Role of non-compatible osmolytes in the stabilization of proteins during heat stress

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This study is a systematic attempt to understand the roles of non-compatible osmolytes, i.e. solutes that have inhibitory effects on enzymes, in the stabilization of proteins against denaturing stress. Thermal denaturation of RNase A, holo- α -lactalbumin, apo- α -lactalbumin, lysozyme and metmyoglobin in the absence and presence of various concentrations of free basic amino acids was studied by observing changes in the absorption coefficients of these proteins. It has been observed that arginine and histidine destabilize all proteins in terms of the midpoint of the transition curve and Gibbs energy change on denaturation. Study of the

heat-induced denaturation of the proteins in the presence of various concentrations of arginine at different pH values demonstrated that arginine binds to the denatured molecules. In contrast with the effect of arginine and histidine on protein stability, it was observed that the effect of lysine on proteins stability is unpredictable, i.e. it may have a stabilizing effect, no effect or a destabilizing effect on proteins during denaturing stress. The results of this study are considered from an evolutionary perspective.

INTRODUCTION

It is a well-known fact that certain organisms that live under conditions of stress, be it heat stress, salt stress, water stress or cold stress, accumulate low-molecular-mass organic compounds collectively called osmolytes. These naturally occurring osmolytes are restricted to three main classes of compounds, namely polyols, free amino acids and their derivatives, and combinations of urea and methylamines [1]. Previous *in vitro* studies have reported the effect of some of these compounds on the activity of several enzymes [2]. The basic observations from these studies are: (i) enzyme activity near room temperature is not perturbed in the presence of most osmolytes, i.e. they are compatible solutes, and (ii) urea and free basic amino acids are not compatible solutes. It seems that organisms have evolved two mechanisms to protect their enzymes from the harmful effects of non-compatible osmolytes: (1) when urea is accumulated, organisms accumulate a second type of osmolyte to counteract the perturbation of proteins by the non-compatible solute [3]; (2) when a basic amino acid is released, organisms immediately convert it into a compatible solute [3].

In their study of the effect of osmolytes on thermal stability of enzymes in terms of T_m (the midpoint of the transition curve), Bowlus and Somero [4] have reported that Arg destabilizes RNase. This destabilizing effect has recently been confirmed [5]. During our studies of the thermal stability of cytochrome *c* in the presence of different concentrations of various free amino acids, we have observed that Arg decreases not only T_m but also ΔG_D° , the Gibbs energy of stabilization of the protein at 25 °C [6]. To see whether the destabilization behaviour of Arg is common to other proteins, we have made a systematic study of the thermodynamics of thermal denaturation of five proteins in the presence of different concentrations of Arg. We report that all proteins are

destabilized in terms of T_m and ΔG_D° in the presence of different concentrations of Arg near neutral pH. Timasheff [7] suggested that Arg can bind to denatured proteins, with its positive charge leading to a shift in the equilibrium, N (native) state $\leftrightarrow$ D (denatured) state, toward the denatured state, the native proteins becoming destabilized. To test more critically Timasheff's concept of binding of Arg to the denatured protein, we studied the thermal denaturation of RNase A in the presence of different concentrations of Arg in the pH range 6.0–1.5. We report that the destabilizing effect of Arg decreases with decreasing pH, i.e. with increasing number of positive charges on the protein. In fact, the destabilizing effect of Arg on RNase A is reversed at pH values below 3.0, i.e. it stabilizes the protein. Thus our results support the earlier conclusion on the binding of Arg to denatured protein [5,7].

Measurements of the Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) of several enzymic reactions in the presence of 0.0–0.1 M Lys have been reported by Bowlus and Somero [4]. They have shown that Lys inhibits enzyme activity. On the other hand, all available data on the effect of Lys on the thermal stability of proteins in terms of T_m suggest that this amino acid is a strong stabilizer of cytochrome *c* [6] and RNase A [5]. These thermal denaturation studies have, however, used higher concentrations of Lys. We thought it would be interesting to see the effect of lower concentrations of Lys on several proteins. We report that (i) Lys has both stabilizing and destabilizing effects on RNase A, (ii) myoglobin stability is unchanged in the presence of Lys, and (iii) Lys enhances the thermal stability of lysozyme, holo- α -lactalbumin and apo- α -lactalbumin.

It has been previously reported that His destabilizes cytochrome *c* [6]. This is the only report on His of which we are aware. We have studied the effect of this amino acid on five other proteins and report that all proteins are destabilized in its

Abbreviations used: ΔG_D , Gibbs energy of thermal denaturation; ΔG_D° , the value of ΔG_D at 25 °C; T_m , midpoint of thermal denaturation; ΔH_m , enthalpy change at T_m ; ΔC_p , constant-pressure heat-capacity change; GdmCl, guanidinium chloride; h-LA, holo- α -lactalbumin; a-LA, apo- α -lactalbumin; Mb, metmyoglobin.

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presence. In fact, on a molar scale, His is a stronger destabilizer than Arg.

