Thermodynamics of Ribonuclease T1 Denaturation†

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ABSTRACT: Differential scanning calorimetry has been used to investigate the thermodynamics of denaturation of ribonuclease T1 as a function of pH over the pH range 2–10, and as a function of NaCl and $MgCl_2$ concentration. At pH 7 in 30 mM PIPES buffer, the thermodynamic parameters are as follows: melting temperature, $T_{1/2}$ = 48.9 ± 0.1 °C; enthalpy change, ΔH = 95.5 ± 0.9 kcal mol^{-1}; heat capacity change, ΔC_p = 1.59 kcal mol^{-1} K^{-1}; free energy change at 25 °C, $\Delta G°$(25 °C) = 5.6 kcal mol^{-1}. Both $T_{1/2}$ = 56.5 °C and ΔH = 106.1 kcal mol^{-1} are maximal near pH 5. The conformational stability of ribonuclease T1 is increased by 3.0 kcal/mol in the presence of 0.6 M NaCl or 0.3 M $MgCl_2$. This stabilization results mainly from the preferential binding of cations to the folded conformation of the protein. The estimates of the conformational stability of ribonuclease T1 from differential scanning calorimetry are shown to be in remarkably good agreement with estimates derived from an analysis of urea denaturation curves.

The conformational stability of a globular protein is generally defined as the free energy change at 25 °C in the absence of a denaturant for the reaction:

folded ↔ unfolded

There is considerable interest in measuring the conformational stability because globular proteins are only biologically active when they are folded (Pace, 1990). It has been clear for many years that the conformational stability of globular proteins is remarkably low, generally between 5 and 15 kcal/mol (Pace, 1975; Privalov, 1979). However, there has been uncertainty in how accurately the conformational stability can be measured. The two methods most often used to estimate the conformational stability are differential scanning calorimetry (DSC) (Jackson & Brandts, 1970; Privalov & Khechinashvili, 1974; Freier & Biltonen, 1978; Privalov & Potekhin, 1986; Becktel & Schellman, 1987; Sturtevant, 1987) and solvent denaturation studies using urea and guanidine hydrochloride (GdnHCl) (Green & Pace, 1974; Pace, 1986; Santoro & Bolen, 1988). Both methods require an extrapolation of the results from the conditions used to promote unfolding to physiological conditions. DSC experiments yield the midpoint of thermal unfolding, $T_{1/2}$, the enthalpy change at this temperature, $\Delta H_{1/2}$, and the difference in heat capacity between the folded and unfolded conformations, ΔC_p. These parameters can then be used to estimate the free energy change at any temperature, $\Delta G°(T)$, with the Gibbs–Helmholtz equation:

$$\Delta G°(T) = \Delta H_{1/2}(1 - T/T_{1/2}) - \Delta C_p[(T_{1/2} - T) + T \ln (T/T_{1/2})] \quad (1)$$

Solvent denaturation experiments yield $\Delta G°$ as a function of denaturant concentration. The dependence of $\Delta G°$ on denaturant concentration is generally linear, and the results are fit to the equation:

$$\Delta G° = \Delta G°(H_2O) - m(\text{denaturant}) \quad (2)$$

where $\Delta G°(H_2O)$ is an estimate of the value of $\Delta G°$ in the absence of denaturant and m is a measure of the dependence of $\Delta G°$ on denaturant concentration. Other methods have also been used to extrapolate $\Delta G°$ to zero concentration of denaturant to obtain $\Delta G°(H_2O)$ (Pace, 1986). Therefore, the most frequently used estimates of the conformational stability of a protein are $\Delta G°$(25 °C) from DSC studies and $\Delta G°(H_2O)$ from solvent denaturation studies. Surprisingly, these estimates have never been compared for the same protein under a variety of conditions. One aim of this paper is to provide such a comparison with one small protein, ribonuclease T1 (RNase T1).

The conformational stability of RNase T1 has previously been measured as a function of salt concentration (Pace & Grimsley, 1988), temperature (Pace & Laurents, 1989), and pH (Pace et al., 1990; McNutt et al., 1990) using urea denaturation. In this paper, DSC is used to determine the thermodynamics of denaturation under the same conditions. The agreement between estimates of the conformational stability from the two approaches is shown to be remarkably good.

Experimental Procedures

Two different forms of RNase T1 are under active study: Gln25-RNase T1 and Lys25-RNase T1. Gln25-RNase T1 was first isolated by Sato and Egami (1957), and is the RNase T1 used for all of the experiments reported here. RNase T1 was prepared from a gene expressed in *Escherichia coli* (Shirley & Laurents, 1990). Concentrations were determined using 1.67 as the absorbance at 278 nm of a 1 mg/mL solution (Pace et al., unpublished observations). The buffers used were 30 mM MOPS for the urea denaturation experiments at pH 7 and 30 mM glycine (pH 2.2, 2.8, 9.0, and 10.0), acetate (pH 4.0 and 5.0), and PIPES (pH 6.0–8.0) for the calorimetry experiments.

