Temperature-Induced Denaturation of Ribonuclease S: A Thermodynamic Study†

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ABSTRACT: In this paper the thermal denaturation of ribonuclease S, the product of mild digestion of ribonuclease A by subtilisin, is deeply investigated by means of DSC and CD measurements. It results that at whatever pH in the range 4−7.5 the process is fully reversible but not well represented by the simple two-state N ↔ D transition. Actually, a two-state model that considers both unfolding and dissociation, NL ↔ D + L*, well accounts for the main features of the process: the tail present in the low-temperature side of DSC peaks and the marked dependence of denaturation temperature on protein concentration. This mechanism is strictly linked to the exact stoichiometry of RNase S. An excess of the protein component of RNase S, the so-called S-protein, shifts the system toward a more complex behavior, that deserves a separate treatment in the accompanying paper [Graziano, G., Catanzano, F., Giancola, C., & Barone, G. (1996) *Biochemistry 35*, 13386−13392]. The thermodynamic analysis leads to the conclusion that the difference in thermal stability between RNase S and RNase A is due to entropic effects, i.e., a greater conformational flexibility of both backbone and side chains in RNase S. The process becomes irreversible at pH 8.0−8.5, probably due to side-reactions occurring at high temperature. Finally, the influence of phosphate ion on the stability of RNase A and RNase S at pH 7.0 is studied and explained in terms of its binding on the active site of ribonucleases. The analysis enables us to obtain an estimate of the apparent association constant and binding enthalpy also.

Thermodynamic investigations of the reversible denaturation of small globular proteins have clarified fundamental features of the native structure stability (Privalov & Gill, 1988; Privalov, 1992; Murphy & Freire, 1992; Makhatadze & Privalov, 1995). Although globular proteins are heteropolymers, comprised of 20 different monomeric units, their thermal denaturation is an all-or-none process that can be regarded as a first-order phase transition (Shakhnovich & Finkelstein, 1989; Karplus & Shakhnovich, 1992). Usually only two thermodynamic states are populate, the native and denaturated ones, without the presence of intermediates. Furthermore, the native structure results marginally stable because the denaturation free energy amounts to 40−50 kJ mol^{-1}. This figure, when normalized to the number of residues, roughly corresponds, at room temperature, to the random thermal energy, RT (Jaenicke, 1991). These findings are at odds, but both are determined by the cooperative three-dimensional network of noncovalent interactions that give rise to the native tertiary structure. The denaturation process of bovine pancreatic ribonuclease A (RNase A)[1] is a very good example of this behavior.

RNase A, under mild digestion conditions, is preferentially hydrolyzed between Ala20 and Ser21, by the bacterial protease subtilisin, with formation of RNase S (Richards & Vithayathil, 1959). No loss of activity occurs as a result of this modification. The N-terminal fragment, S-peptide, and the rest of the molecule, S-protein, remain tightly bound to each other, due to specific noncovalent interactions. The two components of RNase S can be separated at low pH, and neither one shows any enzymatic activity. But when the fragments are mixed in a molar ratio equal or greater than 1, around neutral pH, a fully active enzyme is re-formed.

The three-dimensional structure of RNase S is very similar to that of RNase A, apart small differences near the site of proteolytic cleavage (Wyckoff et al., 1970; Kim et al., 1992; Varadarajan & Richards, 1992). However, the stability of the two enzymes is very different, as already reported in old papers (Sherwood & Potts, 1965; Takahashi et al., 1969). Despite the popularity of this enzyme, a comprehensive thermodynamic study of RNase S denaturation process has not been performed so far. Therefore, it is interesting to investigate the thermal denaturation of RNase S by means of differential scanning calorimetry, DSC, and circular dichroism, CD, measurements at changing both protein concentration and pH. The principal aim is to elucidate the mechanism of the process because the structure is given by the association of two parts. Indeed, the simple N ↔ D model is unable to explain and justify the experimental findings, and a more complex model, considering both unfolding and dissociation of the two components, is proposed. This model well accounts for the experimental results. Moreover, the thermodynamic analysis leads to the conclusion that RNase S denaturation is accompanied by a smaller entropy change than RNase A denaturation. The results bring out that the native state of RNase S has a greater conformational flexibility compared to RNase A, resulting in a smaller conformational entropy gain on unfolding that overwhelms the cratic contribution. This finding explains the great difference in thermal stability between RNase S

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[1] Abbreviations: RNase A, bovine pancreatic ribonuclease; RNase S, product of proteolytic cleavage of bond 20−21 in RNase A; S-protein, protein component of RNase S, residues 21−124; S-peptide, peptide component of RNase S, residues 1−20; DSC, differential scanning calorimetry; CD, circular dichroism; ASA, accessible surface area.

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and RNase A, despite the strong structural similarity. Finally, the influence of phosphate ion on the stability of RNase A and RNase S at pH 7.0 is investigated and explained in terms of the binding of phosphate on the active site of ribonucleases.

MATERIALS AND METHODS

RNase A was type XII-A of Sigma. RNase S was grade XII-S obtained from RNase A type XII-A of Sigma. The purity of both proteins was confirmed by HPLC gel filtration, and the samples were used without further purification. Protein concentration was determined by absorption spectroscopy using a molar extinction coefficient at 277.5 nm of 9800 M^{-1} cm^{-1} (Connelly et al., 1990). Protein solutions for DSC and CD measurements were exhaustively dialyzed by using Spectra Por MW 6000−8000 membranes against buffer solutions at 4 °C. Buffers purchased from Sigma were as follows: at pH 4.0 and 5.0 acetic acid/sodium acetate; at pH 6.0 and 6.5 Mes [2-(*N*-morpholino)ethanesulfonic acid]; at pH 7.0 and 7.5 Mops [3-(*N*-morpholino)propanesulfonic acid]; at pH 8.0 and 8.5 Heppso [*N*-(2-hydroxyethyl)-piperazine-*N*′-(2-hydroxypropanesulfonic acid)]. For all solutions the buffer concentration was 10 mM and the ionic strength was determined by addition of 200 mM NaCl. Doubly deionized water was used throughout. The pH of all solutions was determined with a Radiometer pHmeter model PHM 93 at 25 °C.