MATERIALS AND METHODS

Commercial lyophilized preparations of bovine RNase A (type III A), α -lactalbumin (type I) from bovine milk, horse heart myoglobin and hen's egg-white lysozyme were purchased from Sigma Chemical Co. L-Histidine, L-arginine monohydrochloride and L-lysine monohydrochloride were from Aldrich. Guanidinium chloride (GdmCl) was ultrapure sample from Schwarz/Mann. These and other chemicals which were of analytical grade were used without further purification.

Concentrated stock solutions of RNase A, lysozyme and metmyoglobin (Mb) were prepared as reported previously [8]. To remove salts present in the protein sample, α -lactalbumin was dissolved and dialysed extensively against 0.1 M KCl, pH 7.0. Protein stock solutions were filtered using 0.45 μ m millipore filter paper, and the concentration of each protein was determined experimentally using molar absorption coefficient ($M^{-1} \cdot cm^{-1}$) values of 9800 at 277.5 nm for RNase A [9], 29210 at 280 nm for α -lactalbumin in 0.1 mM $CaCl_2$ [10], 39000 at 280 nm for lysozyme [11] and 171000 at 409 nm for Mb [12]. Apo- α -lactalbumin (a-LA) was prepared by adding 4 mM EGTA to the solution of holoprotein (with Ca^{2+} bound) (h-LA). The concentration of GdmCl stock solution was determined by refractive index measurements [13]. All solutions for optical measurements were prepared in the desired degassed buffer containing 0.1 M KCl.

Thermal denaturation studies were carried out in Jasco V-560 UV/Vis and UVIKON-930 spectrophotometers, with a heating rate of 0.5–1.0 $^{\circ}C/min$. Each sample was heated from 25 to 85 $^{\circ}C$. Change in absorbance with increasing temperature was followed at 287 nm for RNase A, 295 nm for h-LA and a-LA, 292 nm for lysozyme and 409 nm for Mb. The basic observation for each protein was a heat-induced transition curve, i.e. a plot of an optical property against temperature. To obtain values of T_m and ΔH_m (enthalpy change on denaturation at T_m), a non-linear least-squares analysis was used to fit all the data points of a transition curve according to the relation:

$$Y(T) = \frac{(a_N + b_N T) + (a_D + b_D T) \exp[-\Delta H_m / R(1/T - 1/T_m)]}{1 + \exp[-\Delta H_m / R(1/T - 1/T_m)]} \quad (1)$$

where $Y(T)$ is the optical property at temperature T (K), $(a_N + b_N T)$ gives the temperature-dependence of Y_N (the optical property of the native protein), $(a_D + b_D T)$ describes the temperature-dependence of Y_D (the optical property of denatured protein) and R is the gas constant. Using values of T_m , ΔH_m and ΔC_p (constant-pressure heat-capacity change), $\Delta G_D(T)$ (the Gibbs energy change at T) was estimated with the help of the following form of the Gibbs–Helmholtz equation:

$$\Delta G_D(T) = \Delta H_m(1 - T/T_m) - \Delta C_p[(T_m - T) + T \ln T/T_m] \quad (2)$$

To check the reversibility of the thermal denaturation, the protein solution was first heated to a temperature just high enough to cause complete denaturation. The heated protein solution was then cooled to 25 $^{\circ}C$ and its optical property compared with that of native protein.

RESULTS

All denaturation curves were measured at least three times. To convert the reversible heat-induced optical transition data into thermodynamic parameters, the following assumptions were made. First, the transition between N and D states follows a two-

Figure 1 Thermal denaturation curves for RNase A in the presence of different concentrations of His (a), Arg (b) and Lys (c) at pH 5.0

The buffer used was 0.05 M citrate/0.1 M KCl. For the sake of clarity, all data points are not shown. The inset in (b) shows the plot of ΔT_m against pH at Arg concentrations: 0.2 M (Δ), 0.5 M ($\square$), 0.7 M ($\bullet$) and 1.0 M ($\blacksquare$). The inset in (c) shows the plot of ΔT_m against [Lys]: 0.1 M (∇), 0.2 M (Δ), 0.35 M ($\blacktriangledown$), 0.5 M ($\bullet$), 0.7 M ($\blacksquare$), 1.0 M ($\square$) and 2.0 M ($\blacktriangle$).

state mechanism. Second, the temperature-dependences of Y_N and Y_D are linear. Third, osmolytes have no effect on the conformational ΔC_p of a protein. Making use of the first two assumptions, the thermal transition curve was analysed according to eqn. (1). This analysis gave values for ΔH_m and T_m , which along with the previously reported [14,15] value for ΔC_p were used in eqn. (2) to estimate Gibbs energy of stabilization at 25 $^{\circ}C$, ΔG_D° . The change in each of the parameters ($\Delta \Delta H_m$, ΔT_m and $\Delta \Delta G_D^{\circ}$) due to the presence of an osmolyte was obtained from the difference in the thermodynamic property in the presence and absence of the osmolyte.