Urea denaturation curves were determined by measuring the intrinsic fluorescence intensity (278-nm excitation, 320-nm emission) with an SLM 8000 spectrofluorometer of solutions containing ≈0.5 μM RNase T1 in 1-cm cuvettes thermostated to maintain the temperature at ±0.1 °C (Shirley et al., 1992). At lower temperatures, care was taken to assure that the unfolding reaction had reached equilibrium. Since the un-

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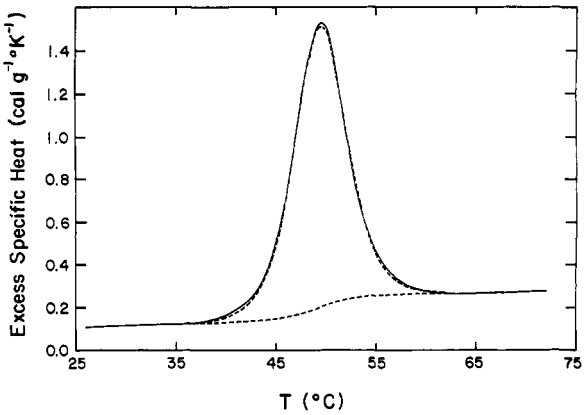

FIGURE 1: Typical DSC curve for the thermal denaturation of RNase T1 at pH 7 in 30 mM PIPES buffer. The RNase T1 concentration was 4.96 mg/mL. The solid curve is the observed excess specific heat capacity, and the dashed curve is based on the following parameters: $T_{1/2}$ = 49.36 °C; Δh_{cal} = 9.10 cal g^{-1}, β = 13 080, and ΔC_p = 0.105 cal g^{-1} K^{-1}.

folding of RNase T1 closely approaches a two-state mechanism (Thomson et al., 1989), the free energy of unfolding, $\Delta G°$, can be calculated using

$$\Delta G° = -RT \ln K = -RT \ln [(y_F - y)/(y - y_U)] \quad (3)$$

where R is the gas constant, T is the absolute temperature, K is the equilibrium constant, y is the observed fluorescence intensity, and y_F and y_U are the fluorescence intensities characteristic of the folded and unfolded states, respectively (Pace et al., 1989).

Three different calorimeters were used for the DSC experiments: an MC-2 from Microcal Inc. and a DASM-1 and a DASM-4 from Biopribor (Privalov, 1980; Privalov & Potekhin, 1986). For all of the DSC scans, the RNase T1 was dialyzed against the experimental buffer using 3500 molecular weight cutoff dialysis tubing, and then a base line was determined by filling both cells of the calorimeter with the dialysis buffer. A scan rate of 1 °C/min was used for base-line and sample runs after it was shown that essentially identical results are obtained at a scan rate of 0.5 °C/min. By rescanning samples a second time, it was shown that thermal denaturation is almost completely reversible except near the isoelectric pH (≈4) where precipitation of denatured RNase T1 is sometimes observed.

To analyze a DSC experiment (Sturtevant, 1987; Connelly et al., 1991), the instrumental base line determined with buffer in both cells was subtracted from the results obtained with the sample. Pre- and posttransition base lines, $C_F = A + BT$ and $C_U = C + DT$ in calories per gram per degree kelvin, are defined by a least-squares analysis of the data before and after the transition, and a chemical base line is calculated as the progress from C_F to C_U in proportion to the increase in the heat absorption during the transition as evaluated in the curve-fitting process. The difference between the observed data and the chemical base line, the excess apparent specific heat, is fitted by a least-squares calculation to a theoretical curve for a modified two-state process, with the adjustable parameters $T_{1/2}$ (the temperature of half-completion of the transition), Δh_{cal} (the specific enthalpy in calories per gram), and $\beta = \Delta H_{vH}/\Delta h_{cal}$ where ΔH_{vH}, the van't Hoff enthalpy, is the quantity which controls the progress of the transition according to the van't Hoff equation:

$$d(\ln K)/d\ T = \Delta H_{vH}/RT^2 \quad (4)$$

A typical DSC experiment is shown in Figure 1. The adequacy of the fit is indicated by the standard deviation of the observed data from the calculated excess apparent specific heat expressed as the percent of the maximal value of the excess specific heat. This quantity ranges from 0.5 to 2.6% with a mean value of 1.0 ± 0.3% for the experiments reported here. The standard deviation for the DSC scan shown in Figure 1 is 0.9%.

Table I: Thermodynamic Parameters Characterizing the Thermal Denaturation of Ribonuclease T1 as a Function of pH[a]

pH[b]	$T_{1/2}$ (°C)	ΔH_{cal} (kcal mol^{-1})	ΔH_{vH} (kcal mol^{-1})	ΔC_p[c] (kcal mol^{-1} K^{-1})	$\Delta\nu$[d]
10.0	39.38 ± 0.19	76.9 ± 0.7	104.3 ± 2.0	−0.40 ± 0.56	−0.98
9.0	40.73 ± 0.12	76.5 ± 0.4	113.9 ± 0.7	0.42 ± 0.23	−1.06
8.0	45.80 ± 0.09	91.6 ± 1.8	116.0 ± 0.6	0.88 ± 0.06	−1.04
7.5	46.74 ± 0.07	93.2 ± 1.1	120.9 ± 1.1	1.71 ± 0.62	−1.07
7.0	48.91 ± 0.11	95.5 ± 0.9	118.9 ± 1.1	1.35 ± 0.15	−1.04
6.0	54.03 ± 0.07	101.0 ± 3.5	115.6 ± 2.3	0.69 ± 0.04	−0.98
5.0	56.54 ± 0.01	106.1 ± 0.4	116.7 ± 0.4	1.74 ± 0.15	−0.98
4.0	56.71 ± 0.11	102.2 ± 0.5	114.1 ± 1.7	2.47 ± 0.66	1.68
2.8	49.05 ± 0.14	95.3 ± 0.7	109.3 ± 0.9	1.64 ± 0.48	1.61
2.2	45.43 ± 0.15	89.6 ± 0.7	113.7 ± 0.9	1.53 ± 0.78	1.78
mean (±standard error)			114.3 ± 1.7	1.20 ± 0.29	

[a] The errors given are standard errors of the mean. [b] The following buffers were used at a concentration of 30 mM: glycine (pH 2.2, 2.8, 9.0, and 10.0); acetate (pH 4.0 and 5.0); PIPES (pH 6.0–8.0). [c] For all 32 DSC experiments, ΔH_{cal} = 16.28 + 1.591($T_{1/2}$) (standard deviation = 2.93; R^2 = 0.893) (Figure 5). [d] For the data from pH 5 to 10: $T_{1/2}$ = 75.03 − 3.675(pH) (standard deviation = 0.88, R^2 = 0.981). For the data from pH 2.2 to 4.0: $T_{1/2}$ = 31.55 + 6.283(pH) (standard deviation = 0.08, R^2 = 1.0). $\Delta\nu = (\Delta H_{cal}/2.303RT_{1/2}^2)(d\ T_{1/2}/d\ pH)$.

