Increased thermal stability of proteins in the presence of amino acids

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This study is a systematic attempt to understand the roles of osmolytes in protecting proteins against denaturing stress. Thermal denaturation of cytochrome *c* has been studied in the presence of various concentrations of all L-amino acids that are more hydrophobic than glycine and have a solubility of 0.1 M or higher in water at 25 °C. The basic observations are as follows. (1) Arginine and histidine destabilize the native protein; both T_m (the midpoint of thermal transition) and $\Delta G_D^{H_2O}$ (25 °C) (the Gibbs energy of stabilization) decrease with increasing amino acid concentration. (2) Isoleucine, leucine and phenylalanine

have no effect on T_m and $\Delta G_D^{H_2O}$ (25 °C). (3) Valine and less hydrophobic amino acids stabilize the protein in terms of T_m but $\Delta G_D^{H_2O}$ (25 °C) is unchanged. This observation was confirmed by the study of isothermal denaturation of cytochrome *c* by guanidinium chloride which suggested that $\Delta G_D^{H_2O}$ is independent of osmolyte concentration, but C_m (the midpoint of transition) is increased in their presence. (4) In the case of stabilizers, change in T_m /mol of amino acid decreases with increasing hydrophobicity of these osmolytes.

INTRODUCTION

Almost all *in vivo* studies on plants and micro-organisms have shown that various low-molecular-mass organic compounds known collectively as osmolytes are accumulated under stress, be it heat stress, salt stress, water stress or cold stress [1]. Naturally occurring osmolytes are chemically restricted to three main classes of compound, namely polyhydric alcohols, free amino acids and their derivatives, and combinations of urea and methylamines [1]. Previous *in vitro* studies [2–10] have reported the effect of some of these compounds on the structure and function of different enzymes and proteins. The basic observations from these studies are: (1) osmolytes may enhance protein stability, their effect being independent of the chemical nature of the protein; (2) osmolytes may protect enzymes against heat inactivation although the enzyme activity is not perturbed in their presence at room temperature; (3) neither source nor type of enzyme is important, as comparable effects were observed with various enzymes from yeast, wheat and chicken.

Plants are basically poikilotherms, incapable of controlling their temperature to any great extent. Crop plants generally grow in the temperature range 15–45 °C, and they may suffer heat injury when temperature exceeds their normal growing range [11]. Earlier studies [1,12] appear to support the premise that osmolytes stabilize protein against heat stress and suggest that a more quantitative and detailed investigation would be useful in establishing the scope and character of the phenomenon. We have therefore investigated the thermodynamic basis for the protection of enzymes and proteins by the compounds that accumulate during heat stress. This is the first report of such a study. We present here measurements of the effects of various L-amino acids on the thermal denaturation of cytochrome *c* (cyt-*c*).

As the source of the protein is not important, and as osmolyte–protein interaction is non-specific and protein behaves as an inert solution component, we chose horse heart cyt-*c* for our study. Other reasons for selecting this protein are: (1) thermal denaturation can be monitored in the visible region where none of the amino acids absorb; (2) microcalorimetric measurements have shown that thermal denaturation of cyt-*c*

follows a two-state mechanism [13,14], which makes analysis of the conformational transition curve easier. From measurements of thermal denaturation of the protein in the presence of amino acids, it has been observed that the effects of amino acids on the stability of the protein in terms of T_m (the midpoint of the thermal transition) can be divided into three groups, namely neutral, stabilizing and destabilizing. The behaviour of cyt-*c* in the presence of various amino acids has been explained in quantitative thermodynamic terms.

MATERIALS AND METHODS

Horse heart cyt-*c* was purchased from Sigma. Amino acids were purchased from Hi-media Laboratories. Ultrapure guanidinium chloride (GdmCl) was obtained from Schwartz/Mann Biotech.

The ferric form of cyt-*c* was prepared by dissolving the protein in about 0.01 % potassium ferricyanide. The mixture was dialysed in the cold against 0.1 M KCl (pH 7.0) in order to remove excess potassium ferricyanide. Cyt-*c* gave a single band on PAGE. Protein concentration was determined using a value of $10.7 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for the molar absorption coefficient at 530 nm [15].