Circular Dichroism. The CD spectra were recorded with a Jasco J-710 spectropolarimeter. The instrument was calibrated with an aqueous solution of d_{10}-(+)-camphorsulfonic acid at 290 nm (Yang et al., 1986). Molar ellipticity at 222 nm is reported as molar ellipticity per mean residue ($[\theta]_{222nm}$ deg cm^2 $dmol^{-1}$) and calculated from the equation $[\theta] = [\theta]_{obs}mrw/10lC$, where $[\theta]_{obs}$ is the ellipticity measured in degrees, mrw is the mean residue molecular weight, 110 Da, C is the protein concentration in g mL^{-1}, and l is the optical path length of the cell in centimeters. CD spectra were measured with a time constant of 4 s, a 2 nm band width, and a scan rate of 5 nm min^{-1}. An 0.1 cm pathlength cell was used for far-UV spectra. Spectra were signal-averaged by adding at least five scans and baseline corrected by subtracting a buffer spectrum. Temperature was regulated with a circulating water bath, and the cell-holder temperature was measured with a termosensor. The thermal unfolding curves of RNase S were determined in the temperature mode at a single wavelength, 222 nm. The scan rate was 0.33 K min^{-1}.

Scanning Calorimetry. Calorimetric measurements were carried out on a second-generation Setaram Micro-DSC apparatus, interfaced with a data translation A/D board for automatic data accumulation. A scan rate of 0.5 K min^{-1} was chosen for the present study. All data analyses were accomplished with software developed in our laboratory (Barone et al., 1992). The raw data were converted to an apparent molar heat capacity by correcting for the instrument calibration curve and the buffer−buffer scanning curve and by dividing each data point by the scan rate and the number of moles of protein in the sample cell. Finally, the excess heat capacity function $\langle \Delta C_p \rangle$ was obtained after baseline subtraction, assuming that the baseline is given by the linear temperature dependence of native state heat capacity (Freire & Biltonen, 1978; Biltonen & Freire, 1978; Freire, 1994). The calorimetric enthalpy was determined by direct integration of the area under the curve, and the van't Hoff enthalpy was calculated with the correct formula for a dissociating system (Sturtevant, 1987):

$$\Delta H^{vH}(T_d) = 5.83RT_d^2[\langle \Delta C_p \rangle_{T_d}/\Delta_d H(T_d)] \quad (1)$$

where T_d is the denaturation temperature and corresponds to the maximum of the DSC peak, $\langle \Delta C_p \rangle_{T_d}$ is the value of the excess heat capacity function at T_d, and $\Delta_d H(T_d)$ represents the calorimetric enthalpy. The close correspondence between the calorimetric enthalpy $\Delta_d H(T_d)$ and the van't Hoff enthalpy $\Delta H^{vH}(T_d)$ is a necessary condition to state that the denaturation is a two-state transition.

Thermodynamic Model. To analyze the DSC curves of RNase S we consider a protein comprised of two parts, N and L, bonded by noncovalent interactions. On thermal denaturation the two parts dissociate and the overall process can be represented as

$$NL \leftrightarrow D + L^* \quad (2)$$

where the star indicates the denaturated form of L. The overall equilibrium constant accounting for both unfolding and dissociation is

$$K = [D][L^*]/[NL] = [D]^2/[NL] \quad (3)$$

It is temperature dependent according to

$$K = \exp -\{[\Delta_d H(T_o)/R][(1/T) - (1/T_o)] + (\Delta_d C_p/R)[1 - (T_o/T) + \ln(T/T_o)]\} \quad (4)$$

where T_o is the temperature at which $K = 1$ and should be independent of concentration, $\Delta_d H(T_o)$ is the denaturation enthalpy change at $T = T_o$, and $\Delta_d C_p$ is the heat capacity difference between denaturated and native states, assumed temperature-independent, and is given by the baseline shift characteristic of globular proteins DSC profiles. The mass balance equation for the total molar concentration of the protein is

$$C_{tot} = [NL] + [D] = [NL] + (K[NL])^{1/2} \quad (5)$$

from which the native protein concentration results:

$$[NL] = [2C_{tot} + K - (K^2 + 4KC_{tot})^{1/2}]/2 \quad (6)$$

and the advancement degree of the process is

$$\alpha = [D]/C_{tot} = (K[NL])^{1/2}/C_{tot} \quad (7)$$

The excess enthalpy function for a two-state process is given by the well-known formula

$$\langle \Delta H \rangle = [\Delta_d H(T_o) + \Delta_d C_p(T - T_o)]\alpha \quad (8)$$

and the excess heat capacity is simply the temperature derivative of $\langle \Delta H \rangle$:

$$\langle \Delta C_p \rangle = \Delta_d H(T_o)(d\alpha/dT) + \alpha\Delta_d C_p \quad (9)$$

From the knowledge of α as a function of T, it is straightforward to determine the excess heat capacity function, using as input parameters the values of C_{tot}, T_o, $\Delta_d H(T_o)$,

and $\Delta_d C_p$. Therefore it is possible to fit the experimental DSC curves by performing a nonlinear regression with respect to eq 9. The nonlinear regression was performed by means of the Levenberg−Marquardt algorithm (Morè, 1977), as implemented in the Optimization Toolbox of MATLAB.

In the calorimetric curves simulated with this model, a marked tail is present in the low-temperature side of DSC peaks because unfolding and dissociation simultaneously occur. Furthermore, it is worth noting that T_d does not coincide with T_o, the temperature where $K = 1$; the maximum of DSC peak happens at a temperature T_d always lower than T_o.

