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THERMAL DENATURATION OF RIBONUCLEASE T1 A DSC study

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The thermal denaturation of microbial Ribonuclease T1 (RNAase T1) as a function of *pH*, was studied by means of DSC microcalorimetry. The midpoint denaturation temperatures, enthalpy changes and heat capacity changes of Ribonuclease T1 were compared with those obtained for pancreatic Ribonuclease A (RNAase A). It was found that the microbial T1 protein undergoes a more complex conformational transition than the simple two-state transition shown by Ribonuclease A. The hypothesis of the presence of a 'molten globule' form is discussed. The conformational stability of RNAase T1 is lower than that of RNAase A at high *pH* values. Indeed, the maximum stability of RNAase T1 occurs at $pH \approx 5$, whereas that of RNAase A occurs at $pH \approx 8$. At $pH = 3.7$ an irreversible aggregation phenomenon was indicated by the existence of a reproducible exothermic peak. The conformational transition of RNAase T1 is reversible in the range of *pH* 4.5–7.0, whereas it becomes irreversible at $pH \geq 8.0$ as for RNAase A.

Keywords: microbial Ribonuclease T1, denaturation temperature, enthalphy change, heat capacity

Introduction

Pancreatic Ribonuclease A is one of the most thoroughly studied enzymes. Crystallographic structures have been exhaustively determined by many laboratories [1]. The mechanism of folding has been recently discussed by Wearne and Creighton, Udgaonkar and Baldwin, and Scheraga [2–5]. Thermal denaturation was studied calorimetrically by Privalov and Kechinashvili [6], Fujita and Noda [7], and more recently by some of us [8, 9].

In the past few years great attention has been paid to Ribonuclease T1 [10–15]. It was originally obtained from commercial preparations of the culture

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medium of the mold fungus *Aspergillus Oryzae* [10] and after prepared from a gene expression in *Escherichia Coli* [17]. This protein has a very low degree of homology with RNAase A, in spite of the fact that its overall shape and catalytic site nature relate well with those of RNAase A. (The active site of RNAase T1 however is specific for the recognition of guanine base and, in part, for that of adenine with respect to the specificity of RNAase A for uracil and cytidine.) RNAase T1 (104 amino acid residues, M_r = 11100) is stabilized by two disulfide bridges (positions 2–10 and 6–103) which connect the ends of the chain. It is a mixture of two isoforms, Gln25-RNAase T1 and Lys25-RNAase T1, differing as regards the two isobaric residues in position 25 [16]. It shows a small content of basic amino acids (one or two Lys, one Arg, three His) and hence has an isoionic *pH* of about 4.5 [18] instead of *pH* = 9.6 as in RNAase A (124 residues, M_r = 13600) [19]. However both these ribonucleases work better at *pH* = 7–7.5. One of the self-adaptation mechanisms to the primary function, in the case of RNAase T1, is that the two histidines 40 and 92 of the active site have pK_a values of 7.8 and 7.9 respectively [20], whereas the histidines 12 and 119 of RNAase A have pK_a = 5.8 and 6.2 respectively [21]. These high pK_a values probably help in the interaction with RNA, permitting the presence of extra charges in the active site, thus compensating the overall acidic nature of the protein.

Experimental

Materials

RNAase T1 (Lys25 or K25 isoform, genetically engineered) was a kind gift of Dr. C. N. Pace of the Texas A & M University. Bovine pancreatic RNAase A was a Sigma product (Fraction XII-A) used without further purification and containing less than 0.01% phosphates (in moles).

The buffers used were 0.1 *M* formiate for *pH* = 3.7, 0.1 *M* acetate for *pH* = 4.5 and 5.0, 0.1 *M* 2-[Morpholino]-ethansulfonate (MES) for *pH* = 5.7 and 6.3, 0.1 *M* 3-[N-Morpholino]-propanesulfonate (MOPS) for *pH* = 7.0 and 0.1 *M* N-[Hydroxyethyl]piperazine-N'-[2-hydroxypropanesulfonate (HEPPSO) for *pH* = 8.0 (all are a mixture of acids and sodium salts).

In all cases deionized water, twice distilled and then filtered on Millipore membranes was used for the preparation of solutions. The solutions were finally equilibrated by dialysis against the buffer solutions which were repeatedly renewed.

