Hydrogen Bonds and the pH Dependence of Ovomucoid Third Domain Stability†

Liskin Swint-Kruse and Andrew D. Robertson*

Department of Biochemistry, The University of Iowa, Iowa City, Iowa 52242

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ABSTRACT: Thermal denaturation of turkey ovomucoid third domain (OMTKY3) has been monitored with differential scanning calorimetry (DSC) and circular dichroism in H_2O and D_2O, pH 1.5 to 5 and ionic strength 0.01 to 0.71. Results from DSC experiments are in good agreement with spectroscopic studies [Swint, L., & Robertson, A. D. (1993) *Protein Sci. 2*, 2037−2049] and fit well to the two-state unfolding model. The average ratio of calorimetric to van't Hoff enthalpies is 0.99 ± 0.03 (n = 16), and the average value of ΔC_p is 620 ± 20 cal/(mol K) (n = 7). The free energy of unfolding (ΔG°_u) increases in the presence of salt at both pH 1.5 and 4.5. This stabilization is not due to ion binding and probably results from screened repulsive interactions between the cationic groups of OMTKY3. At very low ionic strength, the change in ΔG°_u from pH 1.5 to 4.5, $\Delta\Delta G^\circ_{pH}$, is 3.5 ± 0.2 kcal/mol. Few interactions between ionizing groups are affected by the addition of 200 mM KCl; $\Delta\Delta G^\circ_{pH}$ decreases by only 0.4 ± 0.3 kcal/mol. Comparison of $\Delta\Delta G^\circ_{pH}$ with values calculated from the pK_as of all six carboxyl groups in OMTKY3 [Schaller, W. S., & Robertson, A. D. (1995) *Biochemistry 34*, 4714−4723] suggests that some pK_as in the denatured state may be lower than those of model compounds. Moreover, calculated values of $\Delta\Delta G^\circ_{pH}$ are very sensitive to modest changes in the cooperativity of proton binding. Aspartates 7 and 27, whose pK_as appear to be perturbed primarily by involvement in hydrogen bonds, are responsible for most of the pH dependence of OMTKY3 stability.

Experimentalists often treat the Gibbs free energy of protein unfolding (ΔG°_u)[1] as the sum of contributions from many noncovalent interactions (Matthew & Gurd, 1986):

$$\Delta G^\circ_u = \Delta G^\circ_{charge} + \Delta G^\circ_{hyd} + \Delta G^\circ_{conf} + \Delta G^\circ_{v,W} + \Delta G^\circ_{Hbond} \quad (1)$$

where ΔG°_{charge}, ΔG°_{hyd}, ΔG°_{conf}, ΔG°_{vW}, and ΔG°_{Hbond} are the contributions to the native stability from individual residues through charge−charge, hydrophobic, configurational, van der Waals, and hydrogen bond interactions. This approach provides a framework for addressing the question "How much do these interactions contribute to protein stability?" Components of eq 1 may be isolated by mutational analysis [e.g., Horovitz et al. (1990), Serrano et al. (1990), Dao-Pin et al. (1991), and Sali et al. (1991)], by model compound studies (Murphy & Freire, 1992; Privalov & Makhatadze, 1993), or by systematic variation of solution conditions (Horovitz et al., 1990; Pace et al., 1990; Yang & Honig, 1992; Scholtz et al., 1993). The strength of the latter approach is the use of reversible perturbations directly on the protein of interest, which facilitates thermodynamic analysis of the resulting changes in stability. We have used this method to dissect the changes in ΔG°_u for turkey ovomucoid third domain (OMTKY3) that accompany variations in ionic strength and pH (Figure 1).

Only ΔG°_{charge} and ΔG°_{Hbond} (eq 1) are likely to make significant contributions to changes in the ionic strength and pH dependences of ΔG°_u ($\Delta\Delta G^\circ_{salt}$ and $\Delta\Delta G^\circ_{pH}$, respectively). Increasing salt concentrations may screen charge−charge interactions (Russell & Fersht, 1987; Dill, 1990; Horovitz et al., 1990; Scholtz et al., 1993), but effects of submolar concentrations on ΔG°_{hyd} and ΔG°_{vW} are negligible (Tanford, 1973; Lockhart & Kim, 1992, 1993). Changes in ionic strength have little effect on uncharged (Beeson et al., 1993) or singly charged hydrogen bonds (Scholtz et al., 1993), although the larger electrostatic component of doubly charged salt bridges is perturbed (Horovitz et al., 1990; Scholtz et al., 1993). OMTKY3 has no salt bridges (Fujinaga et al., 1987) and, in the absence of ion binding or solvent effects, provides a potentially simple system for analysis of the ionic strength dependence of ΔG°_u: $\Delta\Delta G^\circ_{salt}$ is due to changes in ΔG°_{charge}.

$\Delta\Delta G^\circ_{pH}$ may result from changes in either ΔG°_{charge} or ΔG°_{Hbond}. ΔG°_{charge} is affected by variation in the net charge of the protein or disruption of ion pairs. Formally charged hydrogen bonds are affected by changing pH when the population of the ionized donor or acceptor changes; charged hydrogen bonds are stronger than uncharged bonds (Fersht

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* Fax: (319) 335-9570. E-mail: aroberts@vaxa.weeg.uiowa.edu.

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[1] Abbreviations: ΔG°_u, change in free energy upon protein unfolding; ΔG°_{charge}, ΔG°_{hyd}, ΔG°_{conf}, ΔG°_{vW}, and ΔG°_{Hbond}, contributions to ΔG°_u from charge−charge interaction, hydrophobic, configurational, van der Waals, and hydrogen bond free energies, respectively; $\Delta\Delta G^\circ_{pH}$, change in ΔG°_u with pH; $\Delta\Delta G^\circ_{salt}$, change in free energy resulting from addition of salt; $\Delta\Delta\mathcal{G}^\circ_{pH}$, change in ΔG°_u with change in pH calculated from values of pK_a; $K_{a,N}$ and $K_{a,D}$, acid dissociation constants for an ionizing residue in native and denatured protein, respectively; α_N and α_D, cooperativity coefficients for protein−proton interactions on native and denatured protein, respectively; OMTKY3, turkey ovomucoid third domain; D_2O, deuterium oxide; DSC, differential scanning calorimetry; CD, circular dichroism; T_m, temperature at the midpoint of denaturation; ΔH_m, enthalpy change at the midpoint of denaturation; (T_{mid}, pH_{mid}), δT, and m, coordinates of the inflection point (in parentheses), distance between the baselines, and slope of the transition for plots of T_m versus pH; ΔC_p, change in heat capacity upon denaturation; ΔH_{vH}, van't Hoff enthalpy; $C_{p,max}$, excess heat capacity at T_m; ΔH_{cal}, calorimetric enthalpy; σ, standard deviation; σ^2, variance.

