Energetics of Ribonuclease T1 Structure†

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ABSTRACT: The energetics of thermal denaturation of two isoforms of ribonuclease T1 (Gln25 and Lys25) in various solvents have been studied by differential scanning calorimetry. It has been shown that the thermal transition of both forms of RNase T1 is strongly affected by slow kinetics, which cause an apparent deviation of the transition from a simple two-state model. By decreasing the heating rate or increasing the transition temperature, the denaturation of RNase approaches an equilibrium two-state transition. This permits determination of the thermodynamic parameters characterizing unfolding of the native structure. These thermodynamic parameters were correlated with the structural features of protein. Analysis of different contributions to the stability of RNase T1 shows that van der Waals interactions and hydrogen bonding are the major contributors to the conformational stability of the protein.

The contribution of different forces to the stability of protein molecules is still the subject of controversy (Creighton, 1991). One of the ways to resolve this problem is to analyze a variety of proteins and to correlate the thermodynamic parameters specifying the stability of the native structure, *i.e.*, its unfolding, and the structural features of proteins (Makhatadze & Privalov, 1993; Privalov & Makhatadze, 1993). Success in this analysis largely depends on the choice of protein, its size, the availability of a three-dimensional structure, the completeness and reversibility of its unfolding, and the reliability of the method used for the thermodynamic characterization of unfolding.

Ribonuclease T1 (RNase T1) from *Aspergillus oryzae* is one of the proteins that meets these requirements. It is a small globular protein (104 amino acid residues, molecular weight 11 071) that has been well characterized by numerous methods (Thomson *et al.*, 1989; Kiefhaber *et al.*, 1992a,b). It denatures reversibly in the alkaline solutions upon temperature increasing and does not posses extensive residual structure in the denatured state.

RNase T1 exists in two isoforms which differ by the amino acid at position 25: one isoform (Lys25-RNase T1) has lysine and the other (Gln25-RNase T1) glutamine (Takahashi, 1962; Takahashi & Moore, 1982). The X-ray crystal structures of both isoforms have been determined (Heinemann & Saeger, 1982; Arni *et al.*, 1992).

The thermal denaturation of both isoforms of RNase T1 has been studied by calorimetric techniques—Gln25-RNase T1 (Hu *et al.*, 1992) and Lys25-RNase T1 (Kiefhaber *et al.*, 1990; Plaza del Pino *et al.*, 1992; Barone *et al.*, 1992)—but the results are contradictory. According to Kiefhaber *et al.* (1990), the denaturation of RNase T1 is approximated well by a two-state transition model, whereas all others found apparent deviations from a two-state model. Hu *et al.* (1992) gave no explicit explanation for the apparent deviation of Gln25-RNase T1 denaturation from a two-state transition, stating only that self-association is not likely to be the cause. Plaza del Pino *et al.* (1992) attributed the apparent deviation from a two-state transition to slow kinetics, although they observed this phenomenon only in the presence of guanidinium hydrochloride. Barone *et al.* (1992) hypothesized the existence of a molten globule state as an explanation for deviation from a two-state transition.

To resolve this situation, we undertook a detailed calorimetric study of the thermal denaturation of both isoforms, Gln25-RNase T1 and Lys25-RNase T1, and examined various possibilities that could lead to an apparent deviation from a two-state model for the transition. First, we remeasured the extinction coefficient; second, we studied self-association of the native and unfolded states; and third, we studied the effect of the heating rate on the calorimetric results. This allowed us to obtain self consistent data which correlated with the structural features of RNase T1 according to an approach proposed earlier (Makhatadze & Privalov, 1993; Privalov & Makhatadze, 1993). The contributions of the hydrophobic effect and hydrogen bonding to the stability of the protein were compared with those obtained from an analysis of hydrophobic and hydrogen-bonding mutants (Shirley *et al.*, 1992; Pace, 1992; Sneddon & Tobias, 1992).

MATERIALS AND METHODS

Gln25-RNase T1 and Lys25-RNase T1 were purified from a synthesized gene cloned and expressed in *Escherichia coli* (Shirley & Laurents, 1990). The reduced and carboxymethylated protein (RCOM-RNase T1) was obtained from the Gln25-RNase T1, using the procedures described by Pace and Creighton (1986). The completeness of modification was checked according to Ellman (1959).

The concentration of protein in the solution was determined spectrophotometrically at 278 nm. The extinction coefficient of RNase T1 was determined by two different methods: quantitative nitrogen determination and amino acid analysis. The nitrogen determination was performed following the procedure of Jaenicke (1974). Ammonium sulfate and ribonuclease A were used as standards. The amino acid analysis was carried out at The Johns Hopkins Medical School Protein/Peptide Facility Center and at the American Red Cross Biomedical Research and Development using, in both cases, a PICO-TAG (Millipore-Waters) amino acid analysis

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system. A correction for light scattering was applied as described by Winder and Gent (1971).

The sedimentation equilibrium experiment was performed on a Beckman Model E analytical ultracentrifuge. The protein was dialyzed extensively against buffer, and the protein and buffer solutions were used to fill the two sectors of a 12-mm pathway Epon-filled cell. The solvent conditions studied were 10 mM glycine–HCl, pH 2.0; 10 mM sodium acetate–acetic acid, pH 5.0; and 10 mM sodium cacodylate–HCl, pH 7.0. All buffers contained 80 mM NaCl to reduce nonideality caused by electrostatic repulsion between protein molecules. The initial concentration of the samples was 0.9 mg/mL. Sedimentation experiments were done at three different rotor speeds: 30 000, 33 000, and 36 000 rpm, all at 20 ± 0.05 °C, using an An-F-Ti 4-hole analytical rotor. The concentrations along the centrifuge column were monitored by a photoelectric scanning optical system at 280 and 295 nm, to give the profile of concentration *versus* radius. Data were analyzed by the FORTRAN IV high-speed equilibrium ultracentrifugation program described by Roark and Yphantis (1969) to give the molecular weight at each concentration.