The protein concentrations were $0.9–1.7 \cdot 10^{-4}$ mol·l^{-1} in all the experiments and were determined spectrophotometrically (ε_{27} = 9800 l·cm^{-1}·mol^{-1} for RNAase A [22] and ε_{278} = 18500 l·cm^{-1}·mol^{-1} for RNAase T1 [23]). The use of the last ex-

tinction coefficient (the method having be checked successfully even with other proteins in different conditions [23]) is critical in the evaluation of data. Old literature value [24] can invalidate the conclusions. Small differences in the ionic strength of the buffer solutions do not change appreciably the results. It has been shown that a variation from 0.5 *M* to 0.1 *M* NaCl changes the $\Delta_d H$ of less then 2% at $pH = 7$ [11, 23].

Urea was a C. Erba (Milan) product, crystallized twice from ethanol-water mixtures and dried *under vacuo* on P_2O_5. The concentrations of urea were calculated by weighing the dried substance before to adding protein and buffer solution. Finally the *pH* was adjusted and checked with a Radiometer *pH*-meter.

Instrumentation

A second-generation Setaram MICRO-DSC microcalorimeter was used. It is designed for the study of dilute aqueous solutions of biological macromolecules. Its temperature program covers the range 273–373 K and its scanning rate can be changed to suit widely differing conditions. This microcalorimeter was connected to a PC for the automatic aquisition of data utilizing the MIDAS informatic system developed at our laboratory. The instrument signal was corrected for the calibration curve and for buffer-buffer reference baseline by the help of the THESEUS program [25]. In this manner a consistent baseline was obtained so that the apparent molar heat capacity (C_p^*) was evaluated with minimum registration artifacts.

Based on our experience, a scanning rate of 0.5 deg·min^{-1} was chosen because it allows an increase of temperature slow enough to avoid the signal distortion, but fast enough to avoid maintaining the protein at high temperatures for extended time periods. Spending as little scanning time as possible at high temperatures is especially important when the program operates a counter-scanning to prove that the process is reversible. A prolonged permanence at 80°– 90°C or more, promotes irreversible chemical transformation and inactivation of both the RNAases. At the normal scanning rate used, the denaturation becomes irreversible at $pH \geq 8$.

Result and discussion

Thermodynamics of RNAase T1 denaturation with varying the pH

In Fig. 1 a characteristic normalized registration of the C_p^* is reported as function of temperature. This plot gives some important parameters, such as the initial (T_i) and final (T_f) temperatures of the denaturation process, the temperature of the maximum heat absorption (T_d) assumed to correspond to the midpoint of

denaturation, and the changes in the apparent molar heat capacity ($\Delta_d C_p$). The change of enthalpy associated with the denaturation process is in turn obtained directly by integrating the area enclosed between the transition peak and the reference base line:

$$\Delta_d H = \int_{T_i}^{T_f} C_p^* dT - \int_{T_i}^{T_d} C_p^N dT - \int_{T_d}^{T_f} C_p^D dT \quad (1)$$

where C_P^N and C_P^D are the apparent molar heat capacities of the native and denatured form, extrapolated into the $T_i \rightarrow T_d$ range and $T_f \rightarrow T_d$ range respectively.

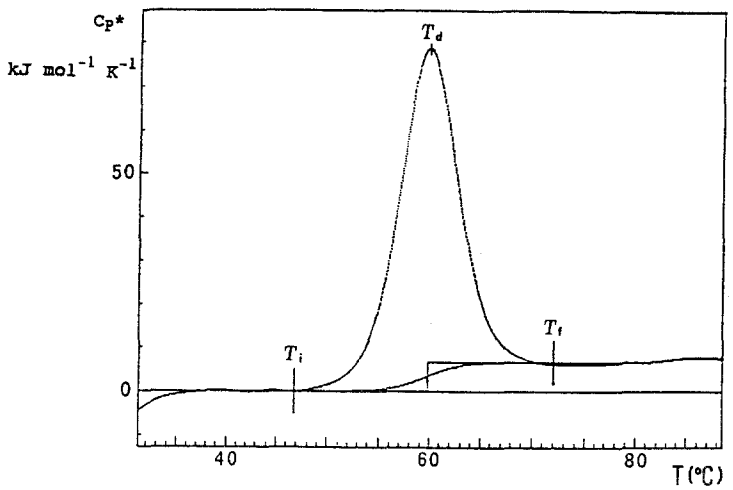

Fig. 1 Characteristic apparent molar heat capacity-temperature plot after the correction for calibration and buffer-buffer reference line