Denaturation of cyt-*c* (in 0.05 M citrate buffer containing 0.1 M KCl) was followed by measuring the change in molar absorption coefficient at 530 nm in a u.v./visible Perkin–Elmer spectrophotometer (model lambda 3B) equipped with thermostatically controlled cell holders. A temperature probe was dipped into the dummy cell kept in one of the cell holders in the same compartment but not in the light path, and temperature was read using a tele-thermometer with an accuracy of ± 0.1 °C.

The denaturation curves were analysed as follows. If an optical property, Y , is used to follow the course of two-state reversible denaturation of a protein, the state of equilibrium is expressed in terms of conformational Gibbs energy change (ΔG_D) using the relation

$$\Delta G_D = -RT \ln \left(\frac{Y - Y_N}{Y_D - Y} \right) \quad (1)$$

where Y_N and Y_D are respectively the properties of the protein in the native (N) and denatured (D) states under the same con-

Figure 1 Thermal denaturation curves of cyt-c

(a) Absorbance spectra at pH 3.0. (b) Heat-denaturation curves at various pH values. Y_N gives the dependence of the optical property of the native protein on temperature. (c) Plot of ΔG_D versus temperature at pH 3.0.

ditions under which Y was measured, R is the gas constant ($8.302 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{degree}^{-1}$), and T is the temperature in kelvin. The values of ΔG_D can be measured most accurately near the midpoint of the denaturation curve, and for values of ΔG_D ($\text{kJ} \cdot \text{mol}^{-1}$) outside the range $-5.4 \leq \Delta G_D \leq 5.4$, the error becomes substantial [16].

RESULTS

Thermal denaturation of cyt-c at different pH values was followed by measuring absorption spectra of the protein in the range 600–480 nm (e.g. see Figure 1a). From these measurements, values of the molar absorption coefficient at 530 nm (ϵ_{530}) were determined and heat-denaturation curves were constructed by plotting values of ϵ_{530} , which measures the haem-globin interaction [17], against temperature. Figure 1(b) shows transition curves at different pH values. It can be seen that (1) transition curves are reversible at all pH values, (2) protein at pH values above 4 cannot be fully denatured even at 85 °C, and (3) the thermal transition curve at pH 3.0 is complete at 75 °C, and analysis of this curve using the method described below suggests that cyt-c is 25% denatured at 25 °C.

The effects on thermal denaturation of cyt-c of different concentrations of L-amino acids that are more hydrophobic than glycine and have a solubility of 0.1 M or higher in water at

25 °C were studied at pH 3.0. This pH was chosen for two obvious reasons. (1) We are interested in measuring the effect of amino acids on the conformational equilibrium $N \rightleftharpoons D$, and (2) the end product thermal denaturation in the presence of amino acids can be observed and measured only at pH 3.0 or below. In order to convert optical data (ϵ_{530}) into ΔG_D values by using eqn. (1), it is necessary to know Y_N and Y_D as a function of composition variables at this pH. The requisite data for Y_N in the absence of amino acids were obtained from the measurements of native cyt-c at pH values in the range 6.0–4.2 (see Figure 1b) and show that the temperature-dependence of Y_N is independent of pH. This dependence is described by eqn. (2), which was assumed to apply in the transition region at pH 3.0 also:

$$Y_N = 11084 (\pm 36) - 14.47 (\pm 0.90) t \quad (2)$$

where t is temperature in °C. It can be seen in Figure 1(b) that Y_D at pH 3.0 is independent of temperature, so a value of $5.12 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ was assumed to apply within the transition region.

A plot of ΔG_D versus temperature is shown in Figure 1(c), from which the value of T_m , the temperature at which $\Delta G_D = 0$, was determined. The value of ΔS_m , the entropy change at T_m , was determined according to eqn. (3) using all results above and below the T_m that fall on a straight line (see Figure 1c).

$$(\partial \Delta G_D / \partial T)_{P, \text{pH}} = -\Delta S \quad (3)$$

Table 1 Thermodynamic parameters for the thermal denaturation of cyt-c at various concentrations of amino acids at pH 3.0

The uncertainties in ΔS_m and ΔH_m are S.D.s, obtained on treating data such as shown in Figure 1(c) by the method of least-squares.

