Molecular Thermodynamics of Heat-Induced Protein Unfolding in Aqueous Media

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The molecular thermodynamic model studied is based on the two-state mechanism of inactivation, in which only native folded and polymorphous unfolded protein forms are present at equilibrium. The influence of solvent on protein stability is described in terms of perturbation of the protein distribution between the two conformational states. An expression derived for the chemical potential of the protein accounts for conformational changes, ideal mixing effects, and interaction of the protein with the surrounding medium.

Thermal unfolding of lysozyme was then studied in the absence or presence of hydroxylic compounds. Ultraviolet difference spectroscopy was used to monitor the conformational changes induced by heating and to determine the melting temperature of the protein. The additives investigated are ethanol, glycols, and natural osmolytes. Media containing ethanol and glycols destabilized lysozyme, whereas sugars increased the conformational stability of the protein. For all of the systems examined the melting temperature was linearly related to the surface tension of the mixed solvent, supporting the ability of the model to describe the influence of the solvent and composition on lysozyme unfolding. Model predictions agreed fairly well with published differential scanning calorimetric data. The influence of hydroxylic additives on protein's conformational stability does not extend to any special property of these components, but to their ability to perturb the surface tension of water. This model can be used to interpret and correlate thermal unfolding data and to solve the problem of protein stabilization.

Introduction

In living organisms nascent polypeptide chains emerging from ribosomes fold spontaneously to acquire the conformation of native, biologically active proteins (Creighton, 1990; Gething and Sambrook, 1992). Although very little is known about how this happens, available evidence indicates that two large and opposing forces drive the transition from the 1-D to the 3-D structure (Sandberg and Terwilliger, 1989; Dill, 1990). The first one, hydrophobicity, stems from the propensity of nonpolar residues to remain buried in the protein core in order to avoid contact with water. The second one arises from the impossibility of two polypeptide segments simultaneously occupying the same volume. In an aqueous environment the hydrophobic interactions favor the compact state (Privalov, 1988), with the hydrophobic side chains tightly packed in the interior. By contrast, steric constraints due to excluded volume effects favor the unfolded state (Dill and Alonso, 1988), characterized by having a considerably higher number of nonpolar residues exposed to solvent. Under physiologic conditions the native-folded and the denatured-unfolded states are in equilibrium. The free-energy change for this equilibrium is typically 5–20 kcal/mol (Aune and Tanford, 1969b; Pace, 1975; Privalov, 1979; Privalov and Gill, 1988), which indicates the remarkably low stability of native proteins. This intrinsic lability demarcates the upper limits of life and poses serious obstacles to the use of proteins in virtually all biotechnological applications.

Environmental factors affecting protein stability include temperature, pressure, pH, ionic strength, mechanical forces, and the presence of a variety of denaturing agents (Lapanje, 1978). From the practical point of view, thermal inactivation represents the most important form of denaturation

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(Klibanov, 1983). The detailed mechanisms of inactivation are still unclear, but it now seems to be agreed that the first, and perhaps the only universal step of thermal denaturation is partial unfolding (Kauzmann, 1959; Schulz and Schirmer, 1978; Creighton, 1990; Pace, 1990). This process is highly cooperative and usually reversible (Tanford, 1968; Privalov et al., 1971), in the sense that the protein becomes active again when the original temperature conditions are restored. On the other hand, the subsequent transformations, including aggregation, incorrect folding, and covalent changes (mainly hydrolysis of the peptide bond, autolysis/proteolysis, and deamidation of amide residues), are irreversible and specific for each individual protein (Ahern and Klibanov, 1985).

Methods for minimizing inactivation can be divided into four main categories (Volkin and Klibanov, 1989; Tomazic, 1991): synthesis of more stable proteins by genetic engineering, chemical modifications, immobilization, and use of water-miscible stabilizing additives. In many instances the latter represents the simplest and most effective approach to hindering protein unfolding (Arakawa and Timasheff, 1982; Gray, 1988; Timasheff and Arakawa, 1989; De Cordt et al., 1994; Cioci and Lavecchia, 1994; Cioci, 1995a,b). The effects of additives on protein stability can be largely described in terms of preferential hydration phenomena (Schellman, 1987). In particular, all the systems so far examined have been found to obey what appears to be a general rule: additives stabilizing the native structure of proteins are preferentially excluded from the protein domain (Gekko and Timasheff, 1981a,b; Arakawa and Timasheff, 1983; Lee and Lee, 1987). The physical explanation of this behavior is to be found in the fact that additives interacting more favorably with the bulk solvent than with the protein increase the chemical potential of the protein (Timasheff, 1992). This rise is proportional to the protein–solvent contact surface. Since unfolded proteins have a much greater surface area exposed to solvent than do native proteins (Creighton, 1994), an increase in the system's free energy induced by a preferentially excluded additive will result in a thermodynamically unfavorable situation and lead to the displacement of the unfolding equilibrium toward the native protein. The mechanisms by which exclusion occurs may be different and include steric hindrance, solvophobic effects, and increases in the surface tension of water (Timasheff and Arakawa, 1989; Cioci et al., 1994).

