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THE EFFECT OF TRIMETHYLAMINE N-OXIDE ON RNase A STABILITY A DSC study

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The thermal stability of bovine pancreatic ribonuclease (RNase A) has been investigated in the presence of trimethylamine N-oxide (TMAO), a naturally occurring osmolyte, by means of differential scanning calorimetry (DSC) and circular dichroism (CD) measurements at neutral and acid pH conditions. It is well known that compatible osmolytes such as TMAO are effective in stabilizing protein structure and counteracting the denaturing the effect of urea and guanidinium hydrochloride (GuHCl). Calorimetric results show that TMAO stabilizes RNase A at pH 7.0 and does not stabilize the protein at pH 4.0. RNase A thermal denaturation in the presence of TMAO is a reversible two-state N⇆D process. We also show that TMAO counteracts the urea and GuHCl denaturing effect at neutral pH, whereas the counteracting ability is lost at acid pH.

Keywords: *circular dichroism, differential scanning calorimetry, osmolytes, thermal stability*

Introduction

Many organisms produce and accumulate small organic solutes, named osmolytes, to counteract the effects of environmental stresses such as high temperature, pressure, high salt concentration and dehydration [1]. All known osmolytes are amino acids and derivatives, polyols and sugars, methylamines, and urea [2]. Osmolytes that do not have inhibitory effect on the functional activity of enzymes and can accumulate *in vivo* without perturbing the macromolecular functions at physiological conditions are called compatible solutes [3]. Some osmolytes stabilize macromolecules and provide protection against different denaturing effects, *e.g.*, methylamines can stabilize proteins against perturbations by urea in elasmobranchs and mammalian kidney, and high hydrostatic pressure in deep-sea animals [4]. Among them, trimethylamine N-oxide (TMAO) which is present in high concentrations in coelacanth and marine elasmobranchs has been widely studied. It has been shown that TMAO is able a) to increase the protein melting temperature [1]; b) to offset the destabilizing effects of urea and GuHCl [2–4]; c) to restore the enzyme activity that is lost upon urea treatment [5]; d) to force the folding of unstructured proteins [6]; e) to counteract *in vivo* the damaging effects of salts and urea [7–9], and hydrostatic pressure [9] on proteins.

The mode of action by which osmolytes confer protection on proteins is still not completely understood, and both direct and indirect actions have been proposed. Many studies have been reported on the mechanisms of interaction of proteins with osmolytes, in particular of the thermodynamic driving forces of destabilization by urea and GuHCl and the stabilization by other osmolytes [10–12]. Lin and Timasheff reported that TMAO acts independently of denaturant and does not affect the binding of denaturant to proteins [13]. Other works demonstrated that TMAO exerts its effect primarily through changes in solvent hydrogen bonding by unfavorable interactions between TMAO and the amide moiety of proteins [14–16]. Thermodynamic studies have shown that the unfavorable interaction between TMAO and the amide unit is the dominant factor in the ability of TMAO to stabilize proteins and molecular dynamic studies have shown that TMAO enhances water structure via an increase in water-water hydrogen bonds, stronger hydrogen bonds, and greater spatial ordering of the water molecules [15]. All these conclusions have been reached from studies of the effect of TMAO on proteins in the pH range 6.0–8.0, where TMAO is in a neutral zwitterionic form. TMAO is a compound that has a pK_a value of 4.6; it can exist in a zwitterionic or positively charged form depending on pH (Scheme 1) [17]. Several studies have been reported describing the effect of TMAO on protein stability at neutral pH [10–14]. Up to now, no calorimetric data are available on protein stability in the presence of TMAO. This paper is the first DSC study on the conformational stability of a small globular protein (RNase A) in the presence of TMAO at

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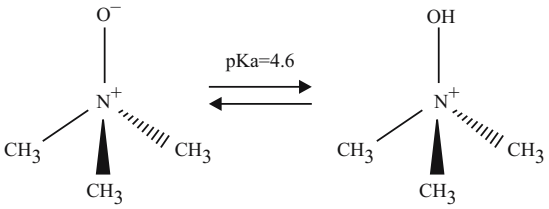

Scheme 1 Chemical structure of TMAO

different pH values. We report direct calorimetric measurements on the effect of TMAO on RNase A thermal denaturation at neutral and acid pH values and its counteracting ability against urea and GuHCl denaturants.