Heat-induced denaturation of RNase A in the presence of different concentrations of osmolytes was followed by observing changes in $\Delta \epsilon_{287}$. Figure 1(a) shows the heat-induced denaturation of RNase A in the presence of different concentrations of His at pH 5.0. In all cases, about 350 data points ($\Delta \epsilon_{287}$, T) were collected. It can be seen that the temperature-dependences of Y_N and Y_D , the pre- and post-transition baselines respectively, do not depend on [His], the molar concentration of His. These temperature-dependences are given in Table 1. To obtain values for ΔH_m and T_m these observations were used in the analyses of transition curves shown in Figure 1(a) using eqn. (1). We obtained a value of 481 kJ $\cdot$ mol $^{-1}$ for ΔH_m at $T_m = 65.0$ $^{\circ}C$ of RNase A in the absence of His. This value for ΔH_m is in excellent agreement with that reported previously [16]. From the measured

Table 1 Coefficients of temperature-dependences of Y_N and Y_D [eqn. (1)]

$Y_N = a_N + b_N T$, and $Y_D = a_D + b_D T$. T is in K. Dimensions of a are $M^{-1} \cdot cm^{-1}$ and those of b are $M^{-1} \cdot cm^{-1} \cdot K^{-1}$. Results are means $\pm$ S.D.

Amino acid	[Amino acid] (M)	a_N	b_N	a_D	b_D
RNase A					
His	0–0.2	1406 $\pm$ 59	–4.693 $\pm$ 0.015	–3397 $\pm$ 132	3.725 $\pm$ 0.014
Arg	0.2	1406 $\pm$ 59	–4.693 $\pm$ 0.015	–3321 $\pm$ 40	3.713 $\pm$ 0.021
	0.5	1406 $\pm$ 59	–4.693 $\pm$ 0.015	–3120 $\pm$ 60	3.225 $\pm$ 0.033
	0.7	1406 $\pm$ 59	–4.693 $\pm$ 0.015	–2916 $\pm$ 35	2.603 $\pm$ 0.061
	1.0	1406 $\pm$ 59	–4.693 $\pm$ 0.015	–2779 $\pm$ 50	2.411 $\pm$ 0.024
Lys	0–1.0	1406 $\pm$ 59	–4.693 $\pm$ 0.015	–3397 $\pm$ 132	3.725 $\pm$ 0.014
	2.0	1406 $\pm$ 59	–4.693 $\pm$ 0.015	–4005 $\pm$ 99	6.148 $\pm$ 0.029
h-LA					
His	0–0.25	–3379 $\pm$ 119	11.367 $\pm$ 0.027	–8383 $\pm$ 77	20.019 $\pm$ 0.022
Arg	0.3–1.0	–3379 $\pm$ 119	11.367 $\pm$ 0.027	–5478 $\pm$ 114	10.964 $\pm$ 0.023
Lys	0.2	–3379 $\pm$ 119	11.367 $\pm$ 0.027	–4134 $\pm$ 150	8.012 $\pm$ 0.031
	0.5	–3379 $\pm$ 119	11.367 $\pm$ 0.027	–3686 $\pm$ 120	7.115 $\pm$ 0.015
	0.7	–3379 $\pm$ 119	11.367 $\pm$ 0.027	–4446 $\pm$ 129	8.113 $\pm$ 0.027
	1.0	–3379 $\pm$ 119	11.367 $\pm$ 0.027	–2451 $\pm$ 166	2.947 $\pm$ 0.013
	2.0	–3379 $\pm$ 119	11.367 $\pm$ 0.027	–5205 $\pm$ 163	11.929 $\pm$ 0.038
a-LA					
His	0–0.25	–3380 $\pm$ 377	11.416 $\pm$ 0.022	–7631 $\pm$ 384	19.042 $\pm$ 0.043
Arg	0–1.0	–3380 $\pm$ 377	11.416 $\pm$ 0.022	–7631 $\pm$ 384	19.042 $\pm$ 0.043
Lys	0–2.0	–3380 $\pm$ 377	11.416 $\pm$ 0.022	–7631 $\pm$ 384	19.042 $\pm$ 0.043
Lysozyme					
His	0–0.15	4481 $\pm$ 50	–15.114 $\pm$ 0.213	1040 $\pm$ 14	–9.610 $\pm$ 0.008
Arg	0–1.0	4481 $\pm$ 50	–15.114 $\pm$ 0.213	1050 $\pm$ 14	–9.610 $\pm$ 0.008
Lys	0–1.0	2787 $\pm$ 100	–9.384 $\pm$ 0.021	–1576 $\pm$ 126	–3.589 $\pm$ 0.016
Mb					
His	0.00	275148 $\pm$ 993	–349.082 $\pm$ 3.219	263830 $\pm$ 340	–701.503 $\pm$ 20.650
	0.05–0.15	275148 $\pm$ 993	–349.082 $\pm$ 3.219	223876 $\pm$ 205	–603.231 $\pm$ 23.170
Arg	0.25	273105 $\pm$ 267	–351.409 $\pm$ 6.103	265997 $\pm$ 152	–695.112 $\pm$ 24.511
	0.50	271562 $\pm$ 482	–345.181 $\pm$ 8.712	267215 $\pm$ 716	–718.001 $\pm$ 30.993
	0.75	268301 $\pm$ 331	–350.009 $\pm$ 3.773	270105 $\pm$ 510	–705.520 $\pm$ 17.211
	1.00	267037 $\pm$ 645	–352.144 $\pm$ 7.161	272113 $\pm$ 429	–689.914 $\pm$ 29.071
Lys	0–0.5	275148 $\pm$ 993	–349.082 $\pm$ 3.219	263830 $\pm$ 340	–701.503 $\pm$ 20.650