Referring to the latter mechanism, which appears to be the most widespread in nature (Yancey, 1982), it should be noticed that the dependence of protein stability on the bulk surface tension of the solvent is in qualitative agreement with the Gibbs adsorption isotherm (Adamson, 1982), according to which an additive increasing the surface tension of water should exhibit a negative excess in the surface layer, that is, be preferentially excluded. No information, however, can be derived on how such exclusion could be put in relation to experimentally accessible quantities connected with the conformational state of the macromolecule.

The central role played by surface tension in a number of solvent effects involving proteins or smaller molecules is also emphasized by the solvophobic theory developed by Sinanoğlu (1968). The theory is based on the use of microthermodynamic surface tension, a quantity accounting for curvature differences between molecular and macroscopic surfaces, for expressing the free energy of cavity formation (Sinanoğlu, 1980, 1981). Application of this theory to heat-induced unfolding, however, is not straightforward. First, it is questionable to assess whether the protein–solvent interface should be treated as microscopic or macroscopic. Second, the temperature dependence of the microthermodynamic surface tension, which is not known *a priori*, should be carefully assumed. Finally, no indication can be given on why in some instances the free energy of cavity formation makes a negligible contribution to stabilization.

Difficulties encountered in overcoming the preceding limitations, along with the complexity of the phenomena under investigation, manifest themselves in the lack of quantitative relationships between macroscopic quantities reflecting the structural state of the protein, on the one hand, and the physical properties of the solvent, on the other. This lack is reflected in the high degree of empiricism affecting the methods for stabilizing proteins by specific as well as nonspecific additives (Gianfreda and Scarfi, 1991).

In this article we address the question of interpreting the stability behavior of proteins in aqueous media in the light of molecular thermodynamics. The main aim of our work was to analyze in quantitative terms the influence of water-miscible compounds on the conformational stability of the macromolecule. We were also interested in evaluating the sensitivity of protein stability to microenvironmental changes attained by the addition of those components. Although the concept of structural stability is unclear (Ahmad and Bigelow, 1986; Becktel and Schellman, 1987), a number of properties related to the conformational state of the protein, and therefore varying during unfolding, can be measured to monitor the conformational changes induced by heating (Robson and Garnier, 1986; Schmid, 1989). We used the melting temperature, namely, the temperature at which the concentrations of the native and denatured conformations are equal, as an index of stability. We concentrated on a globular model protein, lysozyme, because of its well-known three-dimensional structure (Blake et al., 1965) and denaturation mechanism (Tanford et al., 1966; Khechinashvili et al., 1973). Unfolding experiments were performed by heating the protein solution in the presence or absence of additives. The substances investigated included hydroxylic destabilizing agents and some naturally occurring polyhydric osmolytes. Osmolytes are low-molecular-weight solutes produced by living organisms in response to stresses such as heating, dehydration, and extreme of pH (Yancey et al., 1982). Strong evidence exists that these substances are synthesized *in vivo* to protect proteins against inactivation. Their *in-vitro* ability to stabilize proteins is well documented (Santoro et al., 1992; Thakar et al., 1994; Cioci and Lavecchia, 1994; Cioci, 1995a), but detailed mechanisms of stabilization are poorly understood and how they reinforce the structure of proteins without substantive effects on biological activity is still obscure.

The results obtained in this investigation indicate that the melting temperature of lysozyme in media containing destabilizing additives as well as stabilizing osmolytes is linearly related to the bulk surface tension of the mixed solvent. To clarify the physical bases of this phenomenon, we developed a molecular thermodynamic model to account for the additive-induced perturbations of the unfolding equilibrium. It is shown that under particular limiting conditions the model correctly predicts the observed dependence of melting tem-

perature on surface tension. The simple relation between the two quantities can be used to interpret and correlate the thermal unfolding data and, according to expectation, for devising efficient strategies of stabilization by solvent engineering.

Experimental Studies

Materials

Lysozyme (EC 3.2.1.17) from hen egg white was obtained from Fluka Chemie (Buchs, Switzerland) as a lyophilized and dialyzed salt-free powder. The claimed activity was 100,000 U mg^{-1}, where 1 U corresponds to the amount of protein that decreases the absorbance at 450 nm by 0.001 per minute at pH 7 and 25°C, using *Micrococcus luteus* as the substrate. Ethanol, ethanediol, 1,2-propanediol, glucose, sucrose, and sorbitol were purchased from Carlo Erba (Milano, Italy) with a purity $>99\%$. All other chemicals were of reagent grade and used without further purification. Just before use, the lysozyme was dissolved in 0.1-M acetate buffer, pH 4.

Methods

Ultraviolet difference spectroscopy was used to monitor heat-induced unfolding. Protein solutions at the desired concentrations were prepared gravimetrically and passed through a 0.22-μm sterile filter to remove dust and improve the signal-to-noise ratio. Optical measurements were made by a double-beam UV-VIS spectrophotometer (Perkin Elmer; Lambda 5) containing two 1-cm matched quartz cuvettes. Both cuvettes were filled with 1 mL of the same protein solution. The reference cell was heated at 25 ± 0.05°C, whereas the sample was continuously heated by a circulating water bath controlled by a digital temperature programmer (Haake, PG 41). Preliminary experiments were made to calibrate the sample cell temperature against that of the circulating water bath. During calibration, a high-precision electrical resistance thermometer was inserted into the cell filled with bidistilled water. Thermal fluctuations in the sample cell were $< \pm 0.05$°C.

