Protein Stabilisation by Compatible Solutes: Effect of Mannosylglycerate on Unfolding Thermodynamics and Activity of Ribonuclease A

Tiago Q. Faria,[a] Stefan Knapp,[b, c] Rudolf Ladenstein,[b] António L. Maçanita,[a, d] and Helena Santos*[a]

Differential scanning calorimetry, optical spectroscopy, and activity measurements were used to investigate the effect of mannosylglycerate, a negatively charged osmolyte widely distributed among thermophilic and hyperthermophilic archaea and bacteria, on the thermal unfolding of ribonuclease A (RNase A). For comparison, assays in the presence of trehalose, a canonical solute in mesophiles, and potassium chloride were also carried out. A thermodynamic analysis was performed by using differential scanning calorimetry data. The changes in the heat capacity for unfolding were similar for the different solutes examined. Mannosylglycerate was an efficient thermostabiliser of RNase A and induced an increase of 6 °C mole^{-1} in the melting temperature. Moreover, the performance of mannosylglycerate as a stabiliser depended on the net charge of the molecule, with the maximal effect being observed at pH values above 4.5. Analysis of the enthalpic and entropic contributions to unfolding, derived from calorimetric data, revealed that the stabilisation rendered by mannosylglycerate is primarily achieved through a decrease in the unfolding entropy. Also, the number of protons taken up by RNase A upon denaturation in the presence of mannosylglycerate was considerably higher than with other solutes, a result consistent with a more rigid structure of the native protein. Mannosylglycerate (potassium salt) inhibited the activity of RNase A, albeit to a smaller extent than KCl, and acted as an efficient suppressor of aggregation of the denatured protein, thereby having a remarkable beneficial effect on the inactivation of RNase A upon thermal denaturation. The results are discussed in view of the physiological role of this charged compatible solute.

KEYWORDS:

calorimetry · compatible solutes · mannosylglycerate · protein stabilisation · thermodynamics

Introduction

Protein stability and stabilisation mechanisms are key issues in most industrial and pharmaceutical applications involving proteins. In the last decade, an increasing effort has been directed towards the development of highly stable proteins, which should perform efficiently under the severe working conditions in many industrial processes.[1, 2] Extremophiles are microorganisms that can thrive in the most extreme environments on earth[3, 4] and appear to be an ideal source of stable enzymes. In particular, the discovery of hyperthermophiles was a great incentive to investigate the determinants of protein stability. Surprisingly, in addition to intrinsically highly stable proteins, hyperthermophiles also possess intracellular proteins that are not particularly stable; this indicates that alternative strategies are used for their stabilisation in vivo. In fact, thermophiles and hyperthermophiles isolated from saline environments synthesize de novo low-molecular-mass compounds that have not been found or have been rarely encountered in mesophilic organisms; this has led to the view that the compatible solutes of (hyper)thermophiles could play a role in the stabilisation of cell components at high temperature.[5, 6] At least in vitro, these solutes are highly efficient in the protection of enzymes and proteins from thermophilic, as well as mesophilic, origin against thermal inactivation and other stresses.[7–11] Interestingly, the compatible solutes from hyperthermophiles are generally negatively charged, while mesophilic bacteria, yeast, filamentous fungi, and algae accumulate primarily neutral or zwitterionic solutes. The question then arises of whether those charged solutes were selected by organisms adapted to

[a] *Prof. H. Santos, T. Q. Faria, Prof. A. L. Maçanita, Instituto de Tecnologia Química e Biológica Universidade Nova de Lisboa, Rua da Quinta Grande 6 Apartado 127, 2780-156 Oeiras (Portugal) Fax: (+351) 214-428-766 E-mail: santos@itqb.unl.pt*

[b] *Dr. S. Knapp, Prof. R. Ladenstein Karolinska Institute, Department of Biosciences at Novum Center for Structural Biochemistry Hälsovägen 7-9, 14157 Huddinge (Sweden)*

[c] *Dr. S. Knapp, Current address: Pharmacia Corp. Discovery Research Oncology Department of Chemistry 20014 Nerviano (Mi) (Italy)*

[d] *Prof. A. L. Maçanita, Instituto Superior Técnico Universidade Técnica de Lisboa Av. Rovisco Pais, 1049-001, Lisboa (Portugal)*

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grow at high temperatures because their physicochemical properties make them more suitable to protect proteins and other cell components against thermal denaturation. This point was the motivation for the present study, in which we investigate some thermodynamic aspects of the stabilisation of a model protein by 2-*O*-α-mannosylglycerate (MG), a widespread compatible solute amongst microorganisms adapted to grow in hot environments.[12]

The beneficial properties of certain salts (for example, ammonium sulfate) and uncharged osmolytes (glycerol, trehalose, sucrose) on protein stability have been known for a long time, but the molecular principles responsible for this stabilisation are still a matter of controversy and active research.[7, 13–20] Excellent discussions on the principles that control protein stabilisation by salts and electrically neutral molecules are available.[7, 21–23]

In the present work, bovine ribonuclease A (RNase A) was selected as the model enzyme because it is one of the most common systems used in stabilisation studies and it exhibits a good reversibility of unfolding.[24–26] Moreover, many authors report that the thermal denaturation of RNase A occurs in agreement with a two-state model.[21, 24–26] Differential scanning calorimetry (DSC) was used to determine thermodynamic parameters, and optical spectroscopy was employed to assess changes in protein conformation during thermal unfolding. Also, activity measurements were carried out to evaluate the effect of MG on the catalytic performance of RNase A subjected to heat stress. For comparison, parallel experiments were performed in the presence of trehalose, a canonical neutral osmolyte present in many mesophilic organisms, and potassium chloride, since potassium was the counterion of mannosylglycerate.