Amino acid	[Amino acid] (M)	T_m (K)	ΔS_m ($J \cdot mol^{-1} \cdot K^{-1}$)	ΔH_m ($kJ \cdot mol^{-1}$)
None	—	318.2	50 ± 0.7	67 ± 1.0
Gly	0.25	318.5	41 ± 1.4	55 ± 1.9
	0.50	319.2	38 ± 0.9	50 ± 1.2
	0.75	319.5	35 ± 0.9	47 ± 1.3
	1.00	321.5	33 ± 1.6	44 ± 2.1
Ala	0.25	318.6	50 ± 2.0	67 ± 2.6
	0.50	319.0	49 ± 0.3	65 ± 0.4
	0.70	321.2	45 ± 1.6	60 ± 2.3
Lys	0.25	322.6	45 ± 1.7	60 ± 1.7
	0.50	323.2	40 ± 1.2	54 ± 1.6
	0.70	324.7	42 ± 0.7	57 ± 1.0
Ser	0.25	319.3	40 ± 1.0	54 ± 1.4
	0.50	320.1	38 ± 0.8	51 ± 1.0
	0.75	321.2	37 ± 0.7	50 ± 1.0
	1.00	321.7	37 ± 0.6	49 ± 0.8
Thr	0.25	319.2	45 ± 2.5	63 ± 3.4
	0.50	319.9	43 ± 1.5	57 ± 2.0
His	0.09	316.7	47 ± 1.6	62 ± 2.1
	0.17	316.2	36 ± 1.9	47 ± 1.2
Pro	0.25	319.0	50 ± 2.6	66 ± 3.4
	0.50	319.7	49 ± 3.4	65 ± 4.2
	0.75	320.7	45 ± 2.1	61 ± 2.7
	1.00	322.5	42 ± 2.2	56 ± 2.9
Met	0.10	318.7	47 ± 2.1	63 ± 2.8
	0.21	319.0	45 ± 2.7	57 ± 3.6
Val	0.10	319.5	44 ± 1.6	59 ± 2.2
	0.20	320.0	40 ± 1.4	54 ± 1.9
	0.30	320.0	40 ± 1.3	53 ± 1.8
Ile	0.08	318.2	50 ± 2.2	67 ± 3.0
	0.12	318.4	50 ± 1.0	66 ± 1.3
	0.16	318.4	49 ± 2.0	65 ± 2.7
Phe	0.05	318.4	50 ± 1.2	67 ± 1.6
	0.10	318.4	49 ± 1.8	65 ± 2.4
Leu	0.05	318.2	53 ± 3.4	70 ± 4.5
	0.10	318.2	52 ± 1.3	69 ± 1.7
Arg	0.25	315.1	45 ± 0.7	59 ± 1.0
	0.50	313.2	39 ± 1.1	51 ± 1.4
	0.75	313.0	40 ± 1.5	53 ± 2.0

The value of ΔS thus determined is also applicable at T_m . The product of T_m and ΔS_m gives the value of ΔH_m , the enthalpy change at the transition midpoint. Results of these analyses are shown in Table 1.

The effect of amino acids on the thermal denaturation of cyt-c was studied at pH 3.0. It has been observed (1) that denaturation is reversible in the presence of amino acids, (2) that the temperature dependence of Y_N given by eqn. (2) is independent of the amino acid and amino acid concentration, (3) that, except for histidine and arginine, Y_D is independent of temperature, amino acid and amino acid concentration, and (4) that in the cases of histidine and arginine, Y_D varies with the molarity of the amino acids but shows no dependence on temperature. These observations were used in the determination of ΔG_D in the presence of amino acids.