For a true two-state process, β = molecular weight (MW), but in many cases it is found that a satisfactory fit can be obtained only if β/MW is allowed to vary significantly from unity. If the constants B and D expressing the variation of C_F and C_U with temperature are not equal, the apparent value of $\Delta C_p = C_U - C_F$ will vary with temperature, and this variation should be included in the curve-fitting calculation. It is frequently not possible to give a satisfactory explanation for the deviations of β/MW from unity, or for the apparent dependence of ΔC_p on temperature. This latter anomaly, which is not as evident in the data shown in Figure 1 as in many of the other experiments reported here, is particularly puzzling since plots of ΔH_{cal} (=MWΔh_{cal}) vs temperature uniformly indicate that ΔC_p is independent of temperature (see Figure 5).

Results

Table I summarizes results from 32 DSC scans determined over the pH range 2.2–10. Figure 2 shows $T_{1/2}$ as a function of pH from the DSC results reported here and includes $T_{1/2}$ values from thermal denaturation curves determined independently with different spectroscopic techniques in three laboratories. The agreement is reasonably good.

It can be seen in Table I that ΔH_{vH} is constant to well within experimental uncertainty and is greater than ΔH_{cal} over the entire pH range. $\Delta H_{vH} > \Delta H_{cal}$ has also been observed with Lys25-RNase T1 by Kiefhaber et al. (1990) if their results are corrected by using the same extinction coefficient as used here, and by Plaza del Pino and Freire (unpublished observations). $\Delta H_{vH} > \Delta H_{cal}$ is frequently interpreted as being due to self-association of either the folded or the unfolded form of the protein or of both forms (Sturtevant, 1987). The fact that $T_{1/2}$ does not depend on protein concentration up to 10 mg/mL at pH 7 suggests that the extent of self-association would have to be the same for both forms of the protein. This seems unlikely, and, in addition, there is no evidence from gel filtration studies of self-association of folded RNase T1 up to a concentration of ≈5 mg/mL.

Table II: Thermodynamic Parameters Characterizing the Thermal Denaturation of Ribonuclease T1 as a Function of NaCl Concentration[a]

[NaCl] (M)	$T_{1/2}$ (°C)	ΔH_{cal} (kcal mol^{-1})	ΔH_{vH} (kcal mol^{-1})	ΔH_B(app)[b] (kcal mol^{-1})	ΔC_p[c] (kcal mol^{-1} K^{-1})	$\Delta(\Delta G°)$[d] (kcal mol^{-1})
0	48.91	95.5	118.4		1.35	
0.05	51.05	99.5	120.4	1.6	0.93	0.65
0.10	52.06	101.4	118.6	2.4	1.60	0.96
0.20	53.88	105.2	121.0	4.2	1.31	1.54
0.40	56.49	107.4	121.4	3.5	1.40	2.33
0.60	58.49	110.6	121.7	4.5	1.31	2.97
0.80	60.22	110.8	124.1	2.7	2.11	3.45
1.20	63.19	112.4	125.9	1.1	1.42	4.28
1.60	65.59	114.7	125.0	0.7	1.37	4.98
mean (±standard error)			121.8 ± 0.9	2.6 ● 0.6	1.42 ± 0.12	

[a] The concentration of RNase T1 was 2.63 mg/mL, and the buffer was 30 mM PIPES, pH 7. The standard deviations of the fitted curves ranged from 0.7 to 1.1%. [b] $\Delta H_B(app) = \Delta H_{cal} - \Delta C_p[T_{1/2} - T_{1/2}(0\ M\ NaCl)] - \Delta H_{cal}(0\ M\ NaCl)$. A ΔC_p value = 1105 cal mol^{-1} K^{-1} was used for these calculations. [c] For these experiments, ΔC_p = 1105 ± 170 (standard deviation) cal mol^{-1} K^{-1} based on a plot of ΔH_{cal} vs $T_{1/2}$ (e.g., see Figure 5). [d] The $\Delta(\Delta G°)$ values were calculated by using eq 1 with ΔC_p = 1590 cal mol^{-1} K^{-1} to calculate $\Delta G°$ (48.91 °C) at each NaCl concentration. The $\Delta(\Delta G°)$ values depend only slightly on the value of ΔC_p used. For example, in the worst case, 1.6 M NaCl, the $\Delta(\Delta G°)$ value would be increased from 4.98 to 5.19 kcal mol^{-1} (a 4% increase) if ΔC_p = 1105 were used instead of 1590 cal mol^{-1} K^{-1}.

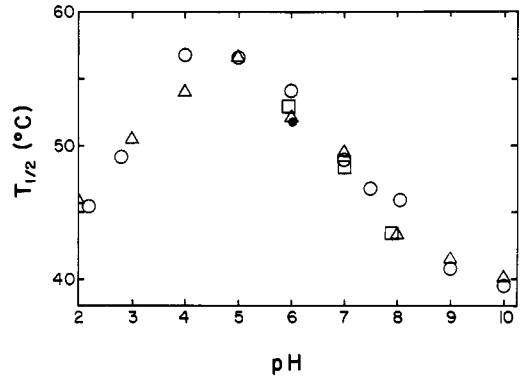

FIGURE 2: $T_{1/2}$ as a function of pH for the thermal unfolding of RNase T1. (○) From the DSC results in Table I; (□) from Pace and Laurents (1989); (△) interpolated values (±0.5 °C) from Figure 5 in Oobatake et al. (1979); (●) from Walz and Kitareewan (1990).