All heat capacity measurements were performed using the differential scanning microcalorimeters developed in The Johns Hopkins University using principles described elsewhere (Privalov & Potekhin, 1986; Privalov & Plotnikov, 1989). Most of the experiments were performed at a heating rate of 1 °C/min, although in some experiments heating rates from 0.2 to 1.7 °C/min were used.

The partial volume of the protein, required for the calculation of the partial molar heat capacity, was computed from its amino acid composition (Makhatadze *et al.*, 1990). The partial volume was found to be 0.70 cm^3/g for RNase T1 and 0.72 cm^3/g for reduced and carboxymethylated RNase T1.

Before each calorimetric scan, the protein was extensively dialyzed against 10 mM buffer with the desired pH followed by centrifugation at 5000*g* for 20 min in order to remove insoluble material. The buffers were chosen to have a minimum temperature dependence of pH. The protein concentration for the experiments varied from 1 to 9 mg/mL.

The water-accessible surface area of RNase T1 was calculated using the computer program CAVT66 (Rashin, 1984) from the structures given by Arni *et al.* (1992) and Heinemann and Saeger (1982). The atomic coordinates of Gln25-RNase T1 (prerelease entry 1rnl) and Lys25-RNase T1 (entry 8rnt) were from the Brookhaven Protein Data Bank (Bernstein *et al.*, 1977). The X-ray diffraction data for Gln25-RNase show that there are three molecules in the asymmetric crystal unit. The structures of these molecules differ slightly, so the surface area was calculated for each of the structures and then these were averaged. The water-accessible surface area for the unfolded state was calculated as described elsewhere (Miller *et al.*, 1987). The changes in water-accessible surface area for different groups for the unfolding of Gln25-RNase T1 and Lys25-RNase T1 are listed in Table 1.

Table 1: Water-Accessible Surface Area Change (ΔASA, Å²) for the Unfolding of RNase T1

type of surface	Gln25-RNase T1	Lys25-RNase T1
aliphatic	4662	4799
aromatic	1767	1825
polar parts of:		
Arg	99	101
Asn	176	156
Asp	78	93
Cys	223	226
Gln	133	97
Glu	257	241
His	106	108
Lys	22	45
Met	0	0
Ser	188	182
Thr	70	78
Trp	27	27
Tyr	282	292
–CONH–	2518	2550

RESULTS

Extinction Coefficient. The results of 10 nitrogen determinations gave an average extinction coefficient of 1.86 ± 0.07 (mg/mL at 278 nm). Three amino acid analyses of Gln25-RNase T1 gave 1.86 ± 0.10. These values are close to the value of 1.9 reported by Takahashi (1962) but differ significantly from the value of 1.67 determined by a dry-weight method (Hu *et al.*, 1992). The same extinction coefficient was used for Lys25-RNase T1.

The effect of pH on the extinction coefficient of RNase T1 was tested by comparing the absorption at 278 nm of samples diluted into buffers with various pH values. It was found that the extinction coefficient was, within experimental error (less than 1%), not dependent on pH in the pH range of 2–10.

It should be noted that an extinction coefficient of 1.9 was used in the calorimetric study of Kiefhaber *et al.* (1990), whereas all others used 1.67 (Hu *et al.*, 1992; Barone *et al.*, 1992; Plaza del Pino *et al.*, 1992).

Sedimentation Equilibrium. The data from sedimentation equilibrium show that the apparent molecular weight of RNase T1 is about 11 kDa over the concentration range from 0 to 1.9 mg/mL at pH 2.0, 5.0, and 7.0 at 25 °C, indicating that the protein exists in monomeric form.

Scanning Calorimetry. The temperature dependencies of the partial molar heat capacity of the RNase T1 at different pH values, obtained at a heating rate of 1.0 °C/min, are presented in Figure 1. The partial molar heat capacity of RCOM-RNase T1 with all of the disulfide bonds reduced and the cysteines carboxymethylated is also presented in Figure 1. The dependence of the transition temperature on pH is presented in Table 2 and Figure 2. The same figure presents the data obtained previously by others (Hu *et al.*, 1992; Kiefhaber *et al.*, 1990; Plaza del Pino *et al.*, 1992; Barone *et al.*, 1992). As can be seen, the data are in good correspondence with each other.

The dependence of the transition temperature on pH can be used for the calculation of the difference between the number of protons bound to the native and denatured states of protein $\Delta_t\nu$ (Privalov *et al.*, 1969) as:

$$\Delta_t\nu = -\frac{\Delta H^{cal}(T_t)}{2.303RT_t^2}\frac{dT_t}{dpH} \quad (1)$$

where $\Delta H^{cal}(T_t)$ is the calorimetrically measured enthalpy at the transition temperature T_t, listed in Table 2, and R is the gas constant. The results suggest that between pH 5 and 10, on the average, 0.76 proton is released upon denaturation of Gln25-RNase T1 and 0.86 from Lys25-RNase T1. This proton release is probably associated with histidine residues