Experimental

Materials

Ribonuclease A (Type XII-A) was purchased from Sigma (St. Louis, Missouri) and used without further purification. Protein solutions for calorimetric and spectroscopic measurements were exhaustively dialyzed against the appropriate buffer solution at 4°C by using Spectra Por MW 6–8 kDa dialysis tubes. Protein concentration was determined spectrophotometrically using an extinction coefficient of 9800 M^{-1} cm^{-1} at 278 nm [18]. Buffer solutions, purchased from Sigma and prepared at 50 mM concentration, were sodium acetate for pH 4.0 and N-[hydroxyethyl] piperazine-N'-[2-ethansulfonate] (HEPES) for pH 7.0. The buffer solutions employed had low protonation enthalpies [19] in order to reduce the pH dependence on temperature. Urea was used after recrystallization from ethanol/water (1:1) mixtures; urea solutions were prepared immediately before use and the concentration determined by refractive index measurements [20]. A commercial (Sigma) 8 M solution was used for GuHCl. The water used for buffer and sample solutions was deionized and filtered through a Millipore Elix3 reagent grade system. The pH was measured at 20°C with a Radiometer pHmeter model PHM93.

Methods

Differential scanning calorimetry

DSC measurements were carried out on a Setaram Micro-DSC III instrument, interfaced with a data translation A/D board for automatic data acquisition. Data analysis was accomplished with in-house programs [21]. A scan rate of 1.0 K min^{-1} was chosen for all experiments, and protein concentration ranged from 1 to 2 mg mL^{-1}. The raw data were converted to an apparent 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 protein molar concentration in the sample cell. Finally, the excess heat capacity function, $<\Delta C_p>$, was obtained by subtraction of the baseline, given by linear extrapolation of the heat capacity of the native state [22]. The van't Hoff enthalpy was calculated by the commonly used formula [23]:

$$\Delta_d H(vH)=4RT_d^2<\Delta C_p(T_d)>/\Delta_d H \quad (1)$$

where T_d is the denaturation temperature corresponding to the maximum of DSC peak, $<\Delta C_p(T_d)>$ is the height of the excess heat capacity at T_d, $\Delta_d H$ is the total denaturation enthalpy change calculated by direct integration of the area of the DSC peak, and R is the gas constant. The finding that the calorimetric to van't Hoff enthalpy ratio is close to one is a necessary condition to state that the denaturation is a two-state transition [24].

Circular dichroism

CD spectra were recorded with a Jasco J-715 spectropolarimeter equipped with a Peltier type temperature control system (Model PTC-348WI). The instrument has been calibrated with an aqueous solution of d-10-(+)-camphorsulfonic acid at 290 nm [25]. Molar ellipticity per mean residue, [θ] in deg cm^2 $dmol^{-1}$, was calculated from the equation: $[\theta]=[\theta]_{obs}mrw/10lC$, where $[\theta]_{obs}$ is the ellipticity measured in degrees, mrw is the mean residue molecular mass, 110.5 Da for RNase A [26], C is the protein concentration in g mL^{-1} and l is the optical path length of the cell in cm. Cuvettes of 0.1 and 1 cm path length and protein concentrations of about 0.3 and 2.5 mg mL^{-1} were used in the far-UV and near-UV region, respectively. CD spectra, recorded at 0°C with a time constant of 4 s, a 2 nm band width and a scan rate of 5 nm min^{-1}, were signal-averaged over at least five scans, and baseline was corrected by subtracting a buffer spectrum.

Results and discussion

We selected a TMAO concentration range of 0–1 M to reproduce the typically intracellular concentration of the osmolytes [1]. In Table 1 are collected the thermodynamic parameters obtained by the analysis of calorimetric curves at pH 7.0 and pH 4.0. It was observed that in 50 mM HEPES buffer at pH 7.0, the denaturation temperature, T_d, increases by increasing TMAO concentrations, passing from 63.9°C in the absence to 67.2°C in the presence of 1 M TMAO. Denaturation enthalpy ($\Delta_d H$) and heat capacity ($\Delta_d C_p$) changes values slightly increase. On the contrary, in

Table 1 Thermodynamic parameters of RNase A in the presence of TMAO in 50 mM HEPES at pH 7.0 and 50 mM sodium acetate at pH 4.0

	TMAO/M	T_d/°C	Δ_dH/kJ mol^{-1}	Δ_dC_p/kJ mol^{-1} K^{-1}	Δ_dG^0/kJ mol^{-1} (298 K)
pH 7.0	0	63.9	467	5.1	42.1
	0.42	64.8	476	5.1	43.7
	0.5	65.5	488	5.1	45.6
	0.63	66.0	490	5.2	46.0
	1	67.2	509	5.2	49.0
	1.2	68.5	510	5.3	49.7
pH 4.0	0	57.5	413	5.7	31.3
	0.5	57.0	408	5.7	30.0
	1	56.0	411	5.7	30.2