The total protein concentration is a significant variable of the model because it is present in the expression of the equilibrium constant K. At raising the protein concentration, the value of T_d increases. This increase can be rationalized in thermodynamic terms. Indeed, Sturtevant (Takahashi & Sturtevant, 1981; Fukada et al., 1983; Sturtevant, 1987), showed that the following equation does hold:

$$\ln C_{tot} = \text{constant} - (\Delta H^{vH}/RT_d) \qquad (10)$$

A plot of ln C_{tot} as a function of $1/T_d$ should be linear with a slope equal to $(-\Delta H^{vH}/R)$. Thus it is possible to determine the value of ΔH^{vH} by performing a least-squares regression on the experimental points.

RESULTS

Thermal Denaturation at pH 7.0 and 7.5. The thermal denaturation of RNase S has been carefully investigated at pH 7.0 and 7.5 because RNase S possesses the same catalytic activity of RNase A at neutral pH. A direct comparison of the denaturation parameters of RNase A and RNase S at pH 7.0, 10 mM Mops buffer, and 200 mM NaCl shows the great difference in thermal stability between the two proteins. The thermodynamic parameters of RNase A denaturation are C_{tot} = 0.140 mM, T_d = 62.8 °C, $\Delta_d H(T_d)$ = 500 kJ mol^{-1}, and $\Delta_d C_p$ = 5.5 kJ K^{-1} mol^{-1}, whereas those of RNase S are C_{tot} = 0.140 mM, T_d = 50.1 °C, $\Delta_d H(T_d)$ = 430 kJ mol^{-1}, and $\Delta_d C_p$ = 5.1 kJ K^{-1}mol^{-1}. Therefore the breaking of the single peptide bond between Ala20 and Ser21, although it does not change the overall structural features of the protein, causes a strong decrease of conformational stability. Furthermore, the DSC profiles of RNase S show a marked tail in the low-temperature side of endothermic peak. This experimental evidence, coupled with the knowledge that RNase S is comprised of two parts, S-peptide and S-protein, bonded by noncovalent interactions, suggested that its thermal denaturation represents two concomitant phenomena: unfolding of the native structure and dissociation of the two parts. The overall process can be schematized according to eq 2, where NL is the RNase S and D represents the denatured form of S-protein. The ligand L, corresponding to the S-peptide, is labeled with a star when separated from S-protein, because it does not maintain its prevailingly α-helix conformation. Figure 1A shows the experimental DSC curve for C_{tot} = 0.300 mM and the best fit obtained with a simple two-state transition N ↔ D: the agreement between the two curves is poor. Figure 1B shows the same experimental curve and that calculated with the model that considers both unfolding and dissociation: the agreement is good, particularly where there is the tail.

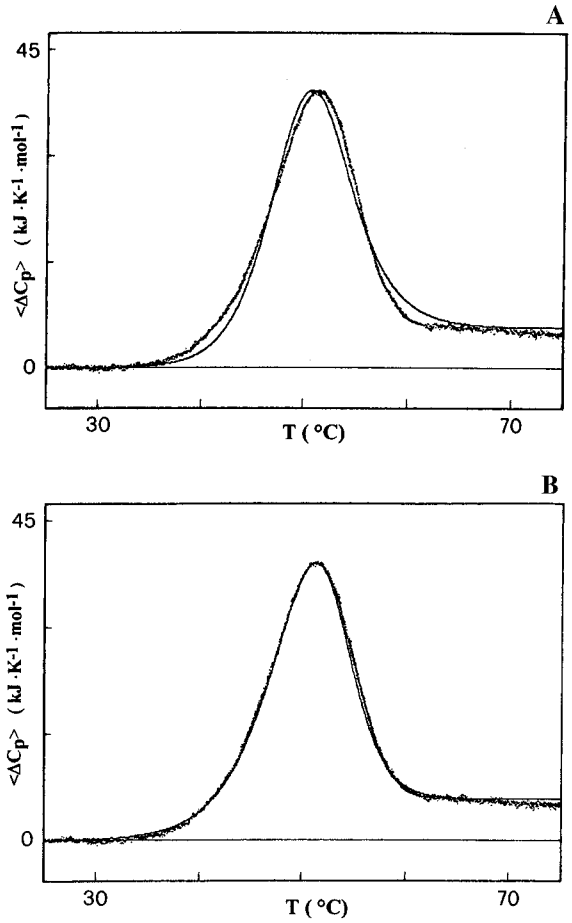

FIGURE 1: (A) Experimental DSC curve of RNase S (0.300 mM) at pH 7.0, 10 mM Mops buffer, and 200 mM NaCl. The solid line represents the best fit according to the two-state N ↔ D model. (B) Experimental DSC curve of RNase S (0.300 mM) at pH 7.0, 10 mM Mops buffer, and 200 mM NaCl. The solid line represents the best fit according to the two-state NL ↔ D + L* model.

According to the thermodynamic model, we tried to bring out a dependence of the denaturation temperature on protein concentration. By performing both CD and DSC measurements, we found that the value of T_d increases as the RNase S concentration rises. Figure 2 shows three CD thermal profiles at different protein concentrations, while Figure 3 shows three DSC curves at increasing protein concentrations. The solid lines reported in these figures are the best fits calculated with the model of eq 2. The experimental values of thermodynamic parameters obtained from both CD and DSC measurements are collected in Table 1. It is worth noting that T_d increases from 43.2 °C at C_{tot} = 0.005 mM to 53.3 °C at C_{tot} = 0.880 mM. The ratio of the calorimetric enthalpy $\Delta_d H(T_d)$ to the van't Hoff enthalpy $\Delta H^{vH}(T_d)$ is always close to unity. This is a strong indication that the thermal denaturation is a two-state transition. The increase of $\Delta_d H(T_d)$ from 420 kJ mol^{-1} at C_{tot} = 0.070 mM to 450 kJ mol^{-1} at C_{tot} = 0.880 mM can be directly ascribed to the increase of T_d and to the positive value of $\Delta_d C_p$. The dependence of T_d on protein concentration has been rationalized according to eq 10. The plot of ln C_{tot} versus $1000/T_d$ with the experimental points obtained at pH 7.0 is reported in Figure 4. The linear regression has given a correlation coefficient of 0.996, a slope equal to −54.0 ± 1.5 K, and an intercept of 158.5 ± 1.8. The plot slope enables to