Lysozyme unfolding is accompanied by a transfer of some strongly absorbing side chains of tryptophan residues ($\epsilon_{max} = 5{,}600\ M^{-1} \cdot cm^{-1}$) from the relatively nonpolar protein interior to the polar solvent (Robson and Garnier, 1986). This change in polarity gives rise to changes in the spectral properties of the absorbing chromophores that lead to a *blue-shift* of the absorption spectrum. As a result, prominent peaks appear in the 285–295-nm region of the difference spectrum. Spectral measurements were performed in this region, using the procedure reported by Pace et al. (1989). Melting curves were determined at constant wavelength ($\lambda = 290$ nm) by plotting the first derivative of the difference absorption spectrum against temperature. These data were then analyzed, as described in the Results section, to evaluate the amount of protein denatured at each temperature (in the range 25–85°C). The reproducibility of spectrophotometric data was within 0.1%.

In all experiments (either in pure buffer or in the presence of additives) the protein concentration was 0.5 $mg \cdot mL^{-1}$. Additions of hydroxylic compounds were made in the range 10–30 wt. %. Most runs were carried out by continuously heating the protein solution at a rate of 0.2°C min^{-1}. Changes in the heating rate or the thermal program, however, did not appreciably affect the results obtained. The reversibility of unfolding was accurately checked by cooling some partially unfolded protein solutions to the pretransition region. The absorbance values measured after cooling were found to be nearly identical to those determined directly upon heating.

Surface tensions of systems containing glucose, sucrose, and sorbitol were measured at 20 ± 0.1°C by a calibrated Traube stalagmometer. Measurements were carried out in bidistilled and degassed water. The observed accuracy was within ± 0.05 $mN \cdot m^{-1}$.

Molecular Thermodynamic Model

Despite the complexity of the molecular events accompanying heat-induced protein unfolding, several experimental studies strongly validate the possibility of using the simple two-state model to describe this process (Tanford, 1968, 1970; Pfeil and Privalov, 1979; Pfeil, 1986; Lavecchia and Zugaro, 1991; Cioci et al., 1994). The model assumes that only the native folded (F) and the polymorphous unfolded (U) protein forms are present at equilibrium. This hypothesis, of course, does not exclude the possibility of conformational oscillations nor that intermediate states could appear that are neither fully folded nor fully unfolded, as some experiments seem to suggest (Blumenfeld, 1981; Kim and Baldwin, 1982; 1990; Chen et al., 1992). It simply implies that, under the conditions in which the transition is studied, intermediate states are so sparsely populated that they can be neglected. Accordingly, protein unfolding can be schematized as $F \rightleftarrows U$. The equilibrium criterion ($dG_{T,P} = 0$) applied to the distribution of the protein between the two conformational states yields

$$\mu_1 = \mu_2, \tag{1}$$

where the subscripts 1 and 2 denote the folded and unfolded protein forms, respectively. In order to express the chemical potential of the two species in terms of intensive properties (temperature, pressure, and composition) we need to evaluate the Gibbs free energy of the system composed of the folded and unfolded proteins and the mixed solvent. For the sake of simplicity, this latter was regarded as a unique pseudocomponent (component 3), either in the absence or in the presence of additives. The Gibbs free energy of the system containing the three species can be written as

$$G = \sum_{i=1}^{3} n_i g_i^0 + \Delta G, \tag{2}$$

where g_i^0 is the molar free energy of the i-component in its reference state (pure components, at temperature T and pressure P). The term ΔG was determined by considering a thermodynamic cycle composed of three steps, as shown in Figure 1. Thus, we have

$$\Delta G = \Delta G^{I} + \Delta G^{II} + \Delta G^{III}. \tag{3}$$

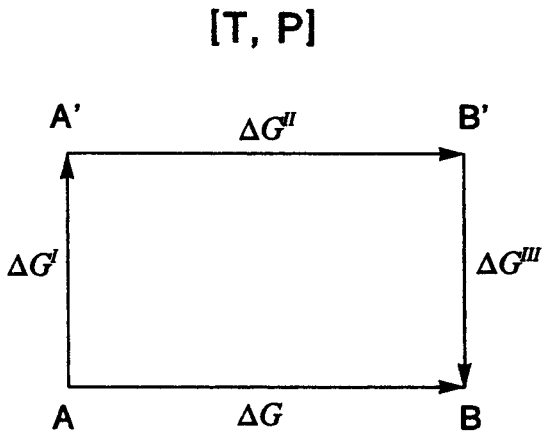

Figure 1. Actual and hypothetical paths for calculation of Gibbs free-energy change.

A: Pure components (1, 2, and 3) in their reference state; A': pure components (1, 2, and 3) with (1, 2) in the conformation assumed in the real mixture; B': (1, 2, and 3) in the ideal mixture; B: (1, 2, and 3) in the real mixture.