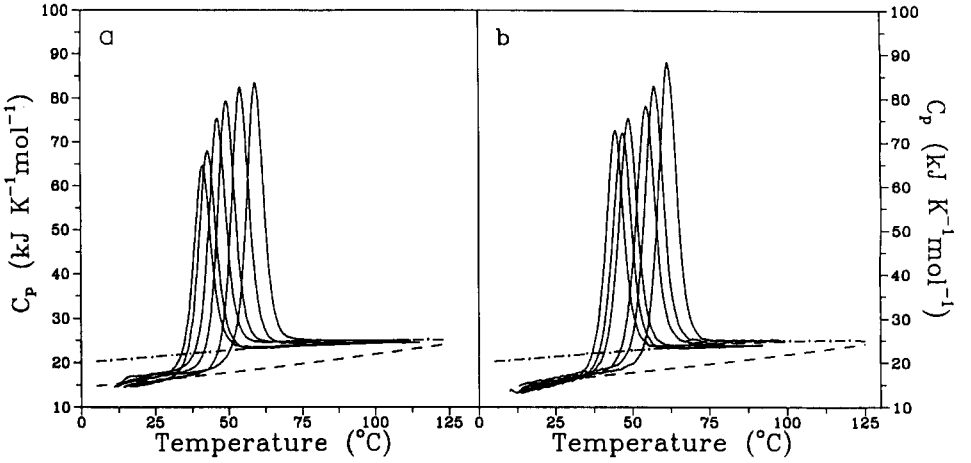

FIGURE 1: Temperature dependence of the partial molar heat capacity of (a) Gln25-RNase T1 at pH 9.7, 9.1, 7.9, 7.0, 6.0, and 5.0 (from left to right) and (b) Lys25-RNase T1 at pH 9.7, 8.6, 8.0, 6.5, 6.0, and 5.0 (from left to right). The dashed-and-dotted line represents the partial molar heat capacity of the carboxymethylated Gln25-RNase T1 at pH 6.0. The dashed line represents the extrapolation of the partial molar heat capacity of the native protein.

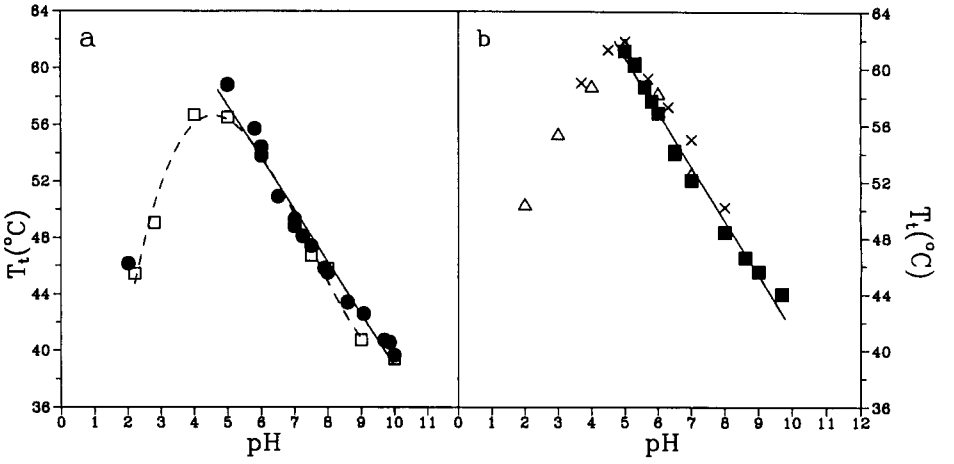

FIGURE 2: Dependence of the transition temperature T_t on pH for Gln25-RNase T1 (a) and Lys25-RNase T1 (b): (●) Gln25-RNase T1 this work, (■) Lys25-RNase T1 this work, (□) Gln25-RNase T1 by Hu *et al.* (1992), (△) Lys25-RNase T1 by Kiefhaber *et al.* (1990), and (×) Lys25-RNase T1 by Barone *et al.* (1993).

which have higher pK values in the native state than in the denatured state (Inagaki *et al.*, 1981; McNutt *et al.*, 1990; Pace *et al.*, 1990).

Together with the calorimetric enthalpies ΔH^{cal}, Table 2 also presents the van't Hoff enthalpies ΔH^{vH} calculated from the shape of calorimetric profiles (Privalov & Khechinashvili, 1974) as:

$$\Delta H^{vH} = \frac{4RT_t^2C_{p,max}}{\Delta H^{cal}} \quad (2)$$

where $C_{p,max}$ is the heat capacity at the transition temperature.

Calorimetric enthalpies obtained in this work are compared with those reported in literature in Figure 3a. There is a good correspondence between our data and the data by Kiefhaber *et al.* (1990). However, there is poor correspondence between our data and those reported by Hu *et al.* (1992), Plaza del Pino *et al.* (1992), and Barone *et al.* (1992), which is caused mainly by the difference in the extinction coefficients. As for the van't Hoff enthalpy, which is independent of the experimental procedure for measuring the concentration, there is a fair agreement with the data reported earlier.

The reversibility of denaturation was checked by reheating the same sample. At pH 2.0, 5.0, and 7.0, and reversibility, judged by the enthalpy, is not less than 99%, while at pH 10.0, and reversibility is about 90%. At pH 3.0 and 3.5, the protein aggregates upon denaturation, probably because these pHs are close to the isoelectric point (pI = 3.8) for RNase T1 (Iida & Ooi, 1969).

DISCUSSION

Mechanism of RNase T1 Denaturation. Figure 4 presents the plot of the ratio of the van't Hoff to calorimetric enthalpy $\Delta H^{vH}/\Delta H^{cal}$ *versus* the transition temperature T_t. As one can see, at high T_t, the ratio is close to 1 (0.97 ± 0.04), indicating that the transition can be well approximated by a simple two-state mechanism. As T_t decreases, this ratio gradually increases to 1.2–1.3. This apparent deviation from a two-state transition could be caused by any of the following: (a) by the dependence of the extinction coefficient on pH, since the transition temperature was perturbed by changing the pH of the solution; (b) by the pH or temperature dependence of the self-association of the protein in the native or denatured states (Sturtevant, 1987); (c) by the existence of a stable intermediate state upon denaturation of the protein (Privalov & Potekhin, 1986); or (d) by nonequilibrium conditions during the calorimetric experiments (*i.e.*, slow equilibration compared to the heating rate) (Lentz *et al.*, 1978; Freire & Biltonen, 1978; Lepock *et al.*, 1992).