The results of DSC measurements, concerning the thermal denaturation process of RNAase T1 in the *pH* range 3.7– 8.0 are reported in Table 1*a* and Figs 2*a*) and *b*). The T_d and $\Delta_d H$ values show maxima at $pH \approx 5.5$ (62°C and ≈460 kJ·mol^{-1} respectively). These data can be compared with those obtained for RNAase A (given in Table 1*b*). For RNAase A no maxima were found up to pH = 8.0 since T_d and $\Delta_d H$ increase monotonically with the *pH*. In Figs 3a and 3b it is shown the approximate linear dependence of the denaturation enthalpies on the midpoint denaturation temperature for both the ribonucleases. The slope of each line gives the best average of $\Delta_d C_P$, assumed independent on the *pH*, as suggested by Privalov and Becktel and Schellman [26, 27]. We found a value of 6.54 kJ·mol^{-1}·K^{-1} for Lys25- RNAase T1 against 6.65 kJ·mol^{-1}· K^{-1} recently reported for the Gln25 form [23]. Comparable value was obtained also in a recent work on the Lys25 isoform [28] but the authors used an uncorrect value for ε.

Table 1

a) Thermodynamic parameters of thermal denaturation of RNAase T1 [1]

pH	T_d/ °C	$\Delta_d H$ / kJ·mol^{-1}	$\Delta_d C_p$ / kJ·mol^{-1}·K^{-1}	$\Delta H^{v.H.}$ / kJ·mol^{-1}	$R^{(o)}$
3.7	59.0	403	4.1	520	0.77
4.5	61.3	440	7.0	533	0.83
5.0	61.9	452	5.9	536	0.84
5.7	59.3	436	5.2	520	0.84
6.3	57.3	402	4.0	536	0.75
7.0	55.0	387	6.6	477	0.81
8.0	50.2	375	6.7	499	0.75

b) Thermodynamic parameters of thermal denaturation of RNAase A [1]

pH	T_d/ °C	$\Delta_d H$ / kJ·mol^{-1}	$\Delta_d C_p$ / kJ·mol^{-1}·K^{-1}	$\Delta H^{v.H.}$ / kJ·mol^{-1}	$R^{(o)}$
2.0	35.2	274	4.8	321	0.85
3.7	56.8	439	6.4	428	1.03
5.0	62.9	492	5.3	465	1.05
6.0	63.1	501	5.6	503	1.00
7.0	63.9	519	5.3	500	1.02
8.0	63.9	524	5.2	516	1.02

[o] $R = \Delta_d H/\Delta H^{v.H.}$

[1] Reported values are the averages on three or four measurements for each *pH*. The standard deviations in $\Delta_d H$ and $\Delta_d C_p$ at each *pH* are of the order of 10–15 per cent of the reported values. Errors on T_d are ±0.2°C

Table 1 reports the van't Hoff denaturation enthalpies, $\Delta H^{v.H.}$ (calculated as suggested by Privalov and Khechinashvili [6]), and the ratio of the experimental calorimetric enthalpy to the enthalpy calculated according to the van't Hoff equation $R = \Delta_d H^{cal}/\Delta H^{v.H.}$. This ratio was regularly lower than unity (ranging between 0.75 and 0.85) at all the pH values investigated for RNAase T1 whereas it is practically equal to unity for RNAase A (except for *pH* = 2). Then we concluded that the denaturation process for the former protein cannot be represented by a simple two-state (one step) process.. However the value of $R < 1$ seems to exclude the presence of a stable intermediate state. A possible explanation of this behaviour can be represented by the existence, besides the native form of RNAase T1, of the so-called 'molten globule', firstly postulated by Kim and Baldwin [29]. The existence of this state has been discussed for several proteins under certain cir-