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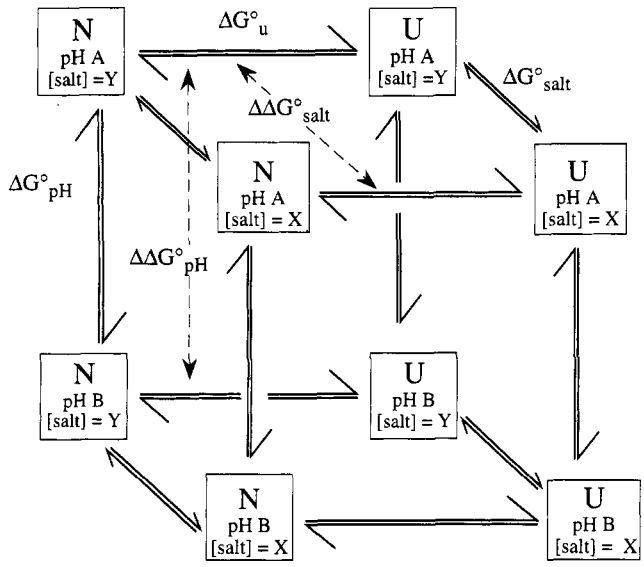

FIGURE 1: Thermodynamic cycles describing the solution matrix of pH and ionic strengths utilized during thermal denaturation experiments. "N" represents folded protein, and "U" is unfolded protein; other parameters are defined in the text.

& Serrano, 1993). Ideally, measurements of $\Delta\Delta G^{\circ}_{pH}$ should be made over a pH range wide enough to encompass complete ionization transitions.

OMTKY3 is especially suited for such experiments because it is folded even at very low pH (Swint & Robertson, 1993). Furthermore, apparent ionization constants and their cooperativity coefficients have been determined at low and high ionic strength for all of the carboxyl groups in native turkey ovomucoid third domain (OMTKY3; Schaller & Robertson, 1995). These allow $\Delta\Delta G^{\circ}_{pH}$ to be analyzed via the linkage relationship between protein stability and proton binding (Tanford, 1970; McNutt et al., 1990; Pace et al., 1990; Hu et al., 1992; Yang & Honig, 1992, 1993). The stability data reported here and the ionization constants (Schaller & Robertson, 1995) have been determined under matched solution conditions and thus provide a unique opportunity to understand macroscopic stability in terms of microscopic ionization events. Moreover, the pH and ionic strength dependences of stability may be interpreted on a molecular level using the crystal (Fujinaga et al., 1987) and solution (Krezel et al., 1994) structures available for OMTKY3.

MATERIALS AND METHODS

Domestic turkey (*Maleagris gallopavo*) eggs were a gift from Theis Farms (New Haven, IA). Purification of OMTKY3 and thermal denaturation monitored with circular dichroism (CD) were performed as described by Swint and Robertson (1993). Deuterium oxide (99.9 atom %) was obtained from Cambridge Isotope Laboratories (Cambridge, MA). The pH of all samples was measured on an Orion Research Model 611 pH meter equipped with a 3-mm Ingold electrode (Wilmad Glass Company, Buena, NJ). The pH meter was calibrated at pH 7 and 4 or pH 7 and 2 with standards from VWR Scientific (West Chester, PA) and Fisher Scientific (Pittsburgh, PA) at room temperature. Samples for experiments in which pH was varied contained 10 mM potassium phosphate and 10 mM potassium acetate. Samples titrated with HNO_3, $HClO_4$, and H_2SO_4 were made by first adjusting the pH of water to 2 with the titrant and then dissolving the protein and readjusting the pH, in order to avoid locally high concentrations of the acids. Volumes of HNO_3 and $HClO_4$ added during titration were carefully tabulated for accurate determination of ionic strength. Values of pH* reported for D_2O solutions represent apparent pH values, without correction for isotope effects.

Differential Scanning Calorimetry. Differential scanning calorimetry (DSC) was performed on a Hart Model 4207 differential scanning calorimeter (Hart Scientific, Pleasant Grove, UT). Samples contained 2−4 mg/mL OMTKY3; protein concentration was determined by making 1:10 dilutions of the sample solution with calibrated pipettes, adjusting the pH to approximately 7, and measuring A_{280}. The extinction coefficient for OMTKY3 at 280 nm, pH 7, is $4.41 \times 10^3\ M^{-1}\ cm^{-1}$ (Swint & Robertson, 1993). Blanks consisted of the same buffer without protein. Sample temperature was changed at a rate of 0.5 °C/min during the experiments (Swint & Robertson, 1993).

DSC data were fit to a two-state model for unfolding using CPPLUS6 for the Macintosh (E. Freire, Johns Hopkins University, Baltimore, MD). Baselines were established by subtracting combinations of first- and second-order polynomials from the data. Data analysis was facilitated by subtracting buffer blanks to generate preliminary baselines, although the quality of the final fits was not affected by the absence of a blank. Each data set was fit with a number of different baselines to determine ΔH_m and T_m; parameters are reported for the fit with the smallest variance. Percent reversibility was calculated from the enthalpies of first ($\Delta H_{m,first}$) and second ($\Delta H_{m,second}$) scans of the same sample using the equation

$$\% \text{ reversibility} = [(\Delta H_{m,second})]/[(\Delta H_{m,first})] \times 100\% \quad (2)$$

Errors of the fit were determined for ΔH_m using F analysis (Bevington, 1969), which is further discussed in the Appendix.

RESULTS

The DSC data in the present study have been collected over wider temperature and pH ranges than previous studies in which thermal denaturation was monitored with CD spectropolarimetry (Swint & Robertson, 1993). The high thermal stability of OMTKY3 restricted the latter experiments to a pH range of 1.5−3 and a 14° spread in T_m. DSC samples are sealed in ampules, thus facilitating data collection at temperatures above 100 °C. Furthermore, determination of the enthalpy is not highly dependent upon pre- and posttransitional baselines (see Appendix), while accurate analysis of spectroscopic data relies upon good baseline extrapolations into the transition region (Pace et al., 1992).

Thermal Denaturation in H_2O and D_2O. A major impetus for studying the thermodynamics of unfolding for OMTKY3 is quantitative interpretation of hydrogen exchange experiments (Swint & Robertson, 1994). For these experiments, protein is dissolved in deuterium oxide (D_2O), which may have significant effects on protein stability and activity (Hermans & Scheraga, 1959; Jencks, 1969; Lemm & Wenzel, 1981; Antonio et al., 1991; Connelly et al., 1993). Therefore, OMTKY3 stability has been examined in both H_2O and D_2O. Figure 2 shows results of OMTKY3 thermal denaturation monitored with DSC; Figure 2a is a plot of raw data minus buffer blanks. Baselines have been corrected by subtracting a second-order polynomial from these data, and