The results of extinction coefficient measurements show that there is no pH dependence of the absorbency of protein

Table 2: Calorimetric Data[a] of RNase T1 Denaturation at a Scan Rate of 1.0 °C/min

pH[b]	T_t (°C)	ΔH^{cal}	ΔH^{vH}	$\Delta H^{vH}/\Delta H^{cal}$	ΔC_p	$\Delta\nu$[c]	ΔH^{ion}[d]	ΔH^{conf}
				Gln25-RNase T1				
5.0	58.8	466	481	1.03	3.71	0.82	22	444
6.0	54.1	465	462	0.99	5.35	0.84	24	441
6.5	50.9	444	467	1.05	5.13	0.82	24	420
7.0	48.8	453	451	1.00	5.05	0.84	24	429
7.5	47.4	410	447	1.09	4.84	0.77	13	397
7.9	45.8	402	444	1.10	4.95	0.76	13	389
8.6	43.4	396	454	1.15	5.02	0.76	12	384
9.1	42.6	362	430	1.19	5.09	0.70	−11	373
9.7	40.7	333	425	1.28	5.08	0.65	−11	344
9.85	40.6	333	436	1.31	5.16	0.65	−11	344
10.0	39.7	373	452	1.21	5.31	0.74	12	361
average					4.97 ± 0.42	0.76		
				Lys25-RNase T1				
5.0	61.2	508	480	0.94	4.78	0.91	26	482
5.3	60.3	484	465	0.96	4.89	0.87	25	459
5.3	60.2	503	458	0.91	3.90	0.91	28	475
5.6	58.7	473	438	0.83	4.07	0.86	27	446
5.8	57.7	480	449	0.94	3.03	0.88	27	453
6.0	56.9	470	462	0.98	4.12	0.87	27	443
6.5	54.0	457	454	0.97	4.98	0.88	27	440
6.5	54.2	462	436	0.94	5.36	0.87	27	435
7.0	52.1	445	412	0.93	5.97	0.84	26	419
8.0	48.4	423	438	1.04	4.90	0.82	13	410
8.6	46.6	408	427	1.05	4.82	0.80	12	396
9.7	44.0	393	439	1.12	5.78	0.78	12	381
average					4.87 ± 0.64	0.86		

[a] All enthalpies are in kJ/mol, and the heat capacity change is in kJ/K mol. [b] A 10 mM concentration of the following buffers was used, pH 5.0–5.3, sodium acetate; pH 5.3–7.0, sodium cacodylate–HCl; pH 7.5–7.9, PIPES–HCl; pH 8.0–9.0, sodium borate HCl; pH 9.1–9.9, glycine–NaOH for Gln-25 RNase T1 and sodium borate–NaOH for Lys-25 RNase T1; and pH 10.0, sodium borate–NaOH. [c] $\Delta\nu$ is the number of protons released upon unfolding, calculated using eq 1. [d] ΔH^{ion} includes heats of ionization for both the protein and the buffer as shown in eq 3.

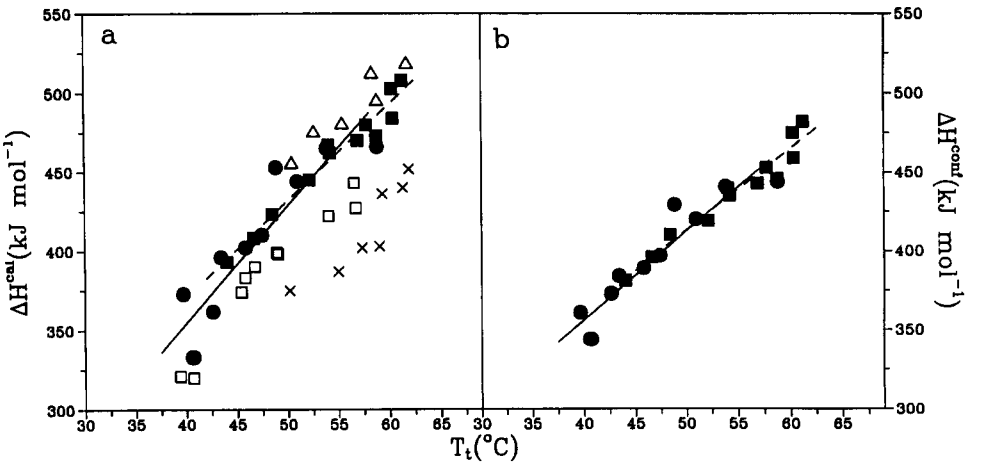

FIGURE 3: (a) Temperature dependence of the calorimetric enthalpies ΔH^{cal} of denaturation of RNase T1: (●) Gln25-RNase T1 this work, (■) Lys25-RNase T1 this work, (□) Gln25-RNase T1 by Hu *et al.* (1992), (△) Lys25-RNase T1 by Kiefhaber *et al.* (1990), and (×) Lys25-RNase T1 by Barone *et al.* (1993). (b) Dependence of the conformational enthalpy ΔH^{conf} on the transition temperature T_t for Gln25-RNase T1 and Lys25-RNase T1 (symbols are the same as in Figure 3a). The solid and dashed lines represent the linear fit of the experimental data for Gln25-RNase T1 and Lys25-RNase T1, respectively. Their slopes give 5.63 and 5.18 kJ/K mol for the heat capacity changes upon unfolding of Gln25-RNase T1 and Lys25-RNase T1, respectively.

at 278 nm. Sedimentation equilibrium experiments show that the native protein exists as a monomer. Similar results were found by Hu *et al.* (1992) using size-exclusion chromatography. There is no evidence of self-association of protein in the denatured state either. This was checked by measuring the effect of protein concentration (1–9 mg/mL) on the transition temperature. No dependence was found which indicates an absence of self-association in the denatured state under the conditions used in the experiments. The presence of stable intermediate states upon unfolding of RNase T1 has been studied by several groups with negative results (Thomson *et al.*, 1989; Kiefhaber *et al.*, 1990; Plaza del Pino *et al.*, 1992).