values of ΔH_m and T_m , values for $\Delta\Delta H_m$ and ΔT_m were estimated in the presence of each [His]. For example, since analyses of the results for RNase A in 0.2 M His gave $\Delta H_m = 458 \text{ kJ} \cdot \text{mol}^{-1}$ and $T_m = 60.7^\circ\text{C}$, the values for $\Delta\Delta H_m$ and ΔT_m are $-23 (= 458-481) \text{ kJ} \cdot \text{mol}^{-1}$ and $-4.3 (= 60.7-65.0)^\circ\text{C}$ at [His] = 0.2 M. Table 2 shows the values of $\Delta\Delta H_m$ and ΔT_m for RNase A in the presence of different concentrations of His. Values of ΔG_D° in the presence of the osmolyte were determined using a value of $5.14 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ for ΔC_p [14] in eqn. (2). Values of $\Delta\Delta G_D^\circ$ for RNase A at different concentrations of His are also shown in Table 2.

Figure 1(b) shows the thermal denaturation of RNase A in the presence of various concentrations of Arg at pH 5.0. It can be seen that Y_N does not show any dependence on [Arg] and there is a shift in Y_D with increasing [Arg]. The temperature-dependence of Y_N and Y_D at each [Arg] is given in Table 1. Using these observations, transition curves in the presence of different concentrations of the osmolyte were analysed according to eqn. (1). From these analyses, values of $\Delta\Delta H_m$ and ΔT_m at different concentrations of Arg were determined (see Table 2). $\Delta\Delta G_D^\circ$ values at different concentrations of the osmolyte are also shown in Table 2.

Figure 1(c) shows thermal denaturation of RNase A in the presence of different concentrations of Lys at pH 5.0. The

temperature-dependence of Y_N and Y_D were found to be independent of [Lys] in the range 0.0–1.0 M. However, the temperature-dependence of Y_D in the presence of 2 M Lys is different from that observed at lower concentrations (see Figure 1c). These temperature-dependences of Y_N and Y_D at each [Lys] are given in Table 1; they were used to analyse transition curves according to eqn. (1). Values of ΔH_m , T_m and ΔG_D° were determined at each [Lys]. Table 2 shows the values of $\Delta\Delta H_m$, ΔT_m and $\Delta\Delta G_D^\circ$.

In addition to pH 5.0, thermal denaturation of RNase A was also studied in the presence of Arg at pH 6.0, 3.0, 2.0 and 1.5 (transition curves not shown). It was observed that the temperature-dependence of Y_N is independent of [Arg] at all pH values. In contrast, Y_D exhibited dependence on temperature, pH and [Arg]. We therefore determined the temperature-dependence of Y_D at each pH and [Arg]. (For the sake of brevity these dependences are not given here.) These observations were used in the analyses of the transition curve at each [Arg] and pH according to eqn. (1). The values of ΔH_m ($\text{kJ} \cdot \text{mol}^{-1}$) at T_m ($^\circ\text{C}$) in the absence of osmolytes are 493 at 65.5, 385 at 46.6, 339 at 36.0 and 318 at 30.5 respectively at pH 6.0, 3.0, 2.0 and 1.5. These values are in excellent agreement with those reported previously [16–18]. Table 3 shows the values of ΔT_m , $\Delta\Delta H_m$ and $\Delta\Delta G_D^\circ$ at different pH values and Arg concentrations.

Table 2 Thermodynamic parameters associated with thermal denaturation of various proteins in the presence of non-compatible osmolytes

For each protein, non-linear least-squares analyses of transition curves using eqn. (1) gave standard deviations in the range 8–12 kJ·mol⁻¹ for ΔH_m and 0.1–0.2 °C for T_m .