The first step corresponds to the structural transformations necessary for the folded and unfolded protein forms to assume the conformations they exhibit in the mixture. Its contribution to the overall free-energy change is

$$\Delta G^{\mathrm{I}} = \sum_{i=1}^{2} n_i \Delta g_i^{\mathrm{conf}}. \tag{4}$$

The second step is related to the ideal mixing of the three components, and leads to a free-energy change equal to

$$\Delta G^{\mathrm{II}} = RT \sum_{i=1}^{3} n_i \ln x_i. \tag{5}$$

Finally, the third step is associated with the introduction of nonideality in the mixture thus obtained. This can be accomplished by considering the various interactions between the protein and the surrounding medium (dispersion forces, dipole–dipole interactions, hydrogen bonding, hydrophobic interactions, etc.) that prevail at the protein–solvent interface (Adamson, 1982). All these interactions are responsible for the work required in separating the protein surface from that of the solvent, and their contribution to the overall free-energy change can be calculated as

$$\Delta G^{\mathrm{III}} = \sum_{i=1}^{2} n_i \gamma_{i,3} a_i, \tag{6}$$

where $\gamma_{i,3}$ is the interfacial free energy between the protein and the solvent, and a_i is the molar surface area of the protein. The term $\gamma_{i,3}$ can be related to the interfacial free energies of pure components by the Dupré equation (Israelachvili, 1985), which yields

$$\gamma_{i,3} = \gamma_3 + \gamma_i - \Delta g_{i,3}^{adh} \qquad (i = 1, 2), \tag{7}$$

where γ_i is the surface energy of the i-component and $\Delta g_{i,3}^{adh}$ is the molar free energy of adhesion between the protein and the surrounding medium per unit protein area. Since there is no way to evaluate the terms γ_i and $\Delta g_{i,3}^{adh}$ separately, it may be convenient to lump them into a residual term, $\gamma_{i,R}$, so as to obtain

$$\gamma_{i,3} = \gamma_3 + \gamma_{i,R} \qquad (i = 1, 2), \tag{8}$$

where $\gamma_{i,R} = \gamma_i - \Delta g_{i,3}^{adh}$. Substitution of Eqs. 3–8 into Eq. 2 gives

$$G = \sum_{i=1}^{3} n_i g_i^0 + \sum_{i=1}^{2} n_i \Delta g_i^{\mathrm{conf}} + RT \sum_{i=1}^{3} n_i \ln x_i + \sum_{i=1}^{2} n_i (\gamma_3 + \gamma_{i,R}) a_i. \tag{9}$$

This equation allows us to express the chemical potential of the i-component, $\mu_i = (\partial G/\partial n_i)_{T,P,nj}$, in the following explicit form:

$$\mu_i = g_i^0 + \Delta g_i^{\mathrm{conf}} + RT \ln x_i + (\gamma_3 + \gamma_{i,R}) a_i. \tag{10}$$

Thus, the equilibrium criterion yields

$$g_1^0 + \Delta g_1^{\mathrm{conf}} + RT \ln x_1 + (\gamma_3 + \gamma_{1,R}) a_1 = g_2^0 + \Delta g_2^{\mathrm{conf}} + RT \ln x_2 + (\gamma_3 + \gamma_{2,3}) a_2. \tag{11}$$

If we define the following quantities

$$\Delta g^0 = g_2^0 - g_1^0 \tag{12}$$

$$\Delta g^{\mathrm{conf}} = \Delta g_2^{\mathrm{conf}} - \Delta g_1^{\mathrm{conf}} \tag{13}$$

$$\Delta(\gamma_R a) = \gamma_{2,R} a_2 - \gamma_{1,R} a_1 \tag{14}$$

$$\Delta a = a_2 - a_1, \tag{15}$$

Eq. 11 takes the form

$$\Delta g^0 + \Delta g^{\mathrm{conf}} + \Delta(\gamma_R a) + \gamma_3 \Delta a + RT \ln \frac{x_2}{x_1} = 0. \tag{16}$$

This equation can also be written as

$$\Delta g^* + \Delta(\gamma_R a) + \gamma_3 \Delta a + RT \ln K_x = 0, \tag{17}$$

where K_x is the equilibrium constant for protein unfolding and $\Delta g^* = \Delta g^0 + \Delta g^{\mathrm{conf}}$. Since Δg^* can be expressed as

$$\Delta g^* = \Delta h^* - T \Delta s^*, \tag{18}$$

Eq. 17 becomes

$$\Delta h^* - T \Delta s^* + \Delta(\gamma_R a) + \gamma_3 \Delta a + RT \ln K_x = 0. \tag{19}$$

At the transition temperature (T_m) the mole fractions of the F and U species are equal, $K_x = 1$, and Eq. 19 reduces to

$$\Delta h^* - T_m \Delta s^* + \Delta(\gamma_R a) + \gamma_3 \Delta a = 0 \qquad (20)$$

or, equivalently,

$$T_m = \varphi^* \gamma_3 + \xi^* \qquad (21)$$

where $\varphi^* = \Delta a/\Delta s^*$ and $\xi^* = [\Delta h^* + \Delta(\gamma_R a)]/\Delta s^*$. This equation indicates that, if the two groups $\Delta a/\Delta s^*$ and $[\Delta h^* + \Delta(\gamma_R a)]/\Delta s^*$ can be assumed constant, that is, temperature- and additive-independent, the transition temperature varies linearly with the surface tension of the mixed solvent. Under such conditions, fitting the experimental data of T_m against γ_3 provides the two parameters φ^* and ξ^*.

