon is that, because Δn_X is very small, association enthalpies may not be large enough to be detected by our techniques. Using the calculated value of $\Delta n = 2$ for the number of GuHCl binding upon denaturation of OMTKY3, ΔH_m and T_m values from thermal denaturation experiments with changing GuHCl concentrations, and denaturant binding enthalpies from Makhatadze and Privalov (1992), a plot of ΔH_m versus T_m in the presence of varied dena-

turant concentration yields a predicted value of approximately 900 cal/(mol·K) for ΔC_p. Assuming 20% error in this calculation, this ΔC_p value is within two standard deviations of the mean value obtained in this study.

The experiment in which pH is varied in samples containing constant denaturant concentration may be of general utility, because it potentially expands the range of temperatures covered when measuring ΔH_m. In the case of OMTKY3, a 14 °C range was covered when T_m was perturbed with pH (Table 2), whereas the range was expanded to 24 °C when T_m was perturbed with combinations of chemical denaturant and changing pH (Table 4). These experiments also expanded the pH range over which complete thermal denaturation curves could be obtained. An extended temperature range greatly increases confidence in the determination of ΔC_p. This technique may therefore prove useful for characterization of other proteins.

We have also determined that, for OMTKY3, extrapolations in GuHCl and urea are linear over the ranges of 1–7 M and 1–8 M, respectively. Most models predict linearity for urea extrapolations (Schellman, 1978; Alonso & Dill, 1991). However, a number of models predict convex curvature in extrapolations of ΔG_u at low GuHCl concentrations (Aune & Tanford, 1969; Tanford, 1970; Pace & Vanderburg, 1979; Alonso & Dill, 1991; Makhatadze & Privalov, 1992). Linear extrapolations would therefore yield a low extrapolated value of ΔG_u. To our knowledge, the only report of extrapolations from direct measurements of ΔG_u in low concentrations of GuHCl is by Santoro and Bolen (1992), who examined thioredoxin in 0–3 M GuHCl. After controlling for ionic strength, they found no curvature, which is consistent with our observations.

A unique feature of our technique is the ability to make direct comparisons between measured and extrapolated values of ΔG_u. For OMTKY3, values of ΔG_u extrapolated from urea solutions are statistically identical to values measured directly, whereas the extrapolated values of ΔG_u in GuHCl are about 1.5 kcal/mol higher. This stabilization of OMTKY3 is probably due to the high ionic strength of GuHCl solutions. We are unaware of any other direct comparisons between extrapolated and measured values of ΔG_u. Santoro and Bolen (1992) made comparisons between the two values, but were required to perform an additional extrapolation with the Gibbs–Helmholtz equation to normalize the temperature. The key to making direct comparisons between extrapolated and directly measured values of ΔG_u is the use of thermal denaturation in the presence of chemical denaturant. This method provides good baselines for determining the equilibrium constant of unfolding, and thus ΔG_u, over a much wider range of temperatures than is obtained from a simple chemical denaturation experiment. We are currently investigating the general utility of this approach in discerning the accuracy of extrapolated ΔG_u values.

The slope from the plot of ΔG_u versus denaturant concentration, m, is proportional to $\Delta\alpha$, the fraction of peptide groups and uncharged side chains that become exposed to solvent upon unfolding (Ahmad & Bigelow, 1986). For OMTKY3, $\Delta\alpha$ in urea is 0.19, while in GuHCl it is 0.28. For other proteins reported in the literature, values of $\Delta\alpha$ in the two denaturants are the same (Pace et al., 1990). In the case of OMTKY3, $\Delta\alpha$(GuHCl) may be artifactually high because of the stabilizing effects discussed previously. $\Delta\alpha$(urea) for OMTKY3 is consistent with the trend of reduced exposure with increased disulfide bond density observed for the proteins studied by Pace and coworkers (1990).