Results

Reversibility of the thermal denaturation of RNase A

The extent of reversibility was assessed by comparing the area under two sequential DSC scans acquired with the same protein solution. On average, the second scan had at least 85% of the area under the heat-absorbance peak of the first scan, thereby permitting an equilibrium thermodynamic analysis of the unfolding process.

Dependence of the melting temperature on the pH value

The melting temperature, T_m, of RNase A varies with the pH value in the acidic region and remains approximately constant above pH 5 either in the absence or presence of solutes (Figures 1). In this study, 0.1 M phosphate was used to counteract the buffering capacity of MG and enable measurements over a wide pH range; T_m values in this buffer alone were slightly higher than those reported in the literature with other buffers and/or lower concentrations.[24, 25] We measured the T_m values of RNase A at two phosphate concentrations, 0.01 and 0.1 M (both at pH 6.9), and obtained values of 61.9 and 63.3 °C, respectively; this shows the effect of increasing phosphate concentration on the stability of this protein.

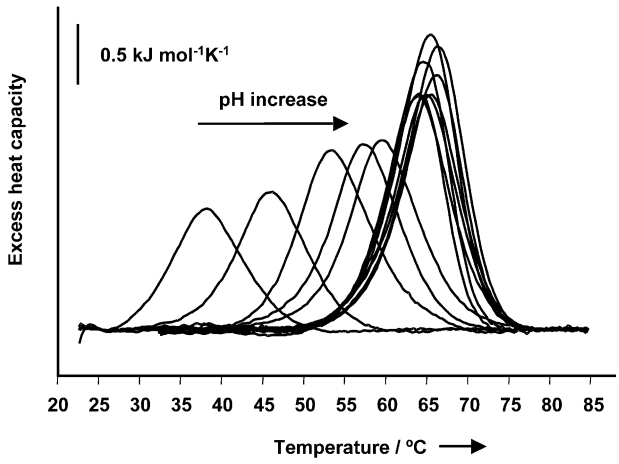

Figure 1. *DSC transition curves of RNase A in 0.1 M phosphate buffer at different pH values in the range 2.0 – 7.5. The individual pH values examined are: 2.0, 2.5, 3.0, 3.4, 3.7, 4.4, 4.7, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5.*

In comparison with no solute addition, the presence of trehalose increased the T_m value of RNase A over the whole pH range examined (Figure 2 A). The profile with KCl exhibited a discontinuity at circa pH 3: at higher pH values KCl destabilised RNase A, but it exerted a protecting effect at pH values lower than 3 (Figure 2 A). The extent of protection rendered by MG

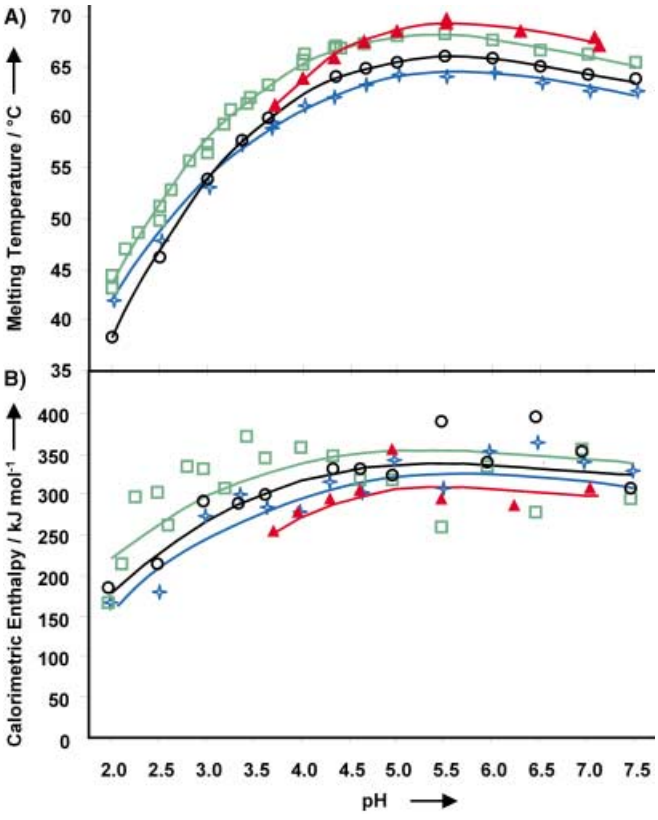

Figure 2. *Dependence of the melting temperature (A) and calorimetric enthalpy (B) of RNase A on the pH value. Scans were performed with RNase A (concentration: 0.2 – 0.5 mg mL^{-1}) in 0.1 M phosphate buffer with no solute added (○) or with 0.5 M mannosylglycerate (▲), KCl (*), or trehalose (□) added.*

appeared to depend on the degree of ionisation of its carboxylic group. From the pH dependence of the NMR chemical shift of the proton at position 2 in the glycerate moiety, a value of 3.2 was determined for the pK_a of MG (data not shown). The effect of MG on the T_m value of RNase A clearly depended on the proportion of dissociated molecules; at pH 3.7 there was no significant stabilisation (as compared to no solute addition) but there was a pronounced improvement at higher pH values. Above pH 5 the stabilising effect of MG was even greater than that of trehalose and the T_m value of RNase A increased by more than 3 °C (Figure 2 A).