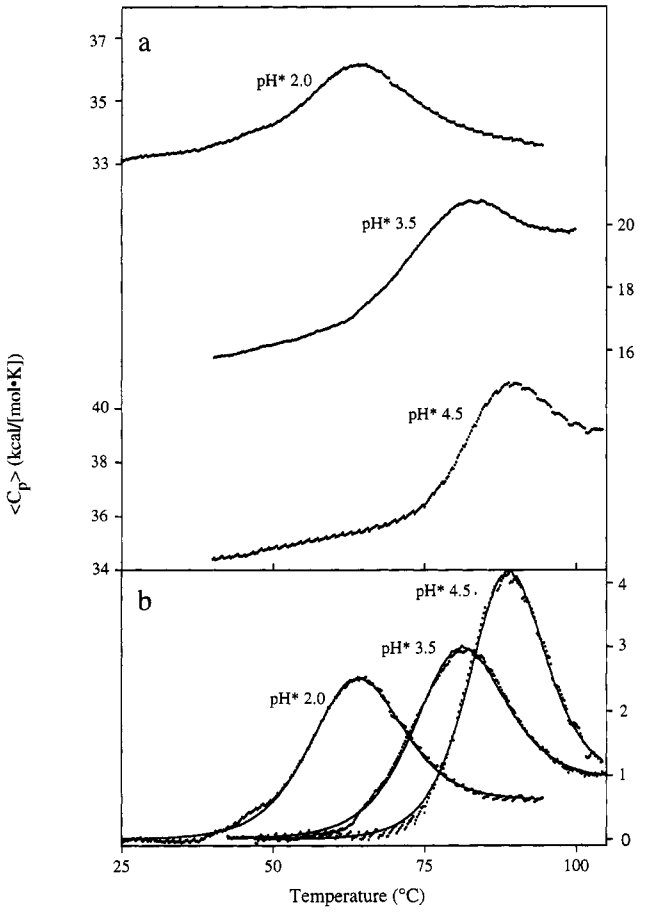

FIGURE 2: (a) Thermal denaturation of OMTKY3 monitored with DSC. Plots represent the results of subtracting a buffer baseline from the raw denaturation data. (b) DSC baseline corrections. First- and second-order baseline corrections were subtracted from the data in panel a. The solid lines represent the best fit of the data to a two-state model.

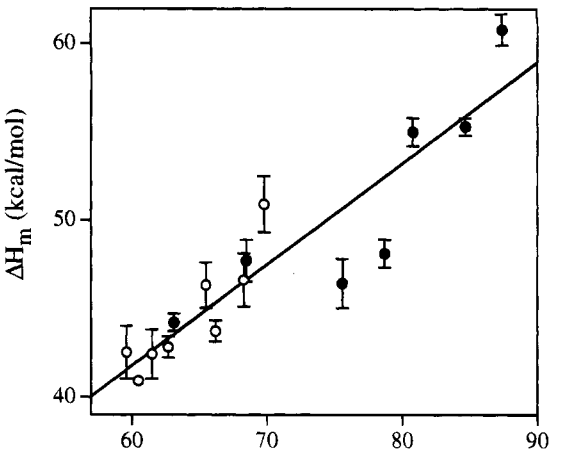

FIGURE 3: ΔH_m versus T_m for all thermal denaturation experiments of OMTKY3 in D_2O. Data were obtained with circular dichroism [○; Swint and Robertson (1993)] and DSC (●). The solid line was determined by linear regression of the data (Table 4).

Table 1: Perturbation of T_m with pH[a]

solvent		pH[b]	ΔH_m (kcal/mol)	T_m (°C)	$\frac{\Delta H_{cal}}{\Delta H_{vH}}$	% reversibility[c]
H_2O	DSC	2.56	45.4(1.1)	65.7	1.00	78
		3.48	50.0(1.3)	78.7	0.98	81
		4.51	57.4(0.5)	85.2	1.02	84
D_2O	CD[d]	1.50	40.9(0.2)	60.5		82
		2.10	42.8(0.6)	62.7		89
		2.50	43.7(0.6)	66.2		85
	DSC	1.99	44.2(0.5)	63.1	0.99	91
		2.52	47.7(1.2)	68.5	0.97	90
		3.17	46.4(1.4)	75.6	0.95	88
		3.46	48.1(0.8)	78.7	0.97	90
		3.51	55.0(0.8)	80.8	0.95	n.d.
		3.94	55.3(0.5)	85.7	1.05	87
		4.50	60.8(0.9)	87.4	1.04	88
		4.96[e]	62.4(n.d.)	87.8	n.d.	100

[a] Experiments are described in Materials and Methods. Numbers in parentheses represent errors of the fit at 1 σ, as described in the Appendix. [b] The pH in D_2O is reported as apparent pH, without correction for isotope effects. [c] Percent reversibity for DSC experiments is discussed in Materials and Methods. Percent reversibility for CD experiments is determined as in Swint and Robertson (1993). [d] Performed in addition to experiments reported in Swint and Robertson (1993). [e] The maximum temperature reached during this experiment was 94 °C, which is only 70% of the denaturation transition. The value of ΔC_p was fixed at 600 cal/(mol K) during the fit.

the results are presented in Figure 2b; solid lines represent the best fits of the data to a two-state denaturation model. The fitted value of ΔC_p is very sensitive to changes in the baseline, and differences between the intercepts of posttransitional baselines in Figure 2b are not statistically significant. Thus, the values of ΔC_p determined from individual endotherms are not included in the reported average. Rather, ΔC_p was determined from plots of ΔH_m versus T_m, which yield values of 580 ± 120 and 740 ± 140 cal/(mol K) in D_2O and H_2O, respectively (Figure 3; Tables 1 and 4).

Both H_2O and D_2O data show good agreement between DSC and CD experiments. Values for the van't Hoff enthalpies, ΔH_{vH}, were calculated from DSC data using the following equation (Privalov & Khechinashvili, 1974):

$$\Delta H_{vH} = \frac{4RT_m^2 C_{p,max}}{\Delta H_{cal}} \quad (3)$$

where R is the ideal gas constant, $C_{p,max}$ is the excess heat capacity at T_m, and ΔH_{cal} is the calorimetric enthalpy. Values of $\Delta H_{cal}/\Delta H_{vH}$ (Table 1) are consistent with the absence of significantly populated intermediates during OMTKY3 denaturation (Swint & Robertson, 1993). Above pH 4.5, the reversibility of the denaturation reaction decreases significantly, and OMTKY3 precipitated during a denaturation experiment at pH* 5.5. Complete reversibility was achieved at pH* 5.0 by heating through only 70% of the denaturation transition. The pI of OMTKY3 is 7.5 ± 0.1, as measured by isoelectric focusing (data not shown), and the decreased reversibility of unfolding is consistent with a general trend of decreased solubility of proteins as pH approaches their pI values. For experiments at all other pHs, the values of T_m from second scans are within 1−2 °C of values from the first scans.

Upon solvation in D_2O, many surface protons on OMTKY3 quickly exchange with deuterons. However, a subset of backbone protons are protected from exchange until the protein structure is perturbed by denaturation (Englander et al., 1972). To test for possible effects of exchange during denaturation experiments in D_2O, the following control was performed. All exchangeable sites in OMTKY3 were deuterated by denaturing the protein in D_2O at pH 2 and 80 °C for 5 minutes, then cooling and lyophilizing the sample. A sample of OMTKY3 in H_2O was treated similarly. When both of these samples were subjected to thermal denaturation in D_2O (monitored with CD), values of T_m and ΔH_m were statistically identical (data not shown). Hence, subsequent

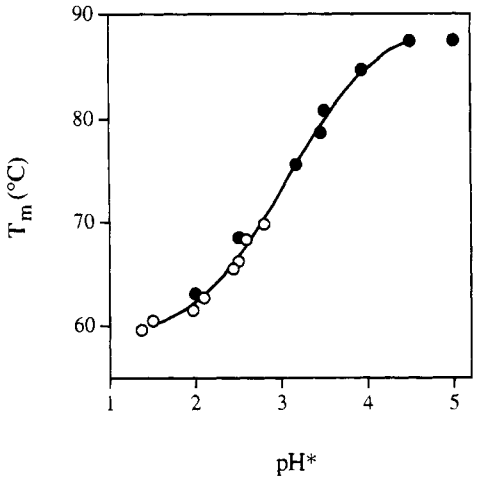

FIGURE 4: T_m versus pH* for all thermal denaturation experiments of OMTKY3 in D_2O. Data were obtained with circular dichroism [○; Swint and Robertson (1993)] and DSC (●). The solid line represents the best fit of the data between pH 1.5 and 4.5 to eq 4; parameters of the fit are described in Table 4.