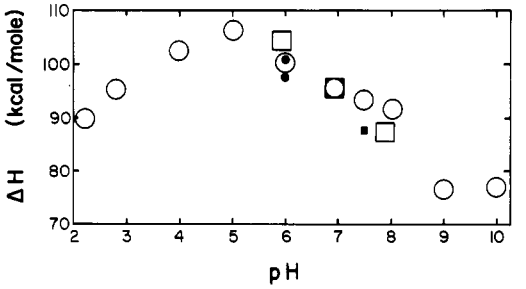

FIGURE 3: ΔH as a function of pH for the thermal unfolding of RNase T1. (○) ΔH_{cal} from the DSC results in Table I; (□) ΔH_{vH} from Pace and Laurents (1989); (●) ΔH_{vH} from Walz and Kitareewan (1990); (■) ΔH_{vH} from Okajima et al. (1990).

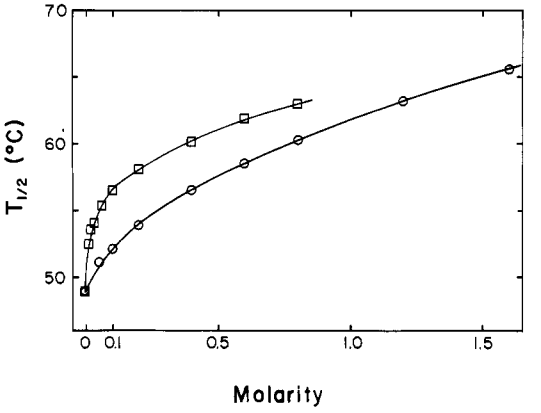

FIGURE 4: $T_{1/2}$ as a function of NaCl (○) or $MgCl_2$ (□) molarity for the thermal denaturation of RNase T1 at pH 7 in 30 mM PIPES buffer. These results are from Tables II and III.

An alternative explanation for $\Delta H_{vH} > \Delta H_{cal}$ has recently been suggested by Xie et al. (1991), who found this situation to occur in the thermal unfolding of apo-α-lactalbumin in the presence of guanidine hydrochloride and attributed it to the partial formation of a "molten globule" form with a reduced calorimetric enthalpy. There is at present no other indication of the existence of a molten globule form of RNase T1.

A further possibility should be mentioned. The ΔH_{cal} value depends on the protein concentration, but the ΔH_{vH} value does not. The extinction coefficient that has been used over the years for RNase T1 is 1.91 at 278 nm for a 1 mg/mL solution (Takahashi, 1962). The method used to determine this extinction coefficient was not reported. For this reason, we have carefully determined the extinction coefficient for RNase T1 using a dry-weight procedure (Hunter, 1966), and find 1.67 ± 0.3 for the absorbance of a 1 mg/mL solution at 278 nm (Pace et al., unpublished observations). Using the same procedure, we get excellent agreement with literature values for the extinction coefficients of bovine serum albumin, ribonuclease A, and lysozyme. Thus, we do not think the discrepancy between ΔH_{cal} and ΔH_{vH} values determined by DSC is the result of errors in the determination of protein concentration.

It is perplexing that the ΔH_{cal} values from DSC, which differ significantly from the ΔH_{vH} values from DSC, are nevertheless in good agreement with ΔH_{vH} values obtained from a two-state analysis of thermal denaturation curves (Figure 3). At pH 7, for example, ΔH_{cal} = 95.5 kcal mol^{-1} from DSC (Table I), and the ΔH_{vH} values from thermal denaturation curves are 96.3–98.5 kcal mol^{-1} using five different techniques to follow unfolding (Thomson et al., 1989), 93 and 96 kcal mol^{-1} (Shirley et al., 1989), and 95.2 kcal mol^{-1} (Pace & Laurents, 1989). Thus, there is excellent agreement between ΔH_{cal} determined by DSC and ΔH_{vH} evaluated from thermal denaturation curves. For this reason, we have used the ΔH_{cal} values from the DSC experiments for further analyses of the data given in the tables and under Discussion. The ΔC_p values and the pH dependence of the thermodynamic parameters will be considered in more detail under Discussion.

Table II summarizes results derived from DSC scans determined as a function of the NaCl concentration, and Table III summarizes results derived from DSC scans as a function of $MgCl_2$ concentration. In Figure 4, $T_{1/2}$ values from the DSC experiments are plotted as a function of salt concentration. The dependence of $T_{1/2}$ on salt concentration is similar to that determined using thermal denaturation by Oobatake

Table III: Thermodynamic Parameters Characterizing the Thermal Denaturation of Ribonuclease T1 as a Function of $MgCl_2$ Concentration[a]

[$MgCl_2$] (M)	$T_{1/2}$ (°C)	ΔH_{cal} (kcal mol^{-1})	ΔH_{vH} (kcal mol^{-1})	ΔH_B(app)[b] (kcal mol^{-1})	ΔC_p[c] (kcal mol^{-1} K^{-1})	$\Delta(\Delta G°)$[d] (kcal mol^{-1})
0	48.91	95.5	118.4		1.35	
0.01	52.44	98.2	116.8	−1.3	1.22	1.03
0.02	53.52	98.9	116.7	−1.9	0.86	1.34
0.03	54.00	99.1	118.9	−2.2	0.94	1.48
0.06	55.39	103.7	120.3	−0.8	1.02	1.94
0.10	56.56	103.5	121.1	−0.7	1.11	2.26
0.20	58.07	104.4	122.1	−1.5	0.71	2.68
0.40	60.13	107.8	121.8	−0.5	1.22	3.33
0.60	61.86	109.4	124.7	−0.9	1.07	3.83
0.80	62.97	110.6	120.6	−0.9	1.14	4.15
mean (±standard error)			120.1 ± 1.9	−1.0 ± 0.3	1.06 ± 0.07	