Histidine				Arginine				Lysine			
[His] (M)	$\Delta\Delta H_m$ (kJ·mol ⁻¹)	ΔT_m (°C)	$\Delta\Delta G_D^\circ$ (kJ·mol ⁻¹)	[Arg] (M)	$\Delta\Delta H_m$ (kJ·mol ⁻¹)	ΔT_m (°C)	$\Delta\Delta G_D^\circ$ (kJ·mol ⁻¹)	[Lys] (M)	$\Delta\Delta H_m$ (kJ·mol ⁻¹)	ΔT_m (°C)	$\Delta\Delta G_D^\circ$ (kJ·mol ⁻¹)
RNase A											
0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0	0	0.0	0.0
0.1	-20	-1.5	-3.5	0.2	-21	-3.6	-4.7	0.1	0	-1.7	-1.1
0.2	-23	-4.3	-5.5	0.5	-59	-4.5	-9.3	0.2	4	-2.2	-1.1
				0.7	-67	-4.5	-10.2	0.35	-13	-1.3	0.2
				1.0	-63	-4.5	-9.8	0.5	-8	-1.2	-1.7
								0.7	0	0.4	0.3
								1.0	-15	1.6	-0.8
								2.0	-13	4.2	0.4
h-LA											
0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0	0	0.0	0.0
0.13	-30	-1.3	-3.3	0.3	-50	-4.3	-5.4	0.2	-7	0.4	0.9
0.25	-27	-2.3	-3.1	0.5	-64	-5.4	-6.9	0.5	2	0.9	0.0
				0.8	-68	-6.8	-7.2	0.7	-11	1.4	1.7
				1.0	-69	-7.9	-7.2	1.0	-4	3.5	0.8
								2.0	-2	3.9	1.7
a-LA											
0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0	0	0.0	0.0
0.13	-13	-0.7	-0.9	0.3	-5	-0.3	-0.4	0.1	-1	2.7	0.7
0.25	-26	-1.1	-1.7	0.5	-18	-0.6	-1.2	0.2	-4	5.6	1.1
				1.0	-23	-2.1	-1.8	0.5	-1	7.2	1.7
								0.7	-3	8.0	1.5
								1.0	-5	9.7	1.6
								2.0	-8	12.1	1.5
Lysozyme											
0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0	0	0.0	0.0
0.05	-13	-0.8	-1.7	0.25	-50	-0.9	-5.4	0.2	5	2.0	1.3
0.15	-84	-3.8	-9.6	0.75	-109	-4.5	-12.1	0.5	4	3.7	1.3
				1.00	-130	-6.1	-14	0.7	4	4.3	1.3
								1.0	5	6.7	1.8
Mb											
0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0	0	0.0	0.0
0.05	-54	-3.7	-4.2	0.25	-54	-3.8	-4.2	0.05	1	0.3	0.0
0.10	-75	-6.6	-5.0	0.50	-75	-6.6	-5.0	0.15	1	0.1	0.1
0.15	-88	-7.4	-5.9	0.75	-100	-8.4	-6.7	0.25	2	0.1	0.3
				1.00	-117	-10.7	-7.1	0.50	5	0.2	0.4

Thermal denaturation of α -lactalbumin preparations (h-LA and a-LA) in the presence of different concentrations of various osmolytes was followed by observing changes in $\Delta\epsilon$ at 295 nm. About 500 data points ($\Delta\epsilon_{295}$, T) were collected for each transition curve. Their thermal denaturations in the presence as well as the absence of all osmolytes were found to be completely reversible. It has been observed (i) that the temperature-dependences of Y_N of both preparations and that of Y_D of a-LA are independent of the nature and concentration of amino acid, and (ii) that the temperature-dependence of Y_D of h-LA does not depend on [His] but shows dependence on [Arg] and [Lys]. These dependences of both preparations are given in Table 1. Using these observations, each transition curve of h-LA and a-LA was analysed by eqn. (1) to obtain values for ΔH_m and T_m . For h-LA a value of 309 (kJ·mol⁻¹) for ΔH_m at 65.3 °C (the value of the observed T_m in the absence of osmolytes) is in excellent agreement with that reported previously [15]. A value of 7.1 kJ·mol⁻¹·K⁻¹ for ΔC_p [15] was used in the estimation of ΔG_D° of the holoprotein, using eqn. (2). The values of $\Delta\Delta H_m$, ΔT_m and $\Delta\Delta G_D^\circ$ at different concentrations of His, Arg and Lys are shown in Table 2. For a-LA in the absence of osmolytes we obtained a value of 217 kJ·mol⁻¹ for

ΔH_m at $T_m = 42.2$ °C, which is in excellent agreement with the value reported previously [15]. A value of 6.18 kJ·mol⁻¹·K⁻¹ for ΔC_p [15] was used to determine ΔG_D° of the apoprotein preparation in the presence of different concentrations of each osmolyte. Table 2 shows the values of $\Delta\Delta H_m$, ΔT_m and $\Delta\Delta G_D^\circ$.

Thermal denaturation of lysozyme in the presence of different concentrations of osmolytes was studied by observing changes in $\Delta\epsilon$ at 292 nm. It has been observed that (i) thermal denaturation of lysozyme at pH 2.50 is reversible in the presence and absence of His and Arg, and (ii) Y_N and Y_D do not depend on [His] and [Arg], but they do depend on temperature. These temperature-dependences of Y_N and Y_D are given in Table 1. In the absence of His and Arg, a value of 380 kJ·mol⁻¹ for ΔH_m at $T_m = 62.3$ °C was obtained at pH 2.50. This value is in good agreement with that reported previously [19]. A value of 6.56 kJ·mol⁻¹·K⁻¹ for ΔC_p [14] was used to determine ΔG_D° values at different osmolyte concentrations. Table 2 shows the values of $\Delta\Delta H_m$, ΔT_m and $\Delta\Delta G_D^\circ$ at different concentrations of His and Arg.