Table 2: Effects of Ionic Strength on OMTKY3 Thermal Denaturation[a]

salt	I[b]	ΔH_m (kcal/mol)	T_m (°C)	% reversibility[c]
0[d,e §]	0.010	40.8(0.3)	58.6(0.4)	>90
0, titrated with HNO_3[§]	0.010	42.0(2.7)	59.2(0.5)	79
0, titrated with $HClO_4$[§]	0.013	39.7(0.5)	60.3(0.1)	84
0, titrated with H_2SO_4[§]	0.014	41.5(0.5)	64.1(0.2)	89
10 mM KCl[§]	0.020	40.3(0.4)	59.5(0.1)	88
20 mM KCl[§]	0.030	41.2(0.5)	59.8(0.1)	87
10 mM KH_2Phosphate, 10 mM KAcetate[§,‡]	0.030	39.7(1.2)[f]	61.3(1.6)[f]	
100 mM KCl[d §]	0.11	42.8(1.8)	63.2(0.4)	95
		42.9(1.3)	63.3(0.4)	97
33.3 mM $MgCl_2$[‡]	0.11	43.1(0.4)[g]	64.0(<2.5)[h]	n.d.[i]
200 mM KCl, 10 mM KH_2Phosphate, 10 mM KAcetate[‡]	0.23	43.6(1.0)[j]	65.7(1.3)[j]	
700 mM KCl[§]	0.71	35.2(1.8)	68.8(0.4)	50[k]

[a] Thermal denaturation of OMTKY3, pH 2.0, in H_2O, was performed as described in Materials and Methods. Unless otherwise indicated, buffer and salt were added to samples before adjusting the pH with HCl. Numbers in parentheses represent errors of the fit at 1 σ, unless reported as experimental error for average values. Denaturation was monitored with CD (§) or with DSC (‡). [b] Includes estimated concentration of counterions added during pH adjustment. [c] Percent reversibilities for CD and DSC experiments are discussed in Swint and Robertson (1993) and Materials and Methods, respectively. [d] Results from Swint and Robertson (1993). [e] Average of 15 thermal denaturation experiments. [f] Calculated from the data in Table 1, as described in the Discussion. [g] $\Delta H_{cal}/\Delta H_{vH} = 0.96$. [h] See the Appendix for the origin of this value. [i] Percent reversibility could not be determined due to technical problems during the second thermal denaturation experiment. [j] Calculated from the data in Table 3, as described in the Discussion. [k] The low value of ΔH_m in 700 mM KCl is probably a fitting artifact due to a short posttransitional baseline of the CD data (not shown).

D_2O experiments were performed using OMTKY3 lyophilized out of H_2O.

In Figure 4, values of T_m from denaturation experiments in D_2O have been plotted versus pH*. A mathematical description of this curve is desirable for quantitative comparison of D_2O and H_2O data, quantitation of experimental error, and interpolation of T_m at any pH between 1.5 and 4.5. In principle, an equation describing the pH-dependence of T_m can be derived from Wyman's theory of linked equilibria (Wyman, 1964), but analysis requires accurate $pK_{a,D}$ values, which are not known (see Discussion). T_m versus pH has also been described by a sigmoidal curve with an inflection point representing a single pK (Renner et al., 1992). For proteins with several ionizing groups, this assumption may not be valid (Yang & Honig, 1993); a distribution of pK_as may lead to broadening of the plots (Freifelder, 1982).

Table 3: Perturbation of T_m with pH in the presence of 200 mM KCl[a]

pH	ΔH_m (kcal/mol)	T_m (°C)	$\frac{\Delta H_{cal}}{\Delta H_{vH}}$	% reversibility[b]
1.50	42.2(0.6)	63.8	0.96	n.d.[c]
2.28	45.2(1.6)	68.0	1.01	93
2.94	47.9(1.0)	73.5	1.01	96
3.24	n.d.[d]	78.0	n.d.	n.d.
3.61	n.d.[d]	83.8	n.d.	n.d.
4.13	56.9(1.3)	86.0	1.00	83
4.51	58.6(0.7)	88.2	1.04	86

[a] Experiments were performed in H_2O and are described in Materials and Methods. Numbers in parentheses represent errors of the fit at 1 σ, as described in the Appendix. [b] Determination of percent reversibility for DSC experiments is described in Materials and Methods. [c] This experiment was performed simultaneously with the experiment at pH 4.51. Therefore, during the first scan, denatured OMTKY3 at pH 1.50 was subjected to high temperatures for nearly 1 h, resulting in complete irreversible denaturation. [d] A problem with the reference cell distorted endotherms at the beginning of the transition. Although values for ΔH_m could not be determined, confidence in T_m is high, as the peak maximum was not affected.

In lieu of a model-based equation, a simple function that fits the data well was chosen to describe the curve:

$$T_m = T_{mid} + \delta T \tanh[m(\mathrm{pH} - pH_{mid})] \qquad (4)$$

A hyperbolic tangent (tanh) describes a symmetric, sigmoidal curve centered at (0,0) with an amplitude and a slope of 1. T_{mid} represents the value of T_m at the inflection point of the curve, which is shifted from the origin. δT is the amplitude of the T_m change with pH. The parameter m reflects deviation in the slope of the transition from 1 and allows for both a spread in pK_as and their Hill coefficients. pH_{mid} describes the pH value at the inflection point of the curve. Although six groups are ionizing over the studied pH range, eq 4 is appropriate because the individual pK_as in OMTKY3 are always within 0.6 pH unit of another value (Schaller & Robertson, 1995). Two binding constants must differ more than 10-fold to be distinguished as separate inflection points (Freifelder, 1982). The results of fits to eq 4 for H_2O and D_2O are reported in Table 4.

Ionic Strength. At low salt concentrations, ionic strength can vary significantly with the amount of acid needed to adjust the pH of a sample. In order to estimate the magnitude of this effect on the stability of OMTKY3, ionic strength was varied systematically at pH 2. This pH was chosen for a number of reasons. First, salt is likely to have larger effects at a pH where OMTKY3 is highly charged (Stigter & Dill, 1990; Antosiewicz et al., 1994). Second, many independent samples were individually adjusted to the same pH, so a value was chosen where minor variations in pH had little effect on thermal denaturation (Figure 4). Third, low values of T_m at pH 2 facilitate measurements of this parameter. Confidence in T_m determined at pH 2 is higher than at pH 4.5, since more complete denaturation transitions are observed (Figure 2). Finally, buffer is unnecessary for maintaining constant pH with varied temperature at pH 2 (Swint & Robertson, 1993), which permits evaluation of

