THE STABILIZATION OF PROTEINS BY OSMOLYTES

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ABSTRACT The preferential interactions of lysozyme with solvent components and the effects of solvent additives on its stability were examined for several neutral osmolytes: L-proline, L-serine, γ-aminobutyric acid, sarcosine, taurine, α-alanine, β-alanine, glycine, betaine, and trimethylamine *N*-oxide. It was shown that all these substances stabilize the protein structure against thermal denaturation and (except for trimethylamine *N*-oxide for which interaction measurements could not be made) are strongly excluded from the protein domain, rendering unlikely their direct binding to proteins. On the other hand, valine, not known as an osmolyte, had no stabilizing effect, although it induced a large protein-preferential hydration. A possible explanation is given for the use of these substances as osmotic-pressure-regulating agents in organisms living under high osmotic pressure.

INTRODUCTION

It is well known that certain organisms that live under conditions of high osmotic pressure accumulate low molecular weight components at moderate to high concentrations to raise the osmotic pressure in the cytoplasm (e.g., Yancey et al., 1982). Except for extremely halotolerant bacteria, most osmolytic bacteria utilize neutral substances such as polyhydric alcohols, sugars, amino acids, and related compounds as regulators of the cytoplasmic osmotic pressure. In a previous paper (Arakawa and Timasheff, 1983), it had been suggested that glycine (Gly)[1] and betaine at 0.7 to 2 M are expected not to be toxic to enzymes in the cytoplasm, since they stabilize protein structure and are excluded extensively from the protein domain, rendering improbable their direct binding to the proteins. Furthermore, sugars and polyhydric alcohols that are similarly excluded from contact with proteins (Lee and Timasheff, 1981; Gekko and Timasheff, 1981*a,b;* Gekko and Morikama, 1981*a,b;* Arakawa and Timasheff, 1982*b*) are also used by these organisms as osmolytes. To test this apparent correlation, the preferential interactions with proteins of a number of other osmolytic substances and their protein structural stabilizing effectiveness were examined.

MATERIALS AND METHODS

The lysozyme used was obtained from Worthington Biochemical Corp. (Freehold, NJ) (lot No. 31A-993). L-Proline, L-serine, γ-aminobutyric acid, sarcosine, taurine, α-alanine, β-alanine, glycine, betaine, and trimethylamine *N*-oxide were purchased from Sigma Chemical Co. (St. Louis, MO). The effect of additives on protein stability was examined by following the thermal denaturation of lysozyme both in the absence and presence of the additives. The thermal denaturation measurements were carried out in a spectrophotometer (model 2600; Gilford Instrument Laboratories, Oberlin, OH), equipped with a thermoprogrammer, by following the absorbance change of the protein with increasing temperature according to the method described previously (Arakawa and Timasheff, 1983). The rate of heating was 0.25°/min. The protein solutions were made in 0.02 M NaCl and adjusted to the desired pH with HCl or NaOH. Three samples, identical in pH, were always run simultaneously, including a control that contained no additive.

Preferential interaction measurements were carried out by densimetry at 20°C as described previously (Lee et al., 1979). Solvents were made in 0.02 M NaCl, pH 6.0. The partial specific volumes of the protein in the different solvents were determined at conditions at which the molality and chemical potential of the diffusible component were, in turn, kept identical in the protein solution and in the reference solvent. Setting component 1 = water, component 2 = protein, and component 3 = additive (Scatchard, 1946; Stockmayer, 1950), the preferential interaction parameter, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, was calculated from the partial specific volumes, ϕ_2^0 and $\phi_2'^0$, of the protein determined, respectively, at constant molality and constant chemical potential conditions, by (Cohen and Eisenberg, 1968)

$$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} = \rho_0(\phi_2^0 - \phi_2'^0)/(1 - \rho_0\bar{v}_3), \qquad (1)$$

where g_i is the concentration of component i in grams per gram of water, T is the thermodynamic (Kelvin) temperature, μ is the chemical potential, ρ_0 is the density of the reference solvent and $\bar{v}_3$ is the partial specific volume of component 3, which was also determined by densimetry. The superscript 0 indicates extrapolation to zero protein concentration. The preferential hydration parameter, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$, was calculated from (Timasheff and Kronman, 1959)

$$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3} = -(1/g_3)\,(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}. \qquad (2)$$

The absorbance values of lysozyme in the presence of the additives were determined as described previously (Arakawa and Timasheff, 1982*a*).