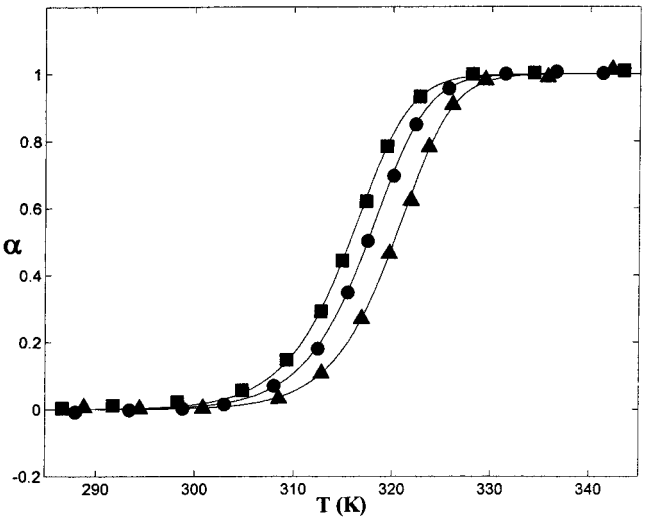

FIGURE 2: Fraction of RNase S denatured at different concentrations as a function of temperature. The concentrations are 0.005 mM (squares), 0.010 mM (circles), and 0.050 mM (triangles). The fraction denatured was monitored by the ellipticity at 222 nm using CD spectroscopy. Experimental data are presented by symbols. The solid lines represent the theoretical curves.

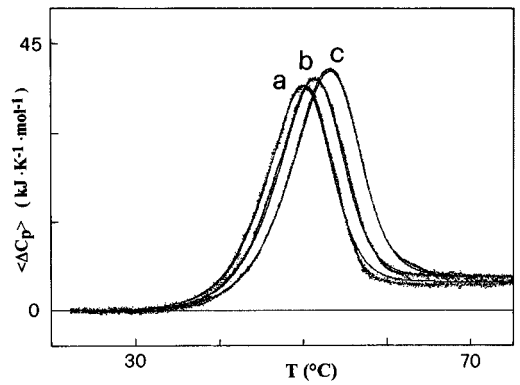

FIGURE 3: DSC profiles of RNase S as a function of concentration. The concentrations are 0.140 mM (curve a), 0.300 mM (curve b), and 0.880 mM (curve c). Experiments are performed at pH 7.0, 10 mM Mops buffer, and 200 mM NaCl.

determine the ΔH^{vH}. The obtained value, 449 ± 15 kJ mol^{-1}, is in agreement with the value, 435 ± 15 kJ mol^{-1}, averaged over all experimental measurements at pH 7.0.

To further validate the proposed model, CD and DSC measurements were performed on RNase S at pH 7.5, 10 mM Mops buffer, and 200 mM NaCl at various protein concentrations. The results are collected in Table 2. Figure 5A shows the experimental curve for $C_{tot} = 0.660$ mM and the best fit obtained with a simple two-state transition N ↔ D. Figure 5B shows the same experimental curve and that calculated according to our model. It is evident that only considering both unfolding and dissociation the agreement between calculated and experimental curve is satisfactory. At raising the protein concentration, the value of T_d increases from 46.0 °C at $C_{tot} = 0.010$ mM to 53.2 °C at $C_{tot} = 0.660$ mM. The plot of ln C_{tot} versus $1000/T_d$ with the experimental points is reported in Figure 6. The least-squares regression has given a correlation coefficient of 0.989, a slope equal to −56.0 ± 3.5, and an intercept of 164.1 ± 10.7. The ΔH^{vH} determined from the plot slope, 465 ± 30 kJ mol^{-1}, is in agreement with the value, 440 ± 20 kJ mol^{-1}, averaged over all experimental measurements at pH 7.5.

Table 1: Thermodynamic Parameters of the Denaturation Process of RNase S at pH 7.0, 10 mM Mops Buffer, and 200 mM NaCl[a]

	C_{tot} (M)	T_d (°C)	T_o (°C)	$\Delta_d H(T_d)$ (kJ mol^{-1})	$\Delta_d C_p$ (kJ K^{-1} mol^{-1})	$\Delta H^{vH}(T_d)$ (kJ mol^{-1})
CD	5.0×10^{-6}	43.2	67.6			395
CD	1.0×10^{-5}	45.0	67.5			400
CD	5.0×10^{-5}	47.5	67.5			415
DSC	7.0×10^{-5}	48.4	67.2	420	4.8	428
DSC	1.0×10^{-4}	49.3	67.2	425	4.9	431
DSC	1.4×10^{-4}	50.1	67.1	430	5.1	425
DSC	2.6×10^{-4}	51.0	67.1	435	5.2	440
DSC	3.0×10^{-4}	51.5	67.4	435	5.1	429
DSC	5.9×10^{-4}	52.0	67.3	440	4.9	444
DSC	7.5×10^{-4}	52.9	67.1	445	5.0	450
DSC	8.8×10^{-4}	53.3	67.3	450	5.4	442

[a] Each figure represents the value averaged over three or four measurements. The error in T_d does not exceed 0.2 °C. The estimated (relative) uncertainties in $\Delta_d H(T_d)$ and $\Delta_d C_p$ amount to 5% and 10%, respectively, of reported values. The values of $\Delta H^{vH}(T_d)$ are calculated according to eq 1 for DSC measurements, while, for CD measurements, the values are calculated from a van't Hoff analysis of experimental data. The values of T_o are obtained from the performed nonlinear regression.