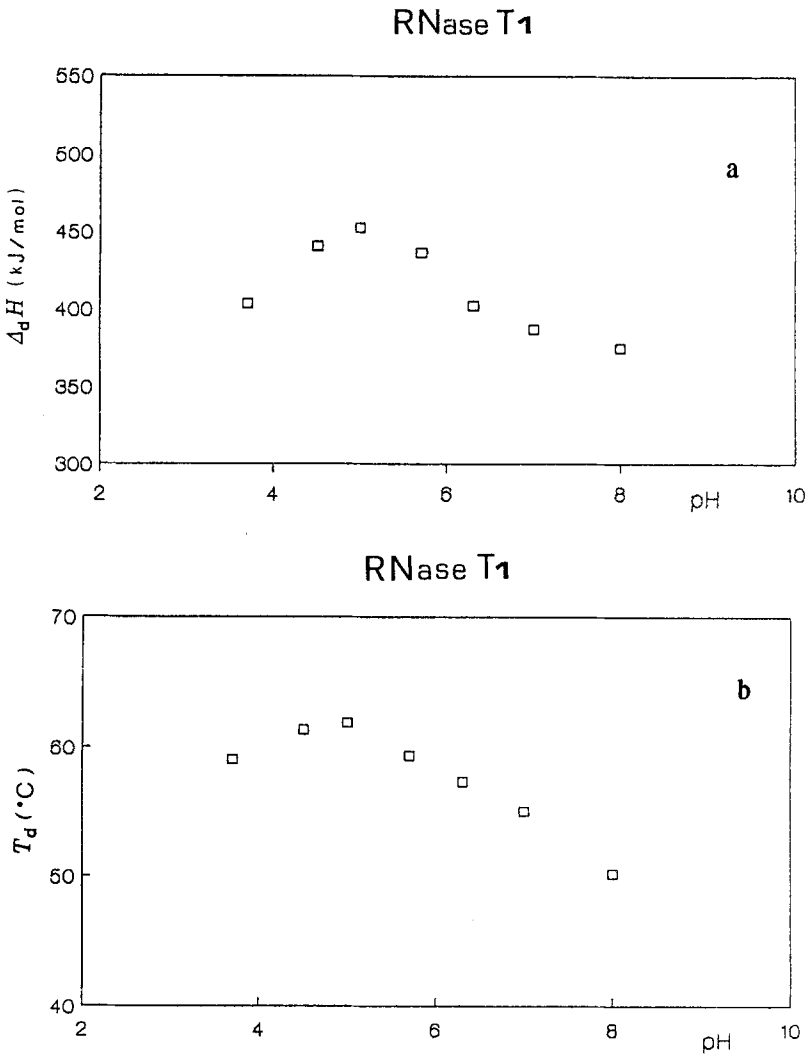

Fig. 2*a-b* Trends of the denaturation enthalpies and midpoint denaturation temperatures as a function of *pH*. Data and units as in Table 1

cumstances [30–33]. It seems to be characterized by a high content of secondary structure and high compactness. Part of intramolecular hydrogen bonds seems to be broken but still hydrophobic forces seem to operate [33]. One hypothesis is that in the present case the molten globule is not a stable intermediate between native and denatured states, but a stable compact form, without specific tertiary structure, that co-exists with the native form at room temperature. It transforms in a poor cooperative and parallel way at increasing temperature into the denatured

