Stability of Recombinant Lys25-Ribonuclease T_1†

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ABSTRACT: The conformational stability of recombinant Lys25-ribonuclease T_1 has been determined by differential scanning microcalorimetry (DSC), UV-monitored thermal denaturation measurements, and isothermal Gdn·HCl unfolding studies. Although rather different extrapolation procedures are involved in calculating the Gibbs free energy of stabilization, there is fair agreement between the $\Delta G°$ values derived from the three different experimental techniques at pH 5, ϑ = 25 °C: DSC, 46.6 ± 2.1 kJ/mol; UV melting curves, 48.7 ± 5 kJ/mol; Gdn·HCl transition curves, 40.8 ± 1.5 kJ/mol. Thermal unfolding of the enzyme is a reversible process, and the ratio of the van't Hoff and calorimetric enthalpy, $\Delta H_{vH}/\Delta H_{cal}$, is 0.97 ± 0.06. This result strongly suggests that the unfolding equilibrium of Lys25-ribonuclease T_1 is adequately described by a simple two-state model. Upon unfolding the heat capacity increases by $\Delta C_p° = 5.1 \pm 0.5$ kJ/(mol·K). Similar values have been found for the unfolding of other small proteins. Surprisingly, this denaturational heat capacity change practically vanishes in the presence of moderate NaCl concentrations. The molecular origin of this effect is not clear; it is not observed to the same extent in the unfolding of bovine pancreatic ribonuclease A, which was employed in control experiments. NaCl stabilizes Lys25-ribonuclease T_1. The transition temperature varies with NaCl activity in a manner that suggests two limiting binding equilibria to be operative. Below approximately 0.2 M NaCl activity unfolding is associated with dissociation of about one ion, whereas above that concentration about four ions are released in the unfolding reaction. The isoenzyme Gln25-ribonuclease T_1 shows lower stability under comparable conditions (Shirley et al., 1989). Energy minimization calculations provide a rationalization for this finding by suggesting the existence of a salt bridge between Glu28 and Lys25 in Lys25-ribonuclease T_1 that cannot form in the Gln25-ribonuclease T_1 isoenzyme.

The present quest for improving understanding of the molecular origins of protein stability stems to a large extent from the practical goals of protein design to synthesize new proteins with improved or unprecedented properties. Prediction of the effects of amino acid exchanges on folding, structure, stability, and function of proteins is still difficult at best. To improve this situation, mutated proteins are an indispensable tool, particularly if high-resolution X-ray structures exist whose mutation-induced differences can be correlated with the changes in the thermodynamic and kinetic properties of the protein (Alber, 1989; Shortle, 1989). Ribonuclease T_1 fulfills the criteria for a good model protein, since it is relatively small and well characterized and designed mutants are becoming available. Ribonuclease T_1 (EC 3.1.27.3) was first characterized by Sato and Egami (1957). The enzyme consists of 104 amino acids and has a molecular weight of 11 085 (Takahashi, 1965, 1985). The protein occurs as a mixture of two isoenzymes. They differ by the amino acid at position 25, which can be either Lys (Lys25-ribonuclease T_1) or Gln (Gln25-ribonuclease T_1). The three-dimensional structures of the Lys25-ribonuclease T_1 with bound inhibitors are known at high resolution (Heinemann & Saenger, 1982; Arni et al., 1988). The first structural data for the Gln25 isoenzyme are now available as well (Sugio et al., 1988). No significant differences were observed in the coordinate sets. An intriguing feature for an RNA processing enzyme is the high ratio of acidic to basic amino acids [6 Glu, 6 Asp to 2 Lys (1 Lys in the Gln25 isoenzyme), 1 Arg, 3 His], which results in an isoelectric point of 2.9 (Takahashi, 1962). The enzyme has two disulfide bridges connecting Cys2–Cys10 and Cys6–Cys103. An increase in ionic strength leads to a surprisingly strong increase in stability. This effect was suggested to result from weak interactions of the ions with the native, folded conformation of the protein (Oobatake et al., 1979; Pace & Grimsly, 1988).

Ribonuclease T_1 exhibits partial activity and conformational stability with one or both disulfide bonds broken and the cysteines carboxymethylated (Pace et al., 1988). The thermal stability of Gln25-ribonuclease T_1 prepared from Taka-Diastase has been investigated by Oobatake et al. (1979) and by Pace and co-workers (Pace, 1986; Pace et al., 1988; Thomson et al., 1989; Shirley et al., 1989) by using spectral methods to measure unfolding. We determined the stability parameters of the Lys25 isoenzyme by three different techniques: by high-sensitivity differential scanning microcalorimetry, by ultraviolet absorption measurements (287 nm) and by isothermal Gdn·HCl unfolding studies. Our studies focus on the recombinant Lys25-ribonuclease T_1, since the three-dimensional structure of this isoenzyme and of numerous mutated proteins is under intensive investigation and since most of the existing stability data refer to the Gln25 isoenzyme.

Materials and Methods

Lys25-ribonuclease T_1 was prepared from a chemically synthesized gene, which was cloned and expressed in *Escherichia coli* as described previously (Quaas et al., 1988). The

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lyophilized enzyme was dissolved in the appropriate buffer and dialyzed to equilibrium against the same buffer. Protein concentrations were determined by using an absorbance of 1.9 at 278 nm, 1-cm light path, for a 1 mg/mL solution (Takahashi et al., 1970). For the different pH ranges, the following buffers were employed at a concentration of 0.1 M: glycine/HCl, pH 2 and 3; acetate/acetic acid, pH 4 and 5; and cacodylate/HCl, pH 6 and 7. Studies on the influence of NaCl concentration were carried out in 10 mM acetate/acetic acid buffer containing the appropriate amounts of NaCl (10 mM–3 M).