These results leave only one explanation for the apparent deviation of the transition from a two-state model: the slow kinetics of the transitions which can cause a distortion of the transition profile (Plaza del Pino *et al.*, 1992; Lepock *et al.*, 1992). In order to check this possibility, we performed calorimetric experiments on RNase T1 at different heating rates ranging from 0.2 to 1.7 °C/min. The effect of heating rate on the thermodynamic parameters of RNase T1 was studied at pH 7.0 and 10.0, and the results are presented in Table 3 and Figure 5. There is a notable difference in the effect of heating rate on the thermodynamic parameters at pH 7.0 and 10.0. The transition temperature at pH 7.0 is

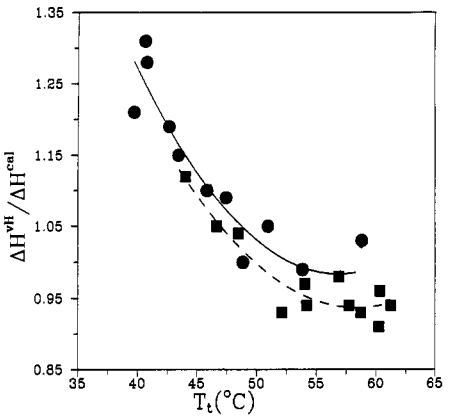

FIGURE 4: Dependence of the ratio of the van't Hoff to calorimetric enthalpy $\Delta H^{vH}/\Delta H^{cal}$ on the transition temperature T_t: Gln25-RNase T1 (●) and Lys25-RNase T1 (■).

independent of scanning rate, whereas at pH 10.0, this dependence is clearly observed. The ratio of the van't Hoff to calorimetric enthalpy is practically independent of the heating rate and is close to 1 at pH 7.0. At pH 10.0, the ratio $\Delta H^{vH}/\Delta H^{cal}$ varies from about 1.05 to 1.20 (Figure 5a). This means that the apparent deviation of the transition mechanism of the RNase T1 from a two-state model at high scanning rates at pH 10.0 is caused by the nonequilibrium conditions of the experiment, due to the slow kinetics of the transition. These kinetics of the transition appear to be strongly dependent on temperature but not on pH. This can be seen by comparing the ratio of the van't Hoff to calorimetric enthalpy for the isoforms of RNase T1: the ratio varies with temperature but not pH. At pH 10.0, where the transition occurs at a lower temperature, the kinetics are slow and the reaction does not reach equilibrium under the conditions of the experiment. Decreasing the pH to 7.0 leads to an increase in the transition temperature by about 10 °C, and this leads to the disappearance of the effect of scan speed on the equilibration during the experiment. Kiefhaber and Schmid (1992b) have suggested that the slow equilibration of RNase T1 in the transition region is due to prolyl isomerization. In contrast to the van't Hoff enthalpy, the calorimetric enthalpy does not dependent on the heating rate (Figure 5b).

Enthalpy of the Conformational Transition. The calorimetrically measured enthalpy of protein denaturation includes not only the enthalpy of the conformational transition $\Delta H^{conf}(T_t)$ but also the heat of ionization of the protein upon transiton $\Delta H^{ion}_{pr}(T_t)$ and the heat of ionization of buffer $\Delta_t\nu\Delta H^{ion}_{buf}(T_t)$:

$$\Delta H^{cal}(T_t) = \Delta H^{conf}(T_t) + \Delta H^{ion}_{pr}(T_t) + \Delta_t\nu\Delta H^{ion}_{buf}(T_t) \qquad (3)$$

where $\Delta_t\nu$ is the difference between the number of protons bound to the native and denatured states of the protein. In the pH range 5–10, $\Delta H^{ion}_{pr}(T_t)$ should be mainly associated with the His residues and can be approximated by the heat of ionization of free histidine (Privalov *et al.*, 1986). The heat of ionization of the buffers used are known (Izatt & Christensen, 1976). The total ionization heats and the enthalpies of the conformational transition of RNase T1, $\Delta H^{conf}(T_t)$, are listed in Table 2. The dependence of the ΔH^{conf} on temperature for Gln25-RNase T1 and Lys25-RNase T1 is presented in Figure 3b. As can be seen, the temperature dependencies of the enthalpies of unfolding for the isoforms are almost identical. In the temperature range studied, ΔH^{conf} varies linearly with temperature and the slope of this function corresponds to the heat capacity change ΔC_p for the conformational transition of RNase T1. The linear fit of the dependence of the conformational enthalpy on temperature gives values for ΔC_p equal to (5.6 ± 0.3) kJ/K mol for Gln25-RNase T1 and (5.2 ± 0.3) kJ/K mol for Lys25-RNase T1. One should note that the lower values reported in literature were obtained from the temperature dependence of the calorimetric enthalpy without correction on the ionization effects and using an incorrect extinction coefficient. When corrected for the error in the extinction coefficient and the heats of ionization, the data of Hu *et al.* (1991) give a value of 5.4 kJ/K mol and the data of Kiefhaber *et al.* (1990) give a value of 5.7 kJ/K mol. Thus, the agreement between the ΔH^{conf} and ΔC_p values from these three laboratories is excellent (Figure 3b).

Heat Capacity Change upon Unfolding. The heat capacity change for the conformational transition of RNase T1 obtained from the temperature dependence of ΔH^{conf} represents the average value for the temperature range 40–59 °C. In order to obtain the heat capacity change for a wider temperature range, one should directly measure the heat capacities of the native and denatured states of the protein over the temperature range of interest. The heat capacity of the native state varies linearly with temperature up to 40 °C, and we assume this continues at higher temperatures. The linear extrapolation of the heat capacity of the native state was justified for a number of proteins with different thermostabilities (Privalov & Makhatadze, 1990, 1992; Wintrode *et al.*, 1993; Makhatadze *et al.*, 1993). The partial molar heat capacity of the native state of RNase T1 is listed in Table 4.

The heat capacity of the denatured state of RNase T1 can be measured in the temperature range 60–125 °C (Figure 1). These values can be compared to the heat capacity of the protein with both disulfide bonds reduced and the cysteine residues carboxymethylated. It was shown earlier (Privalov *et al.*, 1989; Makhatadze *et al.*, 1993) that the reduction of disulfides and their modification in many cases lead to complete unfolding of a protein. The heat capacity of reduced and carboxymethylated RNase T1 is a smooth function of temperature (Figure 1). If we take into account the heat capacity effect of the additional four CH_2COOH groups introduced by modification (Makhatadze & Privalov, 1990), the resultant function will represent the heat capacity of unfolded RNase T1. The values obtained (Table 4) are very close to the heat capacity of denatured RNase T1 in the temperature range 60–125 °C. This means that, judging by the heat capacity criteria, the denatured state is indistinguishable from the unfolded state. This allows us to assign the thermodynamic parameters for denaturation to those of unfolding.