Results

The first point emerging from this experimental study is that lysozyme unfolding is a thermodynamically reversible process, as indicated by spectrophotometric measurements performed on the heat-treated protein solution after lowering the temperature to pretransition values. This makes thermal unfolding data amenable to interpretation by molecular thermodynamics.

The experimental thermal unfolding curves in pure buffer and in the presence of additives were very similar in shape. Typical denaturation profiles relative to systems containing a single additive and mixtures of two additives are plotted in Figures 2 and 3, respectively. For comparison, the stability behavior of lysozyme in pure buffer is also shown. The abrupt increase in absorbance characterizing these curves shows that upon heating a highly cooperative, single-step transition occurs. According to these peculiar features and to previous studies on lysozyme denaturation (Tanford et al., 1966; Aune and Tanford, 1969a; Khechinashvili et al., 1973) we assumed that, under the experimental conditions adopted, only the native, folded and the denatured, unfolded forms were present.

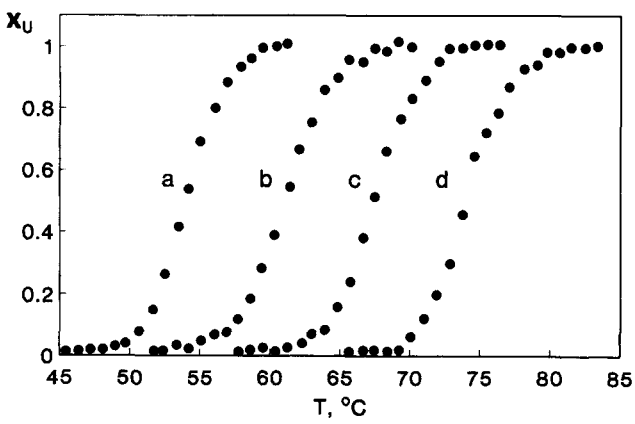

Figure 2. Experimental thermal unfolding curves in systems containing ethanol ((a) 30% w/w; (b) 20%; (c) 10% and in pure buffer (d)); T is the temperature of the protein solution and x_u is the corresponding fraction of protein denatured.

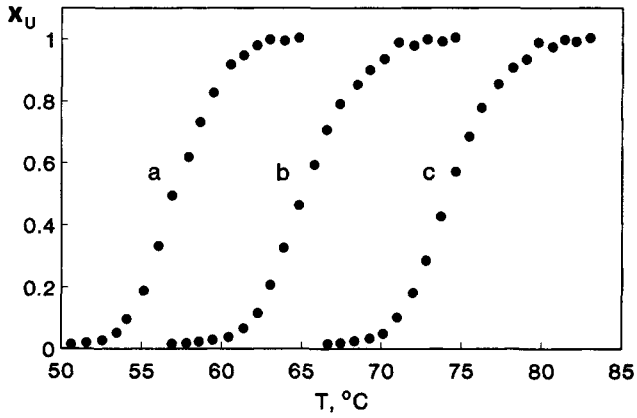

Figure 3. Experimental thermal unfolding curves in systems containing 1,2-propanediol (10% w/w) and a second additive ((a) 20% ethanol; (b) 10% ethanol; (c) 10% ethanediol); T is the temperature of the protein solution and x_u is the corresponding fraction of protein denatured.

Because of the two-state hypothesis, the first derivative of the difference absorption spectrum ($\psi = dA/d\lambda$), which is the observable property chosen to follow unfolding, can be expressed as

$$\psi = x_F \psi_F + x_U \psi_U, \qquad (22)$$

where x_F and x_U denote the fractions of total protein in the folded and unfolded conformations, respectively, while ψ_F and ψ_U represent their corresponding contributions to the overall value of ψ. ψ_F and ψ_U were calculated from the experimental melting curves at $\lambda = 290$ nm, as reported by Pace et al. (1989). In order to account for the effect of temperature on the spectral properties of the F and U forms, the pre- and posttransition base lines were evaluated by a least-square procedure and extrapolated to the transition region. Since at any temperature $x_F + x_U = 1$, Eq. 22 yields

$$x_U = \frac{\psi - \psi_F}{\psi_U - \psi_F}. \qquad (23)$$

The melting temperature (T_m) of the protein was determined at the midpoint of the transition curve, that is, at the point where $x_U = 0.5$. The results are listed in Table 1.