Gdn·HCl-Induced Unfolding Curves. The stability of Lys25-ribonuclease T_1 toward Gdn·HCl was determined by incubating native protein in the presence of various concentrations of Gdn·HCl (0–6 M) at 25 °C. The buffer employed was 0.1 M NaOAc/acetic acid, pH 5. The protein concentration was 13 μM. A Perkin-Elmer Lambda 5 spectrophotometer employing 1-cm cells in a thermostated cuvette holder was used for the absorption measurements. The reference cell contained a protein-free Gdn·HCl solution of the respective concentration. Additionally, the absorbance at 310 nm was measured and subtracted from the values obtained at 287 nm in order to eliminate effects other than protein absorbance. Incubation times of 26 and 48 h produced identical absorbance changes of the samples at 287 nm, indicating that equilibrium had been reached already after 26 h.

Calorimetry. Variation with temperature of the apparent specific heat capacity of the protein solutions was determined with a DASM 4 scanning microcalorimeter. Sample concentrations between 0.5 and 1.5 mg/mL (45–135 μM) were used without effect on the ΔH values. Generally a heating rate of 1 K·min^{-1} was employed, since lower or higher scan rates did not influence the thermodynamic parameters. The instrument is connected to an Olivetti M24 computer via a six-digit Keithley 192 DMM for data acquisition. Temperature and heat capacity values are stored every 0.1 deg. Each sample measurement is preceded by a baseline scan with equlibrium buffer filled cells. Electrical calibration is performed at 50 μW for 5 min. Repeated measurements with the same protein sample demonstrated reproducibility of ΔH values of ≥95%. The areas under the transition curves are determined numerically. Both linear- and step-function baselines are routinely employed for evaluation of the excess heat capacities. The two procedures generally yield identical results for transition enthalpies and temperatures within error limits. Molar thermodynamic quantities have been evaluated on the basis of M = 11 085 g/mol. Van't Hoff enthalpies at T_m have been calculated from the calorimetric transition curves by using the formula for a two-state transition

$$\Delta H_{vH}(T_m) = 4RT_m^2\left(\frac{C_p(T_m)}{\Delta H_{cal}(T_m)}\right) \quad (1)$$

where T_m is the transition temperature. It is defined as the temperature at which 50% of the protein has been converted to the unfolded state. Depending on the limits of integration, transition temperatures can vary by ±0.5 K. $C_p(T_m)$ is the molar excess heat capacity at T_m and $\Delta H_{cal}(T_m)$ the corresponding molar calorimetric transition enthalpy. R is the gas constant [8.314 J/(mol·K)]. The standard Gibbs free energy changes of unfolding have been evaluated by employing the Gibbs–Helmholtz equation:

$$\Delta G°(T) = \Delta H°(T_m)(1 - T/T_m) + \Delta C_p°(T - T_m) - \Delta C_p°T \ln (T/T_m) \quad (2)$$

$\Delta H°(T_m)$ refers to the molar transition enthalpy at T_m, $\Delta C_p°$ is the molar temperature independent heat capacity change associated with unfolding, and T is the absolute temperature. Transition curves obtained in the presence of sodium chloride concentrations larger than 2 M exhibited features characteristic of partial precipitation of the protein after the transition. These curves could be used for reasonable estimates of transition temperatures but gave no reliable values for the transition enthalpies. Therefore, the corresponding T_m values appear in Figure 5A but no corresponding ΔH values have been included in Figure 5B.

Thermal UV-Monitored Denaturation Curves. Thermal denaturation was monitored by the change in absorption at 287 nm with a Gilford 2400S spectrophotometer equipped with a Gilford 2527 thermoprogrammer, temperature-controlled 1-cm cells, and a computerized data collection system. The reference cell contained protein-free buffer. Protein concentrations used were between 0.13 and 1 mg/mL. The heating rate was 1 K/min. Decreasing the heating rate to 0.5 K/min had no effect on the shape of the denaturation curves. Transition temperature, van't Hoff enthalpy, and heat capacity changes were obtained from the ln K vs $1/T$ curves by using a nonlinear least-squares fitting procedure. The equation used for fitting was

$$\ln K(T) = -\frac{\Delta H°_{vH}}{R}\left(\frac{1}{T} - \frac{1}{T_m}\right) - \frac{\Delta C_p°}{R}\ln\frac{T_m}{T} + \frac{\Delta C_p°}{R}\left(\frac{T_m}{T} - 1\right) \quad (3)$$

It is derived from eq 2 by using the relationship $\Delta G°(T) = -RT \ln K(T)$. T_m is the transition temperature at which ln $K(T_m) = 0$, $\Delta H°_{vH}$ the van't Hoff enthalpy at T_m, and $\Delta C_p°$ the molar temperature independent heat capacity change on unfolding. The least-squares calculations were performed on a Comparax 8/85 computer employing the EO4FCF routine of the NAG Fortran library document Mark 13. Reversibility of the denaturation reaction was checked by monitoring heating/cooling cycles with 1 K/min. Overlapping unfolding and refolding curves demonstrated reversibility greater than 95%.

Energy Minimization. To get an idea whether exchange of Gln25 by Lys25 could lead to a salt bridge between Lys25 and the adjacent Glu28, the energy minimization program Discover, version 2.5 (BIOSYM Inc., San Diego), was employed, using the consistent valence force field (G. Böhm, private communication). A total of 1000 steps of steepest descent and 1000 steps of conjugate gradient were carried out. The initial model was the noncharged crystal structure of ribonuclease T_1 as deposited in the Brookhaven Protein Database (File 1RNT), with charges on the carboxyl group of Glu28 and on the ε-amino group of Lys25. In the final structure the two charged groups were located at a distance of 2.7 Å without overlapping of van der Waals radii.

Results

Gdn·HCl-Induced Transition. A typical example of a Gdn·HCl-induced transition curve of Lys25-ribonuclease T_1 at 25 °C, pH 5, is presented in Figure 1A. Absorption at 287 nm is followed as a function of Gdn·HCl concentration. Up to approximately 2.5 M Gdn·HCl no absorption change can be detected; between 2.5 and 4.5 M Gdn·HCl normal sigmoidal decrease to the level of the unfolded species is observed. Assuming a two-state unfolding mechanism, equilibrium constants were calculated from the transition curve (Tanford, 1968) and their negative logarithms plotted against denaturant concentration. The resulting curve is shown in Figure 1B.