Each value is the average over four measurements; the error in T_d does not exceed 0.2°C. The estimated relative uncertainties in Δ_dH and Δ_dC_p amount to 5 and 10%, respectively, of reported values

50 mM acetate buffer pH 4.0 the denaturation temperature T_d , slightly decreases at increasing TMAO concentrations, passing from 57.5°C in the absence to 56.0°C in the presence of 1 M TMAO and the values of denaturation enthalpy and heat capacity changes are unaffected by the presence of TMAO. Figure 1 shows representative DSC curves of RNase A in the absence and at increasing TMAO concentration at pH 7.0 (panel A) and at pH 4.0 (panel B). It is worth noting that the ratio between calorimetric enthalpy and van't Hoff enthalpy, Δ_dH(vH), calculated according to Eq. (1) is close to the unity in all the calorimetric experiments, indicating that a two-state model denaturation occurs in the absence and presence of TMAO at both neutral and acid pH values (data not shown). Thermal denaturation of RNase A was found reversible in the absence and presence of TMAO at both neutral and acid pH values. Our results clearly show that the conformational stability of RNase A is enhanced in the presence of TMAO at pH 7.0. In fact, as shown in Table 1, the standard denaturation Gibbs energy values, Δ_dG^0 (298 K), increase from 42 to 50 kJ mol^{-1} in the studied TMAO concentration range. These quantities represent the protein stability at the selected temperature and were calculated using the Gibbs–Helmholtz equation [23]:

$$\Delta_dG^0(T)=\Delta_dH(T_d)[1-T/T_d]+\Delta_dC_p[T-T_d-T\ln(T/T_d)] \quad (2)$$

in the assumption that Δ_dC_p is temperature-independent. A plot of the standard Gibbs energy of unfolding, Δ_dG^0, as a function of temperature for RNase A in the absence, and in the presence of TMAO is reported in Fig. 2, showing the parabola-like stability. On the contrary, at pH 4.0 the values of Δ_dG^0 (298 K), are not modified by the presence of TMAO. It emerges that at pH 4.0, the stability of RNase A is unaffected by the presence of TMAO. Actually, to compare the stability of a protein

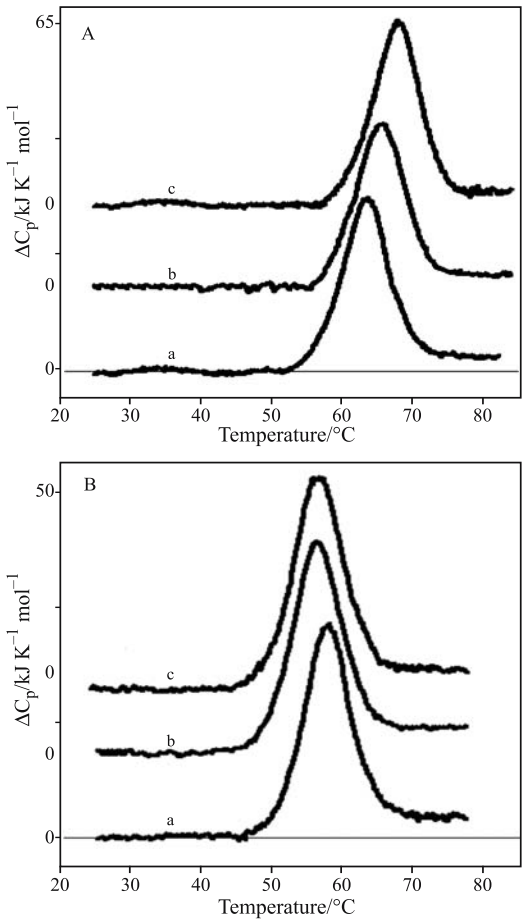

Fig. 1 A – Calorimetric profiles of RNase A a – in the absence, b – in the presence of TMAO at 0.5 M and c – 1 M in 50 mM HEPES buffer at pH 7.0. B – Calorimetric profiles of RNase A a – in the absence, b – in the presence of TMAO at 0.5 M and c – 1 M in 50 mM acetate buffer at pH 4.0

in the presence of TMAO with that in its absence, it is necessary to show that the structural characteristics of the native and denatured states are not affected by the