Heat-induced denaturation of lysozyme in the presence of different concentrations of Lys was found to be irreversible at pH 2.50. It has, however, been observed that thermal

Table 3 Thermodynamic parameters associated with thermal denaturation of RNase A in the presence of Arg at different pH values

For pH 6.0 and 3.0, the buffer used was 0.05 M citrate/0.1 M KCl, and for pH 2.0 and 1.5, it was 0.05 M KCl/HCl with 0.1 M KCl. For each protein, analysis of transition curves using eqn. (1) gave standard deviations in the range 8–12 kJ·mol⁻¹ for ΔH_m and 0.1–0.2 °C for T_m .

pH	[Arg] (M)	$\Delta\Delta H_m$ (kJ·mol ⁻¹)	ΔT_m (°C)	$\Delta\Delta G_D^\circ$ (kJ·mol ⁻¹)
6.0	0.0	0	0.0	0.0
	0.2	-25	-1.3	-2.9
	0.5	-50	-4.3	-8.5
	0.7	-63	-4.3	-9.9
	1.0	-63	-4.3	-9.9
3.0	0.0	0	0.0	0.0
	0.2	-8	0.0	-0.6
	0.5	-29	0.2	-1.8
	0.7	-42	1.2	-2.1
	1.0	-50	1.9	-2.2
2.0	0.0	0	0.0	0.0
	0.2	-8	1.6	1.0
	0.5	-25	3.1	1.5
	0.7	-17	3.5	2.1
	1.0	-17	3.5	1.8
1.5	0.0	0	0.0	0.0
	0.2	-4	5.4	4.6
	0.5	-8	7.2	5.8
	0.7	-13	8.7	6.1
	1.0	-2	8.7	6.1

denaturation of the enzyme is completely reversible in the presence of 1.5 M GdmCl at pH 4.74. The addition of GdmCl at this pH has two advantages. First, it brings down the transition to a measurable temperature range. Secondly, it improves the reversibility in the presence of Lys. It has been observed that Y_N and Y_D do not depend on [Lys] but they show dependence on temperature. The temperature-dependences of Y_N and Y_D are given in Table 1. These observations were used to analyse thermal transition curves according to eqn. (1). We obtained a value of 334 kJ·mol⁻¹ for ΔH_m at $T_m = 63.1$ °C for the protein in the presence of 1.5 M GdmCl at pH 4.74. It has been reported that GdmCl contributes to the measured ΔH for lysozyme because of its preferential binding to the protein [19]. The decrease in ΔH due to this solvation effect of 1.5 M GdmCl is about 63 kJ·mol⁻¹ (see Figure 23 in [19]). Thus we obtained a value of 397 kJ·mol⁻¹ for lysozyme in the absence of the chemical denaturant at pH 4.74, which is in good agreement with the values reported previously [19]. A value of 6.68 kJ·mol⁻¹·K⁻¹ for ΔC_p in the presence of 1.5 M GdmCl [20] was used in eqn. (2) for the determination of ΔG_D° . The values of $\Delta\Delta H_m$, ΔT_m and $\Delta\Delta G_D^\circ$ are given in Table 2.

Thermal denaturation of Mb at pH 5.95 was followed by observing changes in ϵ_{409} . This change reflects changes in the interaction of the haem group with the globin [21]. It has been observed that thermal denaturation of the protein is reversible if the protein solution containing 0.6 M GdmCl is not exposed to high temperatures for a long time. GdmCl not only improves the reversibility of thermal denaturation but also brings down the transition curve to an experimentally measurable temperature range.

In the case of His, it has been observed that Y_N is independent of [His] but depends on temperature. The temperature-dependence of Y_N is given in Table 1. On the other hand, Y_D depends

Figure 2 Thermal denaturation curves of Mb in 0.05 M citrate/0.1 M KCl buffer (pH 5.95) in the presence of different concentrations of Arg

For the sake of clarity, all data points are not shown.

on [His] as well as temperature. The temperature-dependences of Y_D in the absence and presence of His in the concentration range 0.05–0.15 M are also given in Table 1. In order to estimate values of ΔH_m and T_m , each transition curve was analysed using eqn. (1); the observations are given in Table 1. In the absence of His, a value of 339 kJ·mol⁻¹ for ΔH_m was obtained at $T_m = 66.8$ °C. This value is in good agreement with that reported previously [22]. Table 2 shows the values of $\Delta\Delta H_m$ and ΔT_m in the presence of different concentrations of His. Values of ΔG_D° under different conditions were estimated with the help of eqn. (2) using a value of 11.57 kJ·mol⁻¹·K⁻¹ for ΔC_p [14]. Values of $\Delta\Delta G_D^\circ$ are also given in Table 2.