Figure 2(a) shows experimental denaturation curves obtained on heating cyt-c solutions containing 0, 0.5 and 1.0 M proline at pH 3.0; for clarity, transition curves at 0.25 and 0.75 M proline are not shown. Figure 2(b) shows stability plots at different

concentrations of the osmolyte. As in the cases of the thermal-transition curve in the absence of amino acid, the transition curves in the presence of amino acid were analysed for values of T_m , ΔS_m and ΔH_m . Results of this analysis are shown in Table 1. Values of T_m are plotted as a function of proline concentration in Figure 2(c).

Thermal denaturation of cyt-c in the presence of arginine in the concentration range 0–1 M was measured at pH 3.0. Figure 3(a) shows the transition curves at 0, 0.25 and 0.7 M arginine; to maintain clarity, transition curves at other concentrations are not shown. All the ΔG_D versus temperature plots (see, e.g., Figure 3b) were analysed and the values of T_m , ΔS_m and ΔH_m are listed in Table 1. Figure 3(c) shows T_m versus [arginine].

Effects of other amino acids on the thermal denaturation of cyt-c were studied at pH 3.0. These thermal transition curves (not shown here) were analysed as described above for T_m , ΔS_m , and ΔH_m . Results are given in Table 1.

Figure 4(a) shows the isothermal GdmCl denaturation of cyt-c in the absence and presence of alanine and serine at pH 6.0. In the absence of amino acid, denaturation starts at around 1.5 M GdmCl and is complete at 3.25 M (Figure 4a). In the presence of 0.45 M alanine, denaturation starts at 1.75 M and is complete at 3.5 M GdmCl, whereas in the presence of 0.6 M serine denaturation starts at 2.01 M and is complete at 3.6 M. It should be noted that all these transition curves are reversible. In each case the dependence of Y_N and Y_D on GdmCl concentration was determined, as shown in Figure 4(a). These observations were used in the determination of ΔG_D using eqn. (1). The plots of ΔG_D versus [GdmCl] (Fig. 4b) were analysed according to eqn. (4) [18,19], using a least-squares method.

$$\Delta G_D = \Delta G_D^{H_2O} - m[\text{GdmCl}] \quad (4)$$

where $\Delta G_D^{H_2O}$ is the value of ΔG_D in the absence of denaturant, m is the slope of the straight line, and C_m is given by $\Delta G_D^{H_2O}/m$. It can be seen in Table 2 that, within experimental error, $\Delta G_D^{H_2O}$ does not depend on the presence of amino acids but C_m is increased in the presence of amino acids.

DISCUSSION

We have observed that the thermal denaturation of cyt-c in the presence and absence of amino acids is reversible and co-operative. All transition curves have been analysed assuming two-state behaviour of the thermal denaturation of protein [13,14]. In order to get a feel for the accuracy of our analysis of the thermal transition curves obtained at pH 3.0 (0.05 M citrate buffer, 0.1 M KCl), we estimated ΔC_p (heat capacity change at constant pressure) using the Gibbs–Helmholtz equation [(eqn. (5))]. This value is compared with the calorimetrically determined ΔC_p at pH 4.0 (0.05 M acetate buffer, no salt). Two comments are necessary. First, calorimetric ΔC_p of cyt-c is independent of pH [13,20] allowing a comparison between values at pH 3 (this study) and pH 4 [14]. Secondly, ΔC_p of cyt-c is smaller for the transition in the presence of high salt concentrations than that in the presence of low salt concentration [21]; ΔC_p in the presence of 0.1–0.5 M KCl is about 17 % smaller than that in the absence of KCl [21].

$$\Delta G_D = \Delta H_m[(T_m - T)/T_m] - \Delta C_p[(T_m - T) + T \ln(T/T_m)] \quad (5)$$

Inserting $\Delta H_m = 66.92 \pm 1.00 \text{ kJ} \cdot \text{mol}^{-1}$ at $T_m = 318.15 \pm 0.10 \text{ K}$ (see Table 1) and $\Delta G_D = 2.40 \text{ kJ} \cdot \text{mol}^{-1}$ at $T = 298.15 \text{ K}$, obtained from analysis of the transition curve induced by heating cyt-c in the absence of osmolyte at pH 3.0 (see Figure 1c), gives a value of $2.81 \pm 0.12 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ for ΔC_p . As the measurements were made in the presence of 0.1 M KCl, it is expected that