RESULTS

Protein Stabilization

The effectiveness of the various substances examined as stabilizers against denaturation is presented in Table I.

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[1] *Abbreviations used in this paper:* GuHCl, guanidine hydrochloride; TMAO, trimethylamine *N*-oxide; L-Pro, L-proline, L-Ser, L-serine, GABA, γ-aminobutyric acid; Sar, sarcosine, α-Ala, α-alanine; β-Ala, β-alanine; Gly, glycine; Bet, betanine; Val, valine.

TABLE I
TRANSITION TEMPERATURE OF LYSOZYME IN AQUEOUS SOLUTIONS*

Additive	Transition temperature		$\Delta T_m/M$ of additive	
	No GuHCl	1.2 M GuHCl	No GuHCl	1.2 GuHCl
	°C			
None (control)‡	68–80	56–68	—	—
TMAO (0.5 M)	70–81 (pH 6.74)	58–69 (pH 6.97)	3	3
TMAO	71– (pH 7.9)	61–71 (pH 7.03)	—	6
L-Pro	72–84	59–71	4	4
L-Ser	74–86	61–74	7	8
Taurine (pH 6.8)	72–84 (0.45 M)	60–71 (0.35 M)	9	10
GABA (1.3 M)	75–89 (pH 6.8)	—	6	—
GABA	73–86	60–72	6	6
Sar	73–86	60–72	6	6
α-Ala	71–84	59–71	4	4
β-Ala	73–85	60–71	6	5
Gly	75–85	60–72	7	6
Bet	72–83	59–70	4	4
Val	67–80 (0.25 M)	56–68 (0.38 M)	−2	0
	67–81 (0.5 M)	—	0	—

*All samples contained 0.02 M NaCl. The pH of the solutions was 6.0 and the additive concentrations were 0.9 M and 0.7 M with and without 1.2 M GuHCl, respectively, except where indicated. The results of the last two columns were obtained from the transition data given in the second and third columns.
‡The transition results were independent from the solvent pH between 6 and 7, both in the absence and the presence of 1.2 M GuHCl.

The experiments were carried out both in the absence and presence of 1.2 M GuHCl, which was added to decrease the transition temperature to an experimentally more accessible range. All the additives listed in Table I, except Val, are osmolytes found in organisms living under low water potential. The results for Val are shown for comparison. Note that addition of the osmolytes increased significantly the initial and final temperatures of the transition. The breadth of the transition, which is the difference between the initial and final temperatures above, is ~12°C for all the solvent systems, suggesting that the nature of the alterations in the protein structure is not affected by the additives, i.e., there are no specific effects. The same breadth of the transition, within experimental error, was obtained whether 1.2 M GuHCl was present or not, suggesting that the observed conformational change is the same in all cases.

The effectiveness of the additives as protein-structure stabilizers can be expressed in terms of the molar increase in the transition temperature, $\Delta T_m/M$, where $\Delta T_m = (T_m$ [in the presence of additive] $- T_m$ [control]); T_m is the midpoint of the transition and M is the molar concentration of the additive. These values, listed in the fourth and fifth columns of Table I, have positive values of similar magnitude for all the osmolytes, $\Delta T_m/M$ ranging from 4 to 6°C. This indicates that addition of the osmolytes increases the thermal stability of lysozyme, with L-Ser and Gly being somewhat more effective than the others and taurine being the most effective one. This increment is essentially identical whether GuHCl is present or not, meaning that the effect of GuHCl is additive, with identical reduction of protein stability in all cases. These results, obtained with lysozyme, confirm the previous observation made with BSA (Arakawa and Timasheff, 1983) that Gly and Bet are protein structure stabilizers, with Gly being the stronger one, even though both have an almost identical effect on the chemical potential of the protein. In contrast to all the other substances, Val, which is not an osmolyte, had essentially no effect on protein stability.