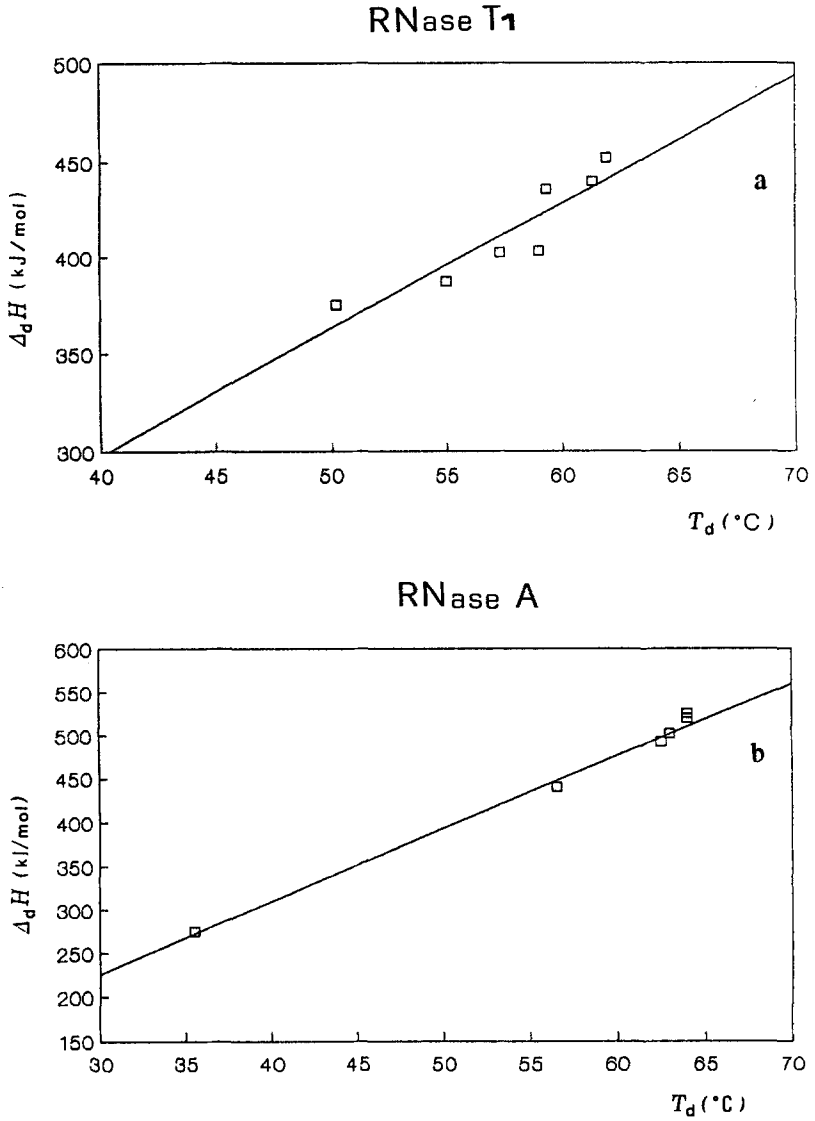

Fig. 3*a-b* Trends of the denaturation enthalpies as a function of the midpoint denaturation temperatures. *a*) RNAase T1; *b*) RNAase A

state. The poor cooperativity do not allow to deconvolute the heat contribution due to this transformation. A systematic error will then affect our present results and those reported in other works [23, 28]. The preceding considerations, however, permit to assume the validity of the two-state model for the prevailing fraction of molecules in the native form. The evaluation of the $\Delta_d G^\circ(T)$ as function of

temperature is then possible by using the calculated $\Delta_d C_P$ value, according to the classical thermodynamic relationships:

$$\Delta_d H^\circ(T) = \Delta_d H^\circ(T_d) + \int_{T_d}^{T} \Delta_d C_p \, dT \tag{2}$$

$$\Delta_d S^\circ(T) = \frac{\Delta_d H^\circ(T_d)}{T_d} + \int_{T_d}^{T} \frac{\Delta_d C_p}{T} dT \tag{3}$$