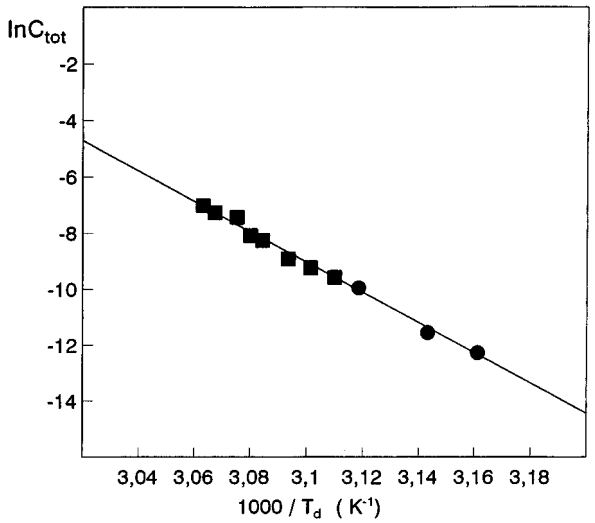

FIGURE 4: Variation of reciprocal denaturation temperature with the natural logarithm of the total protein concentration. All measurements were carried out at pH 7.0, 10 mM Mops buffer, and 200 mM NaCl. Data taken from DSC scans are marked by squares; those from CD measurements by circles. The protein concentration ranged from 0.005 to 0.880 mM. The solid line is the result of the linear least-squares regression.

The value of the denaturation heat capacity change, averaged over all measurements performed at pH 7.0 and 7.5, is 5.0 kJ K^{-1}mol^{-1}. This figure is slightly lower than the value obtained for RNase A, 5.5 kJ K^{-1}mol^{-1} (Barone et al., 1992). The difference may be attributed to the larger exposure of nonpolar accessible surface area near the site of proteolytic cleavage for RNase S with respect to RNase A. In any case both these values are slightly lower than the figure calculated with the models developed by various authors (Murphy & Freire, 1992; Barone et al., 1995). The discrepancy may be due to the relative compactness of the thermally denatured state of RNase A (Sosnick & Trewella, 1992)

Influence of pH. The thermal stability of RNase S was also characterized as a function of pH of the aqueous solution and the results are collected in Table 3. The protein concentration is a significant variable of the process, and,

Table 2: Thermodynamic Parameters of the Denaturation Process of RNase S at pH 7.5, 10 mM Mops Buffer, and 200 mM NaCl[a]

	C_{tot} (M)	T_d (°C)	T_o (°C)	$\Delta_dH(T_d)$ (kJ mol^{-1})	Δ_dC_p (kJ K^{-1} mol^{-1})	$\Delta H^{vH}(T_d)$ (kJ mol^{-1})
CD	1.0×10^{-5}	46.0	67.6			415
CD	6.0×10^{-5}	48.2	67.7			420
DSC	1.0×10^{-4}	49.8	67.5	430	4.9	426
DSC	1.4×10^{-4}	50.6	67.4	435	5.0	440
DSC	1.7×10^{-4}	51.0	67.4	435	5.2	447
DSC	2.4×10^{-4}	51.9	67.5	440	4.9	452
DSC	4.1×10^{-4}	52.5	67.3	445	5.1	460
DSC	6.6×10^{-4}	53.2	67.6	450	5.0	442

[a] Each figure represents the value averaged over three or four measurements. The error in T_d does not exceed 0.2 °C. The estimated (relative) uncertainties in $\Delta_dH(T_d)$ and Δ_dC_p amount to 5% and 10%, respectively, of reported values. The values of $\Delta H^{vH}(T_d)$ are calculated according to eq 1 for DSC measurements, while, for CD measurements, the values are calculated from a van't Hoff analysis of experimental data. The values of T_o are obtained from the performed nonlinear regression.

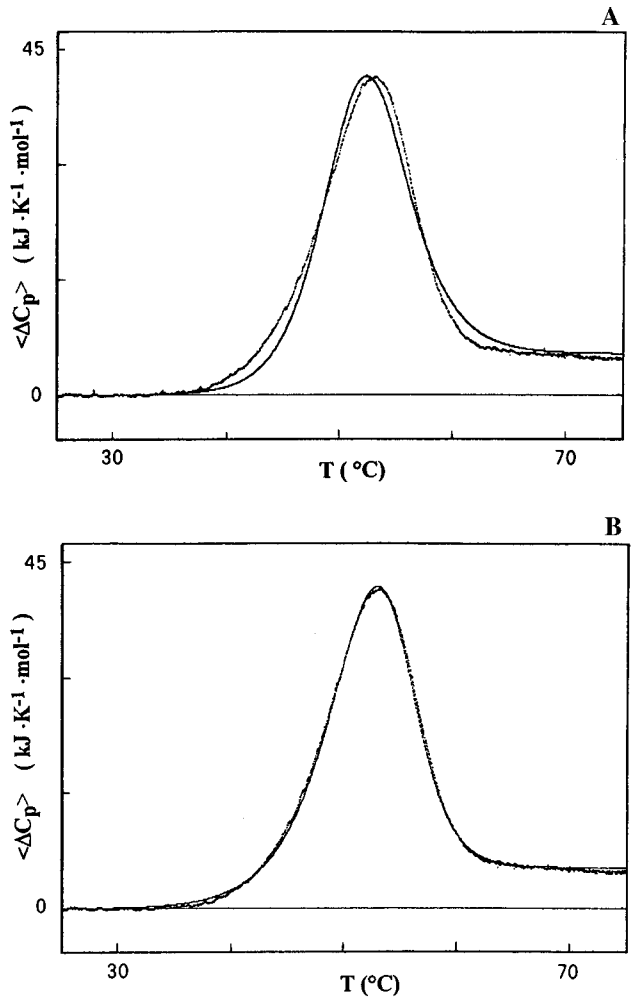

FIGURE 5: (A) Experimental DSC curve of RNase S (0.660 mM) at pH 7.5, 10 mM Mops buffer, and 200 mM NaCl. The solid line represents the best fit according to the two state N ↔ D model. (B) Experimental DSC curve of RNase S (0.660 mM) at pH 7.5, 10 mM Mops buffer, and 200 mM NaCl. The solid line represents the best fit according to the two-state NL ↔ D + L* model.