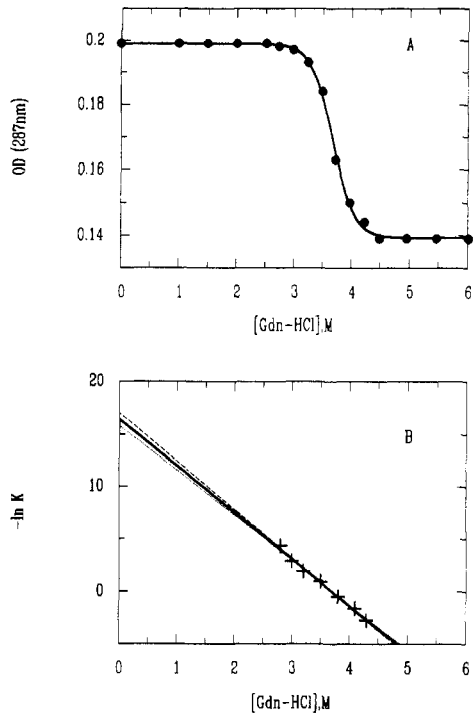

FIGURE 1: (A) Gdn·HCl-induced transition curve of Lys25-ribonuclease T_1 at 25 °C in 0.1 M sodium acetate/acetic acid buffer, pH 5. [Lys25-ribonuclease T_1] = 13 μM. (B) Negative logarithms of the equilibrium constants determined from the transition curve shown in (A) plotted versus the Gdn·HCl concentration. Linear extrapolation of the least-squares fit of the data to zero guanidine concentration results in the equilibrium constant characteristic for unfolding in the absence of denaturant. The equation that fits the data best is $-\ln K = (4.45 \pm 0.17)[\text{Gdn·HCl}] + 16.44 \pm 0.62$; the correlation coefficient is 0.99625.

Linear extrapolation to zero Gdn·HCl concentration (Schellman, 1978; Pace, 1986; Ahmad & Bigelow, 1982; Thomson & Bigelow, 1986; Santaro & Bolen, 1988; Bolen & Santaro, 1988; Chen & Schellman, 1989) yielded a $\Delta G_D^{H_2O}$ value of 40.8 ± 1.5 kJ/mol for the Gibbs free energy of unfolding in the absence of the denaturant (Figure 1B). The dashed and dotted curves illustrate the error limits of the fit.

Thermal Unfolding in the Absence of Salt. (A) Absorption Studies. Figure 2A shows thermal transition curves of Lys25-ribonuclease T_1 in 0.1 M NaOAc buffer, pH 5, and in 0.1 M glycine buffer, pH 2. Protein concentrations were 87 and 82 μM, respectively. The heating rate was identical with that employed in the calorimetric studies (1 K·min^{-1}). It is worth noting that the molar change in absorption, $\Delta\epsilon$ = 3600 M^{-1}·cm^{-1}, resulting from thermal denaturation is smaller than the corresponding change, $\Delta\epsilon$ = 4800 M^{-1}·cm^{-1}, observed with Gdn·HCl unfolding.

Thermal transition curves have been analyzed, assuming a two-state mechanism of unfolding, to obtain equilibrium constants. The logarithms of the equilibrium constants have been plotted vs reciprocal absolute temperature according to the van't Hoff relationship, and these plots are shown in Figure 2B. Nonlinear least-squares curve fitting using eq 3 resulted in the following transition parameters:

pH 2: T_m = 48.5 °C; $\Delta H°_{vH}$ = 448 kJ/mol; $\Delta C_p°$ = 6.9 kJ/(mol·K)

pH 5: T_m = 61.2 °C; $\Delta H°_{vH}$ = 531 kJ/mol; $\Delta C_p°$ = 4.3 kJ/(mol·K)

The errors in the T_m estimates are ≤0.1 °C; the errors in $\Delta H°_{vH}$ and $\Delta C_p°$ are correlated; reasonable estimates of the

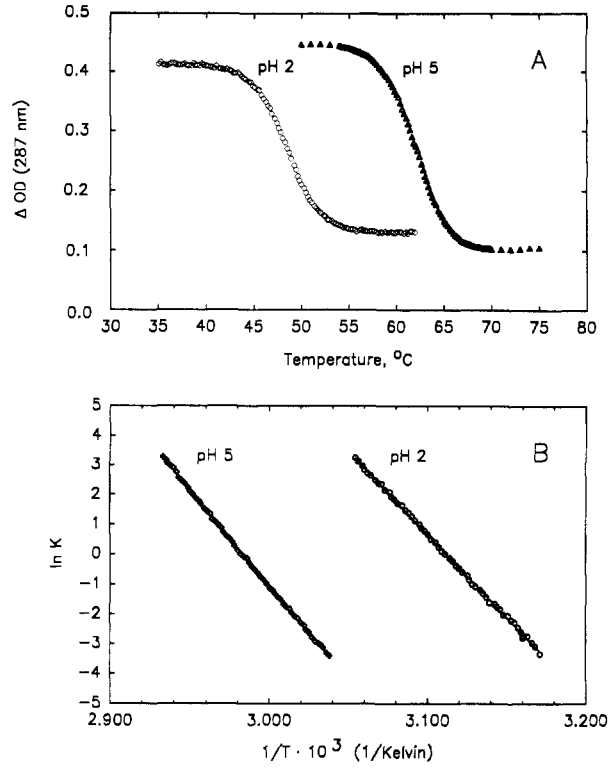

FIGURE 2: (A) Temperature-induced transition curves of Lys25-ribonuclease T_1 (87 μM) in 0.1 M sodium acetate buffer, pH 5, and in 0.1 M glycine/HCl buffer, pH 2, monitored by UV absorption at 287 nm. Protein concentrations were 87 and 82 μM, respectively. The heating rate was 1 K/min; lower or higher heating rates had no influence on the transition properties, thus demonstrating equilibrium unfolding conditions. (B) Van't Hoff plots of transition curves at pH 2 and 5. The curves through the data points are calculated by using eq 3 with the following parameters: at pH 2, T_m = 48.5 °C, $\Delta H°_{vH}$ = 448 kJ/mol, and $\Delta C_p°$ = 6.9 kJ/(mol·K); at pH 5, T_m = 61.2 °C, $\Delta H°_{vH}$ = 531 kJ/mol, and $\Delta C_p°$ = 4.3 kJ/(mol·K).