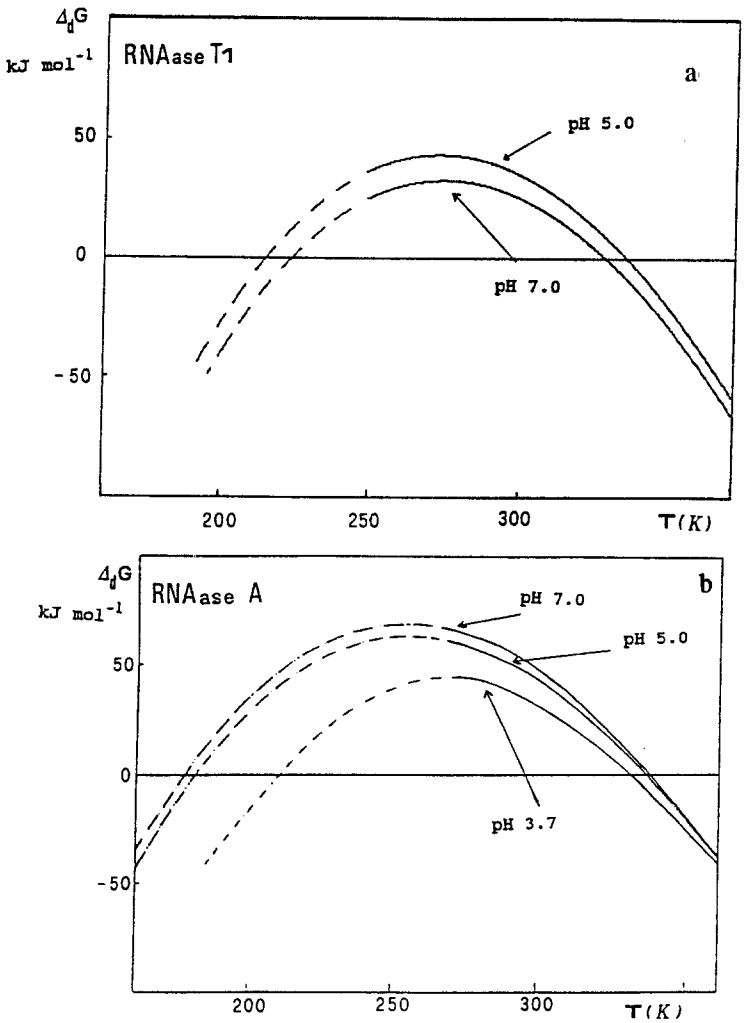

Fig. 4*a-b* Trends of the Gibbs standard energies as a function of the temperature at different *pH*. *a*) RNAase T1; *b*) RNAase A. Data from Table 1 and Eqs. (2–4)

$$\Delta_d G^\circ(T) = \Delta_d H^\circ(T) - T\Delta_d S^\circ(T) \quad (4)$$

The resulting curves, calculated and plotted with help of THESEUS program for some of the explorated *pH*, are given in the Figs 4a) and 4b) for RNAase T1 and RNAase A respectively. Both these diagrams show that the maxima of stability occur at temperatures lower than the room temperature, and that the hypotetical 'cold denaturation temperature' T_d' (where it is $\Delta_d G^\circ = 0$ as at T_d) occurs in a region that cannot be attained even under the extreme condition of water undercooling.

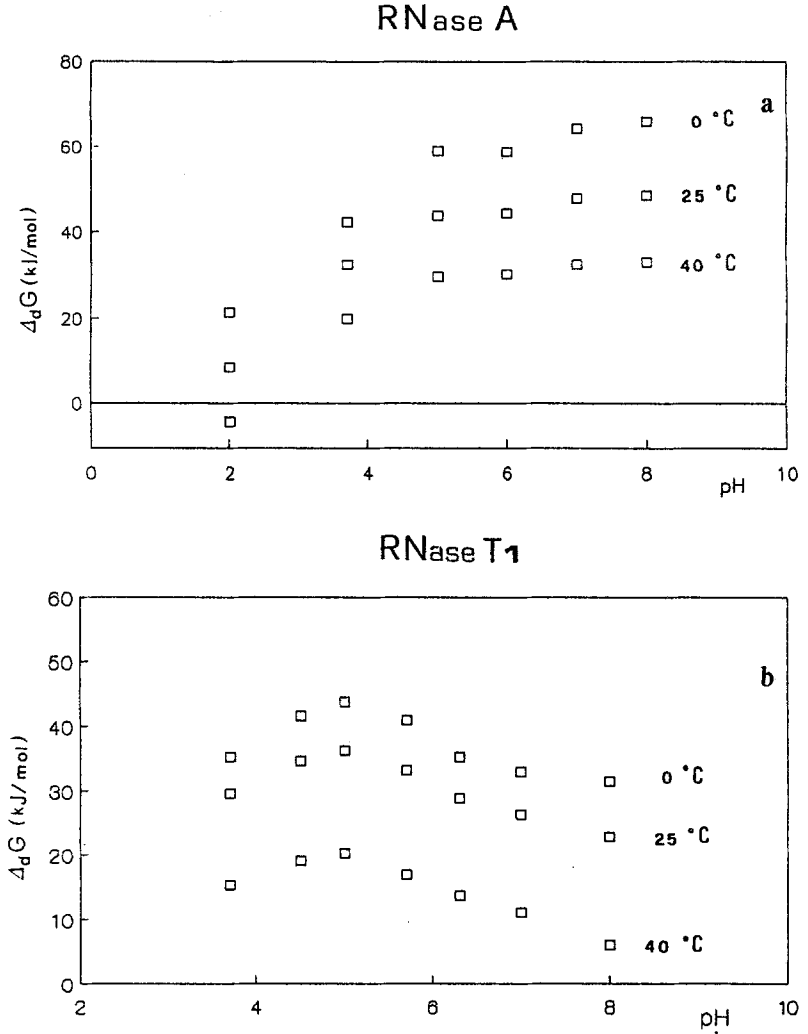

Fig.5*a-b* Trends of the Gibbs standard energies as a function of the *pH* at different temperatures. *a*) RNAase T1; *b*) RNAase A. Data from Table 1 and Eqs. (2–4)