The conformational stability of lysozyme in the absence of additives, which can be estimated from the melting temperature of the protein in pure buffer ($T_m = 74.2$°C), is consistent with reports in the literature (Khechinashvili et al., 1973; Gekko, 1982; Johnston and Castelli, 1989). When hydroxylic cosolvents were added to the protein solution, a detectable change in T_m occurred. In all media this quantity was found to vary monotonously with the additive concentration. The results reported in Table 1 clearly demonstrate that some additives exert a detrimental action on the conformational stability of lysozyme, whereas some others are effective stabilizers. Destabilizing additives include ethanol, glycols (ethanediol and 1,2-propanediol), and their binary mixtures; stabilizing additives comprise sugars (glucose, sucrose, and

Table 1. Effect of Hydroxylic Additives on the Melting Temperature (T_m) of Lysozyme

Additive	% w/w	T_m (°C)
None	—	74.2
Ethanol	10	67.2
	20	61.1
	30	54.1
Ethanediol	10	72.9
	20	72.0
	30	71.1
1,2-Propanediol	10	72.5
	20	70.6
	30	69.3
Ethanediol + 1,2-propanediol	10 + 10	73.8
Ethanol + 1,2-propanediol	10 + 10	65.0
Ethanol + 1,2-propanediol	10 + 20	61.4
Ethanol + ethanediol	20 + 10	58.3
Ethanol + 1,2-propanediol	20 + 10	57.1
Sucrose	10	76.8
	20	79.0
	30	80.5
Glucose	10	76.4
	20	78.6
	30	80.5
Sorbitol	10	76.7
	20	79.2
	30	81.4

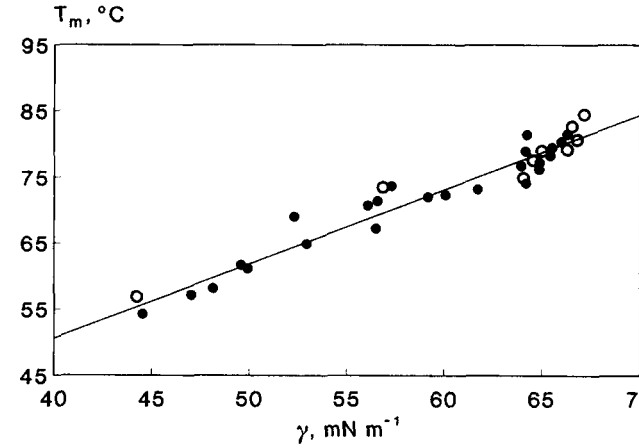

Figure 4. Melting temperature of lysozyme (T_m) as a function of the surface tension of the mixed solvent (γ).

Solid symbols represent experimental data obtained from the present study; open symbols are relative to experiments performed by Gekko (1982) on aqueous solutions of methanol, ethylene glycol, erythritol, xylitol, and sorbitol.

sorbitol). Media containing 30% ethanol resulted in the highest destabilization, decreasing the melting temperature by more than 20°C. In the opposite direction, the most significant increase in stability ($\Delta T_m = 7.4$°C) was attained in the presence of 30% sorbitol. The stabilizing power of the three sugars, however, was nearly the same. On the whole, the effect of these additives on the conformational stability of lysozyme is in qualitative agreement with data in the available literature that are pertinent to different proteins, such as α-amylase (Asther and Meunier, 1990), glucose oxidase (Cioci et al., 1994; Cioci, 1995b), ribonuclease (Gerlsma, 1968; Lee and Lee, 1987), β-galactosidase (Izutsu et al., 1990), α-chymotrypsin (Lozano et al., 1994), chymotrypsinogen, and β-lactoglobulin (Lee and Lee, 1987).

An effort was made to correlate the changes in T_m with some property of the mixed solvent. Attention was first focused on three commonly used indicators of hydrophobicity: the Hildebrand solubility parameter, the dielectric constant, and the partition coefficient between octanol and water (Laane et al., 1987). In fact, even though no general rule can be given on the influence of the hydrophobic features of the protein environment on the macromolecule, the available experience appears to suggest that in some instances the activity and stability of a protein can be affected by the hydrophobicity of the solvent (Nagamoto et al., 1986; Fernandez et al., 1991; Ulbrich and Selisko, 1993). The explanation of this effect is to be found in the fact that solvents exhibiting hydrophobic properties interact more favorably with the hydrophobic side chains of amino-acid residues buried in the protein core than with the external hydrophilic groups. Since during unfolding some of the residues shielded in the protein interior are exposed to the solvent, a hydrophobic medium would result in the stabilization of the unfolded state. Conversely, nonhydrophobic media would be expected to stabilize the native state and increase the melting temperature of the protein. However, poor correlations were found when the melting temperature of lysozyme was plotted against each of the three hydrophobicity indicators. The best result was achieved with the partition coefficient between octanol and water (P). Using the following expression for the mixture property: $(\log P)_{mix} = \Sigma_i z_i \log P_i$, a correlation coefficient of 0.612 was determined. Thus, it can be inferred that solvent hydrophobicity is not the dominant factor involved in stabilization. Conversely, lysozyme stability was found to be strictly related to the surface tension of the mixed solvent, as can be appreciated from Figure 4, where the melting temperature of the protein is plotted against surface tension.