Thermodynamic parameters of the thermal unfolding of RNase A from DSC data

To determine the unfolding thermodynamics of RNase A, the data were fitted to a non two-state denaturation model in which the calorimetric enthalpy (ΔH_{cal}), the van't Hoff enthalpy (ΔH_{vh}), and the T_m values are calculated. Therefore, no specific model was imposed for the data analysis and the calorimetric and van't Hoff enthalpies were determined independently. A plot of ΔH_{cal} as a function of pH value is shown in Figure 2 B. The change in the heat capacity (ΔC_p) was derived by using the Kirchoff relation, $\Delta C_p = (\partial \Delta H_{cal}/\partial T)_P$. The calorimetric enthalpies measured at different pH values were plotted as a function of the melting temperature in the absence and presence of the different solutes (Figure 3). In general, the calorimetric enthalpies for RNase A without solute were lower than those reported

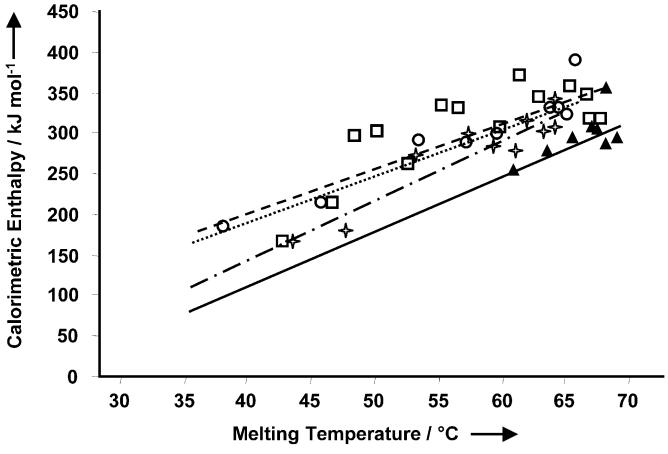

Figure 3. Temperature dependence of the unfolding calorimetric enthalpy of RNase A. Enthalpy values were obtained by numerical integration of DSC endotherms and the average values obtained in independent experiments (up to three) are shown. RNase A was dissolved in 0.1 M phosphate buffer at different pH values in the absence of solutes (●●●●○) or with 0.5 M mannosylglycerate (——▲), KCl (●–●–), or trehalose (- - - -□).*

in the literature, which range from 475 – 493 kJ mol^{-1}.[18, 24, 27] However, significantly lower values (370 kJ mol^{-1}) have been reported as well.[26] This is not totally unexpected given the conclusions of a recent IUPAC study involving six laboratories worldwide with the objective to derive useful recommendations for DSC measurement procedures: the unfolding enthalpy of lysozyme ranged from 377 – 439 kJ mol^{-1}.[28] However, in the case of RNase A we noticed that the higher values correlated with studies involving protein purchased from Sigma, whereas the lower values arose when the protein was supplied by Boehringer Mannheim. By performing parallel DSC measurements with RNase A from the two commercial sources we verified that the unfolding enthalpy for the two preparations differed by 70 kJ mol^{-1}, despite the fact that the two preparations were pure as judged by sodium dodecylsulfate (SDS) PAGE (data not shown).

The reasons for the high dispersion of values obtained for the protein dissolved in trehalose solutions are not clear. The presence of solutes did not significantly affect the values of ΔC_p associated with the unfolding of RNase A (Table 1) and the changes in the heat capacity for RNase A unfolding are in good

Table 1. *Heat capacity change of RNase A thermal unfolding in the presence of potassium mannosylglycerate, trehalose, or KCl at 0.5 M concentration.*

	ΔC_p [kJ mol^{-1} K^{-1}]	$\Delta H_{vh}/\Delta H_{cal}$
no solute	5.7 ± 0.4	1.2 ± 0.2
KCl	7.4 ± 0.7	1.3 ± 0.3
trehalose	5.5 ± 1.1	1.2 ± 0.3
mannosylglycerate	6.8 ± 1.6	1.4 ± 0.2

agreement with those reported in other experimental works[24, 26] as well as with theoretical calculations.[29] For each data point the ratio between the van't Hoff and calorimetric enthalpies was calculated (Table 1); this ratio slightly deviated from the pure two-state condition (1.0) when MG was present.

To calculate the entropy change associated with RNase A denaturation, the approach previously proposed by Plaza-del-Pino and Sanchez-Ruiz was followed.[24] A brief description is presented in the Materials and Methods section. The number of protons, $\Delta\nu$, taken up from the solvent upon denaturation was calculated from the polynomial fit to the pH dependence of T_m by using Equation (1) (Figure 4). At low pH values, carboxylic