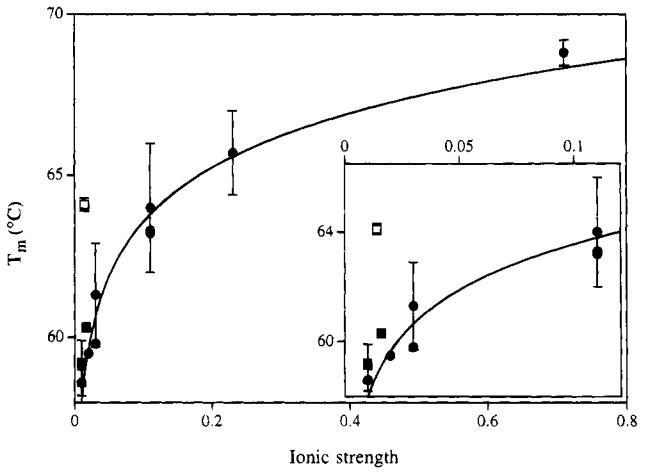

FIGURE 5: Variation of T_m with ionic strength for thermal denaturation of OMTKY3. (●) Samples in H_2O were adjusted to pH 2 with HCl, and ionic strength was varied with KCl or $MgCl_2$. (■) Samples in H_2O were titrated with either $HClO_4$ or HNO_3. (□) Sample was titrated with H_2SO_4. Error bars for all data are described in Table 3. The solid line is a visual aid only and has no physical significance.

possible differences between the DSC buffer (10 mM potassium phosphate and 10 mM potassium acetate) and conditions of NMR titrations (unbuffered 10 mM KCl).

In studies of ionic strength effects, KCl and HCl were utilized in most experiments in an attempt to minimize possible Hofmeister effects, which are dominated by the anion. Cl^- has the smallest effect on water structure (Collins & Washabaugh, 1985). Several other acids were used as titrants to control for specific chloride effects. $MgCl_2$ was added to one experiment to control for specific cation effects.

At pH 2, OMTKY3 is stabilized with increasing ionic strength (Table 2; Figure 5). Little difference is observed between experiments in DSC buffer and unbuffered 10 mM KCl, which permits direct comparison of the stability and NMR titration studies. Most of the effect is evident at 200 mM KCl, although further stabilization is seen at 700 mM KCl. Values of T_m from titrations with HNO_3, $HClO_4$ (Figure 5, ■), and $MgCl_2$ follow the same trend as samples titrated with HCl, which indicates that stabilization is not due to chloride binding or specific cation effects. Data for H_2SO_4, however, suggest that the sulfate dianion may bind native OMTKY3 at pH 2 (Figure 5, □).

Results of thermal denaturation experiments in 200 mM KCl with varied pH are presented in Table 3. The effects of this ionic strength are significant (Table 2; Figure 5), while reversibility of the unfolding reaction is still high (Tables 2 and 3; Swint & Robertson, 1993). The $\Delta H_{cal}/\Delta H_{vH}$ ratios are also consistent with two-state transitions. Values of T_m versus pH were fit with eq 4 (Table 4). T_{mid} is the only parameter statistically different from those of H_2O data and reflects the general trend of thermal stabilization in the presence of salt. Linear regression of ΔH_m versus T_m yields a ΔC_p of 650 ± 30 cal/(mol K) (Table 4).

Calculation of ΔG^o_u. The average value of ΔC_p reported by Swint and Robertson (1993), 590 ± 120 cal/(mol K), has been revised to 620 ± 20 cal/(mol K) using the data of Tables 1, 3, and 4. The weighted average is comprised of seven independent determinations; its low standard deviation is dominated by two ΔC_p values with especially low standard deviations, 580 ± 30 [Figure 4 of Swint and Robertson (1993)] and 650 ± 30 (Table 4). The spread of the seven ΔC_p values is less than 200 cal/(mol K), and their 67% confidence limits envelop those of the average. Furthermore, ΔC_p appears linear over a 55° temperature range and a variety of solution conditions.

For comparison of solution conditions and analysis via Figure 1, data from H_2O, D_2O, and 200 mM KCl experiments [Tables 1 and 3; Table 2 in Swint and Robertson (1993)] were used to calculate the free energy of unfolding, ΔG°_u, at 30 °C from the modified Gibbs–Helmholtz equation:

$$\Delta G^\circ_u = \Delta H_m(1 - T/T_m) - \Delta C_p[(T_m - T)] + [T \ln(T/T_m)] \quad (5)$$

where ΔC_p is the average value, 620 ± 20 cal/(mol K). Values of T_m were calculated from eq 4 and used to compute values of ΔH_m from the appropriate slopes and intercepts (Table 4). Calculations were performed over the pH range of 1.5–4.5. All calculations and error propagation are presented in Table 4 and are further discussed in the Appendix.

DISCUSSION

Isotope Effects on OMTKY3 Stability. Native OMTKY3 is stabilized in D_2O relative to H_2O, as indicated by increases in T_m (T_{mid}, Table 4), ΔH_m (Table 1), and ΔG°_u (Table 4). Solvation in D_2O versus H_2O may affect thermodynamic parameters through (1) hydrogen exchange accompanying denaturation, (2) changes in protonation states, or (3) changes in the hydrophobic effect and hydrogen bonding (Connelly et al., 1993). The ΔH_m increase in D_2O relative to H_2O is not due to the enthalpy of hydrogen exchange, since the experiment in which both protonated and deuterated OMTKY3 were denatured in D_2O yields the same results. The second possibility, that the protonation state is different in the two solvents, is unlikely, because the parameters δT, m, and pH_{mid} are the same in D_2O and H_2O (Table 4). The simplest interpretation of this observation is that the ionization states of OMTKY3 in D_2O and H_2O are the same when pH* equals pH (Bundi & Wüthrich, 1979). Any isotope effects on pK_a values for ionizing groups in OMTKY3 are compensated by the effects of D_2O on glass pH electrodes, as has been observed in many other instances (Glasoe & Long, 1960; Appel & Yang, 1965; Markley, 1975; Bundi & Wüthrich, 1979; Englander et al., 1979). Therefore, OMTKY3 stabilization in D_2O is probably due to isotope effects on hydrophobic interactions and hydrogen bonding.

Ionic Strength Effects on OMTKY3 Stability: Calculation of $\Delta\Delta G^o_{salt}$. OMTKY3 is stabilized in 200 mM KCl over the entire pH range studied. At pH 1.5, the value of T_m increases about 5 °C and $\Delta\Delta G^\circ_{salt}$ (Figure 1) is 0.7 ± 0.2 kcal/mol. At pH 4.5 the difference is less pronounced: T_m increases roughly 3 °C and $\Delta\Delta G^\circ_{salt}$ is 0.3 ± 0.3 kcal/mol. As discussed previously, $\Delta\Delta G^\circ_{salt}$ for OMTKY3 is due to change in ΔG°_{charge}, in the absence of solvent effects or ion binding. Hofmeister effects are probably not responsible for stabilization, since the chloride anion usually has no effect on protein stability (Collins & Washabaugh, 1985) and chaotropic Mg^{2+} exhibited the same behavior as kosmotropic K^+ at constant ionic strength. The value of T_m is not sensitive to the various monovalent anions, so OMTKY3 does not appear to specifically bind chloride ions (Table 2; Figure 5). Therefore, all effects of salt are probably due to changes in charge–charge interactions. The small magni-

tudes of $\Delta\Delta G^{o}_{salt}$ are consistent with the results from a number of laboratories, which demonstrate that there are few interactions between solvent-exposed charged groups (Hollecker & Creighton, 1982; Dao-Pin et al., 1991; Sali et al., 1991).