to perform a correct comparison, the RNase S concentration was fixed at 0.140 mM. At all investigated pH values the DSC peaks show the tail in the low-temperature side, and the ratio of the calorimetric enthalpy $\Delta_dH(T_d)$ to the van't Hoff enthalpy $\Delta H^{vH}(T_d)$, calculated with eq 1, is always close

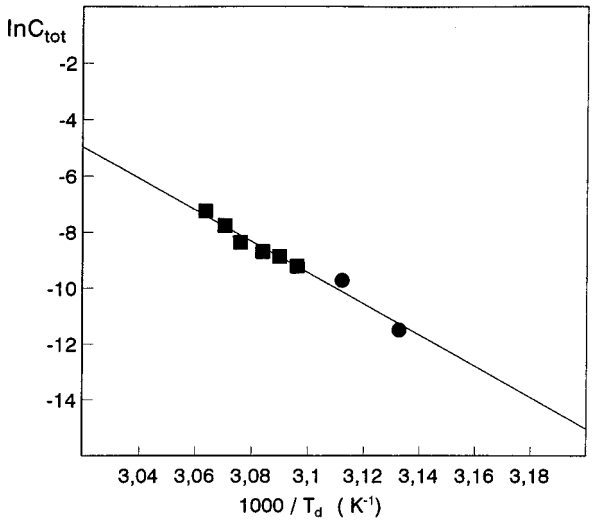

FIGURE 6: Variation of reciprocal denaturation temperature with the natural logarithm of the total protein concentration. All measurements were carried out at pH 7.5, 10 mM Mops buffer, and 200 mM NaCl. Data taken from DSC scans are marked by squares; those from CD measurements by circles. The protein concentration ranged from 0.010 to 0.660 mM. The solid line is the result of the linear least-squares regression.

Table 3: Thermodynamic Parameters of the Thermal Denaturation Process of RNase S at Different pH values and 200 mM NaCl, by Fixing the Protein Concentration at 0.140 mM[a]

pH	T_d (°C)	$\Delta_dH(T_d)$ (kJ mol^{-1})	Δ_dC_p (kJ K^{-1} mol^{-1})	$\Delta H^{vH}(T_d)$ (kJ mol^{-1})
4.0	44.6	395	5.2	410
5.0	45.5	400	5.0	405
6.0	46.8	410	4.9	420
6.5	47.2	415	5.1	420
7.0	50.1	430	5.1	425
7.5	50.6	435	5.0	440
8.0	51.0	415	4.6	420
8.5	49.8	400	4.7	390

[a] See Table 1 footnote.

to unity. Therefore, the proposed two-state model of denaturation remains correct in all investigated conditions.

The process results are reversible, as demonstated by the recovery of the original signal in repeated scans of the same sample, except at pH 8.0 and 8.5. Probably side reactions, involving side chains, happen at high temperature and high pH values, as demonstrated for RNase A (Zale & Klibanov, 1986), and do not allow the correct refolding of the two parts.

The maximum value of T_d , 51.0 °C, happens at pH 8.0, while the maximum value of $\Delta_dH(T_d)$, 430 kJ mol^{-1}, happens at pH 7.0−7.5. These results can be satisfactorily explained in physicochemical terms. It is well established that the maximum thermodynamic stability of a globular protein occurs near its isoelectric point (Dill, 1990). Indeed, according to the classical Linderstrom−Lang approach (1924), the electrostatic repulsion between charged groups at pH values far away from the isoelectric point can be diminished by destroying the folded conformation, in order to increase the distance between charges. The isoelectric point of RNase A, and then of RNase S, is about 9, and this explains the obtained results. On the other hand, the irreversibility of the denaturation at pH 8.0 and 8.5 would account for the observed decrease of denaturation enthalpy with respect to pH 7.0−7.5.

Table 4: (Top Section) Thermodynamic Parameters for the Thermal Denaturation of RNase A as a Function of Phosphate Concentration at pH 7.0 and (Bottom Section) Thermodynamic Parameters for the Thermal Denaturation of RNase S as a Function of Phosphate Concentration at pH 7.0 and $C_{tot} = 0.140$ mM[a]

pH	T_d (°C)	$\Delta_d H(T_d)$ (kJ mol^{-1})	$\Delta_d C_p$ (kJ K^{-1} mol^{-1})	$\Delta H^{vH}(T_d)$ (kJ mol^{-1})	
7.0	62.8	500	5.5	505	10 mM Mops
7.0	64.8	525	5.6	515	30 mM phosphate
7.0	66.1	550	5.5	560	100 mM phosphate
pH	T_d (°C)	$\Delta_d H(T_d)$ (kJ mol^{-1})	$\Delta_d C_p$ (kJ K^{-1} mol^{-1})	$\Delta H^{vH}(T_d)$ (kJ mol^{-1})	
7.0	50.1	430	5.1	425	10 mM Mops
7.0	51.7	455	5.0	460	30 mM phosphate
7.0	52.9	485	5.1	480	100 mM phosphate

[a] See Table 1 footnote.