Surface-tension data for aqueous mixtures containing glucose, sucrose, and sorbitol at 20°C are reported in Table 2. In all of these systems, the surface tension of the solvent increases linearly with the additive concentration. These data were therefore correlated as

$$\gamma = \gamma_b + \beta w, \tag{24}$$

where γ_b is the surface tension of pure buffer and w is the weight fraction of the additive. The parameter β was estimated by a least-square procedure, yielding $\beta = 3.4$, 10.6, and

Table 2. Experimental Surface-Tension Values for Systems Containing a Solid Additive

Additive	% w/w	γ_{293} (mN·m^{-1})
Glucose	10	73.55
	20	74.58
	30	75.71
Sucrose	10	72.84
	20	73.21
	30	73.50
Sorbitol	10	73.68
	20	74.79
	30	76.03

γ_{293} is the surface tension of the water-additive mixture at 20°C.

11. 7 $mN \cdot m^{-1}$ for sucrose, glucose, and sorbitol, respectively.

We used the Guggenheim equation (Adamson, 1982) to determine the temperature dependence of surface tension

$$\gamma(T) = \gamma_0 \left[\frac{T_c - T}{T_c - T_0} \right]^{\delta}, \tag{25}$$

where γ_0 is the value of surface tension at the temperature T_0, T_c is the critical temperature of the solvent, and δ is an empiric parameter depending on the class of components considered. We assumed that the properties of pure buffer were equal to those of water ($T_c = 647.3$ K, $\delta = 0.73$).

Surface-tension data relative to water and pure liquid additives were taken from the literature (Lide, 1993). Equation 25 was used for the temperature dependence of surface tension, assuming $\delta = 0.73$ for water and $\delta = 1.22$ for all other components. Surface tensions of aqueous mixtures of liquid additives were estimated by the following relationship (Reid et al., 1988), after checking its accuracy on a limited number of systems

$$\gamma_{mix}^{1/4} = \rho_{mix} \sum_{i=1}^{n} \frac{z_i \gamma_i^{1/4}}{\rho_i}, \tag{26}$$

where γ, ρ, and z represent the surface tension, molar density, and mole fraction, respectively; ρ_{mix} is the density of the mixed solvent—it was calculated as $\rho_{mix} = \Sigma_i z_i \rho_i$. The density and surface tension of water and those of liquid additives are reported in Table 3.

As follows from the experimental data plotted in Figure 4, the melting temperature of lysozyme closely parallels the changes in surface tension induced by either stabilizing or destabilizing additives. The linear relationship between T_m and γ_3, moreover, supports the independence of φ^* and ξ^* on temperature and on the additive nature. A least-square analysis of the experimental data yielded the following estimates: $\varphi^* = 1{,}124$ $m \cdot N^{-1} \cdot K$, $\xi^* = 278.8$ K, and a correlation coefficient of 0.933.

In order to assess the physical significance of this correlation, the experimental data published by Gekko (1982) were analyzed. This author studied the thermal denaturation of lysozyme in aqueous solutions of hydroxylic additives by differential scanning calorimetry. Figure 4 shows the melting temperatures determined by Gekko in media containing methanol, ethylene, glycol, erythritol, xylitol, and sorbitol at concentrations of up to 40 wt. %. As can be seen, the agreement between experimental and calculated melting temperatures is fairly good, with observed differences within ± 4°C. It seems worth noting that most of the systems investigated by Gekko are different from those considered in our study: the calculated melting temperatures can therefore be regarded as pure predictions.

Once the parameters φ^* and ξ^* are estimated, the sensitivity and lysozyme stability to microenvironmental changes induced by the additives can also be determined. This requires evaluation of the function $\vartheta(w) = dT_m/dw$. For solid additives combining Eqs. 21, 24 and 25 yields

$$\vartheta(w) = \frac{\beta\varphi^* \left[\frac{T_c - T_m}{T_c - T_0} \right]^{\delta}}{1 + \delta \left[\frac{T_m - \xi^*}{T_c - T_m} \right]}. \tag{27}$$

The trends of the function $\vartheta(w)$ so calculated showed that ϑ is almost independent of the additive concentration. We obtained $\vartheta = 2.86$, 8.87 and 9.97 K for sucrose, glucose, and sorbitol, respectively. These results indicate that lysozyme is more sensitive to glucose and sorbitol than to sucrose, even though the stabilizing power of these components is comparable, as shown by the T_m data summarized in Table 1.

Table 3. Experimental Surface Tensions and Other Properties of Pure Components

Component	*MW*	T_c (K)	ρ_{298} ($kg \cdot m^{-3}$)	γ_{298} ($mN \cdot m^{-1}$)
Water	18	647.3	1,000	71.99
Ethanol	45	516.2	789	21.97
Ethanediol	62	645.0	1,114	47.99
1,2-Propanediol	76	625.0	1,036	35.86

Source: Data were taken from Liley et al. (1984) and from Lide (1993). *MW* is the molecular weight, T_c is the critical temperature, ρ_{298} and γ_{298} are the liquid density and surface tension, respectively, at 25°C.