Figure 2 shows the thermal transition of Mb in the presence of different concentrations of Arg at pH 5.95. Y_N and Y_D depend on [Arg] as well as on temperature. The dependences of Y_N and Y_D on the composition variables are given in Table 1. These observations were used in the analysis of each transition curve. Values for $\Delta\Delta H_m$, ΔT_m and $\Delta\Delta G_D^\circ$ in the presence of different concentrations of Arg are given in Table 2.

Thermal denaturation of Mb in the presence of various concentrations of Lys at pH 5.95 was also measured. No effect of Lys in the concentration range 0.0–0.5 M was observed on the thermal denaturation of Mb. We also attempted to see the effect of Lys at concentrations higher than 0.5 M, but heat-induced denaturation of the protein at these higher concentrations led to precipitation of the protein.

DISCUSSION

The estimation of Gibbs energy of stabilization and analysis of denaturation curves are based on certain assumptions. It is therefore necessary to make a few comments. First, in the estimation of ΔG_D (25 °C) using eqn. (2), it has been assumed that the values of ΔC_p associated with the conformational transition between N and D states of each protein in the absence and presence of different osmolytes remains unchanged. We have checked the validity of this assumption by estimating this parameter in the cases of a few proteins. The recommended procedure for the estimation of ΔC_p involves (i) measurement of heat denaturation of the protein at different pH values, and (ii) determination of ΔC_p from the ΔH_m versus T_m plot [19]. Such data are available for RNase A at different Arg concentrations and pH values. Plots of ΔH_m against T_m for RNase A at different

Figure 3 Temperature-dependence of the enthalpy change of RNase A in the presence of different concentrations of Arg

Symbols have the same meaning as in Figure 1(b).

Arg concentrations are shown in Figure 3. A linear least-squares analysis of each plot gave the value of $\Delta C_p = (\partial \Delta H_m / \partial T_m)_p$. Values of ΔC_p at different Arg concentrations are given in Figure 3. It can be seen that the conformational ΔC_p value is, within experimental error, not only independent of [Arg] but also identical with the calorimetric value of $5.14 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ reported previously [14]. A direct determination of the ΔC_p for protein is also possible from extrapolation of pre- and post-transition baselines of the constant-pressure heat capacity versus temperature plot [19]. It should, however, be noted that ΔC_p thus determined (i) shows strong dependence on the choice of baseline extrapolation and hence involves large errors, and (ii) includes contributions from conformational change, solvent, etc. [19], and thus is apparent. It has been observed that the apparent ΔC_p values of h-LA and a-LA thus determined are, within experimental error, the same in the absence and presence of different concentrations of Arg. His and other osmolytes (F. Ahmad and W. Pfeil, unpublished work). Secondly, in the analyses of transition curves using eqn. (1), it has been assumed that the heat-induced denaturation of proteins in the absence and presence of osmolytes follows a two-state mechanism. This is indeed true in the absence of osmolytes [14,15]. A comparison of van't Hoff ΔH_m (this study) with the calorimetric value (F. Ahmad and W. Pfeil, unpublished work) suggests that thermal denaturation of h-LA and a-LA is a two-state process, as both estimates of ΔH_m at any osmolyte concentrations are the same within experimental error. It is interesting to note that two-state behaviour of heat-induced denaturation of RNase A and lysozyme in the presence of different osmolytes has been reported by Santoro et al. [16]. It would therefore appear that the use of a two-state assumption in the analysis of our results is reasonable.

Measurements of K_m and V_{max} of enzymes in the presence of different concentrations of Arg suggest that Arg has inhibitory effects on all enzymes [4,23]. They also show that RNase is destabilized in the presence of Arg, as the T_m of the protein decreases with increasing [osmolyte]. A decrease in the thermal stability of cytochrome *c* in the presence of Arg has also been reported [6]. In order to understand the mechanism of destabilization of proteins by Arg, Lin and Timasheff [5] examined the role of the preferential hydration effect (surface tension effect) and the preferential binding effect in protein stability using RNase A as a model compound. Their measurements of preferential interactions of the protein with Arg showed that (i) the native protein is preferentially hydrated, indicating that Arg stabilizes the native protein, and (ii) the preferential hydration decreases with increasing [Arg], indicating that the amino acid binds weakly to the protein. Thus two counteracting effects, namely the surface tension effect and the preferential binding effect, act on the equilibrium $N \leftrightarrow D$ that exists in the thermal transition region of the protein in the presence of Arg [5,24]. The preferential binding effect, which is a critical factor for the destabilization of proteins [25], stems from the fact that Arg has potential to interact directly with the protein, e.g. by hydrogen-bonding to the peptide group [26]. Lin and Timasheff [5] argued that a decrease in the T_m of RNase A on the addition of Arg to the protein solution occurred because, during denaturation, the additional Arg binding was sufficient to counteract the surface tension effect, which is a critical factor in the stabilization of proteins.