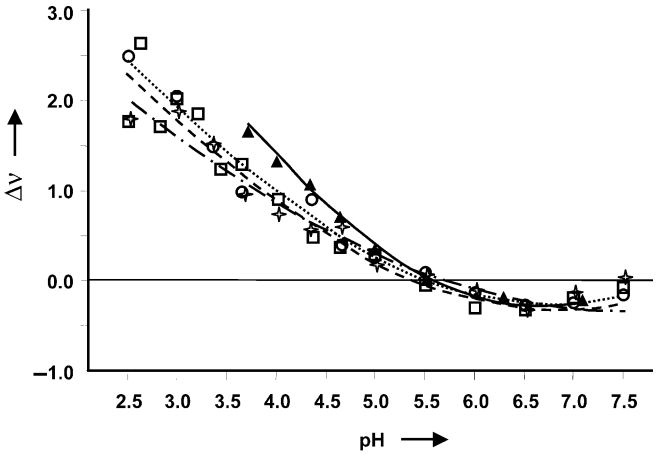

Figure 4. Number of protons taken up from the buffer upon RNase A unfolding in 0.1 M phosphate buffer at different pH values in the absence of solutes (●●●●○) or with 0.5 M mannosylglycerate (——▲), KCl (●–●–), or trehalose (- - - -□).*

groups in the interior of the protein become protonated upon exposure to the solvent in the unfolded state. The profile of $\Delta\nu$ as a function of pH value is in accordance with that reported by Plaza-del-Pino and Sanchez-Ruiz.[24] Above pH 5, $\Delta\nu$ was essentially constant regardless of the presence or absence of solutes. At lower pH values, the number of protons taken up upon denaturation was clearly higher when MG was present, but the curves were essentially coincident with trehalose, no solutes, and KCl. Entropy changes for RNase A unfolding at each temperature were corrected for the difference in pH value by using Equation (2) (Table 2). pH values of 3.7 and 7.0 were chosen as reference values to assess the importance of the degree of ionization of MG on the extent of RNase A stabilisation.

The temperature dependence of the Gibbs energy associated to RNase A denaturation was calculated at the reference pH values by using the Gibbs function, $\Delta G = \Delta H - T\Delta S$ (Figures 5 A, B). The change in the Gibbs energy, $\Delta\Delta G$, defined as the difference between the Gibbs energy with and without solute, indicates the stabilising (if positive) or destabilising (if negative) effect of the solutes on the RNase A structure. The values of $\Delta\Delta G$ for MG, KCl, and trehalose at two pH values (3.7 and 7.0) as a function of temperature are represented in Figures 5 C and D. The difference of the enthalpic, $\Delta\Delta H$, and entropic, $\Delta(T\Delta S)$, contributions in the presence and absence of solutes is shown in Table 2.

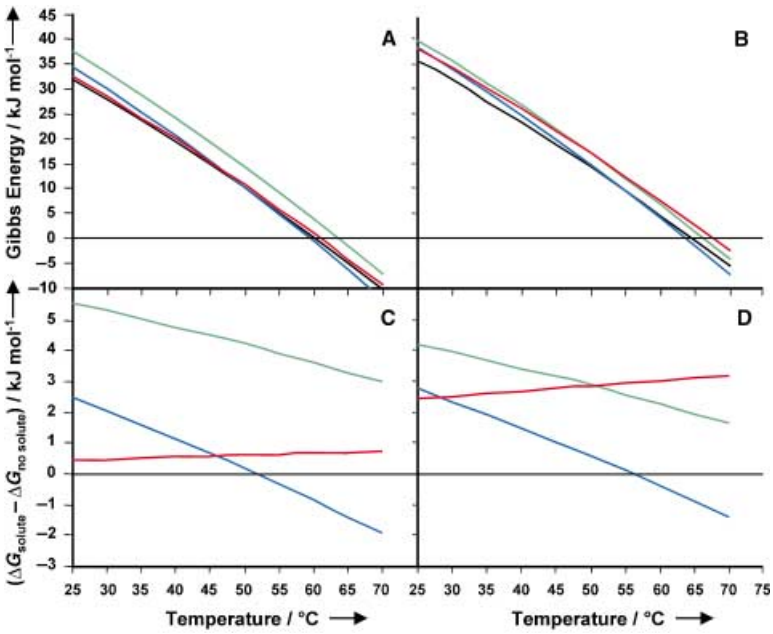

Figure 5. *Gibbs energy change for RNase A unfolding. Curves were calculated with pH 3.7 (A and C) or pH 7.0 (B and D) as the reference state. Panels C and D show the differences between the Gibbs energy with and without solute. Black: absence of solutes; red: with 0.5 M mannosylglycerate; blue: with 0.5 M KCl; green: with 0.5 M trehalose.*

Table 2. *Solute effect on the enthalpic and entropic contributions for RNase A unfolding.*[a]

Temperature [°C]	40	50	60	70	40	50	60	70
	ΔH				$\Delta\Delta H$			
no solute	189.0	246.3	303.6	360.9	–	–	–	–
KCl	142.1	216.3	290.5	364.7	−46.9	−30.0	−13.1	3.8
trehalose	200.4	255.6	310.8	366.0	11.4	9.3	7.2	5.1
MG	111.4	179.0	246.5	314.1	−77.6	−67.3	−57.1	−46.8
	$T\Delta S$ at pH_{ref} 3.7				$\Delta(T\Delta S)$ at pH_{ref} 3.7			
no solute	169.7	236.3	303.4	371.1	–	–	–	–
KCl	121.7	206.1	291.1	376.8	−48.0	−30.2	−12.2	5.7
trehalose	176.3	241.3	306.9	373.2	6.6	5.1	3.5	2.1
MG	91.6	168.3	245.7	323.6	−78.1	−67.9	−57.7	−47.5
	$T\Delta S$ at pH_{ref} 7.0				$\Delta(T\Delta S)$ at pH_{ref} 7.0			
no solute	165.8	232.2	299.2	366.9	–	–	–	–
KCl	117.4	201.6	286.5	372.1	−48.4	−30.6	−12.7	5.2
trehalose	173.7	238.6	304.2	370.3	8.0	6.4	4.9	3.4
MG	85.5	162.1	239.2	316.9	−80.2	−70.2	−60.1	−50.0

[a] Solute concentration = 0.5 M. $\Delta\Delta H$ and $\Delta(T\Delta S)$ are the difference between the unfolding enthalpy and entropic contribution of RNase A in the presence and absence of solute, respectively. The uncertainty of the values presented is circa 13%. All values are given in kJ mol^{-1}.