The difference between the heat capacity functions for the native and unfolded states is the heat capacity change for RNase T1 unfolding and is presented in Table 4. It can be seen that ΔC_p decreases with increasing temperature. At 50 °C, the heat capacity change is 5.0 kJ/K mol which is comparable to the heat capacity change obtained from the temperature dependence of ΔH^{conf}.

Thermodynamic Functions Characterizing the Unfolding of RNase T1. Having the temperature dependence of the heat capacity change and the conformational enthalpy at the transition temperature, we can compute all of the thermodynamic functions for the conformational transition of RNase T1 in the temperature range 5–125 °C using

$$\Delta^U_N H(T) = \Delta^U_N H(T_t) + \int_{T_t}^{T} \Delta^U_N C_p(T)\, dT \qquad (4)$$

Table 3: Calorimetric Data for Gln25-RNase T1 Denaturation Obtained at Different Scan Rates[a]

	pH 7.0				pH 10.0			
scan rate (°C/min)	T_t (°C)	ΔH^{cal}	ΔH^{vH}	$\Delta H^{vH}/\Delta H^{cal}$	T_t (°C)	ΔH^{cal}	ΔH^{vH}	$\Delta H^{vH}/\Delta H^{cal}$
1.67	49.8	451 (445)	455	1.01	41.9	374 (357)	436	1.17
1.00	48.8	453 (452)	451	1.00	39.8	373 (367)	452	1.21
0.37	48.2	444 (446)	422	0.95	38.5	369 (370)	402	1.09
0.19	48.7	428 (428)	402	0.94	38.5	361 (361)	380	1.05

[a] Values in the parentheses are calorimetric enthalpies normalized to the transition temperature of the lowest scan rate, using ΔC_p values given in Table 2. The buffers used were, pH 7.0, 10 mM sodium cacodylate–HCl, and pH 10.0, 10 mM sodium borate–NaOH. The enthalpies are in kJ/mol.

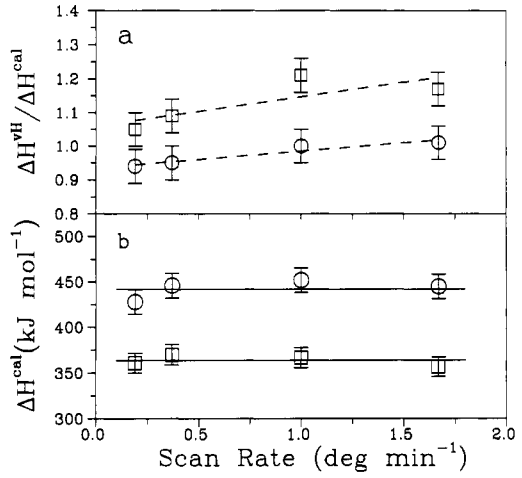

FIGURE 5: (a) Heating rate dependence of the ratio of the van't Hoff to calorimetric enthalpy $\Delta H^{vH}/\Delta H^{cal}$ at pH 7.0 (○) and pH 10.0 (□) for Gln25-RNase T1. Bars show the estimated 5% error. (b) Heating rate dependence of the calorimetric enthalpy at pH 7.0 (○) and pH 10.0 (□) for RNase T1. Bars show the 3% error. The solid lines represent the average values of the calorimetric enthalpies at pH 7.0 and 10.0, respectively.

$$\Delta_N^U S(T,\mathrm{pH}) = \frac{\Delta_N^U H(T_t,\mathrm{pH})}{T_t} + \int_{T_t}^{T} \Delta_N^U C_p(T)\, \mathrm{d} \ln T \quad (5)$$

$$\Delta_N^U G(T,\mathrm{pH}) = \Delta_N^U H(T) - T\Delta_N^U S(T,\mathrm{pH}) \quad (6)$$

The enthalpy and entropy functions are given in Table 4 for pH 5.0 which is the pH of maximum stability. These give a Gibbs energy of stabilization of RNase T1 at pH 5.0 and 25 °C of about 38 kJ/mol for Gln25-RNase T1 and 40 kJ/mol for Lys25-RNase T1. This 5% difference in stability of the two isoforms of RNase T1 reflects the difference in their thermostabilities at the same solvent conditions. Since the enthalpy functions for these two isoforms appear to be similar, one might assume that the difference in stability of the considered two isoforms of RNase T1 is due to the entropic factor. This conclusion, however, is not unequivocal because the difference in stability of two proteins is of the order of resolution of their enthalpy functions (3%).

It should be noted that the found values of the Gibbs energy of stabilization are in fair correspondence with the values obtained under the same conditions by analysis of the urea-induced unfolding which is 37 kJ/mol for Gln25-RNase T1 and 41 kJ/mol for Lys25-RNase T1 according to Pace (1990).

Contribution of Hydrogen Bonding and Hydrophobic Effect to the Stability of RNase T1. By combining the thermodynamic functions for RNase T1 with structural information on the protein, one can make conclusions concerning the relative contribution of different forces to the stabilization of the native structure of this protein. The formalism of this approach was described in detail elsehwere (Makhatadze & Privalov, 1993; Privalov & Makhatadze, 1993).