In conclusion, we have determined a value of 590 ± 120 cal/(mol·K) for ΔC_p of OMTKY3 using van't Hoff analysis and 640 ± 110 cal/(mol·K) from a global fit of the data. We can now calculate ΔG_u at any temperature after determining ΔH_m and T_m from a single thermal denaturation experiment. Additionally, ΔC_p is invariant over a wide range of solution conditions within the limits of our confidence intervals. We have also ascertained that, for OMTKY3, extrapolations of ΔG_u versus denaturant concentration are linear over the ranges of 1–7 M GuHCl and 1–8 M urea. Extrapolated values of ΔG_u in GuHCl are higher than values measured directly, probably due to stabilization of OMTKY3 in solutions of high ionic strength, but urea extrapolated values are statistically identical to determinations made in the absence of chemical denaturant.

Materials and methods

Purification of OMTKY3

OMTKY3 was purified as described by Laskowski et al. (1987), with the following modifications: (1) whole turkey ovomucoid was sometimes purchased from Sigma Chemical Co.; (2) the purification step for whole ovomucoid involving CM-Sepharose was omitted; (3) limited proteolysis of whole ovomucoid was followed with SDS-PAGE on 17% acrylamide, 10% glycerol gels, pH 8.8, with a 5% acrylamide stacking gel, pH 6.8; and (4) all size-exclusion chromatography was performed with Sephadex G-50 columns using 0.1 M NH_4HCO_3 as the eluate.

The resulting OMTKY3 was greater than 95% pure, as judged by amino acid analysis and amino-terminal sequencing (Protein Structure Facility, University of Iowa), as well as analytical reverse-phase HPLC. The latter was performed on a Beckman System Gold HPLC using a Vydac C-4 2.1 × 50-mm column. OMTKY3 was eluted with a linear gradient starting with 98% buffer A (0.06% TFA in H_2O) and 2% buffer B (0.056% TFA in 80/20 acetonitrile/H_2O) that changed over a 10-min time period to 100% buffer B. Elution was monitored by UV absorbance at 214 and 280 nm. OMTKY3 purity was also checked with two-dimensional proton NMR spectroscopy

(Aue et al., 1976), which detects peptide and protein contaminants at levels exceeding 10%.

Determination of protein concentration

The concentration of OMTKY3 was determined by measuring absorbance at 280 nm (A_{280}) on a Hitachi U-2000 spectrophotometer and using a molar extinction coefficient (ϵ_{280}) of 4.4×10^3 $M^{-1}cm^{-1}$ at pH 7.0 and 22 °C. The ϵ_{280} was calculated by first determining ϵ_{205} by the method of Scopes (1974) and then measuring A_{205} and A_{280} of the same stock solution of OMTKY3, after appropriate dilutions. The estimated uncertainty in ϵ_{280} is ± 5%.

Chemical denaturants

Guanidine hydrochloride (Heico Chemical Co., Delaware Water Gap, Pennsylvania) and urea (Boehringer Mannheim, Indianapolis, Indiana) were used without further purification. Concentrations of aqueous denaturant solutions were determined by refractometry (Pace, 1986).

CD spectropolarimetry

All CD measurements were performed on an AVIV 62 DS spectrometer equipped with a thermoelectric temperature controller and an immersible thermocouple, accurate to ±0.4 °C, for direct monitoring of the sample temperature. Far- and near-UV CD spectra were collected on unbuffered samples containing 0.1 mg/mL and 2.0 mg/mL OMTKY3, respectively, at pH 2.0. The pH of the sample varied less than 0.1 units between 0 and 80 °C. Spectra were collected at 0.25-nm intervals with an averaging time of 1–2 s per point; each spectrum was the average of three scans. Raw data were exported to an Apple Macintosh computer for processing and presentation using Cricket Graph (Computer Associates International, Inc., West Conshohocken, Pennsylvania). The spectra were corrected for DC offset by subtracting the average ellipticity value between 350 and 360 nm, where no signals from protein are expected, from the rest of the spectrum. The spectra were smoothed once over an interval of five data points prior to plotting.