A marked decrease of T_d and $\Delta_d H(T_d)$ for RNase S occurs passing from pH 7.0 to 5.0, whereas for RNase A in the same pH range the values of denaturation temperature and enthalpy little change (Barone et al., 1992). Schreier and Baldwin (1976), performing measurements of hydrogen exchange kinetics of ^{3}H-labeled S-peptide in RNase S, found a strong pH dependence of the equilibrium association constant K_b of S-peptide to S-protein. The value of K_b at 0 °C decreases from 5.0×10^9 M^{-1} at pH 7.0 to 8.3×10^7 M^{-1} at pH 4.3. This finding is caused by the proton release upon reassociation of the two parts. Indeed, several ionizable residues in the S-peptide and S-protein whose p*K* values are shifted to lower values in RNAse S have been identified (Richards & Wyckoff, 1971). The strong decrease of the association constant at lowering the pH from neutrality can reasonably explain the observed parallel decrease in thermal stability of RNase S.

Influence of Phosphate at pH 7.0. The interaction of phosphate ion with RNase A and RNase S is well established: His12 and His119 in the active site of ribonucleases are involved in the binding (Aguilar et al., 1992; Coopley & Barton, 1994). The commercial samples usually contain phosphate in the active site. However, RNase A, type XII-A from Sigma used in this work is phosphate-free, as demonstrated by the detailed crystallographic study of Wlodawer and co-workers (1988). The RNase S sample used is derived from RNase A type XII-A and thus is phosphate-free also (Wlodawer et al., 1988).

Parallel DSC measurements were performed on RNase A and RNase S at pH 7.0 in the following conditions: (i) 10 mM Mops buffer and 200 mM NaCl; (ii) 30 mM phosphate buffer; (iii) 100 mM phosphate buffer. In the latter cases the ionic strength of the solution is adjusted to the value of case (i) by proper amounts of NaCl in order to make a right comparison. The results are collected in Table 4. The stabilizing effect of phosphate ion is evident. For RNase A the denaturation temperature increases from 62.8 °C in 10 mM Mops to 66.1 °C in 100 mM phosphate and the denaturation enthalpy rises from 500 to 550 kJ mol^{-1}. For RNase S at $C_{tot} = 0.140$ mM the denaturation temperature increases from 50.1 °C in 10 mM Mops to 53.1 °C in 100 mM phosphate, and the denaturation enthalpy rises from 430 to 485 kJ mol^{-1}.

To gain more information on the thermodynamics of phosphate binding, we analyzed the DSC curves of RNase A with a two-state model that considers the existence of one binding site on native form (Brandts & Lin, 1990). For more details on this thermodynamic model see the accompanying paper. To fit the DSC curves, we keep fixed the parameters of RNase A denaturation in Mops buffer and calculate with a nonlinear regression the values of the apparent association constant at 25 °C, K_b(25 °C), and the apparent binding enthalpy, $\Delta_b H$. A fair agreement between simulated and experimental curves is obtained by putting $K_b(25\ °C) = 160 \pm 20$ M^{-1} and $\Delta_b H = (-28 \pm 5)$ kJ mol^{-1}. These figures are comparable with literature data, determined by means of different approaches (Anderson et al., 1968; Flogel et al., 1975). The apparent binding constant is not high, but in the presence of an excess of phosphate ion the site is occupied and the protein stabilized. A similar analysis performed in the case of RNase S leads to the conclusion that the energetics of phosphate binding on RNase S is practically identical to that on RNase A.

DISCUSSION

At the best of our knowledge, only three quantitative works on the thermal denaturation of RNase S exist in the literature: two from Sturtevant's group (Tsong et al., 1970; Hearn et al., 1971) and that of Labhardt (1981). Sturtevant and co-workers performed DSC measurements on unbuffered solutions of RNase S at pH 7.0 and 300 mM NaCl, in the same concentration range investigated by us. Apart from large differences in the values of $\Delta_d H(T_d)$, probably due to the performance of their hand-made DSC instrument, and the reported irreversibility of thermal denaturation, these authors found that the denaturation temperature increases at raising the protein concentration by quantities comparable to those here reported. In fact, they measured a raise of about 2.5 °C in T_d passing from 0.330 to 0.830 mM and, tentatively, ascribed it to dissociation of the two parts. Therefore, the results of Sturtevant and co-workers are in agreement with ours. In contrast, Labhardt (1981), by investigating the thermal stability of RNase S by means of CD measurements, emphasized that the denaturation temperature varies approximately linearly with ln C_{tot}, at raising the protein concentration, but in a range lower than that explored by us (i.e., C_{tot} ranging from 0.001 to 0.100 mM). For a further increase of protein concentration (i.e., C_{tot} ranging from 0.240 to 1.0 mM), the denaturation temperature did not seem to change. On the basis of these findings, Labhardt proposed a model of RNase S thermal denaturation that predicts the formation of a thermodynamic intermediate state in which the two parts, although denatured, are still bonded together, forming a noncovalent complex.

Actually, we have found an increase of denaturation temperature in the concentration range where Labhardt measured it constant. The present results do not support the existence of such thermodynamic intermediate state in the unfolding pathway of RNase S. The CD and DSC measurements strongly suggest that the temperature-induced denaturation is a two-state process, representing two concomitant phenomena: unfolding and dissociation of the two parts. In fact, the simple two-state model NL $\leftrightarrow$ D + L* used to analyze the experimental curves accounts well for the main features of the thermal denaturation of RNase S: (i) the marked tail in the low-temperature side of DSC peak; (ii) the dependence of T_d on the protein concentration. For these reasons it can be assumed as a good representation of the actual process. The absence of intermediate states in the

thermal denaturation has been carefully emphasized for a certain number of dimeric globular proteins (Tamura et al., 1991; Steif et al., 1993; Thompson et al., 1993). All these dimeric globular proteins, such as RNase S, are constituted by the noncovalent association of two parts. Additionally, the temperature-induced denaturation of some tetrameric structures is a two-state process, without intermediates (Hagihara et al., 1994; Johnson et al., 1995).