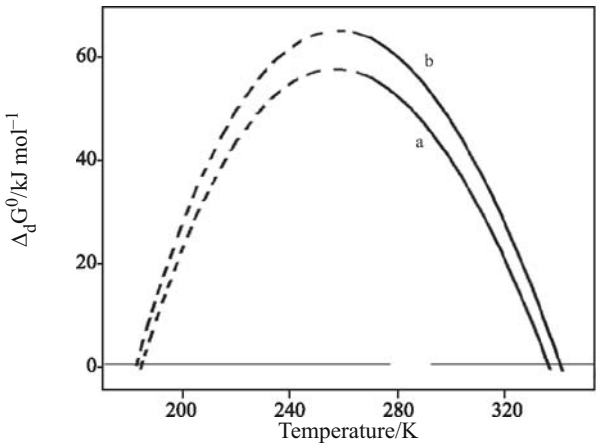

Fig. 2 Change in the standard Gibbs energy of unfolding, $\Delta_d G^0$, as a function of temperature for RNase A a – in the absence, and b – in the presence of 1.2 M TMAO in 50 mM HEPES buffer at pH 7.0

presence of the osmolyte. In Fig. 3 are reported the (A) far-UV CD and (B) near-UV CD spectra of both native and thermally denatured RNase A in the absence and presence of TMAO, indicating that the structure of RNase A does not change on adding TMAO to the aqueous solution. Amhad and coworkers [17] have found that near room temperature TMAO destabilizes proteins at pH values below its pK_a whereas it stabilizes proteins at pH values above its pK_a. The different behaviour observed at acid pH was attributed to the presence of a positive charge on the TMAO molecule. Our direct calorimetric results are in partially agreement with the conclusions of Amhad and coworkers. In fact, we confirmed the stabilization ability of TMAO on RNase A at pH 7.0, but we detected only a small decrease in denaturation temperature and practically constant values of $\Delta_d H$ at pH 4.0. The stabilizing (or destabilizing) effect of TMAO on protein stability is strictly connected with the ability to counteract the perturbing effect of urea and GuHCl. DSC measurements of RNase A in the presence of urea/TMAO or GuHCl/TMAO mixed solvents were performed at pH 7.0 and pH 4.0 and the thermodynamic parameters are reported in Table 2. The calorimetric profiles of RNase A in the absence and in the urea/TMAO or GuHCl/TMAO mixed solvent, at pH 7.0 and pH 4.0, are collected in Fig. 4.

In line with literature data [13, 1] we found that at a molar ratio of [urea]/[TMAO]=2/1, the denaturing effect of urea is balanced by the stabilizing effect of TMAO at pH 7.0. Moreover, at pH 7.0 a molar ratio of [GuHCl]/[TMAO]=1/1 is necessary to counteract the denaturing ability of GuHCl confirming the major efficiency of GuHCl with respect to urea. In contrast, at pH 4.0 the denaturing effects of urea and GuHCl are not affected by the presence of TMAO, in