The conformational enthalpy of protein unfolding can be dissected into the contribution from the enthalpy of hydration of internal polar, $\Delta_N^U H_{pol}^{hyd}$, and nonpolar, $\Delta_N^U H_{npl}^{hyd}$, groups, the enthalpy of van der Waals interactions between the groups packed in the protein interior, $\Delta_N^U H^{vdW}$, and the enthalpy of intramolecular hydrogen bonding, $\Delta_N^U H^{HB}$:

$$\Delta_N^U H^{conf} = \Delta_N^U H_{pol}^{hyd} + \Delta_N^U H_{npl}^{hyd} + \Delta_N^U H^{vdW} + \Delta_N^U H^{HB} \quad (7)$$

where the right-side terms are related to the structural properties of proteins as

$$\Delta_N^U H_{pol}^{hyd} = \sum_i \Delta \hat{H}_i^{hyd} \Delta_N^U \mathrm{ASA}_i \quad (8)$$

$$\Delta_N^U H_{npl}^{hyd} = \Delta \hat{H}_{arm}^{hyd} \Delta_N^U \mathrm{ASA}_{arm} + \Delta \hat{H}_{alp}^{hyd} \Delta_N^U \mathrm{ASA}_{alp} \quad (9)$$

$$\Delta_N^U H^{vdW} = \Delta \hat{H}_{arm}^{int} \Delta_N^U \mathrm{ASA}_{arm} + \Delta \hat{H}_{alp}^{int} \Delta_N^U \mathrm{ASA}_{alp} \quad (10)$$

$$\Delta_N^U H^{HB} = N_{HB} \Delta h^{HB} \quad (11)$$

In eqs 8–11, $\Delta \hat{H}_i^{hyd}$ is the reduced enthalpy of hydration of a given type of surface area, listed in Table 5 of Makhatadze and Privalov (1993); $\Delta \hat{H}^{int}$ is the reduced enthalpy of van der Waals interaction, listed in Table 7 of Makhatadze and Privalov (1993); $\Delta_N^U \mathrm{ASA}_i$ is the change of a given type of surface area upon unfolding of the protein, listed in Table 1 of this paper; Δh^{HB} is the average enthalpy of an intramolecular hydrogen bond; and N_{HB} is the number of hydrogen bonds in the RNase T1 molecule. The enthalpies computed according to eqs 8–10 are presented in Table 4. Knowledge of these enthalpies allows us to calculate the only unknown parameter in eq 7—$\Delta_N^U H^{HB}$. The values obtained for the enthalpy of hydrogen bonding in the RNase T1 molecule are given in Table 4. According to Stickle *et al.* (1992), there are 104 intramolecular hydrogen bonds in folded RNase T1. Dividing the enthalpy of hydrogen bonding by the number of hydrogen bonds in the molecule, we find that the contribution of one internal hydrogen bond to the enthalpy of stabilization of RNase T1 is about 55 kJ/mol.

Near pH 5.0, RNase T1 unfolding does not depend on pH, *i.e.*, there is no additional protonation, so the entropy determined by eq 5 is the entropy of the conformational transitionto the unfolded state $\Delta_N^U S^{conf}$ which can be represented as:

$$\Delta_N^U S^{conf} = \Delta_N^U S_{npl}^{hyd} + \Delta_N^U S_{pol}^{hyd} + \Delta_N^U S^{cnfg} \quad (12)$$

where $\Delta_N^U S_{npl}^{hyd}$ and $\Delta_N^U S_{pol}^{hyd}$ are the entropies of hydration of nonpolar and polar groups exposed to water upon unfolding, respectively, and $\Delta_N^U S^{cnfg}$ is the configurational entropy. The configurational entropy includes the contributions of unfreezing of the backbone chain, unpacking the side chain

Table 4: Thermodynamic Functions for the Unfolding of RNase T1

	temperature (°C)					
	5	25	50	75	100	125
	Heat Capacity (kJ/K mol)					
C_p^N	14.6	16.1	17.8	19.7	21.9	24.3
C_p^U	20.1	21.4	22.8	24.0	24.9	25.1
$\Delta^U_N C_p$	5.5	5.3	5.0	4.3	3.0	0.8
	Gln25-RNase T1, Enthalpy (kJ/mol)					
$\Delta^U_N H^{conf}$	173	281	410	528	621	672
$\Delta^U_N H^{hyd}_{npl}$	−1092	−830	−522	−234	40	290
$\Delta^U_N H^{hyd}_{pol}$	−5449	−5573	−5705	−5820	−5923	−6018
$\Delta^U_N H^{VW}$	911	898	879	849	803	736
$\Delta^U_N H^{HB}$	5803	5786	5758	5733	5702	5664
Δh^{HB}	56	56	55	55	55	55
	Entropy (J/K mol)					
$\Delta^U_N S^{exp}$	444	817	1233	1584	1845	1976
$\Delta^U_N S^{hyd}_{npl}$	−4163	−3258	−2257	−1399	−646	7
$\Delta^U_N S^{hyd}_{pol}$	−3120	−3541	−3960	−4296	−4579	−4820
$\Delta^U_N S^{cnfg}$	7726	7617	7451	7280	7070	6789
	Lys25-RNase T1, Enthalpy (kJ/mol)					
$\Delta^U_N H^{conf}$	178	286	415	533	626	677
$\Delta^U_N H^{hyd}_{npl}$	−1125	−856	−539	−241	41	298
$\Delta^U_N H^{hyd}_{pol}$	−5493	−5619	−5752	−5870	−5976	−6072
$\Delta^U_N H^{VW}$	939	926	906	875	827	759
$\Delta^U_N H^{HB}$	5857	5834	5800	5770	5734	5692
Δh^{HB}	56	56	56	55	55	55
	Entropy (J/K mol)					
$\Delta^U_N S^{exp}$	450	823	1239	1590	1851	1982
$\Delta^U_N S^{hyd}_{npl}$	−4288	−3356	−2326	−1441	−665	8
$\Delta^U_N S^{hyd}_{pol}$	−3133	−3560	−3986	−4329	−4619	−4865
$\Delta^U_N S^{cnfg}$	7868	7739	7555	7361	7136	6839