The similarity between the values of $\Delta\Delta G^{o}_{salt}$ at pH 1.5 and 4.5 was unexpected given that the formal charge on OMTKY3 changes from more than +6 to less than +2. The net increase in stability across the entire pH range may be dominated by screened repulsive interactions between cationic residues. Stabilization by increased ionic strength has been observed for a variety of other proteins, including barnase (Horovitz et al., 1990; Sali et al., 1991), binase (Serrano et al., 1990), and RNaseT1 (Pace & Grimsley, 1988; Hu et al., 1992). The data in the latter two papers were interpreted at constant chloride concentration as evidence for a cation binding site on RNaseT1. An alternative interpretation could be general ionic strength effects; when compared at constant ionic strength, the RNaseT1 data (at less than 1 M salt) show no specific effects for any of six cations.

Determination of $\Delta\Delta G^{o}_{pH}$. Changes in OMTKY3 stability with pH are due to changes in ΔG^{o}_{charge} and ΔG^{o}_{Hbond}. Since salt diminishes ΔG^{o}_{charge} and does not affect ΔG^{o}_{Hbond}, a lower limit for the former may be obtained by measuring the difference between $\Delta\Delta G^{o}_{pH}$ at low and high ionic strengths (Figure 1). $\Delta\Delta G^{o}_{pH}$ in low ionic strength is 3.5 ± 0.2 kcal/mol, which is not much different from the value in 200 mM KCl, 3.1 ± 0.2 kcal/mol (Table 4). Therefore at least 0.4 ± 0.3 kcal/mol of the change in ΔG^{o}_{u} with pH at low ionic strength is due to change in ΔG^{o}_{charge}.

The true contribution of ΔG^{o}_{charge} to $\Delta\Delta G^{o}_{pH}$ may be a larger value for several reasons. First, through-protein charge−charge interactions may be insensitive to ionic strength and not detected by these experiments (Thomas et al., 1985; Russell et al., 1987; Rodgers & Sligar, 1991; Jackson & Fersht, 1993; Antosiewicz et al., 1994). Second, the present analysis ignores possible effects of salt on the denatured state of OMTKY3 (Stigter & Dill, 1990; Stigter et al., 1991), although the similarity of ΔC_p values in low and high salt suggests that solvent-accessible surface areas of the denatured states are comparable (Murphy & Gill, 1991). Third, in low salt, ionic strength increases from 0.02 to 0.05 in going from pH 4.5 to 1.5, which could screen charge−charge interactions at the lower pH. The data in Table 2 and Figure 5 indicate that effects of such changes on OMTKY3 stability are within the error of our measurements.

Contributions of Ionizing Groups to $\Delta\Delta G^{o}_{pH}$. The present study of OMTKY3 stability covers the range of pH values in which all carboxyl groups ionize, and experimental conditions are very similar to those in which the apparent pK_as of these groups were measured (Schaller & Robertson, 1995). Therefore, we have a unique opportunity to understand macroscopic stability in terms of microscopic ionization events. Furthermore, we may attempt to interpret these changes at the molecular level with the aid of crystal and solution structures (Fujinaga et al., 1987; Krezel et al., 1994).

Changing ionization states result from changes in ligand (i.e., proton) concentration and affect protein stability through thermodynamic linkage (Wyman, 1964; Tanford, 1970). Differences in proton affinities between native and denatured protein, reflected in differences in proton dissociation constants or cooperativity coefficients, give rise to $\Delta\Delta G^{o}_{pH}$ according to the following equation (Wyman, 1964; Tanford, 1970, eqs 26−31; Pace et al., 1990, 1992):

$$\Delta\mathcal{G}^{o}_{p,\mathcal{H}} = \Delta G^{+}_{u} - RT\sum_{i} \ln \frac{\left(1 + \dfrac{K_{i,D}^{\alpha_{i,D}}}{[H^{+}]^{\alpha_{i,D}}}\right)}{\left(1 + \dfrac{K_{i,N}^{\alpha_{i,N}}}{[H^{+}]^{\alpha_{i,N}}}\right)} \qquad (6)$$

where $\Delta\mathcal{G}^{o}_{p,\mathcal{H}}$ is the change in stability between a protein with infinite charge (ΔG^{+}_{u}) and the same protein bearing finite charge at known pH. $\Delta\Delta\mathcal{G}^{o}_{p,\mathcal{H}}$ is the difference between two values of $\Delta\mathcal{G}^{o}_{p,\mathcal{H}}$; script letters are used to distinguish this value from $\Delta\Delta G^{o}_{pH}$ defined in Figure 1. ΔG^{+}_{u} is not measurable but cancels in the calculation of $\Delta\Delta\mathcal{G}^{o}_{p,\mathcal{H}}$. R is the gas constant, T is the temperature in Kelvin, "i" represents an ionizing group on the protein, and $[H^{+}]$ is the hydrogen ion activity. $\alpha_{i,D}$ and $\alpha_{i,N}$ are Hill cooperativity coefficients for denatured and native protein; they reflect electrostatic interactions between charged protein and a proton, which vary with pH (Linderstrøm-Lang, 1924; Edsall & Wyman, 1958; Tanford, 1962, 1970). $K_{i,D}$ and $K_{i,N}$ are the acid dissociation constants for ionizing groups in denatured and native protein forms, respectively.

In calculations of $\Delta\Delta\mathcal{G}^{o}_{p,\mathcal{H}}$ (eq 6), temperature is 30 °C and all $\alpha_{i,N}$ are initially assigned values of 1 (Figure 6, gray region). Values of $K_{i,N}$ for the acidic groups of OMTKY3 are from Schaller and Robertson (1995) and are listed in the caption to Figure 6. The single histidine in OMTKY3, His 52, has a p$K_{a,N}$ around 7.5 (Markley, 1973; Ogino et al., 1982). Since it is not titrating in the pH range covered by the present work, this pK_a is not included in the following calculations. For denatured OMTKY3, all α_D are set to 1 (Tanford, 1970). Average values of p$K_{a,D}$ for aspartates (3.9−4.1) and glutamates (4.3−4.5) are from Pace et al. (1990) and Creighton (1993). The p$K_{a,D}$ for the carboxyl terminus of a protein is usually assigned a value of 3.5−4.0 (Rawn, 1983; Matthews & van Holde, 1990). However, the terminal residue of OMTKY3, Cys 56, is involved in a disulfide bond, which may decrease the pK_a by 0.4−0.5 unit (Edsall & Wyman, 1958; Greenstein & Winitz, 1961). Therefore, a value of 3.1 for the carboxy terminus of Cys 56 is used in calculations.