Effect of solutes on the thermal unfolding of RNase A: UV differential spectroscopy

Thermal unfolding of RNase A was also monitored by UV differential spectroscopy. DSC assays suggested that the net charge of MG plays an important role in the extent of stabilisation rendered by this solute, and therefore, we deemed it important to examine this effect at two pH values by using a different technique. The melting temperature of RNase A (T_m) at pH 4.5 and 7.5 and the difference in the change of the Gibbs energy with and without solute at 60 °C ($\Delta\Delta G^{60\,°C}$) are shown in Table 3.

Effect of solutes on the temperature profile of RNase A activity

The structural information obtained with DSC and UV differential spectroscopy during thermal unfolding of RNase A was complemented with activity measurements to gain insight into the interrelationship between structural stability and catalytic performance. The temperature profile for activity of RNase A was essentially unaltered in the presence of trehalose, but all the ionic solutes caused considerable inhibitory effects (Figure 6). KCl caused a decrease of approximately 55% in the activity of the enzyme at the optimum temperature, and the activity loss was even greater with NaCl (65%). Interestingly, the inhibitory effect of MG was much weaker (27%) than that of KCl, despite the presence of potassium as a counterion. Furthermore, it is worth noting the shift induced in the temperature for optimum activity by the presence of MG (from 57 to 60 °C).

Table 3. *Effect of various solutes (at 0.5 M concentration) on the melting temperature of RNase A and on the change of the unfolding Gibbs energy. Data from DSC and UV differential spectroscopy.*

	pH 4.5				pH 7.5			
	DSC		UV		DSC		UV	
	T_m [°C]	$\Delta\Delta G^{60\,°C}$[a]	T_m [°C]	$\Delta\Delta G^{60\,°C}$[a]	T_m [°C]	$\Delta\Delta G^{60\,°C}$[a]	T_m [°C]	$\Delta\Delta G^{60\,°C}$[a]
no solute	64.4	–	64.1	–	63.6	–	62.6	–
KCl	62.8	−1.4	60.3	−4.4	62.2	0	61.5	−1.7
trehalose	67.0	3.2	65.5	1.6	65.2	2.3	64.6	1.9
MG	66.8	2.5	64.9	1.0	67.7[b]	3.0[b]	66.2	4.0

[a] $\Delta\Delta G^{60\,°C}$ is the difference between the unfolding Gibbs energy of RNase A in the presence and absence of solute. Values are given in kJ mol^{-1}. [b] Values determined at pH 7.0.

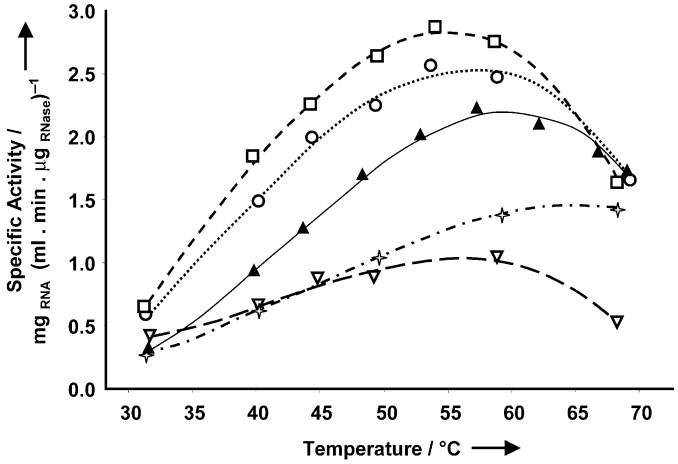

Figure 6. *Solute effect on RNase A activity. Values shown are the average of 2–10 independent measurements performed in 0.1 M phosphate buffer (pH 7.5) without addition of solutes (•••○) or with 0.5 M mannosylglycerate (——▲), KCl (•–•–*), trehalose (- - -□), or NaCl (– – –▽).*

Thermal inactivation of RNase A

The time course for the thermal inactivation of RNase A was monitored to investigate the ability of MG to prevent deleterious, irreversible processes associated with heat denaturation. The half-life for inactivation of RNase A in a dilute solution (15 µg mL^{-1}) was only slightly affected by the different solutes (Table 4). At a higher protein concentration (645 µg mL^{-1}) the half-life decreased for KCl, trehalose, or no additions, but increased in the presence of MG.