Preferential Interactions

Values of the partial specific volume of lysozyme in aqueous solutions of the various additives are given in Table II. The absorptivities determined in the presence of the additives are listed in the last column of Table II. Their near identity suggests that addition of the osmolytes does not affect significantly the protein structure. The preferen-

TABLE II
INTERACTION PARAMETERS AND ABSORPTIVITY OF LYSOZYME AT pH 6.0

Solvent	v_3	$\bar{v}_3$	ϕ_2^0	$\phi_2'^0$	$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$	$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$	Absorptivity*
	ml/g	*g/g*	*ml/g*	*ml/g*	*g/g*	*g/g*	*dl g^{-1} cm^{-1}*
1 M TMAO‡	0.96	—	—	—	—	—	—
1 M L-Pro	0.721	0.126	0.711 ± 0.002	0.721 ± 0.0003	−0.0406 ± 0.0093	0.322 ± 0.074	26.9§
1 M L-Ser	0.595	0.112	0.710 ± 0.0005	0.728 ± 0.0006	−0.0497 ± 0.0030	0.444 ± 0.027	27.6
0.667 M Taurine	0.573	0.0878	0.705 ± 0.001	0.718 ± 0.001	−0.0331 ± 0.0051	0.377 ± 0.058	27.4
1 M GABA	0.727	0.111	0.710 ± 0.001	0.727 ± 0.001	−0.0699 ± 0.0082	0.629 ± 0.074	27.5
1 M Sar	0.713	0.0950	0.711 ± 0.0005	0.723 ± 0.0005	−0.0461 ± 0.0038	0.485 ± 0.040	27.4
0.5 M Val	0.762	0.0615	0.709 ± 0.0005	0.720 ± 0.0005	−0.0487 ± 0.0089	0.792 ± 0.144	27.5

*27.4 dl g^{-1} cm^{-1} in dilute salt (Roxby and Tanford, 1971).
‡pH 7.0.
§In 6 M GuHCl.

tial interaction parameter, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, was calculated by Eq. 1 using values of $\bar{v}_3$ given in Table II. Since the $\bar{v}_3$ value for TMAO is very close to that of water, densimetry cannot be used to measure the preferential interaction parameter for this compound. All the other values of $\bar{v}_3$ were significantly smaller than that of water. The values of $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, given in the sixth column of Table II, were found to be negative in all cases including Val, i.e., all the additives were excluded from the protein domain. The values of $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$, calculated by Eq. 2 with the g_3 values of column 3, are listed in the seventh column. The protein is strongly preferentially hydrated in these systems.

The preferential interaction parameter is related to the total bindings of components 1 and 3 to the protein by (Inoue and Timasheff, 1972)

$$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} = A_3 - g_3 A_1, \qquad (3)$$

where A_i is grams of component i bound to one gram of protein. Assuming, as a limit, total exclusion of component 3, i.e., setting $A_3 = 0$, A_1 becomes equal to $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$. As shown in Table II, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ for all additives is 0.2–0.4 g/g, the usually accepted hydration values for most proteins (Bull and Breese, 1968; Kuntz, 1971; Kuntz and Kauzmann, 1974), indicating that these additives are indeed extensively excluded from the protein domain. Although the preferential hydration does not vary much among the additives, its values for Val and GABA appear to be somewhat larger than for the others. It may be of interest to point out that, similarly to betaine, Val [$(CH_3)_2CHCH(NH_3)^+COO^-$], and GABA ($NH_3^+CH_2CH_2CH_2COO^-$), have large alkyl groups.