[a] The concentration of RNase T1 was 1.06–1.56 mg/mL, and the buffer was 30 mM PIPES, pH 7. The standard deviation of the fitted curves ranged from 0.7 to 1.1%. [b] $\Delta H_B(app) = \Delta H_{cal} - \Delta C_p[T_{1/2} - T_{1/2}(0\ M\ MgCl_2)] - \Delta H_{cal}(0\ M\ MgCl_2)$. A ΔC_p value = 1140 cal mol^{-1} K^{-1} was used for these calculations. [c] For these experiments, ΔC_p = 1140 ± 115 (standard deviation) cal mol^{-1} K^{-1} based on a plot of ΔH_{cal} vs $T_{1/2}$. [d] The $\Delta(\Delta G°)$ values were calculated by using eq 1 with ΔC_p = 1590 cal mol^{-1} K^{-1} to calculate $\Delta G°$(48.91 °C) at each $MgCl_2$ concentration. The $\Delta(\Delta G°)$ values depend only slightly on the ΔC_p value used (See Table II).

Table IV: Parameters Characterizing Urea Denaturation Curves for Ribonuclease T1 Determined in 30 mM MOPS Buffer, pH 7[a]

temp (°C)	m[b] (cal mol^{-1} M^{-1})	$(urea)_{1/2}$ (M)	$\Delta G°(H_2O)$[b] (kcal mol^{-1})
9.98	1170	5.86	7.10
14.90	1260	5.65	6.84
19.20	1210	5.26	6.37
21.10	1100	4.98	6.03
23.05	1200	4.86	5.89
25.00	1200	4.58	5.55
27.00	1200	4.14	5.01
29.00	1265	3.93	4.76
34.75	1220	2.92	3.54
40.10	1280	2.13	2.58

[a] $\Delta G°$ was calculated as a function of urea concentration for the points in the transition region as described by Pace et al. (1989), and these data were fit to eq 2 using least squares. The midpoint of the urea unfolding curve, $(urea)_{1/2}$, $= \Delta G°(H_2O)/m$. We estimate the error to be ±0.05 M for the $(urea)_{1/2}$ values and ±50 cal mol^{-1} M^{-1} for the m values. [b] The average of the m values is 1211 ± 36 (average deviation) cal mol^{-1} M^{-1}. Since the m values do not appear to vary with temperature, the average m value was used to calculate the $\Delta G°(H_2O)$ values.

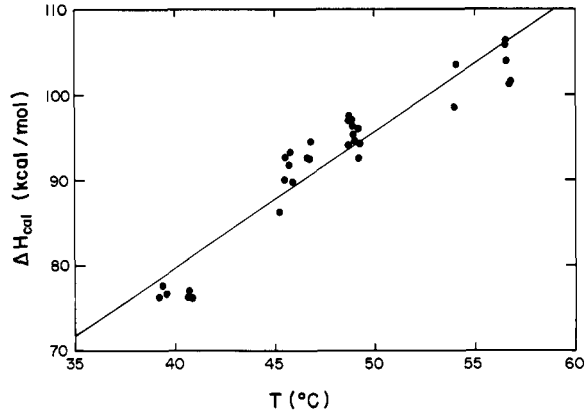

FIGURE 5: ΔH_{cal} as a function of $T_{1/2}$ as determined in 32 DSC experiments. A least-squares analysis gives ΔC_p = 1.59 ± 0.10 (standard error) kcal mol^{-1} K^{-1}. The experimental points deviate from the least-squares line with a standard deviation of 2.9 kcal mol^{-1}.

et al. (1979) at pH 5 for NaCl, and by Walz and Kitareewan (1990) at pH 6 for NaCl and $MgCl_2$. In addition, the stabilization of Lys25-RNase T1 at pH 5 by NaCl is very similar to the stabilization of Gln25-RNase T1 at pH 7 that we observe (Kiefhaber et al., 1990). The marked stabilization of RNase T1 by NaCl and $MgCl_2$ has been shown to result mainly from the preferential binding of cations to the folded conformation of RNase T1 (Pace & Grimsley, 1988).

Ten urea denaturation curves were determined at temperatures ranging from 10 to 40 °C in order to get estimates of the conformational stability for comparison with estimates from the DSC experiments. These curves were analyzed and fit to eq 2 as described by Pace et al. (1989). The results are summarized in Table IV. The m values do not vary significantly with temperature, and the average m value is 1211 ± 36 cal mol^{-1} M^{-1}. The $\Delta G°(H_2O)$ values were calculated with this average m value rather than the individual m values determined at each temperature. Note that the $\Delta G°(H_2O)$ values decrease by over 4.5 kcal mol^{-1} over this temperature range.

Discussion

To use the DSC results to estimate $\Delta G°$(25 °C) requires a value for ΔC_p as shown by eq 1. ΔC_p values can be estimated from individual DSC scans, and these are the values given in Table I. The average of these values is 1200 ± 290 cal mol^{-1} K^{-1}. However, difficulties with the posttransition base lines limit the accuracy of this method, and it is generally regarded as more reliable to estimate ΔC_p by taking the slope of a plot of ΔH_{cal} vs $T_{1/2}$, where $T_{1/2}$ has been varied by changing the pH (Privalov, 1979; Becktel & Schellman, 1987). This gives ΔC_p = 1590 cal mol^{-1} K^{-1} (Figure 5), in good agreement with a value of 1650 ± 200 cal mol^{-1} K^{-1} derived from urea and thermal unfolding studies of RNase T1 (Pace & Laurents, 1989). Kiefhaber et al. (1990) estimated values of ΔC_p for Lys25-RNase T1 of 1030, 1220, 1554, and 1650 cal mol^{-1} K^{-1} using different approaches.