Table 4. *Effect of protein concentration and presence of solutes on the half-life times for inactivation of RNase A at 80 °C.*

	Half-life [min]	
	RNase A: 15 µg mL^{-1}	RNase A: 645 µg mL^{-1}
no solute	33 ± 1	21 ± 1
KCl	49 ± 2	28 ± 1
trehalose	39 ± 1	22 ± 2
mannosylglycerate	41 ± 2	60 ± 3

Discussion

Differential scanning calorimetry, optical spectroscopy, and activity measurements were used to investigate the effect of MG, a compatible solute of (hyper)thermophiles, on the thermal unfolding of RNase A. This combination of techniques was used to study different aspects of the unfolding process, namely the thermodynamic parameters, the changes in the dielectric environment of tyrosine residues in the protein, and the impact of MG on the catalytic activity, respectively.

The T_m value of RNase A increased with the pH value up to around 5 and remained approximately constant at higher values (Figure 2). This is a typical profile for RNase A[24, 26, 30] and reflects the decrease of the net positive charge of the protein, which has an isoelectric point of 9.3.[31]

MG exerted a clear stabilisation of the protein structure, but the pH value had a pronounced effect on the degree of stabilisation rendered by the solute. At pH 7, when the carboxylic acid group of MG is fully ionised, the presence of MG caused an increase of about 3 °C in the T_m value of the protein; however, at the lowest pH value examined with this solute (pH 3.7), the protein unfolded at a temperature that was independent of the presence of MG. In contrast, the neutral solute, trehalose, exerted greater stabilisation at low pH values: at pH 2 the T_m value of RNase A was 4.5 °C higher than without any solute, while the enhancement in T_m at pH 7 was less than 2 °C, in accordance with previous studies.[19] Decreasing degrees of RNase A stabilisation with increasing pH value were also observed upon addition of sorbitol and several magnesium salts.[25, 30] The clearly contrasting behaviour observed with MG reflects the importance of the net charge of the molecule in the mechanism of protein stabilisation, with full ionisation being required for maximal stabilisation. For example, at pH 7.0, MG was a better stabiliser than trehalose and induced a change of 3.2 °C in the T_m value of RNase A, compared with a change of 1.7 °C caused by trehalose. Also favourable to MG was the comparison with other organic osmolytes at an identical concentration (0.5 M). Sorbitol increases the RNase A melting temperature by 1.5 °C at pH 5.5;[25] glycine, sarcosine, dimethylglycine, and betaine were reported to affect the T_m value of the same protein by about 1 °C at pH 6.0.[18] Sarcosine increases T_m by about 1.3 °C at pH 4.5[24] and hydroxyectoine induces an increase of 2.1 °C at pH 5.5.[26]

From the thermodynamic analysis, the calorimetric enthalpy associated with the unfolding of RNase A in the presence of MG was lower than the values determined for the other cases examined (trehalose, KCl, no solute; Figure 3). Interestingly, the other stabilising osmolyte examined, trehalose, provoked an increase in the denaturation enthalpy when compared to the no solute case. A similar pattern for the relative magnitudes of the entropy changes ($T\Delta S$) was observed: a decrease of the unfolding entropy was observed with MG as compared to no solute addition, while trehalose led to an increase of this parameter. In the case of MG, thermodynamic stabilisation of the

protein was achieved, since the decrease in the entropic term exceeded that of the enthalpic term. Our results suggest that protein stabilisation is governed by entropy changes in the denaturation process. However, the dispersion of the experimental data is high and, as pointed out by Santoro et al.,[18] molecular interpretations of the unfolding process taken from the thermodynamic analysis are rather uncertain.

The Gibbs free energy is the thermodynamic parameter that provides a reliable evaluation of protein stability. Surprisingly, even highly stable proteins present only marginal free energies of stabilisation, in the order of 50 kJ mol^{-1}, equivalent to a few hydrogen bonds, ion pairs, or hydrophobic interactions.[32] The free energy associated with RNase A unfolding falls in this range of values (Figure 5). From the temperature dependence of the Gibbs free energy for unfolding in the presence of the different solutes examined, we conclude that the stabilising capacity of MG was most effective at high pH values. In contrast to KCl and trehalose, the Gibbs energy difference for unfolding in the presence and in the absence of MG was higher at a higher pH value, thus reinforcing the relevance of MG charge on the stabilisation process.

Highly remarkable is the increase in the number of protons taken up from the medium upon unfolding of RNase A when MG is present, as compared to other conditions examined (Figure 4). This observation could suggest a more rigid structure of the native protein holding a higher number of nonaccessible ionised groups that become protonated upon thermal unfolding. Indeed, rigidification of protein structures induced by osmolytes has been reported in the literature.[33]

RNase A has six tyrosine residues; some tyrosines are solvent-accessible while others are buried.[29] Upon unfolding these tyrosines become exposed and there is a blue shift on the absorbance spectrum that makes them suitable sensors of the polarity of the environment. The results on T_m and changes in the Gibbs free energy for unfolding obtained by this method are in good agreement with those obtained by DSC, a fact supporting the view that unfolding occurs in a single step. Deviations from a two-state model have been recently reported for the thermal unfolding of RNase A in phosphate buffer,[27] but in our study no clear evidence for such a deviation was found, at least in the absence of solutes (Figure 1). The fit of the UV experimental data to a two-state model was very good (not shown); however, the ratios between the van't Hoff and the calorimetric enthalpies ($\Delta H_{vh}/\Delta H_{cal}$) were in the range 1.2 – 1.4, which indicates some deviation from the theoretical single-step denaturation at least in the presence of MG (Table 1). Nevertheless, the conclusions derived from our analysis are not affected by potential deviations from a pure two-state model since they are based on total calorimetric enthalpies.