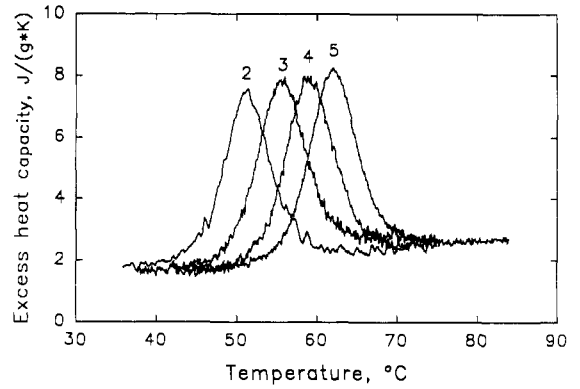

FIGURE 3: Microcalorimetric transition curves of Lys25-ribonuclease T_1 at different pH values. Curve 2: 0.1 M glycine/HCl, pH 2; enzyme concentration 0.72 mg/mL; heating rate 1 K/min. Curve 3: 0.1 M glycine/HCl, pH 3; enzyme concentration 0.51 mg/mL; heating rate 1 K/min. Curve 4: 0.1 M acetate/acetic acid, pH 4; enzyme concentration 0.65 mg/mL; heating rate 1 K/min. Curve 5: 0.1 M acetate/acetic acid, pH 5; enzyme concentration 0.96 mg/mL; heating rate 1 K/min.

respective errors derived from calculations using different data sets are as follows: $\Delta H°_{vH}$, ±20 kJ/mol; $\Delta C_p°$, ±1 kJ/(mol·K). A rough estimate of the average change in heat capacity between 48.5 and 61.2 °C can be obtained from the difference of the transition enthalpies at these temperatures. The corresponding number is 6.5 kJ/(mol·K), which is consistent with the individual values above. Combining the higher $\Delta H°_{vH}$ value with the lower $\Delta C_p°$ estimate in eq 2 and vice versa results in $\Delta G°$(25 °C) variations of ±5 kJ/mol.

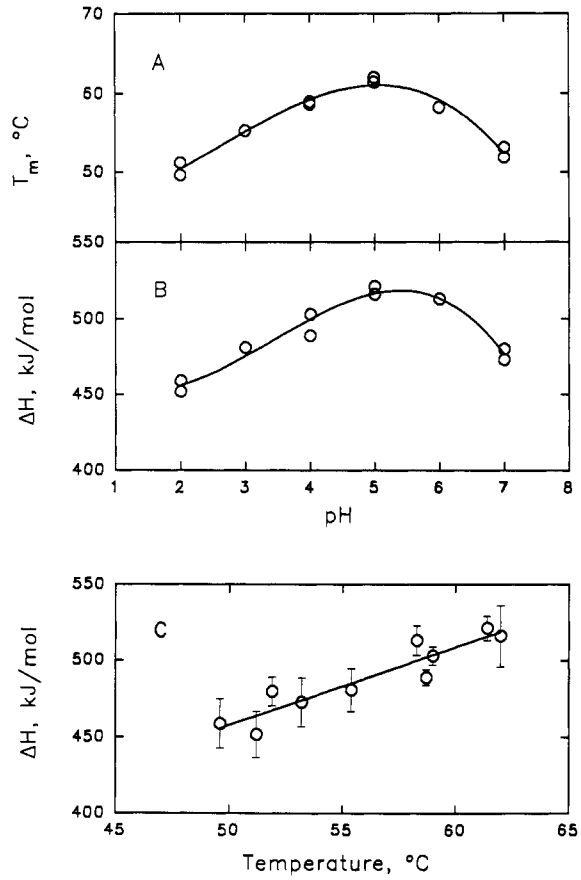

FIGURE 4: (A) Variation with pH of the transition temperature T_m of Lys25-ribonuclease T_1. The data have been derived from DSC studies. The experimental points have been connected by a spline interpolation procedure. Errors in the transition temperatures are typically ≤0.5 deg. (B) Variation with pH of the corresponding calorimetric transition enthalpies. The experimental points have been connected by a spline interpolation procedure. (C) Plot of the calorimetric transition enthalpies versus the transition temperature. The open circles are the experimental values; the straight line has been calculated according to eq 4. Error bars illustrate standard deviations as specified in Table I.

Table I: Thermodynamic Parameters of Lys25-Ribonuclease T_1[a]

pH	T_m (°C)	ΔH (kJ/mol)	pH	T_m (°C)	ΔH (kJ/mol)
2	49.6	459 ± 16	5	62.0	516 ± 20
2	51.2	452 ± 15	5	61.4	521 ± 8
3	55.4	481 ± 14	6	58.3	513 ± 10
4	58.7	489 ± 5	7	51.9	480 ± 9
4	59.0	503 ± 6	7	53.2	473 ± 16

[a] The ΔH values refer to measurements done in the absence of NaCl. The 0.1 M buffers were used as specified under Materials and Methods. The ΔH values are given with standard deviations. These refer to different evaluations of the individual measurements using linear- or step-function baselines and different starting and ending temperatures for the integration of the transition peaks. The same deviations are shown in Figure 4C.

(B) Microcalorimetric Measurements. Figure 3 shows heat capacity vs temperature curves for pH values 2, 3, 4, and 5 in the buffers specified under Materials and Methods. Transition temperatures, T_m, are defined as temperatures where the area under the transition peak curves assumes 50% of its total value. The corresponding data have been summarized in Table I. Panels A and B of Figure 4 present the variation with pH of the transition temperature, T_m, and the transition enthalpy, ΔH_{cal}, respectively. Both curves are bell shaped and have a pronounced maximum around pH 5.

The corresponding variation with temperature of the calorimetric transition enthalpies, ΔH_{cal}, is shown in Figure 4C.