The solid line in Figure 6 shows the variation of $\Delta G°(T)$ calculated with eq 1 using the results from DSC: $T_{1/2}$ = 48.9 °C, ΔH_{cal} = 95.5 kcal mol^{-1}, and ΔC_p = 1590 cal mol^{-1} K^{-1}. The open circles show the $\Delta G°(H_2O)$ values from urea denaturation curves given in Table IV. The average deviation of the 10 $\Delta G°(H_2O)$ values from the $\Delta G°(T)$ values from DSC is ± 0.22 kcal mol^{-1}. It should be noted that these are completely independent experiments. In a previous paper, we had a plot similar to Figure 6 in which the $\Delta G°(T)$ values were based on $T_{1/2}$ = 48.3 °C and ΔH_{vH} = 95.2 kcal mol^{-1} from thermal denaturation experiments and ΔC_p = 1650 cal mol^{-1} K^{-1} on the basis of these results plus results from urea denaturation curves (Pace & Laurents, 1989). The average deviation of the 10 $\Delta G°(H_2O)$ values from these results is ±0.11 kcal mol^{-1}. Thus, the conformational stability estimates from DSC, thermal denaturation curves, and urea denaturation curves at temperatures in the range 10–40 °C, are in astonishingly good agreement. [The error in the $\Delta G°(H_2O)$ values

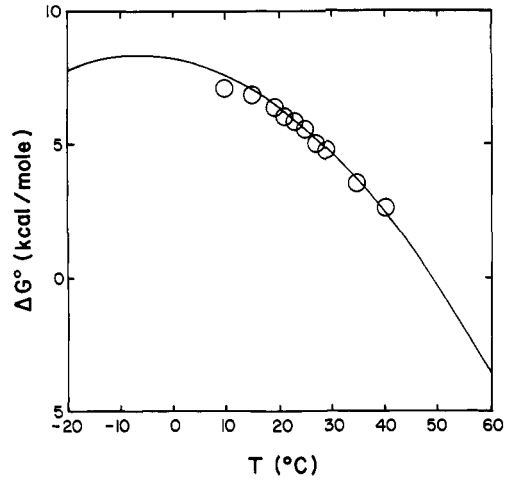

FIGURE 6: $\Delta G°$ as a function of temperature for the denaturation of RNase T1 at pH 7. The solid curve was calculated using eq 1 and the following parameters derived from the DSC results in Table I: $T_{1/2}$ = 48.91 °C; ΔH_{cal} = 95.5 kcal mol^{-1}; ΔC_p = 1590 cal mol^{-1} K^{-1}. The open circles are the $\Delta G°(H_2O)$ values derived from the urea denaturation curves described in Table IV.

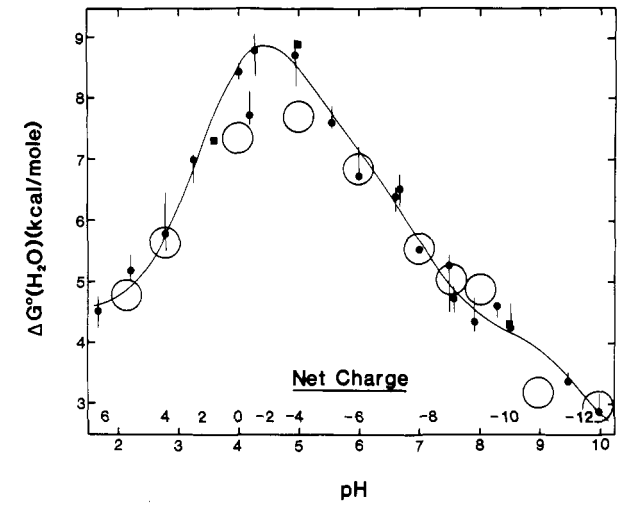

FIGURE 7: $\Delta G°$ as a function of pH for the denaturation of RNase T1 at 25 °C. The solid circles and squares are $\Delta G°(H_2O)$ values from urea denaturation experiments published previously (Pace et al., 1990). The open circles are values for $\Delta G°$(25 °C) calculated with eq 1 using the results from the DSC experiments reported in Table I. The $T_{1/2}$ and ΔH_{cal} values used were taken from the appropriate pH value in Table I, and ΔC_p = 1590 cal mol^{-1} K^{-1} from Figure 5 was used. The net charge given is based on titration studies of RNase T1 by Iida and Ooi (1969).

from urea denaturation experiments is generally estimated at ±0.2–0.3 kcal mol^{-1} (Shirley et al., 1992).] Thus, at least in this case, the use of linear extrapolation to estimate $\Delta G°(H_2O)$ leads to good agreement with $\Delta G°(T)$ values from DSC experiments. Bolen and co-workers have results from other approaches that also support the validity of the linear extrapolation model for estimating the conformational stability of a protein (Santoro & Bolen, 1988; Bolen et al., unpublished observations). This is especially interesting because the product of thermal denaturation is generally thought to be less completely unfolded than the product of urea denaturation (Tanford, 1968). However, it has been noted before that this does not seem to significantly affect the thermodynamics of denaturation (Pfiel & Privalov, 1976; Privalov, 1979; Privalov et al., 1989).