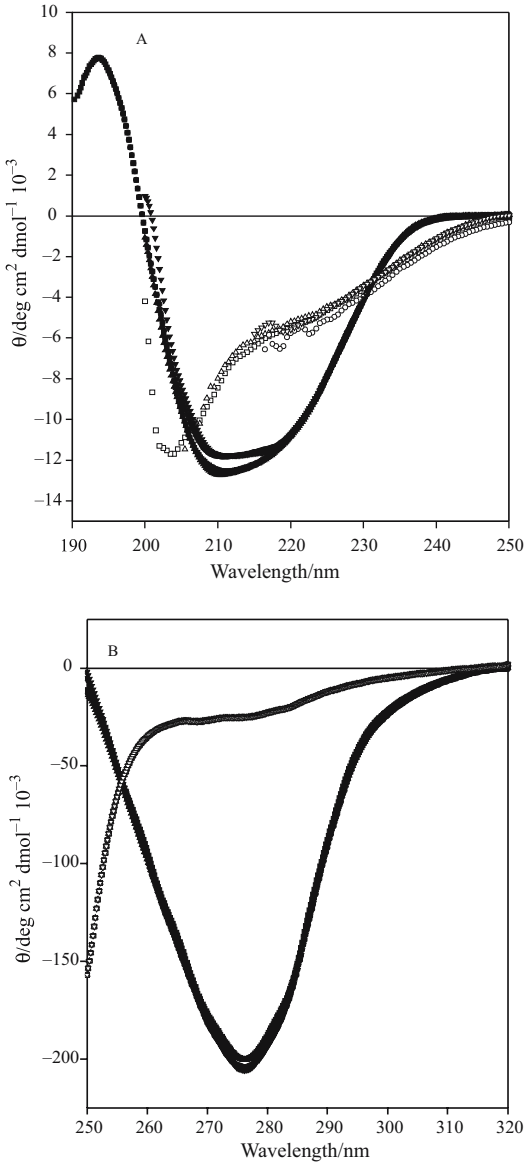

Fig. 3 A – Far-UV CD spectra of RNase A ○ – in absence, ▽ – in presence of TMAO at 0.5 M in 50 mM HEPES buffer at pH 7.0, and □ – in absence and △ – in presence of TMAO at 0.5 M in 50 mM acetate buffer at pH 4.0, recorded at 0°C (filled symbols) and 85°C (open symbols). B – Near-UV CD spectra of RNase A ○ – in absence, ▽ – in presence of TMAO at 0.5 M, □ – in 50 mM HEPES buffer at pH 7.0, and △ – in absence and in presence of TMAO at 0.5 M in 50 mM acetate buffer at pH 4.0, recorded at 0°C (filled symbols) and 85°C (open symbols)

line with our observation that TMAO has no stabilizing effect at pH 4.0 (Table 1). We can conclude that TMAO stabilize RNase A at neutral pH *vs*. both thermal and denaturant-induced unfolding, whereas at acid pH the protein stability is not affected by TMAO, in fact in these conditions the T_d and $\Delta_d H$ values are equal to those in aqueous solution (Table 2).

Table 2 Thermodynamic parameters of RNase A thermal denaturation in the presence of mixed solvents in 50 mM HEPES at pH 7.0 and 50 mM sodium acetate at pH 4.0

	TMAO/M	GuHCl/M	Urea/M	T_d/°C	Δ_dH/kJ mol^{-1}	Δ_dC_p/kJ mol^{-1} K^{-1}
pH 7.0	–	–	–	63.9	467	5.1
	–	–	1	60.7	435	5.2
	0.5	–	1	63.7	460	5.1
	–	0.5	–	58.5	444	5.4
	0.5	0.5		61.7	460	5.2
pH 4.0	–	–	–	57.5	413	5.7
	–	–	1	52.0	370	5.7
	0.5	–	1	52.0	374	5.7

Each value is the average over four measurements; the error in T_d does not exceed 0.2°C. The estimated relative uncertainties in Δ_dH and Δ_dC_p amount to 5 and 10%, respectively, of reported values

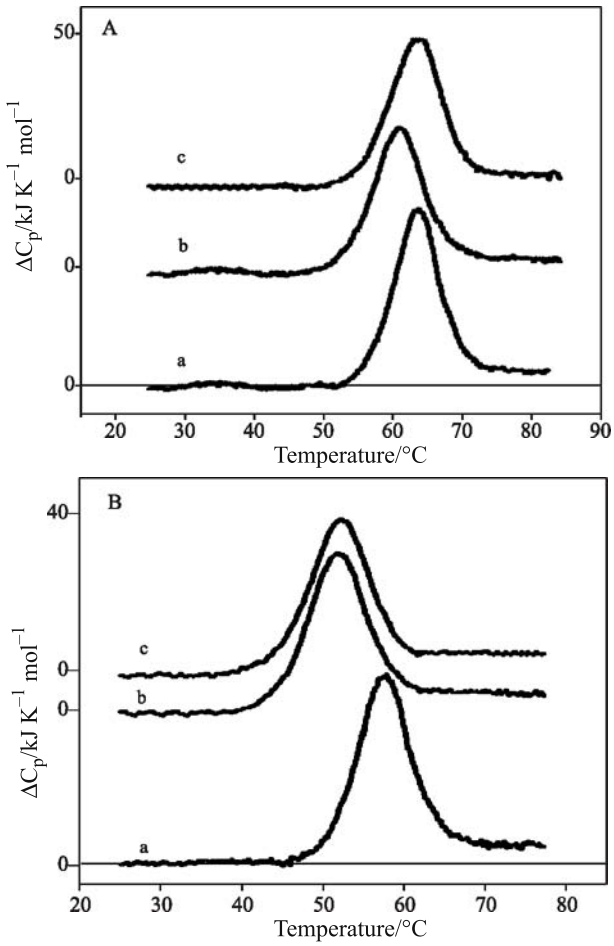

Fig. 4 A – Calorimetric profiles of RNase A in a – 50 mM HEPES buffer at pH 7.0; b – in the presence of 1 M urea; c – in the presence of 1 M urea and 0.5 M TMAO. B – Calorimetric profiles of RNase A in a – 50 mM acetate buffer at pH 4.0; b – in the presence of 1 M urea; and c – in the presence of 1 M urea and 0.5 M TMAO

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