Both RNase A and Arg are positively charged at pH 6.7, the experimental conditions used previously [5]. The positive charge density increases on decreasing the pH of the solution, as their pI values are greater than 7. It is therefore expected that Arg-protein interactions decrease significantly if the pH is lowered from 7. To test this hypothesis we studied the thermal denaturation of RNase A in the presence of various fixed concentrations of Arg at pH 6.0, 5.0, 3.0, 2.0 and 1.5 (see Tables 2 and 3). A plot of ΔT_m against pH is shown in Figure 1(B) (see the inset); as expected, the destabilization effect of Arg decreases non-linearly with decreasing pH. It can also be seen that the amino acid behaves as a stabilizer (i.e. $\Delta T_m > 0$) below pH 3, which means that the surface tension effect overcomes the preferential binding effect. Our results for RNase A therefore support the earlier conclusion that the observed T_m of the protein in the presence of Arg is a combination of increased surface tension and weak binding [5].

To see whether RNase A destabilization by Arg during denaturation is a general effect of the amino acid on proteins, we studied the heat-induced denaturation of four further systems, namely Mb, h-LA, a-LA and lysozyme, in the presence of various concentrations of Arg. We concluded that Arg destabilizes all proteins, as both ΔT_m and $\Delta \Delta G_D^\circ$ were found to be negative (see Table 2).

Like the effect of Arg on proteins, Lys inhibits enzyme function [4,23], preferentially hydrates native proteins [5,24], binds to denatured proteins [5], and has the potential to interact with peptide backbone through hydrogen-bonding [26]. On the other hand, unlike the destabilizing effect of Arg on thermal denaturation of proteins, all available data suggest that Lys stabilizes proteins against heat denaturation [5,6,26]. In the earlier studies, enzyme activity was measured in the presence of Lys at a concentration of 0.1 M or lower [4,23], whereas all heat-induced denaturations were measured in the presence of much higher concentrations [5,6,26]. In the case of RNase A, for instance, Lin and Timasheff [5] reported T_m measurements at Lys concentrations of 0.5 M or higher. Does this mean that higher concentrations of Lys ($> 0.1 \text{ M}$) have a stabilizing effect

on proteins and lower concentrations (< 0.1 M) have a destabilizing effect which is responsible for the loss of enzyme activity? To check this possibility, we studied thermal denaturation of RNase A in the presence of different concentrations of Lys (0–2 M). All the thermodynamic data are given in Table 2, and a plot of T_m against [Lys] is shown in Figure 1(c) (see the inset). It can be seen that, as expected, lower concentrations of the amino acid destabilize the protein ($\Delta T_m < 0$), whereas it is stabilized in the presence of higher concentrations of Lys. The latter observation is consistent with the results reported previously [5].

To see whether the effect of Lys on RNase A is non-specific, i.e. independent of the protein used, we studied heat-induced denaturation of four further systems. It can be seen in Table 2 that (i) Lys protects h-LA, a-LA and lysozyme against thermal denaturation, as $\Delta T_m > 0$ at each [amino acid], and (ii) Lys up to 0.5 M has no effect on the thermal stability of Mb. We conclude that Lys may have a stabilizing effect, no effect or a destabilizing effect on proteins during denaturing stress.

Unlike for Arg and Lys, no data are available on the preferential hydration of native proteins in the presence of His and the preferential interaction of denatured proteins with His, nor on the effect of His on enzyme activity. It has, however, been shown that His destabilizes cytochrome *c* during thermal denaturation [6]. A possible explanation for this destabilizing behaviour, based on the observations of Clarke and Zounce [27], is that ions that denature proteins and the side chains of organic solutes that accumulate during various stress situations have some similarity. If one accepts this hypothesis, all proteins should be destabilized by His during denaturation. To test this, thermal denaturation of several proteins was studied in the presence of different concentrations of His. The thermodynamic data are shown in Table 2: His destabilizes all of these proteins, as $\Delta T_m < 0$ and $\Delta \Delta G_D^\circ < 0$ at each [His]. A comparison of the destabilizing effect of His with that of Arg suggests that, on a molar scale, His is a more effective destabilizer of all proteins (see Table 2).

If we consider the results of this study and those reported previously [4–6,23] from an evolutionary perspective, we can argue that free Arg and His cannot be accumulated by organisms, as they have deleterious effects on proteins, and that, if they are produced in the cell, evolution must have produced systems to counteract the deleterious effects of these amino acids. It is interesting to note that, in the case of release of free Arg, organisms have evolved an enzyme, octopine dehydrogenase, that converts Arg into a compatible solute, octopine [4,23]. It is also interesting to note that the presence of histidinebetaine has been shown in many organisms that evolved to have enzymes that convert free His to its betaine derivative [28]. Whether the histidinebetaine is a compatible solute is not known. However, since all the betaines that have been studied for their effects on

enzyme activity are compatible solutes, and since histidinebetaine is accumulated in several organisms [4,23,28], it is expected that it does behave as a compatible osmolyte. As far as Lys is concerned, we are not aware of any evidence for the accumulation of Lys or its derivative(s) by any organisms under condition of stress. There could be at least two reasons for not utilizing Lys as an osmolyte: (1) it has inhibitory effects on enzyme function [4,23]; (2) the effect of Lys on protein stability is unpredictable, i.e. it may have a stabilizing effect, no effect or a destabilizing effect during denaturing stress.

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