DISCUSSION

In previous studies it had been shown for a number of substances that the stabilization of the native protein structure is accompanied by the preferential hydration of the proteins. This observation is fully supported by a comparison of Tables I and II, which suggests that structure stabilization by the additives listed in Table I can be interpreted in terms of the same mechanism as that proposed for sugars (Lee and Timasheff, 1981; Arakawa and Timasheff, 1982*a*), polyhydric alcohols (Gekko and Timasheff, 1981*b*), salts (Arakawa and Timasheff, 1982*b*), and certain amino acids (Arakawa and Timasheff, 1983). According to this mechanism, the unfavorable interactions of these substances with the proteins, manifested by negative values of $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, is a reflection of an increase in the surface free energy of water induced by these additives, and hence of the surface tension of water. According to the Gibbs adsorption isotherm, this must result in the exclusion of the additive from the water-macromolecule interface. Since this is a thermodynamically unfavorable situation, by the Le Chatelier principle the system should tend toward a state minimizing it, i.e., toward a minimization of the area of the water-protein interface. Globular proteins in the native state have a smaller surface area than in the denatured state. Hence, contact with these solvent systems should displace the native $\rightleftharpoons$ denatured equilibrium to the left, with resulting stabilization of the native structure.

Valine is an exception to this rule, since its effect on protein stability is insignificant, although it displays a large preferential hydration of the native protein. This lack of stabilizing action may be due to its considerably more hydrophobic nature than those of the other additives. Its large nonpolar side chain should interact favorably with the newly exposed nonpolar residues of proteins in the denatured state. These favorable interactions may balance the increase in the unfavorable one stemming from the increased surface area of the protein upon unfolding (Arakawa and Timasheff, 1982*a*). As a result, according to the Wyman (1964) linkage theory, Val should not be effective as a native globular protein-structure stabilizer.

Except for Val, the results given in Tables I and II are essentially identical to those previously obtained for Gly and Bet (Arakawa and Timasheff, 1983). Their interactions with proteins are such that they do not decrease the protein concentration in the native, active form, while they are excluded from the protein domain, making very unlikely direct binding to proteins. Gly and Bet are not toxic to cytoplasmic enzymes, suggesting that the compounds of the present study should also not be toxic.

At this point, it seems pertinent to mention some considerations on the selection of certain substances as osmolytes and the exclusion of others: (*a*) Although Val is not different from the other substances examined in that it is excluded from contact with the protein, it differs from them in its lack of stabilizing power of the native protein structure. Thus, even though Val may not be toxic to enzymes in the cytoplasm, this, along with its relatively low solubility, may be an important reason why organisms do not use it as an osmotic pressure-raising substance. (*b*) It is known that certain strong electrolytes are more or less identical to the neutral amino acids and the related compounds studied here in their preferential interactions with proteins and in their structure-stabilizing effectiveness. Why is it, then, that the osmolytic organisms employ the neutral compounds rather than the strong electrolytes? Salts are toxic to enzyme proteins in these organisms (Yancy et al., 1982). This toxicity may be related to the strong perturbation by salts of the electrostatic properties of both enzymes and substrates, whereas any such perturbations by neutral compounds would be very weak. Extremely halotolerant bacteria, however, accumulate salts instead of neutral compounds. This may be dictated by the environment in which these bacteria live. Salts are soluble in water up to much higher concentrations that, in these bacteria, may be required for maintaining the native structure of the enzymes. These bacteria may, therefore, have reached a balance between the stabilization of the

active protein structure and a sacrifice of some enzymic activity due to their altered electrostatic properties. On the other hand, the enzyme proteins of the other organisms are native at low salt concentration. These organisms, therefore, can utilize neutral compounds, such as those listed in Table I, to raise the osmotic pressure in the cytoplasm.

We have proposed that the preferential hydration of proteins in aqueous solutions of sugars, some amino acids, and salts is related to their increments of the surface tension of water, i.e., to the enhancement of the cohesive force of water. By analogy, we have extended this concept of the cohesive force of water to the osmolytic compounds examined in this study. No direct correlation can be made for them, however, between the surface tension effect and the preferential hydration, since surface tension data for these compounds are not available. It is certain, however, that the strong polarity of these compounds must enhance the cohesive force of water and, hence, is responsible for the observed preferential hydration.

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