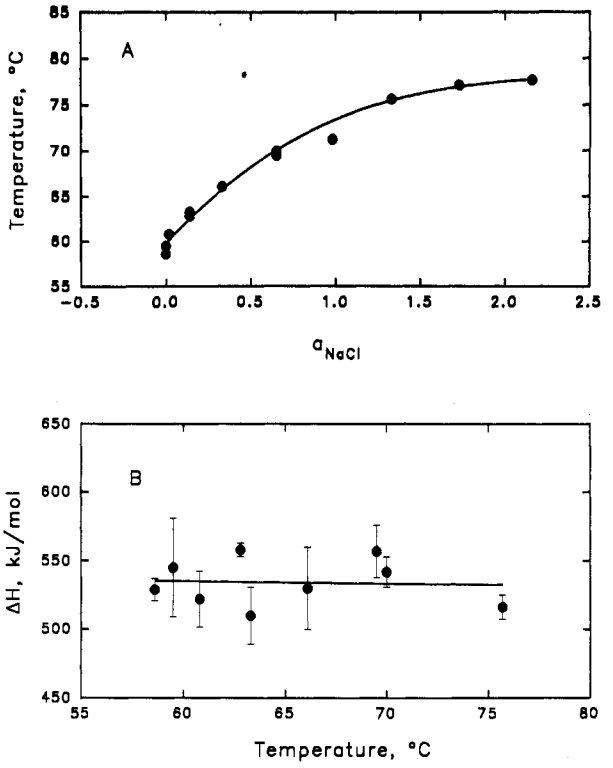

FIGURE 5: (A) Dependence of the transition temperature of Lys25-ribonuclease T_1 on the activity of sodium chloride (10 mM acetate/acetic acid buffer, pH 5). Activity values have been calculated by using the tables in Robinson and Stokes (1968). The solid line represents a least-squares fit by a polynomial of the third degree. (B) Dependence of the transition enthalpy on the transition temperature in the presence of sodium chloride. The straight line has been calculated by using the equation $\Delta H = -0.14\vartheta + 544$ kJ/mol; ϑ refers to 0 °C.

The experimental points can be well represented by the equation

$$\Delta H°_{cal}(T) = 5.1\vartheta + 205 \text{ kJ/mol} \quad (4)$$

indicating a molar heat capacity change on unfolding of 5.1 ± 0.5 kJ/(mol·K), which corresponds to a specific heat capacity change of $\Delta c_p = 0.46$ J/(g·K). The latter value has been obtained by division by the molecular weight. ϑ is the temperature in °C.

(C) Influence of NaCl on the Conformational Stability of Lys25-Ribonuclease T_1. The enzyme has been known to experience remarkable stabilization by the interaction with mono- and divalent salts (Oobatake et al., 1979; Pace & Grimsley, 1988). Particularly with monovalent salts it is not quite clear whether the stabilization results from weak preferential binding or whether it is a general ionic strength phenomenon. We performed microcalorimetric measurements on a series of Lys25-ribonuclease T_1 solutions to obtain T_m values and transition enthalpies as a function of NaCl concentration. If the stabilization is due to weak binding of cations and anions, increasing salt concentration should increase T_m and also ΔH_{cal}, provided binding of ions is associated with a measurable enthalpy. The results of these studies are presented in Figure 5 and Table II. T_m increases with increasing NaCl activity, while the transition enthalpy appears to be independent of salt concentration within error limits of the measurements. The experimental points can be fitted to the linear equation $\Delta H°(\text{NaCl},\vartheta) = -0.14\vartheta + 544$ kJ/mol, where ϑ refers to °C.

Plotting T_m vs the logarithm of the sodium chloride activity reveals a biphasic curve (Figure 6) that can be approximated by two straight lines. This result suggests that below and above

Table II: Thermodynamic Parameters of Lys25-Ribonuclease T_1[a]

[NaCl] (M)	T_m (°C)	ΔH (kJ/mol)	[NaCl] (M)	T_m (°C)	ΔH (kJ/mol)
0	58.6	529 ± 8	0.5	66.1	530 ± 30
0	59.5	545 ± 36	1	69.5	557 ± 13
0.02	60.8	522 ± 21	1	70.0	542 ± 11
0.2	63.3	510 ± 21	2	75.7	516 ± 9
0.2	62.8	558 ± 5			

[a] The ΔH values refer to measurements done in the presence of NaCl in 0.01 M NaOAc buffer, pH 5. The standard deviations of ΔH result from different evaluations of individual measurements as explained under Table I.

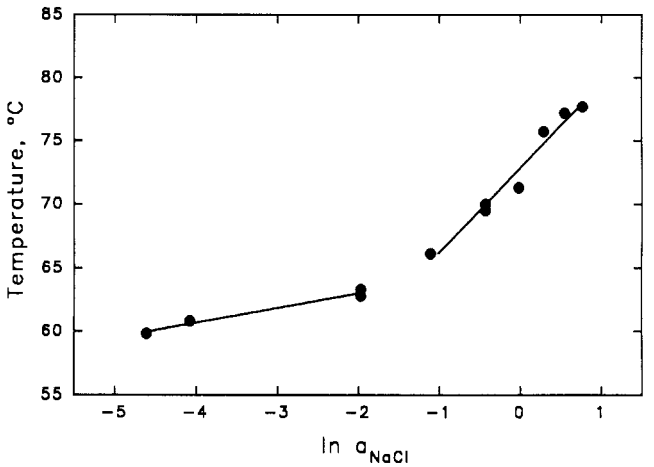

FIGURE 6: Dependence of the transition temperature T_m on the logarithm of the activity of sodium chloride. Solid circles refer to experimental points. The straight lines through the two data subsets represent calculated curves. The equation for the low-salt range is $T_m = 1.37 \ln a + 66.2$ °C, and that for the high-salt range is $T_m = 6.54 \ln a + 72.2$ °C.

a sodium chloride activity of approximately 0.2 M different numbers of ions are preferentially bound to the native protein.