Values of $\Delta\Delta G^{o}_{pH}$ and $\Delta\Delta\mathcal{G}^{o}_{p,\mathcal{H}}$, normalized at pH 1.5, are presented in Figure 6. $\Delta\Delta G^{o}_{pH}$ values and their associated errors are represented by the crosshatched region, and determinations of $\Delta\Delta\mathcal{G}^{o}_{p,\mathcal{H}}$ calculated using the entire range of p$K_{a,D}$s are represented by the hatched region. $\Delta\Delta\mathcal{G}^{o}_{p,\mathcal{H}}$ is at least 1.9 kcal/mol higher than $\Delta\Delta G^{o}_{pH}$ (Table 4). Experimental evidence suggests that some α_Ns may be less than 1 and that p$K_{a,D}$ for Asp 27 may be lower than model compound values (Schaller, 1993; P. M. Bowers and A. D. Robertson, unpublished results). When possible negative cooperativity for Asp 7 (α_N = 0.78 ± 0.11), Asp 27 (α_N = 0.92 ± 0.10), and Cys 56 (α_N = 0.95 ± 0.29) (Schaller, 1993) is included in the calculations, $\Delta\Delta\mathcal{G}^{o}_{p,\mathcal{H}}$ is reduced by 0.9 kcal/mol (Figure 6, Δ). The pK_a of the aspartate residue in an unstructured peptide representing residues 26−32 for OMTKY3 is 3.6 ± 0.1 (P. M. Bowers and A. D. Robertson,

Table 4: Results of Fits and Calculations[a]

	Parameter	D_2O	H_2O	200 mM KCl
T_m versus pH[b]	T_{mid}	74.0(1.1)	71.9(1.7)	76.3(1.5)
	δT	15.7(1.9)	15.4(2.6)	12.9(2.2)
	m	0.904(0.174)	0.865(0.254)	1.07(0.39)
	pH_{mid}	3.06(0.11)	2.97(0.17)	3.09(0.15)
ΔH_m versus T_m[c]	ΔC_p	580(120)	740(140)	650(30)
	intercept	6.9(8.7)	−5.6(9.3)	7.7(2.5)
Gibbs free energy	$\Delta G^\circ_{u,p\ 1.5}$[d]	3.0(0.1)	2.5(0.1)	3.2(0.1)
	$\Delta\Delta G^\circ_{salt,pH\ 1.5}$		0.7(0.2)[e]	
	$\Delta G^\circ_{u,pH\ 4.5}$[d]	6.3(0.2)	6.0(0.2)	6.3(0.2)
	$\Delta\Delta G^\circ_{salt,pH\ 4.5}$		0.3(0.3)[e]	
	$\Delta\Delta G^\circ_{pH}$[f]	3.3(0.2)	3.5(0.2)	3.1(0.2)

[a] Data utilized in fits and calculations are presented in Tables 1 and 3 of this paper and Table 2 of Swint and Robertson (1993). Numbers in parentheses represent 1 σ of the mean. Error determination is described in the Appendix. Units: T_{mid} and δT, °C; ΔC_p, cal/(mol K); intercept and all Gibbs free energies, kcal/mol. [b] Equation 4. [c] Parameters were determined by linear regression with a weighting factor for each data point as the larger of $1/(\sigma_{\Delta H_m})^2$ or $1/(2\%_{\Delta H_m})^2$. [d] Equation 5. [e] Figure 1; $\Delta\Delta G^\circ_{salt} = \Delta G^\circ_{m,200mM\ KCl} - \Delta G^\circ_{u,H_2O}$. [f] Figure 1; $\Delta\Delta G^\circ_{pH} = \Delta G^\circ_{u,pH\ 4.5} - \Delta G^\circ_{u,pH\ 1.5}$.

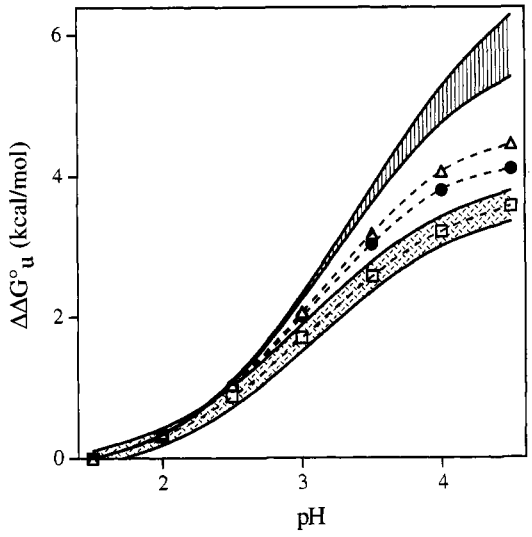

FIGURE 6: $\Delta\Delta G^\circ_{pH}$ and $\Delta\Delta\mathcal{G}^\circ_{p\mathcal{H}}$ for OMTKY3 represented as differences between the value at a specific pH and the value at pH 1.5. (□) Values of $\Delta\Delta G^\circ_{pH}$ in H_2O [Table 1; Table 2 in Swint and Robertson (1993)]. The crosshatched region indicates the confidence limits of these measurements. The hatched zone indicates various calculations of $\Delta\Delta\mathcal{G}^\circ_{p\mathcal{H}}$ (eq 6), using the data of Schaller and Robertson (1995) for values of $pK_{a,N}$ and the range of possible $pK_{a,D}$ values determined from model compounds (Greenstein & Winitz, 1961; Pace et al., 1990; Creighton, 1993). Specifically, the values of $pK_{a,N}$ are as follows: Asp 7, 2.77; Glu 10, 4.18; Glu 19, 3.23; Asp 27, 2.28; Glu 43, 4.67; Cys 56, 2.38. Because values of $pK_{a,N}$ for Asp 7 and 27 are upper estimates, $\Delta\Delta\mathcal{G}^\circ_{p\mathcal{H}}$ is a low estimate. (△) Effects of possible negative cooperativity on $\Delta\Delta\mathcal{G}^\circ_{p\mathcal{H}}$. α_N values are as follows: Asp 7, 0.78; Asp 27, 0.92; Cys 56, 0.95. (●) Effects of both negative cooperativity (△) and a perturbed $pK_{a,D}$ (3.6) for Asp 27, as determined from the peptide OMTKY3[26−32]. Dotted lines are the results of interpolation between data points.

unpublished results). Changing the $pK_{a,D}$ for Asp 27 to this value reduces $\Delta\Delta\mathcal{G}^\circ_{p\mathcal{H}}$ by an additional 0.4 kcal/mol (Figure 6, ●). Uncertainties in α_N and $pK_{a,D}$ do not permit unambiguous resolution of the discrepancy between $\Delta\Delta G^\circ_{pH}$ and $\Delta\Delta\mathcal{G}^\circ_{p\mathcal{H}}$ but the calculations demonstrate the importance of considering such effects.

The discrepancy between the two values of $\Delta\Delta G^\circ$ may be explained by many possible combinations of $pK_{a,D}$ and α_N, but we hypothesize that Glu 19 is a significant source of the discrepancy. $\Delta\Delta G^\circ_{pH}$ is relatively insensitive to changes in ionic strength, as are the pK_as of all residues but Glu 19 (Schaller & Robertson, 1995). Furthermore, Glu 19 is part of the reactive site loop, which in itself is a stable structure but varies with respect to the rest of the molecule (Krezel et al., 1994). Perhaps interactions responsible for lowering its $pK_{a,N}$ persist in denatured protein and lower its $pK_{a,D}$. A prediction following from this hypothesis is that a mutation at Glu 19 would have no effect on $\Delta\Delta G^\circ_{pH}$.

The difference between $pK_{a,N}$ and $pK_{a,D}$ determines the contribution of each residue to $\Delta\Delta\mathcal{G}^\circ_{p\mathcal{H}}$. If noncovalent interactions persist in denatured OMTKY3 at Glu 19, then it contributes little to the pH dependence of stability. The $pK_{a,N}$s for Glu 10 and Glu 43 are similar to model compound values; therefore these residues are not major contributors to $\Delta\Delta G^\circ_{pH}$. Elimination of the three glutamate residues focuses attention on the perturbed $pK_{a,N}$s for Asp 7, Asp 27, and Cys 56.