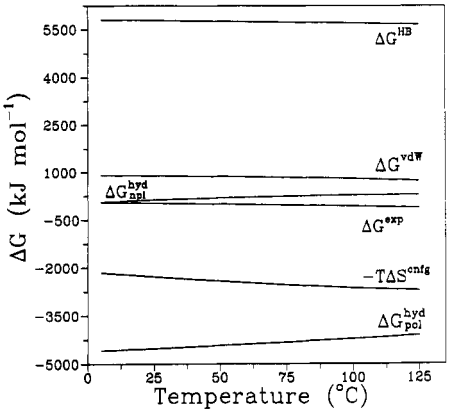

FIGURE 6: Contributions of different forces to the stability of Gln25-RNase T1: ΔG^{exp}, the experimentally obtained Gibbs energy of stabilization of protein at pH 5.0; ΔG^{vdW}, the Gibbs energy of van der Waals interaction; ΔG^{hyd}_{npl}, the Gibbs energy of hydration of nonpolar groups; ΔG^{hyd}_{pol}, the Gibbs energy of hydration of polar groups; ΔG^{HB}, the Gibbs energy of hydrogen bonding; and $-T\Delta S^{cnfg}$, the contribution to the Gibbs energy of the configurational entropy of the backbone and side chains upon unfolding.

groups, and the entropies of van der Waals interactions and intramolecular hydrogen bonding. The hydration terms can be calculated as:

$$\Delta^U_N S^{hyd}_{pol} = \sum_i \Delta \hat{S}^{hyd}_i \Delta^U_N ASA_i \quad (13)$$

$$\Delta^U_N S^{hyd}_{npl} = \Delta \hat{S}^{hyd}_{arm} \Delta^U_N ASA_{arm} + \Delta \hat{S}^{hyd}_{alp} \Delta^U_N ASA_{alp} \quad (14)$$

using the hydration entropies, reduced/Å^2, of a given type of surface area, $\Delta \hat{S}^{hyd}_i$ (Table 4 of Privalov and Makhatadze, 1993). The hydration entropies are listed in Table 4. Knowing the entropies of hydration and the experimental entropies of

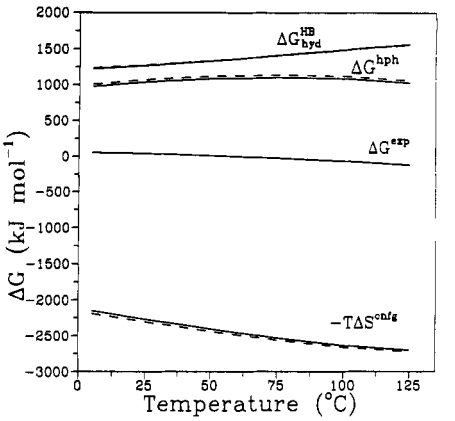

FIGURE 7: Contribution of hydrogen bonding and hydrophobic interactions to the stability of Gln25-RNase T1 (solid) and Lys25-RNase T1 (dashed), both at pH 5.0: ΔG^{hph}, the Gibbs energy of hydrophobic interactions; ΔG^{HB}_{hyd}, the Gibbs energy of hydrogen bonding; and $-T\Delta S^{cnfg}$, the contribution to the Gibbs energy from the increase of configurational entropy of the backbone and side chains upon unfolding.

RNase T1 unfolding, one can get $\Delta^U_N S^{cnfg}$ using eq 12. The values of $\Delta^U_N S^{cnfg}$ obtained are listed in Table 4.

The Gibbs energy difference of the unfolded and native states can be represented as a sum:

$$\Delta^U_N G = \Delta^U_N G^{hyd}_{npl} + \Delta^U_N G^{hyd}_{pol} + \Delta^U_N G^{vdW} + \Delta^U_N G^{HB} - T\Delta^U_N S^{cnfg}$$
$$= \Delta^U_N G^{HB}_{hyd} + \Delta^U_N G^{hph} - T\Delta^U_N S^{cnfg} \quad (15)$$

where ΔG^{hyd}_{npl} and ΔG^{hyd}_{pol} are the Gibbs energies of hydration of nonpolar and polar groups exposed to water upon unfolding; ΔG^{vdW} is the Gibbs energy of van der Waals interactions, which has only the enthalpic term $\Delta^U_N H^{vdW}$; ΔG^{HB} is the Gibbs energy of hydrogen bonding without hydration effects; $\Delta G^{HB}_{hyd} = \Delta G^{HB} + \Delta G^{hyd}_{pol}$ is the Gibbs energy of hydrogen bonding with dehydration of the corresponding polar groups; $\Delta^U_N G^{hph} = \Delta^U_N G^{hyd}_{npl} + \Delta^U_N G^{vdW}$ is the hydrophobic effect; and $-T\Delta^U_N S^{cnfg}$ is the effect of the increase of configurational entropy.

The complete balance of contributions of various forces to the conformational stability of RNase T1 at pH 5.0 is presented in Figure 6. As can be seen, the native state of RNase T1 is stabilized mainly by hydrogen bonding and van der Waals interactions between groups in the protein interior. The hydration of nonpolar groups makes a small contribution to the stability, but the hydration of polar groups has a large destabilizing effect. The significant contribution of van der Waals interactions to the stability of RNase T1 was also emphasized by Sneddon and Tobias (1992).

The Gibbs energy of hydrophobic interactions, $\Delta^U_N G^{hph}$, under the formalism considered, corresponds to the sum of the Gibbs energy of hydration of nonpolar groups and the Gibbs energy of van der Waals interactions. In aqueous media, the contribution of hydrogen bonding to the Gibbs energy of stabilization of protein structure, ΔG^{hyd}_{HB}, includes the enthalpy for disruption of intramolecular hydrogen bonds and the Gibbs energy of hydration of the polar groups exposed to water by unfolding, $\Delta G^{HB}_{hyd} = \Delta^U_N H^{HB} + \Delta^U_N G^{hyd}_{pol}$. This gives the value of ΔG^{hyd}_{HB} per one hydrogen bond in the RNase T1 structure of 12 kJ/mol at 25 °C. As we see in Figure 7, the contributions of hydrogen bonding and hydrophobic interactions to the stabilization of the compact native state of RNase T1 are comparable in magnitude. A similar conclusion was

reached previously on the basis of studies of the stabilities of mutant proteins (Shirley *et al.*, 1992; Pace, 1992).

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