Knowledge of transition temperatures and transition enthalpies as a function of NaCl concentration was used to calculate the number of ions released on unfolding according to the equation

$$dT_m/d(\ln [a]) = -\Delta n(RT_m^2/\Delta H(T_m)) \quad (5)$$

where a is the mean ionic activity of NaCl at the corresponding temperature (Robinson & Stokes, 1968) and $\Delta H(T_m)$ the transition enthalpy at the transition temperature T_m. R is the gas constant, 8.314 J/(mol·K), and $\Delta n = n_u - n_f$ is the denaturational change in the number of bound ions. n_u refers to the number of ions bound to the unfolded protein, and n_f is the corresponding number of the folded protein. Taking 61 and 70 °C, respectively, as characteristic average temperatures of the two linear parts of the biphasic curve to which the enthalpies ΔH = 540 and 534 kJ/mol correspond, the Δn values estimated according to eq 5 are as folllows: 61° C, Δn = −0.8; 70 °C, Δn = −3.6. This result indicates that unfolding of ribonuclease T_1 at low ionic strength is associated with the release of approximately one ion, while at high ionic strength about four ions come off the protein.

Discussion

In the present study on Lys25-ribonuclease T_1 we performed a thorough thermodynamic analysis of the reversible unfolding reaction of the enzyme by using three different techniques and a variety of solvent conditions. Employing scanning microcalorimetry, we measured directly the model-free transition enthalpies, $\Delta H°_{cal}$, the transition entropies, $\Delta S°$, the transition temperatures, T_m, and the denaturational heat capacity change $\Delta C_p°$. Curve analysis of the heat capacity profiles on the basis of a two-state transition model demonstrated that the van't Hoff enthalpy is identical with the calorimetric values within error limits ($\Delta H°_{vH}/\Delta H_{cal}$ = 0.97 ± 0.06). The UV-monitored thermal transition curves were analyzed for the first time in terms of $\Delta H°_{vH}$ and $\Delta C_p°$ by using eq 3 in a nonlinear least-squares fitting procedure. Such an analysis is statistically significant, because of the large number of data points accumulated for each transition profile. The Gdn·HCl-induced isothermal unfolding reaction was studied to permit a comparison between the Gibbs free energy estimates obtained from denaturant unfolding and those calculated from thermal unfolding. The stability data of the Lys25 isoenzyme differ from those determined previously for the Gln25 isoenzyme, even if one takes the different experimental conditions into account. The three-dimensional structures of the two isoenzymes show, however, a high degree of similarity, suggesting that the exchange of Gln by Lys does not cause great alterations in the overall spatial arrangement of the amino acids (Heinemann & Saenger, 1982; Sugio et al., 1988). Therefore, one has to rationalize the existence of energetic perturbations in the absence of visible structural changes.

Dependence on pH and Temperature of the Stability Parameters. The transition temperature, T_m, derived from the heat capacity measurements exhibits the usual bell-shaped pH profile (Figure 4A). The maximum occurs around pH 5, which is close to the value observed by Oobatake et al. (1979) for the Gln25 isoenzyme. We find a transition temperature of 61 ± 0.5 °C at pH 5 in 0.1 M sodium acetate buffer for the Lys25-ribonuclease T_1, which is higher than the value of 56 °C reported by Oobatake et al. (1979) for the Gln25 isoenzyme. Part of the difference can be explained by the lower buffer concentration used in their experiments. However, even if one accounts for the salt dependence of T_m (Figure 5A), the transition temperature of the Lys25 isoenzyme is still higher by approximately 3 K. The increase in stability reflected in the increased transition temperature corresponds to a $\Delta\Delta G°$ value of 4.6 kJ/mol according to the relation

$$\Delta\Delta G° = -\Delta S°\Delta T_m \quad (6)$$

$\Delta S°$ can be calculated from $\Delta H°_{cal}/T_m$, and ΔT_m is the difference between the transition temperatures. The value of $\Delta\Delta G°$ corresponds well with the results of Shirley et al. (1989), who obtained a $\Delta\Delta G°$ value of about 3.8 kJ/mol for the two forms of ribonuclease T_1 under slightly different experimental conditions (30 mM MOPS buffer, pH 7). One possibility to rationalize the additional stability of the Lys25-ribonuclease T_1 is to assume that the presence of lysine in position 25 near the C-terminal end of the α-helix increases the transition temperature by decreasing the negative charge density at the C-terminus of the helix. This interpretation is in line with the observations that appropriately charged residues near the ends of a helix can favorably interact with the helical dipole and stabilize the helix (Ihara et al., 1982; Mitchinson & Baldwin, 1986; Marquese & Baldwin, 1988; Shoemaker et al., 1985, 1987; Hol et al., 1981; Wada, 1977; Blagdon & Goodman, 1975). It should be mentioned that, in principle, destabilization of the denatured state would have the same thermodynamic consequence as stabilization of the native state. We give preference to the stabilization mechanism since it appears that charged Lys25 compared with Gln25 rather stabilizes than destabilizes the unfolded state.

Another mechanism of stabilization could also be operative. It has been shown by energy minimization calculations (G. Böhm, private communication) that the ϵ-amino group of Lys25 and the carboxyl group of Glu28 can be brought into

a vicinity (2.7 Å) close enough to form an ion pair. This ion pair is not visible in the original coordinate set. To invoke such an interaction as a possible explanation for the increased stability is nevertheless not unreasonable, since in view of the difference between the solution conditions used in the crystallization procedure and those used in our studies, the intrahelical ion pair may not exist in the crystal; it might, however, occur under our experimental conditions. The increase in $\Delta G°$ of 4.6 kJ/mol is similar to the value observed for a solvent-exposed salt bridge between the termini of BPTI (4.2 kJ/mol) in 6 M guanidine hydrochloride at pH 6 and 72 °C (Brown et al., 1978).

The analysis of the thermal unfolding transitions led Oobatake et al. (1979) to assume that the thermodynamic functions $\Delta H°$ and $\Delta S°$ of Gln25-ribonuclease T_1 remain constant between pH 1 and 10, $\Delta H°$ assuming a value of approximately 500 kJ/mol independent of pH and transition temperature. This value is almost identical with $\Delta H° = 523 \pm 15$ kJ/mol obtained in this study for the Lys25 isoenzyme in 0.1 M sodium acetate buffer, pH 5. However, the direct microcalorimetric measurements demonstrated clearly that the transition enthalpy varies linearly with temperature. The temperature coefficient, i.e., the heat capacity change on unfolding, is equal to 5.1 ± 0.5 kJ/(mol·K) and appears to be independent of transition temperature in the temperature range studied.