In a previous study, we used urea denaturation studies to estimate the conformational stability of RNase T1 as a function of pH. These results are shown by the solid circles and squares in Figure 7. The open circles in Figure 7 were calculated from the $T_{1/2}$ and ΔH_{cal} values at each pH from Table I along with ΔC_p = 1590 cal mol^{-1} K^{-1}. The agreement is very good at both high and low pH, but not as good near the isoelectric point. We think that the DSC estimates are somewhat lower here because the unfolded state tends to aggregate slightly at the higher concentrations used in the DSC experiments. The urea denaturation experiments were done at concentrations of ≈0.005 mg/mL while the concentrations for the DSC experiments were generally between 1 and 5 mg/mL. Thus, again, the agreement between estimates of the conformational stability from urea denaturation and DSC is good.

It is clear from Figures 2 and 7 that the maximum stability of RNase T1 occurs near pH 5. This is slightly higher than the isoionic pH of ≈4 (Iida & Ooi, 1969). The last column in Table I gives an estimate of the number of protons bound or released when RNase T1 unfolds on the basis of DSC results. On the basis of our results with urea denaturation, we estimated that ≈1.7 protons are bound on unfolding at low pH and that ≈1.2 protons are released on unfolding above pH 5 (Pace et al., 1990). These estimates are in good agreement with the values from DSC in Table I. The binding of protons on unfolding at low pH probably results because some of the carboxyl groups will have lower pK's when the protein is folded that when it is unfolded (Tanford, 1961). We have shown previously that the release of protons on unfolding in the pH range 5–10 results mainly because His40 has a pK = 7.8 and His92 has a pK = 7.7 in folded RNase T1, but both would have a pK ≈ 6.6 in unfolded RNase T1 (McNutt et al., 1990). Thus, these His residues bind protons more tightly when RNase T1 is folded. It can be seen in Figure 3 that ΔH_{cal} depends on pH, and goes through a maximum near pH 5. For Lys25-RNase T1, $T_{1/2}$ and ΔH_{cal} are also both maximal at pH 5 (Kiefhaber et al., 1990).

Oobatake et al. (1979) first showed that RNase T1 is stabilized by the addition of NaCl and other salts. Pace and Grimsley (1988) suggested that the stabilization results mainly because ions are bound preferentially to the folded conformation of the protein. More recently, Walz and Kitareewan (1990) showed that tetraprotonated spermine also stabilizes RNase T1 by preferential binding to folded RNase T1. Therefore, folded RNase T1 has a site or sites that bind Na^+, Mg^{2+}, spermine, and other cations. In addition, the active site of RNase T1 is known to bind phosphate (Pace & Grimsley, 1988; Walz & Kitareewan, 1990), as does the active site of RNase A (Anderson et al., 1968).

In our previous paper, we used results from urea denaturation experiments to characterize the binding of cations to RNase T1. When a ligand, L, binds preferentially to either the folded or the unfolded conformation of a protein, folded ↔ unfolded + (Δn)L, then

$$d(\ln K)/d(\ln a_L) = n_F - n_U = \Delta n \quad (5)$$

where K is the equilibrium constant for unfolding, a_L is the activity of L, and n_F and n_U are the number of ligand molecules bound by the folded and unfolded conformations of RNase T1, respectively (Record et al., 1978). Thus, measurements of K as a function of a_L allow Δn to be estimated. Assuming that the entire stabilization by salts results from the preferential binding of Δn ions at identical and independent binding sites on folded RNase T1, the relationship among $\Delta(\Delta G°)$, a_L, and the binding constant, K_B, is given by (Schellman, 1975)

$$\Delta(\Delta G°) = \Delta G°(L) - \Delta G°(L=0) = (\Delta n)RT \ln (1 + K_B a_L) \quad (6)$$

Table V: Parameters Characterizing the Binding of Cations to Ribonuclease T1 Derived from Urea Denaturation Curves and Differential Scanning Calorimetry

	NaCl		$MgCl_2$	
method	Δn	K_B	Δn	K_B
UDC[a]	−2.2	8.8	−1.1	200
DSC[b]	−2.1 ± 0.4	14.6 ± 1.1	−1.3 ± 0.1	240 ± 23
DSC[c]	−1.1	≈45		
	−3.6	≈7		

[a] The results from urea denaturation curves (UDC) were obtained at 25 °C in 30 mM MOPS buffer, pH 7, in the presence of 4–7 M urea [from Pace and Grimsley (1988)]. [b] The results from differential scanning calorimetry (DSC) were obtained at temperatures in the range 48–65 °C in 30 mM PIPES buffer, pH 7 (Tables II and III). The $\Delta(\Delta G°)$ values were used with eq 5 to calculate Δn, and with eq 6 to calculate K_B. All of the data were used to obtain Δn, but just the data below 0.6 M were used to calculate the values of K_B, as was done by Pace and Grimsley (1988). [c] The plots resulting when the data were analyzed with eq 5 were nonlinear, indicating two classes of Na^+ binding sites. The first row gives the Δn value for the data at 0.2 M NaCl and below, and the second row gives the Δn value for the data above 0.2 M NaCl. The K_B values were then calculated from the data used to determine the Δn values with eq 6.

(For all of the analyses with eq 5 and 6, the mean ion activities of the salts determined at 25 °C were used for a_L.) Plots according to eq 5 for the NaCl data from Table II are decidedly nonlinear. This was not clear with the less extensive data available from urea denaturation curves (Pace & Grimsley, 1988). Consequently, we first analyzed the DSC results just as we had done in our previous study, and these are the results shown in the first row under DSC in Table V. The nonlinearity is evident from the large error in the Δn value. The results are in reasonably good agreement when it is considered that the conditions for the urea denaturation experiments are 25 °C in the presence of 4–7 M urea and the conditions for the DSC experiments are 48–66 °C in the absence of urea.