Further it is interesting to compare the stability of the two proteins as a function of pH at some selected temperatures. These results, obtained directly from Eqs (2–4), are plotted in the Figs. 5a) and 5b) where a clear difference of behavior between RNAase T1 and A appears. The first protein has a maximum at $pH \approx 5.5$ and the second a maximum at $pH > 8$. These values are clearly correlated to, but not coincident with the isoionic *pH*s where the overall charge is zero (the isoionic point for RNAase A is 9.6 against 4.5 for RNAase T1 [18]). The isoionic points are regulated by the number of protonable groups, by the relative pK_a of these groups (between the native and denatured forms) and by the relative distances between each pair of groups*.

The compensation of the overall electrostatic repulsions at zero net charge is only one of the factors that contribute to the stability of the native conformation of an amphiphilic macromolecule. A high absolute number of net charges on the surface of a biopolymer contributes to the hydration and solubility. However the distribution of hydrated charged groups and the prevalence of those having the same sign, perturb in a complex manner the solvation of the other unionized polar groups and the cages of water surrounding the hydrophobic side chains.

Pace and colleagues [14, 16] have discussed in detail the Gibbs energy of RNAase T1 as a function of *pH*, based on the titration data of Iida and Ooi [18]. It is worth noting that our results on the stability dependence either on temperature or on pH are in good agreement with the data determined by these authors.

Aggregation

At $pH = 3.7$ the denatured RNAase T1 undergoes a reproducible but irreversible exothermic phenomenon of aggregation. This occurs at the temperature range 72°–80°C with a peak centered at ≈ 75°C.

Effect of urea

A few measurements were attempted in the presence of an increasing concentration of urea at $pH = 5.0$. When compared to the data reported in Table 1, at $pH = 5.0$, the addition of urea promotes a decrease of both $\Delta_d H$ and T_d. The denaturation enthalpy change is ≈ 377 kJ·mol^{-1} at 2 *M* urea, and ≈ 269 kJ·mol^{-1} at 4 *M* urea, whereas, in water it is ≈ 452 kJ·mol^{-1}. The T_d values are: ≈ 54° and ≈45°C, respectively, against ≈ 62°C in water. The denaturation peaks are followed

* NOTE. It must be remembered that while for Lys25-RNAase T1 there are two Lys and one Arg against 11 carboxylic groups, in the case of RNAase A there are 16 Lys and Arg residues against only eight carboxylic groups.

by a consistent, gradual and continuous decrease of the apparent heat capacity owing to a non-cooperative phenomenon which needs further investigations to clarify its nature. The urea concentration is a critical variable for the thermal protein refolding. In fact in 2 *M* urea solution, the RNAase T1 denaturation process is partially reversible while in 4 *M* urea solution it is completely irreversible.