The different result is not due to the use of different isoenzymes, since Pace and Laurents (1989) conclude from an analysis of thermal transitions in the presence of different urea concentrations that unfolding of the Gln25 isoenzyme is associated with an increase in heat capacity as well. The specific heat capacity change of 0.46 J/(g·K) of ribonuclease T_1 is identical with that observed for lysozyme under comparable conditions, and it lies within the upper range of Δc_p values obtained for other small proteins (Pfeil, 1986). The Δc_p value reflects to a large extent the change in the degree of exposure of hydrophobic residues caused by unfolding the protein (Privalov & Gill, 1989). Hydrophobic hydration is the major origin of the increased heat capacity of the unfolded state with but a minor contribution to heat capacity of the larger number of degrees of freedom of the extended polypeptide chain (Sturtevant, 1977; Velicelebi & Sturtevant, 1979).

Since the heat capacity function of unfolded proteins does not vary linearly with temperature (Privalov & Gill, 1989) while that of the native state appears to be a linear function of temperature with temperature coefficients of approximately $(2.2 \pm 0.5) \times 10^{-3}$ cal/(g·K) (Kitamura & Sturtevant, 1989; Privalov & Khechinashvili, 1974), Δc_p values should be temperature dependent. The apparent invariance with temperature of Δc_p observed so far in the majority of studies is probably due to the fact that no large enough temperature ranges have been investigated. It should be obvious from the studies of Gill and others that with the disappearance of hydrophobic hydration around 150 °C also the denaturational Δc_p should vanish (Gill et al., 1985a,b; Shinoda & Fujihara, 1968; Shinoda, 1977). Therefore, Δc_p values should always be compared at the same temperature unless temperature independence has been clearly established. The relatively large Δc_p values of Lys25-ribonuclease T_1 suggest that the unfolding reaction starts from a compact structure in which the hydrophobic moieties are rather well sequestered from contact with water and proceeds to a coiled polypeptide that is highly solvated.

It was mentioned under Results that thermal unfolding involves a smaller change in the molar UV absorption coefficient than Gdn·HCl unfolding. This phenomenon has been discussed as being suggestive of residual structure in the thermally unfolded state (Nozaki & Tanford, 1971; Tanford, 1968). However, all attempts to demonstrate by direct microcalorimetric measurements a difference in heat capacity between proteins that have been denatured by heat, Gdn·HCl, urea, or reductive removal of disulfide bridges have failed (Pfeil & Privalov, 1976; Privalov & Gill, 1989). Thus even if the unfolded state produced by Gdn·HCl is the most extended one and has the least short range order as shown by Tanford (1968), there is no thermodynamic criterion that could differentiate between thermally and denaturant unfolded states. We assume the point of view recently expressed by Chen and Schellman (1989) that, operationally, the unfolded states producd in the various processes are different manifestations of the same macrostate of the protein. This conclusion gains also support from nonthermodynamic measurements. Comparative NMR studies on heat-denatured BPTI in the absence and presence of 3 M Gdn·HCl have shown identical residue mobilities, which means that the addition of Gdn·HCl does not detectably influence the unfolded state of BPTI under those experimental conditions (Roder et al., 1985).

Stabilization by Salt. Stabilization of Gln25-ribonuclease T_1 by salts has been first reported by Oobatake et al. (1979) and has been studied in more detail by Pace and Grimsley (1988). Oobatake et al. (1979) found a linear relationship between the T_m values and the logarithm of salt concentration, in agreement with our results (Figure 6) at high salt concentrations. The low-salt region was not studied by Oobatake et al. (1979). The low salt concentrations employed in our investigations are similar to those used by Pace and Grimsley in their isothermal urea unfolding studies.

Analysis of the low-salt part of the curve displayed in Figure 6 indicated that about one ion is released upon thermal unfolding in the presence of sodium chloride concentrations not exceeding ≈0.2 M. Pace and Grimsley (1988) obtained a value of $\Delta n = -2.2$ for the denaturational ion release in the presence of NaCl from their urea unfolding studies at 25 °C. The smaller number derived from our thermal unfolding measurements may reflect the influence of urea on the ion binding equilibrium. Alternatively, the different results could arise from the fact that the Δn value calculated in the present study refers to the transition temperature, that of Pace and Grimsley to 25 °C. Thus, if dissociation of ions from the folded states is favored by an increase in temperature, the apparent difference in the two results may well disappear. Apart from this unresolved question, our measurements provide additional qualitative support for the hypothesis that ribonuclease T_1 is stabilized predominantly by preferential ion binding and not by a general ionic strength effect.

The present study on ribonuclease T_1 gave the intriguing result that the transition enthalpy is independent of the NaCl concentration while the transition temperature increases with it. The constancy of ΔH leads to the conclusion that unfolding of the protein in the presence of NaCl at pH 5 does not involve any measurable change in heat capacity. Unfolding in the absence of NaCl is, however, associated with a $\Delta C_p°$ value of 5.1 kJ/(mol·K). Thus addition of NaCl appears to abolish the denaturational heat capacity change. At pH 5 the unfolding enthalpies at low salt and at 2 M NaCl are practically identical. Since a larger number of ions are released upon unfolding above 0.2 M NaCl than below, the invariability of ΔH implies that their dissociation is not associated with a significant positive enthalpy change. Inspection of Figure 5b even suggests a minute decrease of the transition enthalpies with increasing sodium chloride concentration that could be

indicative of slightly negative dissociation enthalpies of the ions at the transition temperatures. The present observation of salt-dependent transition temperatures and salt-independent enthalpies is in line with the general idea that electrostatic interaction does not involve large enthalpies (Kauzmann, 1959).