Figure 2 Thermodynamic parameters for the thermal denaturation of cyt-c in the presence of proline

(a) Thermal denaturation curves of cyt-c at pH 3.0 in the presence of various concentrations of proline: $\circ$, zero; $\bullet$, 0.5 M; $\triangle$, 1 M. Y_N has the same meaning as in Figure 1(b). Note that (i) some data points on each curve are not shown for clarity and (ii) for the same reason transition curves at 0.25, 0.75 M are omitted from this graph. (b) ΔG_D versus temperature for cyt-c determined using eqn. (1). (c) T_m versus [proline] plot using data obtained from the analysis of all the transition curves.

this value would be about 17% lower than the calorimetric value, determined in the absence of salt [14]. Taking this effect of salt on ΔC_p into account, our result is in good agreement with the $3.76 \pm 0.42 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ observed calorimetrically [14].

The current explanation for the effect of osmolytes on protein stability is based on two facts, the preferential exclusion of osmolytes from the folded protein domain and preferential interaction of osmolytes with the unfolded protein [22]. The preferential exclusion of osmolytes, i.e. the preferential hydration of the protein, increases the surface tension of water and tends to oppose denaturation which results in an increase in surface area of the protein. On the other hand, the preferential interaction of osmolytes with the unfolded protein tends to favour denaturation, and this tendency increases with increasing osmolyte hydrophobicity, because denaturation results in the exposure of buried hydrophobic side chains in the protein. Thus the observed effect depends on the fine balance between these two opposing forces. If we accept this hypothesis, we can understand the dependence of T_m , the most commonly used index for protein stability, on the hydrophobicity of amino acid side chains given in Table 3. We have used the hydrophobicity scale proposed by Bull and Breese [24]. A few comments may be necessary. Many scales of hydrophobicity of amino acid side chains have been

proposed [23–29]. Significant differences exist between these scales in ranking hydrophobicities of amino acids. These differences could be due to differences in the reference states assumed to serve as a model for the interior of the folded protein. As the effect of amino acids on the thermal denaturation of cyt-c has been studied in the presence of 0.1 M KCl, we have used the hydrophobic scale proposed by Bull and Breese [24] who made all measurements in the presence of 0.1 M NaCl at 30 °C. As the temperature coefficient of free energy of transfer of amino acid side chains to the protein surface is small [24], it is assumed that the hydrophobic scale given in Table 3 is also applicable at 25 °C.

Alanine and more hydrophobic amino acids are expected to oppose the preferential hydration effect [22], resulting in smaller ΔT_m . The rank order of compatible osmolytes with respect to ΔT_m on the molar scale is consistent with this explanation (see Table 3). In the cases of isoleucine, phenylalanine and leucine, the two effects, one involving stabilization of native protein as a result of preferential hydration and the other involving destabilization caused by favourable interaction of exposed hydrophobic side chains with the apolar part of the osmolytes, appear to be equal in magnitude, resulting in no change in ΔT_m . However, glycine which is expected to have a value for $\Delta T_m/M$ greater than alanine is an exception.

Figure 3 Thermodynamic parameters for the thermal denaturation of cyt-c in the presence of arginine

(a) Thermal denaturation curves of cyt-c in the presence of various concentrations of arginine: $\circ$, zero; ∇ , 0.25 M; $\triangle$, 0.7 M. Y_N has the same meaning as in Figure 1(b). Note that data points for 0.5 M arginine are not shown for clarity. (b) ΔG_D versus temperature for cyt-c determined by using eqn. (1). (c) T_m versus [arginine] plot using data obtained from analysis of all the transition curves.