Thermal denaturation of OMTKY3 was performed by heating the sample at a constant rate while monitoring the ellipticity at the desired wavelength. The pH of all solutions was found to vary less than 0.1 units between room temperature and 80 °C. After denaturation, the sample was immediately cooled to measure reversibility of the reaction (see below). The sample was stirred in a 3-mL stoppered cuvette throughout the experiment. All solution blanks showed no change in ellipticity with temperature and were therefore neglected during data analysis. Other details concerning sample preparation are found in the figure legends and footnotes to the tables.

Data were fit by nonlinear regression to an equation in which both the baselines and the transition region of the curve are fit simultaneously (Santoro & Bolen, 1988). Assuming that denaturation is a two-state process and that the effects of ΔC_p on the enthalpy of unfolding are negligible over the narrow temperature range of the transition, the equilibrium constant of the unfolding reaction, K, is defined as

$$-RT \ln K = \Delta H_m - T\Delta S_m, \tag{8}$$

$$K = \exp[(-\Delta H_m/RT) + (\Delta S_m/R)], \tag{9}$$

where ΔH_m is as defined previously, T is the temperature in Kelvin, R is the gas constant, and ΔS_m is the entropy of unfolding at the midpoint of the thermal transition.

When monitoring a two-state process with an experimental observable, y_{obs}, the equilibrium constant for the reaction is

$$K = \frac{y_{obs} - (y_n + m_n T)}{(y_d + m_d T) - y_{obs}}, \tag{10}$$

where the parameters y_n and y_d refer to the y-intercepts of the native and denatured baselines, respectively, while m_n and m_d are the slopes of the baselines. By setting Equation 9 equal to Equation 10 and substituting the quantity $(\Delta H_m/T_m)$ for ΔS_m, the final equation for y_{obs} is Equation 2 (presented in Results).

Raw data were fit to Equation 2 using the modified Gauss–Newton method described by Johnson and Faunt (1992). Fits were performed using the NonLin program developed by M.L. Johnson (Johnson & Frasier, 1985; Brumbaugh & Huang, 1992; Johnson & Faunt, 1992) for a Silicon Graphics Personal Iris computer or a version modified to run on the Apple Macintosh computer by Robert J. Brenstein (Robelko Software, Carbondale, Illinois). Confidence intervals for the fits are estimated by a search through asymmetrical, nonlinear variance space and are reported at one standard deviation.

Global fits were performed with NonLin in a manner analogous to the individual fits. Equation 3 (presented in Results) was derived by setting Equation 1 equal to $-RT \ln K$ and making appropriate substitutions. Each of the individual experiments within a global set was fit to unique values of the parameters y_n, m_n, y_d, m_d, ΔH_m, and T_m, whereas the parameter ΔC_p was common to all experiments.

Reversibility of the denaturation reaction was measured as percent protein renaturation after heating. For this measurement, ellipticities for the native, y_n, and denatured, y_d, protein were compared at 0 °C; y_n was either measured directly or extrapolated back to 0 °C from the native baseline and y_d was determined by extrapolation of the denatured baseline back to 0 °C. The difference between the two values is Δy.

$$\Delta y = [y_n(0\,°C)] - [y_d(0\,°C)] \tag{11}$$

At the end of the denaturation experiment, the sample was cooled to 0 °C; the difference in this new native ellipticity, y'_n (0 °C), and the value previously extrapolated from the denatured baseline, y_d (0 °C), is $\Delta y'$. Percent renaturation is defined by

$$\% \text{ Renaturation} = [\Delta y'/\Delta y] * 100 \quad (12)$$

In most instances, the slopes of the native and denatured baselines were of the same sign, whereas the slope of the denatured baseline tended to be larger. Therefore, small differences between y_n and y_d at 0 °C often lead to large differences in Δy and $\Delta y'$. We thus believe that the reported values for percent renaturation are low estimates.

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