The decrease in $\Delta C_p°$ as a result of salt addition is not easy to rationalize. One possible explanation is the following. As mentioned before, the positive $\Delta C_p°$ associated with protein denaturation reflects predominantly hydrophobic hydration of the unpolar residues in the unfolded polypeptide chain. The decrease of $\Delta C_p°$ in the presence of high concentrations of NaCl suggests that the characteristic water structure around the hydrophobic residues is perturbed by the ions (Frank & Evans, 1945; Privalov & Gill, 1989; Gill et al., 1985). Electrolytes in general exert a solvent structure breaking effect (Pacquette & Jolicoeur, 1977; Conway, 1981). Therefore, the structure-forming tendency of the hydrophobic residues of the unfolded chain would be counteracted by the structure breaking of Na^+ and Cl^- ions. The overall effect would be a decrease of the temperature coefficient of the transition enthalpy.

To test the correctness of this interpretation, calorimetric measurements on ribonuclease A have been performed at pH 2 and 5 in the absence and presence of 2 M NaCl. Large protein concentrations (6–7 mg/mL) have been employed to reduce the noise level. Therefore, direct determination of Δc_p values from the transition profiles was feasible. Ribonuclease A was chosen, since it had been shown that the stabilizing or destabilizing effects of salts on this enzyme are of the solvent-mediated type rather than resulting from direct binding interactions (Schrier & Schrier, 1967). Measurements in 10 mM potassium phosphate buffer, pH 2 and 5, without and with 2 M NaCl gave equivocal results. At pH 2 in the absence of NaCl Δc_p was 0.43 ± 0.04 J/(g·K) [5891 J/(mol·K)]; in the presence of 2 M NaCl a smaller Δc_p value of 0.31 ± 0.03 J/(g·K) [4247 J/(mol·K)] was determined. However, at pH 5 the Δc_p values are identical within error limits: 0.25 J/(g·K) [3.4 kJ/(mol·K)] in the absence of salt and 0.23 J/(g·K) [3.2 kJ/(mol·K)] in the presence of 2 M NaCl. Thus we arrive at the conclusion that at low pH values the unfolding studies of ribonuclease A appear to support the notion that the presence of salts causes a decrease in Δc_p, whereas at pH 5 the heat capacity change associated with ribonuclease A denaturation is apparently not influenced by the presence of salts. The absence of a measurable effect at pH 5 may be due to the smaller Δc_p value at the higher pH, which renders any change resulting from the presence of salt more difficult to detect. The trend of the Δc_p value of ribonuclease A to decrease between pH 2 and 5 has been observed before. Brandts and Hunt (1967) reported a molar value of $\Delta C_p°$ = 8.42 kJ/(mol·K) for pH 2.5; Fujita and Noda (1984a,b) reported $\Delta C_p°$ = 5.02 and 4.57 kJ/(mol·K) for pH 3 and 5, respectively. This finding by itself could be interpreted as an indication of the effect of charged groups to reduce the heat capacity change.

Another explanation for the invariance of ΔH with temperature in the presence of NaCl would be the assumption of compensatory negative heat effects involved in dissociation of ions. However, the present experiments do not allow partitioning of the overall transition enthalpy into such contributions. Therefore, resolution of this question must await further research.

Conformational Stability of Lys25-Ribonuclease T_1. An appropriate measure of conformational stability of proteins is the Gibbs free energy change associated with unfolding the native structure. Three methods were employed in the present study to estimate the stability of Lys25-ribonuclease T_1. The calorimetric determination of the transition temperatures together with the corresponding transition enthalpies and heat capacity changes provides a model-free basis for evaluation of $\Delta G°(T)$ values. In addition, curve fitting of the van't Hoff plots, derived from the UV-monitored thermal transition curves, yielded estimates for $\Delta H°_{vH}$ and $\Delta C_p°$ values that were used to calculate $\Delta G°$ according to eq 2. Finally, linear extrapolation of Gibbs free energy values calculated from Gdn·HCl transition curves led to an estimate of enzyme stability, $\Delta G_D^{H_2O}$, in the absence of denaturant according to the equation (Schellman, 1978; Pace, 1986)

$$\Delta G_D = \Delta G_D^{H_2O} - m[\text{Gdn·HCl}] \quad (7)$$

ΔG_D is the Gibbs free energy of protein unfolding at a given guanidine concentration; m is the change in ΔG_D per mol/L Gdn·HCl added.

The following Gibbs free energy values have been derived from the three different methods. They refer to 25 °C and 0.1 M sodium acetate buffer, pH 5. DSC: $\Delta G°$(25 °C) = 46.6 ± 2.1 kJ/mol. UV melting curve: $\Delta G°$(25 °C) = 48.7 ± 5 kJ/mol. Gdn·HCl transition curve: $\Delta G_D^{H_2O}$(25 °C) = 40.8 ± 1.5 kJ/mol. There is fair agreement between the stability parameters calculated from the temperature-induced transition curves; the $\Delta G_D^{H_2O}$ derived from the Gdn·HCl transition curves value appears to be somewhat lower than the other two. This observation is in line with the trend shown by $\Delta G°$ parameters in a recent compilation of literature data (Pfeil, 1986). Consistently the $\Delta G_D^{H_2O}$ values calculated from Gdn·HCl unfolding curves by linear extrapolation are lower than the parameters determined by direct calorimetric measurements.

Pace has given $\Delta G_D^{H_2O}$ estimates for ribonuclease T_1 in 0.1 M Tris buffer, pH 8.05, and at 30 °C that range from 15.1 to 17.2 kJ/mol (Pace, 1986). They have been obtained from urea denaturation curves. We have not performed any measurements under similar conditions, since Tris buffer is rather inadequate for measurements where temperature is changed within wide ranges, due to the high heat of ionization of Tris and the concomitant pH changes. However, inspection of Figure 4A indicates that the transition temperature at pH 8 is likely to be rather similar to that observed at pH 2. On the basis of the calorimetrically determined $\Delta H°$ and $\Delta C_p°$ values, calculation of $\Delta G°$ for 30 °C and pH 2 results in a value of 27.1 kJ/mol, which is somewhat higher than the $\Delta G_D^{H_2O}$ values cited by Pace. In view of the very approximate nature of this comparison, it is difficult to judge whether or not the difference between the two quantities is of significance.

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