Kiefhaber et al. (1990) used DSC to study the influence of NaCl on the conformational stability of Lys25-RNase T1. The dependence of $T_{1/2}$ on [NaCl] that they observe is very similar to that shown for Gln25-RNase T1 in Figure 4. They noted that plots equivalent to those using eq 5 were nonlinear, and they found $\Delta n = -0.8$ and -3.6 at low and high NaCl concentrations. As shown in Table V, our results yield similar estimates, and show that the binding constant for the tighter binding site is ≈45 and the binding constants for the weaker binding sites are ≈7.

In one aspect, our results do differ from those of Kiefhaber et al. (1990). They observed that ΔH_{cal} does not depend on NaCl concentration and concluded "...that unfolding of the protein in the presence of NaCl at pH 5 does not involve any measurable change in heat capacity". In contrast, we observe a substantial ΔC_p of 1420 cal mol^{-1} K^{-1} from the individual experiments in NaCl (Table II), and 1105 from a plot of ΔH_{cal} vs $T_{1/2}$. In the presence of $MgCl_2$, we observe ΔC_p = 1060 cal mol^{-1} K^{-1} from the individual experiments, and 1140 from a plot of ΔH_{cal} vs $T_{1/2}$. Thus, salt may reduce ΔC_p, but for Gln25-RNase T1 at pH 7, ΔC_p is still large and positive.

The value of ΔC_p has only a small effect on the $\Delta(\Delta G°)$ values given in Tables II and III (see footnote *d* in Table II). However, it becomes very important in attempting to estimate the enthalpy change for the binding of Na^+ and Mg^{2+} ions, ΔH_B. Since there is evidence that ΔC_p is decreased in the presence of salt, we have used the values of 1105 cal mol^{-1} K^{-1} for NaCl and 1140 cal mol^{-1} K^{-1} for $MgCl_2$ in order to estimate ΔH_B. The results are shown in Tables II and III. It is interesting that ΔH_B values for the binding of Na^+, 2.6 ± 0.6 kcal mol^{-1}, and Mg^{2+}, −1.0 ± 0.3 kcal mol^{-1}, differ in sign. However, the uncertainty in these estimates is obviously large, and ΔH_B should be measured by a better suited technique such as titration calorimetry.

In summary, the most important finding in this paper is that estimates of the conformational stability of RNase T1 from urea denaturation curves, from thermal denaturation curves, and from DSC are in good agreement under a variety of conditions. Earlier we showed that estimates of the differences in stability between wild-type and mutant proteins, $\Delta(\Delta G°)$ values, from urea and thermal denaturation experiments were in good agreement (Shirley et al., 1992). Our preliminary results on these mutants with DSC give excellent agreement with our previous results (Gajiwala and Pace, unpublished observations). It will be interesting to see if the agreement is equally good for some of the other proteins currently under active study.

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Registry No. Na, 7440-23-5; Mg, 7439-95-4; ribonuclease T1, 9026-12-4.

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Identification of the Three-Dimensional Thioredoxin Motif: Related Structure in the ORF3 Protein of the *Staphylococcus aureus mer* Operon†

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ABSTRACT: We have developed a computerized search pattern for recognition of the three-dimensional redox site of thioredoxins based on primary and predicted secondary structure. This pattern, developed in the ARIADNE protein expert system, is used to search for thioredoxin-like tertiary structural motif among proteins for which the only structural information is the primary sequence. The pattern was trained on 102 protein sequences (25 functionals and 77 controls); it matches all 25 members of the functional set under cutoff conditions that include only 2 members of the control set, for a sensitivity of 1.0 and a specificity of 0.97. The pattern matches only one of the two thioredoxin-like domains in protein disulfide isomerases (PDIs) and their analogues, suggesting that the C-terminal domain is more structurally similar to thioredoxin than the N-terminal domain. The *Escherichia coli* DsbA protein, a possible PDI analogue, appears to be more structurally similar to the N-terminal thioredoxin-like domain of PDIs. Thioredoxin-like redox functionality has been proposed for lutropin and follitropin, in part on the basis of their having -Cys-X-Pro-Cys- sequences. None match our pattern; all lack a predicted α-helix pattern element immediately after the active site. Hypothetical proteins in the National Biomedical Research Foundation Protein Identification Resource database were searched for matches to the pattern. The most interesting match was a hypothetical protein (161 residues) from the third open reading frame in the *Staphylococcus aureus mer* operon, which is involved in mercury detoxification. The match to our pattern and the hydrophobicity distribution in aligned elements of secondary structure not in our pattern strongly suggest that it has thioredoxin-like structure.

Prediction of tertiary structure and biological function from primary sequence alone is a central challenge in protein chemistry. Experimental determination of tertiary structure by X-ray crystallography or NMR requires months, while automated protein and gene sequencing techniques are increasingly efficient. This results in an ever-widening gap between the number of known protein primary sequences and the number of known three-dimensional structures. For example, while there are 39 533 loci (potential primary sequences) in GenBank[1] 65.0, as of January 1991 there were only 622 structures in the Protein Data Bank, and many of the latter are redundant. Further, the number of hypothetical proteins predicted from nucleotide sequences is increasing faster than that of proteins with known function.

To address the primary sequence–tertiary structure question, computer-based tools are being developed in many labs to correlate and recognize the tertiary structural information which resides in protein sequences. One common approach to prediction of the tertiary structure of a sequence is to search for primary sequence similarity to one or more proteins with known structure. When such similarity is found, the unknown protein can be aligned with the protein(s) of known structure.

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[1] Abbreviations: GenBank, NIH nucleic acid sequence data bank; PIR, protein identification resource data bank; Swiss-Prot, University of Geneva protein sequence data bank; EMBL, European Molecular Biology Laboratory; PDI, protein disulfide isomerase; ORF, open reading frame.

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