In proteins, hydrogen bonds, buried charges, and charge−charge interactions may shift pK_as away from model compound values (Tanford & Hauenstein, 1956; Hermans & Scheraga, 1961; Tanford, 1961; McNutt et al., 1990; Mauk et al., 1991). The $pK_{a,N}$s for the two aspartate residues are probably perturbed by hydrogen bonds (Schaller & Robertson, 1995). Estimated values for $pK_{a,D}$s in OMTKY3 (3.9, 3.6, and 3.1, for Asp 7, Asp 27, and Cys 56, respectively) and lower limits of α_N (0.78, 0.92, and 0.95) yield contributions of 0.8, 1.4, and 0.8 kcal/mol to $\Delta\Delta G^\circ_{pH}$ in low salt. Combined, the glutamates contribute only 0.5 kcal/mol. The proposed role of hydrogen bonds in the pH dependence of OMTKY3 stability is also consistent with the observation that salt has little effect on $\Delta\Delta G^\circ_{pH}$.

The conclusions regarding perturbations of aspartate pK_as by hydrogen bonds are similar to those for histidine residues in RNaseT1 (McNutt et al., 1990) and apomyoglobin (Barrick et al., 1994). Many attempts have been made to model both the effects of protein structure on values of pK_a for individual amino acid residues and concomitant effects of pH on protein stability [for a review, see Antosiewicz et al. (1994)]. Present results demonstrate the importance of considering contributions from both hydrogen bonds and cooperativity of proton binding in such models.

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APPENDIX: STATISTICAL ANALYSIS

Fitting errors for ΔH_m were determined using F analysis, as described by Bevington (1969). First, the best value of

ΔH_m ($\Delta H_{m,best}$) was determined by changing the baseline to minimize the variance of the fit; then the baseline was held constant while the fit was repeated with ΔH_m fixed at values differing by 2−10% from $\Delta H_{m,best}$. The variance ratio of each new fit was plotted versus ΔH_m to generate an F plot. The target variance ratio corresponding to a 95% confidence level was identified using a table of F values (Brase & Brase, 1987). Confidence limits corresponding to the target F value were interpolated from the plot and are reported as 1 SD (σ), which is one-half of the difference between the interpolated and best values. If the F plot was asymmetric, the larger standard deviation is reported.

Further statistical testing was performed on a representative experiment in order to determine the level of correlation between ΔH_m and the chosen baseline. During fits in which ΔH_m was fixed at values differing by 2−10% from $\Delta H_{m,best}$, the baseline was changed to further minimize the variance. The new baselines never resulted in further reduction in the variance of the original fit, in which all parameters were optimized. F analysis was then repeated. The shape of the new F plot is wider than the original, but the effect is significant only at values greater than $\Delta H_{m,best} \pm 2\sigma$. Therefore, the choice of baseline has little effect on the value of ΔH_m.

The value of ΔC_p is highly dependent upon the baseline correction. During baseline optimization, the value of ΔC_p could be varied up to 3-fold while maintaining comparable variance values. In fact, values of ΔH_m and T_m for fits in which ΔC_p is fixed at zero (with an appropriate baseline) vary little from other determinations, and the data fit reasonably well to a two-state model, although the variance of the fit is worse. Since an exhaustive search of all baselines is computationally impractical, values of ΔC_p determined from fits of individual experiments are disregarded. However, ΔC_p for the unfolding of OMTKY3 is greater than zero (Swint & Robertson, 1993), baseline corrections for a fit are less involved when ΔC_p is greater than zero, and fitted values of ΔC_p are in the range of 800 ± 400 cal/(mol K), comparable to the average value of 620 ± 20 cal/(mol K). Therefore, the parameter ΔC_p is always included during the fit, even though its value cannot be determined directly with a high degree of confidence. The value of T_m usually varied by less than 1 °C as the baseline was adjusted. The maximum deviation observed for fits with comparable variance was 2.5 °C.

Experimental error in T_m, ΔH_m, and ΔC_p was propagated through ΔG_u^o calculations in the following manner. First, plots of T_m versus pH were constructed and fit to eq 4 using NonLin for the Iris (Johnson & Frasier, 1985; Brumbaugh & Huang, 1992; Johnson & Faunt, 1992). The variance (σ^2) of T_m at a given pH was determined by the equation

$$\sigma^2_{T_m} = \sum_{i,j} \sigma^2_{i,j} \frac{dT_m}{di} \frac{dT_m}{dj} \quad (7)$$

where (i,j) is all possible combinations of T_{mid}, δT, m, and pH_{mid};

$$\frac{dT_m}{dT_{mid}} = 1 \quad (8)$$

$$\frac{dT_m}{d\delta T} = \tanh[m(pH - pH_{mid})] \quad (9)$$

$$\frac{dT_m}{dm} = \delta T\{\mathrm{sech}[m(pH - pH_{mid})]\}^2(pH - pH_{mid}) \quad (10)$$

and

$$\frac{dT_m}{d(pH_{mid})} = -\delta T\{\mathrm{sech}[m(pH - pH_{mid})]\}^2 \cdot m \quad (11)$$

Next, a plot of ΔH_m versus T_m was constructed from the data (Table 1), and the best line describing these data was determined (Table 4) with NonLin for the Macintosh (adapted from M. L. Johnson's NonLin by Robert J. Brenstein, Robelko Software, Carbondale, IL). The slope of this line is ΔC_p. At any T_m, a calculated value of ΔH_m can be obtained with this equation, and the variance is determined by

$$\sigma^2_{\Delta H_m} = \sigma^2_{T_m}\left(\frac{d\Delta H_m}{dT_m}\right)^2 + \sigma^2_{\Delta C_p}\left(\frac{d\Delta H_m}{d\Delta C_p}\right)^2 + \sigma^2_{\mathrm{intercept}}\left(\frac{d\Delta H_m}{d(\mathrm{intercept})}\right)^2 + 2\sigma^2_{\Delta C_p,\mathrm{intercept}} \frac{d\Delta H_m}{d\Delta C_p} \frac{d\Delta H_m}{d(\mathrm{intercept})} \quad (12)$$

where

$$\frac{d\Delta H_m}{dT_m} = \Delta C_p \quad (13)$$

$$\frac{d\Delta H_m}{d\Delta C_p} = T_m \quad (14)$$

and

$$\frac{d\Delta H_m}{d(\mathrm{intercept})} = 1 \quad (15)$$

Finally, the value of ΔG_u^o was calculated with the modified Gibbs−Helmholtz equation (eq 5). In this calculation, the value of ΔC_p is the weighted average, 620 ± 20 cal/(mol K). The variance of ΔG_u^o was calcualted from the equation

$$\sigma^2_{\Delta G_u^o} = \sigma^2_{\Delta H_m}\left(\frac{d\Delta G_u^o}{d\Delta H_m}\right)^2 + \sigma^2_{\Delta C_p}\left(\frac{d\Delta G_u^o}{d\Delta C_p}\right)^2 \quad (16)$$

where

$$\frac{d\Delta G_u^o}{d\Delta H_m} = 1 - T/T_m \quad (17)$$

and

$$\frac{d\Delta G_u^o}{d\Delta C_p} = (T - T_m) - [T \ln(T/T_m)] \quad (18)$$

Contributions from T_m to the error in ΔG_u^o are assumed to be propagated through the contributions from ΔH_m.

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