* * *

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References

1 A. Wlodawer in F.A.Jurnak, A. McPherson (Eds) 'Biological Macromolecules & Assemblies, vol.2 Nucleic Acids and Interactive Proteins' Wiley, New York 1985, p. 393.
2 S. J. Wearne and T. E. Creighton, Proteins Struct., Funct., Genet., 4 (1988) 251.
3 J. B. Udgaonkar and R. L. Baldwin, Nature, 335 (1988) 694.
4 T. E. Creighton, Proc. Natl. Acad. Sci. USA, 85 (1988) 5082.
5 D. M. Rothwarf and H. A. Scheraga, J. Am. Chem. Soc., 113 (1991) 6293.
6 P. L. Privalov and N. N. Khechinashvili, J. Mol. Biol. 86 (1974) 665.
7 Y. Fujita and Y. Noda, Bull. Chem. Soc. Jpn. 57 (1984) 1891.
8 G. Barone, P. Del Vecchio, C. Giancola and G. Notaro, Thermochim. Acta, 199 (1992) 189.
9 G. Barone, P. Del Vecchio, D. Fessas, C. Giancola, G. Graziano and A. Verdoliva, to be published
10 C. N. Pace, Methods Enzymol., 131 (1986) 266.
11 C. N. Pace and G. R. Grimsley, Biochemistry, 27 (1988) 3242.
12 C. N. Pace and D. V. Laurents, Biochemistry, 28 (1989) 2520.
13 C. N. Pace, B. A. Shirley and J. A. Thomson, in T. E. Creighton Ed. (1989): Protein Structure: a Practical Approach, IRL Press Oxford.
14 C. N. Pace, D. V. Laurents and J. A. Thomson, Biochemistry, 29 (1990) 2565.
15 F. G. Walz and S. Kitareewan, J. Biol. Chem., 265 (1990) 7127.
16 C. N. Pace, N. Heinemann, H. Hahn and W. Saenger, Angew. Chemie. Intern. Ed. 30 (1991) 343.
17 B. A. Shirley, P. Stanssens, J. Steyaert and C. N. Pace, J. Biol. Chem., 264 (1989) 11621.
18 S. Iida and T. Ooi, Biochemistry, 8 (1969) 3897.
19 C. Tanford and J. D. Hauenstein, J. Am. Chem. Soc., 78 (1956) 5287.
20 F. Inagaki, Y. Kawano, I. Shimada, K. Takahasi and T. Miyazawa, J. Biochem. (Tokio) 89 (1981) 1185.
21 J. B. Matthew and F. M. Richards, Biochemistry, 21 (1982) 4989.
22 M. Sela and C. B. Anfinsen, Biochem. Biophys. Acta, 24 (1970) 229.
23 C.-Q. Hu, J. M. Sturtevant, J. A. Thomson, R. E. Erickson and C. N. Pace, Biochemistry, 31 (1992) in press.
24 K. Takahashi, J. Biochem. (Tokyo), 72 (1972) 1469.
25 G. Barone, P. Del Vecchio, D. Fessas, C. Giancola and G. Graziano, J. Thermal Anal., This Issue.
26 P. L. Privalov, Adv. Protein Chem., 33 (1979) 167.
27 W. Becktel and J. Schellman, Biopolymers, 26 (1987) 1859.
28 T. Kiefhaber, F. X. Schmid, M. Renner and H.-J. Hinz, Biochemistry, 29 (1990) 8250.
29 P. S. Kim and R. L. Baldwin, Annu. Rev. Biochem., 51 (1982) 459.

30 F. M. Hughson, P. E. Wright and R. L. Baldwin, Science, 249 (1990) 1544.
31 H. Christensen and R. H. Pain, Eur. Biophys. J., 19 (1991) 221.
32 J. J. Ewbank and T. Creighton, Nature, 350 (1991) 518.
33 D. Xie, V. Bhakuni and E. Freire, Biochemistry, 30 (1991) 10673.

Zusammenfassung — Mittels DSC Mikrokalorimetrie wurde die thermische Denaturierung von mikrobieller Ribonuklease T1 (RNAase T1) als eine Funktion des *pH*-Wertes untersucht. Die mittleren Denaturierungstemperaturen, Enthalpieänderungen und Wärmekapazitätsänderungen von Ribonuklease T1 wurden mit denen von pankreatischer Ribonuklease A (RNAase A) verglichen. Man fand, daß das mikrobielle Protein T1 einen komplizierteren Konformationsübergang zeigt, als die bei Ribonuklease A auftretende einfache Zweizustandsänderung. Es wird weiterhin die Hypothese des Auftretens einer "geschmolzenen Kügelchen" Form diskutiert. Die Konformationsbeständigkeit von RNAase T1 ist bei hohen pH-Werten niedriger als die von RNAase A. Die größte Stabilität von RNAase T1 liegt bei einem *pH*-Wert von etwa 5, während die von RNAase A bei etwa *pH*=8. Bei einem *pH*-Wert von 3.7 wird durch einen reproduzierbaren exothermen Peak das Auftreten einer irreversiblen Aggregation angezeigt. Die Konformationsänderungen von RNAase T1 sind im *pH*-Wertbereich 4.5 bis 7.0 reversibel, die von RNAase A sind irreversibel bei einem *pH*-Wert von mindestens 8.0 .