Two amino acids, namely arginine and histidine, are found to be 'incompatible', i.e. they destabilize protein structure (see Table 1). Two explanations have been given for the behaviour of incompatible solutes. The older explanation comes from Clarke and Zounce [30] who pointed out the similarity between ions that denature proteins and side chains of organic solutes that accumulate during stress. If one accepts their hypothesis, then arginine and histidine should behave as denaturants because part of their side chains is structurally similar to the guanidinium ion and urea. The other explanation is from Timasheff [22] who suggested that the nature of the interaction of denatured proteins with arginine side chains is similar to that of the guanidinium ion which binds to peptide groups and aromatic side chains [31]. This is what causes arginine to behave as a denaturant. The same may be true for the histidine side chain.

It can be concluded that, of the amino acids studied, arginine and histidine are the only ones that cannot be used to stabilize proteins, nor can other amino acids that contain large hydrophobic side chains. It is noteworthy that in cases where arginine is released, it is immediately converted into the compatible solute octopine [32].

It is dangerous to draw conclusions about protein stability, defined as $\Delta G_D^{\text{H}_2\text{O}} \text{ (25 }^\circ\text{C)}$ for the reaction $\text{N} \rightleftharpoons \text{D}$ in water (or dilute buffer), based solely on T_m values. For instance, differential-scanning measurements gave values of 78.5, 90.0 and

100.0 $^\circ\text{C}$ for T_m of hens'-egg lysozyme, carp parvalbumin and bovine trypsin inhibitor respectively, whereas the $\Delta G_D^{\text{H}_2\text{O}} \text{ (25 }^\circ\text{C)}$ values are identical, within experimental error, for all these proteins [33]. In addition to T_m , we have therefore estimated $\Delta G_D^{\text{H}_2\text{O}} \text{ (25 }^\circ\text{C)}$ over an extended osmolyte concentration range. It has been observed that osmolytes that raise the T_m have no effect on $\Delta G_D^{\text{H}_2\text{O}} \text{ (25 }^\circ\text{C)}$ (see Table 3). In order to confirm this observation, isothermal denaturation of cyt-c by GdmCl was studied in the presence of serine and alanine at 25 $^\circ\text{C}$ and the results summarized in Table 2. As observed during thermal denaturation, $\Delta G_D^{\text{H}_2\text{O}} \text{ (25 }^\circ\text{C)}$ is independent of osmolyte concentration but the value of C_m is increased in the presence of these osmolytes. Both types of measurement, i.e. isothermal denaturation by GdmCl and thermally induced denaturation, appear to suggest that protein stabilization in the presence of amino acids is under entropic and enthalpic controls.

As amino acids preferentially hydrate native proteins, increasing the chemical potential of the protein with increasing osmolyte concentration, and as preferential hydration opposes an increase in surface area, the $\text{N} \rightleftharpoons \text{D}$ equilibrium should therefore favour the more compact native state. The tendency of increased osmolyte concentration to decrease the surface area should progressively make the unfolded ensemble more compact, decreasing the conformational entropy. This view provides a rationale for expecting an entropy decrease in the $\text{N} \rightleftharpoons \text{D}$

Figure 4 (a) Isothermal denaturation of cyt-c by GdmCl at pH 6.0 and 25 ± 0.05 °C followed by measuring the change in ϵ_{405} as a function of GdmCl concentration (○, no amino acid; □, 0.45 M alanine; ●, 0.6 M serine) and (b) plot of ΔG_D versus GdmCl concentration

Symbols in (b) have the same meaning as in (a). In the determination of ΔG_D using eqn. (1), values of Y_N and Y_D were obtained from the observed dependence of the optical property in the pre- and post-transition regions on [GdmCl]: in the absence of osmolyte $Y_N = 2.07 (\pm 0.16)$ [GdmCl] + $2.08 (\pm 0.37)$, $Y_D = 2.74 (\pm 0.24)$ [GdmCl] + $25.94 (\pm 0.98)$; at 0.45 M alanine, $Y_N = 0.42 (\pm 0.23)$ [GdmCl] + $1.68 (\pm 0.29)$, $Y_D = 2.26 (\pm 0.15)$ [GdmCl] + $25.94 (\pm 1.02)$; at 0.6 M serine, $Y_N = 1.01 (\pm 0.16)$ [GdmCl] + $0.64 (\pm 0.22)$ and $Y_D = 1.62 (\pm 0.62)$ [GdmCl] + $24.74 (\pm 2.48)$.