Now we are in the right position to compare the thermal stability of RNase A and RNase S. The parameter of greatest interest is the denaturation entropy change, $\Delta_d S$, which is usually calculated as $\Delta_d H(T_d)/T_d$. While $\Delta_d S$ is independent of concentration for RNase A, whose denaturation process is well represented by the two-state N ↔ D model, it is dependent on concentration for RNase S. In order to correctly compare these proteins, the standard state $\Delta_d S°$ (i.e., at unit M concentration) must be compared. The concentration dependence of $\Delta_d G$ is given by

$$\Delta_d G = \Delta_d G° + RT \ln Q \quad (11)$$

where Q is the reaction quotient and in this case Q = [D]-[L*]/[NL]. The concentration dependence of $\Delta_d S$, being the entropy the negative temperature derivative of the Gibbs energy, results

$$\Delta_d S = \Delta_d S° - R \ln Q \quad (12)$$

Under the experimental conditions selected, $C_{tot} = 0.140$ mM, so that Q at $T = T_d$ (i.e., where the advancement degree of the process is 0.5) is equal to 7.0×10^{-5}. From the eq 12 we can solve for $\Delta_d S°$ and get

$$\Delta_d S°(T_d) = \Delta_d H(T_d)/T_d + R \ln Q = 1.33 - 0.08 = 1.25 \text{ kJ K}^{-1} \text{ mol}^{-1} \quad (13)$$

It is also possible to calculate $\Delta_d S°(T_d)$ from the results of the nonlinear regression performed according to the developed thermodynamic model. Indeed, at $T = T_o$ the overall equilibrium constant $K = 1$ (see eqs 3 and 4), and $\Delta_d G°(T_d) = 0$. Therefore $\Delta_d S°(T_o) = \Delta_d H(T_o)/T_o$. For $C_{tot} = 0.140$ mM the nonlinear regression has given $T_o = 340.25$ K, $\Delta_d H(T_o) = 515.0$ kJ mol^{-1}, and $\Delta_d C_p = 5.1$ kJ K^{-1} mol^{-1}. Thus $\Delta_d S°(T_o) = 1.51$ kJ K^{-1} mol^{-1}. Estrapolating at $T = T_d = 323.25$ K, under the assumption that $\Delta_d C_p$ is constant, it results

$$\Delta_d S°(T_d) = 1.51 + 5.1 \ln(323.25/340.25) = 1.51 - 0.08 = 1.25 \text{ kJ K}^{-1} \text{ mol}^{-1} \quad (14)$$

The two estimates are in perfect agreement, and this may be considered a proof of the internal consistency of our analysis. The thermodynamic parameters for the two proteins at pH 7.0 are as follows. RNase A: $C_{tot} = 0.140$ mM, $T_d = 62.8$ °C, $\Delta_d H(T_d) = 500$ kJ mol^{-1}, $\Delta_d S°(T_d) = 1.49$ kJ K^{-1} mol^{-1}, and $\Delta_d C_p = 5.5$ kJ K^{-1} mol^{-1}. RNase S: $C_{tot} = 0.140$ mM, $T_d = 50.1$ °C, $\Delta_d H(T_d) = 430$ kJ mol^{-1}, $\Delta_d S°(T_d) = 1.25$ kJ K^{-1} mol^{-1}, and $\Delta_d C_p = 5.1$ kJ K^{-1} mol^{-1}.

To perform a correct thermodynamic comparison, the denaturation enthalpy and entropy changes must be referred to the same temperature. We selected the denaturation temperature of RNase S, 50.1 °C, as a reference. The denaturation enthalpy and entropy changes of RNase A, calculated under the assumption that $\Delta_d C_p$ is constant, at this temperature result in $\Delta_d H(50.1\ °C) = 430$ kJ mol^{-1} and $\Delta_d S°(50.1\ °C) = 1.28$ kJ K^{-1} mol^{-1}. Therefore, there is no real difference in the denaturation enthalpy between the two proteins. The apparent difference is completely due to the shift of denaturation temperature and to the positive value of $\Delta_d C_p$. Instead for the denaturation entropy, it results that, by comparing the two values at the same temperature, the denaturation of RNase S is accompanied by a smaller increase of entropy, amounting to 30 J K^{-1} mol^{-1}. This relatively small quantity has a dramatic effect on the value of T_d.

The value of $\Delta_d S°(T_d)$ associated with the thermal denaturation of RNase S also contains the contribution from the cratic entropy (Gurney, 1953; Kauzmann, 1959), amounting to 33.5 J K^{-1} mol^{-1} for a unit molar standard state, and arising from the gain of translational entropy due to the dissociation of the two parts, linked by noncovalent interactions, on unfolding. Therefore, it seems that the native state of RNase S has a greater conformational entropy than the native state of RNase A. The results of the performed thermodynamic analysis suggest that an increased flexibility of the backbone and side chains in RNase S (especially for the residues near the site of proteolytic cleavage) gives rise to a smaller conformational contribution to $\Delta_d S°$ which more than compensates for the cratic contribution.

This result enables to partially explain the puzzle raised by Richards and colleagues (Kim et al., 1992): "The origin of the substantial differences between RNase A and RNase S in stability to both acid and temperature denaturation and in susceptibility to proteolysis at neutral pH is not obvious in our visual comparison of these two structures". Indeed, the energetic interactions in RNase S are practically identical to those existing in RNase A, as emphasized by the close correspondence of the denaturation enthalpy values. The differences around the site of proteolytic cleavage seem to be not relevant probably because the so-called "hinge peptide" (i.e., residues 16−21 of RNase A) is exposed to water even in RNase A. In contrast, the entropy changes associated with the thermal denaturation of the two proteins are different because the native state of RNase S seems to possess a greater conformational flexibility. Therefore, the lower thermodynamic stability of RNase S with respect to RNase A should originate from larger structural fluctuations, which can also explain its higher susceptibility toward acids and proteolysis. This analysis demonstrates that for a correct evaluation of the thermodynamic stability of globular proteins a reliable determination of the denaturation entropy change is always necessary.

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