Brown[34] originally defined compatible solutes as small organic compounds used for osmotic adjustment that do not interfere with cell function. In particular, these compounds should be compatible with the normal functioning of enzymes in the cell. Thus, it was deemed interesting to evaluate the effect of MG on the catalytic activity of RNase A, as well as on the time course for inactivation of the enzyme when subjected to heat stress. The activity of RNase A was inhibited by salts (KCl or NaCl) since these interfere with the electrostatic interactions that play a major role in the formation of the complex between the cationic catalytic site of the enzyme and the highly anionic substrate.[35] Therefore, the potassium salt of MG also inhibited the enzyme activity, but the inhibitory effect was considerably lower than that exerted by the other salt of potassium examined (KCl). The negative effect of MG on the activity of RNase A does not hurt its ability to act as a compatible solute in the natural producers. In fact, many (hyper)thermophiles accumulate high concentrations of potassium as counterions of negatively charged solutes[12] and, therefore, the intracellular enzymes in these organisms are not expected to be vulnerable to inhibition by potassium salts. Interestingly, cyclic 2,3-bisphosphoglycerate, a solute that is accumulated in the molar range of concentrations by the hyperthermophile *Methanopyrus kandleri*, has been shown not only to stabilise but also to activate several enzymes isolated from this organism.[36]

Mannosylglycerate had a beneficial effect in the protection of RNase A activity when the enzyme was subjected to thermal denaturation and the effect was especially remarkable at high protein concentrations (Table 4). The mechanisms responsible for the loss of activity associated with thermal denaturation are not completely understood, but the aggregation of unfolded hydrophobic regions, the misfolding during renaturation, and chemical modifications of the protein residues are believed to be relevant processes.[37] In particular, the aggregation of exposed hydrophobic segments is expected to be highly dependent on protein concentration. Therefore, we propose that MG is very effective in the suppression of aggregation, which is probably the major cause of inactivation at high protein concentration. On the basis of these results we speculate that in vivo MG could play an important role in the prevention of aggregation of nascent or partially unfolded proteins at the huge protein concentrations present in the intracellular milieu.

Materials and Methods

Materials: Bovine pancreatic ribonuclease A ($M_W = 13\,700$ Da) was purchased from Boehringer Mannheim and used without further purification after judging its purity by SDS-PAGE. Protein concentration was determined by optical spectroscopy, with an extinction coefficient of 9800 L mol^{-1} cm^{-1} at 278 nm. We verified that this value was independent of the presence of the different solutes examined in this work. Yeast RNA (Boehringer Mannheim) from a single batch was used as the substrate for RNase A in all the activity assays. All other chemicals were of the highest purity available.

Purification of mannosylglycerate: Mannosylglycerate was purified from *Rhodothermus obamensis* cells grown at 70 °C in a 300-L fermentor on Degryse 162 medium, as described by Silva et al.,[38] containing 5% NaCl. The extraction and purification procedure was essentially as described by Silva et al.[38] for the isolation of MG from *R. marinus*. Fractions eluted from the QAE-Sephadex A25 column (Amersham Pharmacia) were analysed by thin-layer chromatography on silica gel coated aluminium sheets (silica gel 60 F_{254}, Merck) to detect the elution of the three carbohydrates present in the extract (MG, trehalose, and glucose). The solvent system was composed of methanol/chloroform/acetic acid/water (25:10:4:2, v/v). Sugar spots were visualised by spraying with α-naphthol/sulfuric acid solution

followed by charring at 120 °C. Fractions containing MG were pooled and lyophilised. To remove sodium and bicarbonate ions, the samples were loaded onto an activated Dowex AG 50WX8 resin (BioRad, 10 × 2.5 cm) in the H^+ form and eluted with distilled water. Subsequently, the fractions were pooled, degassed under vacuum, and titrated with ultra pure 1 M potassium hydroxide solution. After lyophilisation the sample had a yellowish colour which was removed by molecular filtration in a Sephadex G10 column (Amersham Pharmacia Biotech, 1.6 × 55 cm) eluted with water. The sugar-containing fractions were pooled and lyophilised. MG was quantified by ^{1}H NMR spectroscopy and potassium was determined by plasma emission spectroscopy on a Jobin Yvon spectrometer (model JY24). ^{1}H NMR spectra were recorded at 300.14 MHz on a Bruker AMX 300 spectrometer with a 5 mm inverse probe head. For quantification purposes, spectra were acquired with a repetition delay of 60 s. Formate and acetate were added as concentration standards. In this work, samples with purity higher than 98% and containing a 1:1 proportion of MG and potassium were used.

Differential scanning calorimetry: DSC scans were performed on a MicroCal VP-DSC MicroCalorimeter controlled by the VP-viewer program and equipped with 0.51-mL cells. Calibration of temperature and heat flow were carried out according to the MicroCal instructions. Stock solutions of RNase A were prepared by dissolving the protein in 0.1 M phosphate buffer (pH 6.0) and extensively "washing" with the same buffer in a centricon tube (molecular weight cut-off of 10 kDa). Solutions of the different solutes (KCl, trehalose, or MG) at a concentration of 0.5 M were prepared by dissolving these compounds in 0.1 M HPO_4^{2-} or H_3PO_4 solutions; the desired final pH value was obtained by combination of these solutions in a suitable proportion. An aliquot of the stock solution of RNase A was added to the solute solution (2 mL) and concentrated by ultrafiltration to the desired final protein concentration (0.2 – 0.5 mg mL^{-1}). The concentrated protein solution was used to fill up the calorimeter cell, whereas the liquid that passed through the membrane was used to fill the reference cell. The effect of the pH value was examined over the range 2.0 – 7.5. The high buffering capacity of MG precluded the preparation of solutions at pH values lower than 3.7. Prior to the calorimetric analysis, RNase A and reference solutions were degassed for 8 min. In the DSC scans the temperature was increased from 20 to 85 °C at a constant rate of 1 °C min^{-1}. An overpressure of about 2 atm was applied to the calorimeter cells to prevent bubble formation during heating. To assess reversibility of the transition, a second scan was performed with the same conditions as the first one.