Table 2 Thermodynamic parameters for the isothermal GdmCl denaturation of cyt-c in the presence of amino acids at pH 6.0 and 25 °C

Uncertainties in $\Delta G_D^{H_2O}$ and m are S.D.s in determining them by method of least-squares according to eqn. (4).

	$\Delta G_D^{H_2O}$ (kJ·mol ⁻¹)	m (kJ·mol ⁻¹ ·M ⁻¹)	C_m (M)
Control	35.11 ± 0.54	14.21 ± 0.58	2.49
+ 0.6 M serine	34.70 ± 0.54	12.12 ± 0.16	2.83
+ 0.45 M alanine	36.30 ± 1.25	13.37 ± 0.42	2.75

equilibrium in the presence of osmolytes relative to the equilibrium in the absence of osmolytes. This expectation is borne out by the results given in Table 1. It can also be seen in Table 1 that the decrease in ΔS_m with increasing amino acid concentration is compensated (for) by a decrease in ΔH_m .

Recently Santoro et al. [10] performed differential-scanning calorimetry experiments on RNAase A and lysozyme in the presence of various concentrations of glycine-based osmolytes. They observed that (1) these osmolytes raise the thermal

Table 3 Changes in the stability of cyt-c in the presence of various amino acids

$\Delta\Delta G_D^{H_2O}$ (25 °C) is the difference between Gibbs energies of cyt-c at 25 °C in the presence and absence of the amino acid. ΔT_m is the difference between T_m values of the protein in the absence and presence of 1 M amino acid. Hydrophobicity data were taken from ref. [23].

Amino acid	$\Delta\Delta G_D^{H_2O}$ (25 °C) (J·mol ⁻¹)	ΔT_m (°C/M)	Hydrophobicity (J·mol ⁻¹)
Leu	0	0	-10283
Phe	0	0	-9739
Ile	0	0	-9447
Val	0	1.8	-6521
Met	0	2.6	-6145
Pro	0	3.1	-4096
Thr	0	3.4	-2173
Ser	0	3.7	-1630
Lys	0	6.0	-1463
Ala	0	9.0	-836
His	-148	-2.6	-502
Arg	-253	-5.2	-502
Gly	0	3.3	0

unfolding transition temperature, and the extent of increase in ΔT_m , on a molar scale, decreases with increasing hydrophobicity, (2) ΔS_m decreases with an increase in osmolyte concentration, and (3) the presence of osmolytes results in an increase in apparent Gibbs energy change. It is gratifying to note that the conclusions reached from our equilibrium measurements on cytc agree with observations (1) and (2) obtained from the calorimetric measurements. Contrary to observation (3), however, the equilibrium results suggest that $\Delta G_D^{H_2O}$ (25 °C) is unchanged by the presence of amino acids that protect the protein against heat denaturation (see Table 1), and that even up to 37 °C these amino acids produce insignificant effects on the Gibbs energy change on denaturation. This discrepancy could well be due to the fact that the calorimetric Gibbs energy change was derived from the apparent enthalpy and constant-pressure heat-capacity changes, which also include, besides the contribution from conformational transition, a possible contribution from the presence of preferentially interacting osmolytes (for correction for preferential binding, see Privalov [20]).

The conformational stability of almost all naturally occurring globular proteins is between 20 and 65 kJ·mol⁻¹ [34]. It appears to be advantageous to living organisms to have proteins for which the folded conformation is only marginally more stable than the unfolded conformation. The reason for this is still not clear, but may involve protein turnover [35]. This may be one of the reasons why micro-organisms and plants prefer to produce only those low-molecular mass compounds that do not affect the conformational stability of proteins, but, on the other hand, protect against denaturing stress by altering enthalpy and entropy so as to increase the midpoint of the unfolding transition.

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