DSC data analysis: Raw calorimetric data were converted into the excess heat capacity of the RNase A unfolding by subtracting the instrumental baseline recorded under identical conditions and dividing it by the scan rate and by the sample protein concentration. The calorimetric (ΔH_{cal}) and van't Hoff (ΔH_{vh}) enthalpies were calculated by using the software supplied with the instrument. The calorimetric enthalpy is the experimental value of the unfolding energy process determined by numerical integration of the area below the endothermic peak, while the van't Hoff enthalpy is based on the peak shape and calculated by the van't Hoff equation. The ratio between the van't Hoff and calorimetric enthalpies is equal to 1.0 in the case of a two-state unfolding process. For cooperative processes involving more than one molecule, this ratio is greater than 1.0. When there are intermediate species in the unfolding mechanism, this ratio has a value lower.[39] The heat capacity change upon unfolding, ΔC_p, was shown to be constant in the temperature range studied[24, 26] and was determined from the slopes of the calorimetric enthalpy as a function of temperature.

The number of protons taken up from the buffer, $\Delta\nu$, was calculated according to Equation (1), where $\frac{\partial T_m}{\partial \mathrm{pH}}$ was calculated from a polynomial fit to the experimental values determined.[24]

$$\Delta\nu = \frac{\Delta H_{vh}(T_m)\partial T_m}{\ln 10 R T_m^2\, \partial \mathrm{pH}} \quad (1)$$

The entropy associated to the unfolding process of RNase A was corrected to a reference pH value by using Equation (2), where the subscript 0 refers to the reference pH value chosen.[24]

$$\Delta S_0 = \frac{\Delta H_{cal}(T_m)}{T_m} - \ln 10 R \int_{pH_m}^{pH_0} \Delta\nu \partial \mathrm{pH} \quad (2)$$

The Gibbs free energy change for unfolding at temperature T is given by the Gibbs equation, $\Delta G = \Delta H - T\Delta S$

UV differential spectroscopy: The thermal denaturation of RNase A was monitored by the change in absorbance at 278 nm by using an Olis UV-Vis DW2 spectrophotometer with a cuvette with a 1-cm pathlength. After an initial thermal equilibration, the sample and reference cuvettes were heated by a circulating bath and the sample temperature was monitored with a thermometer placed inside the cuvette but away from the light beam. The average heating rate was about 6 °C min^{-1}. The integration time for each data point was 0.1 min.

Thermal denaturation curves monitored by UV spectroscopy were analysed with the assumption of a two-state denaturation process.[40] The data points within each assay were subtracted from the absorbance value at 40 °C to correct for potential differences in the protein concentration. The fraction of the protein in the denatured state (α) and in the native state, ($1-\alpha$), were determined as a function of temperature. The equilibrium constant ($K = \alpha/(1-\alpha)$) and the Gibbs energy change of this process ($\Delta G = -RT\ln K$) were calculated for each temperature. The melting temperature of the protein was obtained by a linear regression of the Gibbs energy change within the unfolding temperature range.

Activity assays: The endonuclease activity of RNase A was measured indirectly by using a modification of the process described by Greiner-Stoeffele et al.[41] Methylene blue is an acridine derivative that, when intercalated in the RNA molecules, shifts its absorbance maximum and can thus be used to monitor the digestion of RNA by the enzyme. We verified that the absorbance at 694 nm was linearly related with the RNA concentration. This proportionality was temperature dependent and this was taken into account in the activity calculation. Yeast RNA solution (15 mg mL^{-1}) was prepared in 0.1 M phosphate buffer (pH 7.5), and the solution was kept cold to prevent RNA degradation. The methylene blue solution was prepared in the same buffer to give an absorbance of 1.0 at 694 nm and kept in the dark. One volume of the yeast RNA solution was mixed with nine volumes of the methylene blue solution and incubated at the assay temperature until the absorbance reached a constant value (usually 10 – 15 min). The reaction was started by addition of circa 0.06 μg of RNase A and the progress of the reaction was evaluated from the change in absorbance at 694 nm.

Heat inactivation of RNase A: The long-term thermostability of RNase A was assessed in 0.1 M phosphate buffer (pH 7.5), in the absence of solutes and in the presence of KCl, trehalose, and MG (final concentration: 0.5 M). The effect of the protein concentration was also studied by using solutions with an RNase A concentration of 15 or 645 μg mL^{-1}. The protein solution was incubated at 80 °C;

samples were withdrawn at different time intervals and immediately assayed for RNase A activity at 50 °C as described above. In the case of the concentrated protein solution, prior to the activity measurement a suitable dilution was performed at the same temperature as the stress in order to avoid any effects other than the dilution. The half-life values were determined by an exponential decay fit to the inactivation profile.

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