Discussion

Experimental results relative to lysozyme unfolding strongly suggest that Eq. 21 accurately describes the additive-induced changes in stability. The dependence of the melting temperature on the bulk surface tension of the mixed solvent provides additional evidence for the key role played by this property in protein stabilization. According to our molecular thermodynamic model, this result could be interpreted in terms of the close relation of surface tension to the interfacial free energy between the protein and the solvent. In particular, the following points can be made:

1. The primary factor governing protein unfolding is the interfacial free energy between the protein and the surrounding medium ($\gamma_{i,3}$). Because of the increased protein–solvent area of contact resulting from unfolding, additives increasing the interfacial free energy should make the transition to the denatured form less thermodynamically favorable than in water, that is, stabilize the protein, while additives decreasing the interfacial free energy should destabilize the macromolecule.

2. The interfacial free energy between the protein and the solvent can be expressed as the sum of two terms: the bulk surface tension of the solvent (γ_3) and the residual free energy ($\gamma_{i,R}$). The first of these is fully nonspecific, while the second is dependent on the chemical nature of both the protein surface and the additive.

3. As long as the residual term can be considered temperature- and additive-independent, an increase in surface tension should cause a parallel increase in the interfacial free energy. Under these conditions the stability behavior of the protein would yield a characteristic straight line on the $T_m - \gamma_3$ plot.

4. When condition (3) is not fulfilled, anomalies can appear in the $T_m - \gamma_3$ plot.

According to this view, we can reasonably assume that the action of the investigated additives relies exclusively on their ability to perturb the surface tension of water. In particular, additives increasing the surface tension of water are also capable of stabilizing the protein. Addition of these components to the protein solution results in increased chemical potential of the protein that is proportional to the extent of the protein–solvent interface. During unfolding the system would tend to relieve this thermodynamically unfavorable situation by reducing the protein–solvent interface, that is, by displacing the unfolding equilibrium toward the compact, native protein. Conversely, the presence of an additive decreasing the surface tension of water would cause the unfolding equilibrium to shift toward the more extended, denatured conformation. In both cases, however, protein unfolding would result from a perturbation of the properties of the solvent rather than from specific interactions (attractive or repulsive) with the protein, which can be assumed to be basically inert. It is interesting to note that under these conditions the free energy of cavity formation should be the main stabilizing force. Accordingly, the additive action could be directly related to the extent to which it perturbs the surface tension of water.

If mechanisms other than surface tension are simultaneously involved, the situation could change considerably. This is the case, for instance, of glycerol, an additive that slightly decreases the surface tension of water. As indicated by the results summarized in Table 4, this component stabilizes lysozyme, raising the melting temperature by almost 7°C at the highest concentration. In view of the preceding considerations, we must therefore assume that glycerol can affect the residual free-energy term, and in particular the free energy of adhesion at the protein–solvent interface, thereby overcoming the reduced surface tension caused by its presence. The balance between these terms would result in an increased interfacial free energy, causing a displacement of the unfolding equilibrium toward the native form.

Of course, the molecular thermodynamic model cannot say anything about the nature of the factors responsible for any similar behavior. The only conclusion that one can reasonably draw is that, whatever occurs in the microenvironment surrounding the protein, glycerol should be excluded from the protein domain. Since the surface-tension mechanism would lead to an enrichment in the additive molecules of the surface layer, the other mechanism(s) should cause this component to migrate into the bulk solvent. According to the peculiar features of glycerol, we can hypothesize that the solvophobic effect, namely, the enhancement of solvent ordering resulting from the stabilization of networks of structured water, is directly involved (Gekko and Timasheff, 1981a). Because of this increased solvent ordering, the hydrophobic groups present on the surface of the macromolecule would tend to migrate into the protein interior (Murphy et al.,1990; Makhatadze and Privalov, 1994). Such migration, however, is sterically hindered by the tight packing of the three-dimensional structure of the protein and also by the fact that hydrophobic groups are covalently bound to the polypeptide backbone (Schulz and Schirmer, 1978; Creighton, 1994). As a result, only the converse could take place, that is, the water and glycerol molecules would redistribute themselves in order to avoid contact between glycerol and the protein. During unfolding this situation would become even more unfavorable thermodynamically, since additional hydrophobic residues shielded in the protein core would be exposed to solvent. The net result would be stabilization of the innate structure of lysozyme despite the reduction in surface tension accompanying glycerol addition.

Table 4. Thermal Unfolding of Lysozyme in Aqueous Media Containing Glycerol

Glycerol Conc. (wt. %)	T_m^{exp} (°C)	γ (mN·m^{-1})	T_m^{calc} (°C)	ΔT_m (°C)
0	74.8	64.1	77.2	−2.4
10	75.9	63.0	76.3	−0.4
20	76.6	61.9	75.4	1.2
30	77.8	60.7	74.5	3.3
40	79.3	59.5	73.5	5.8
50	81.7	58.1	72.5	9.2

Source: Experimental data taken from Gekko (1982).
*Estimated by Eq. 26.
T_m^{exp} is the experimental melting temperature, γ is the surface tension of water–glycerol mixtures at the temperature T_m^{exp}, T_m^{calc} is the melting temperature predicted by the molecular thermodynamic model, ΔT_m is the difference between experimental and calculated melting temperatures.

Simlar arguments could be made to explain the influence of some nonhydroxylic additives such as urea, which increases the surface tension of water but favors unfolding (Tanford, 1968; Prakash et al., 1981), or betaine, which lowers the surface tension of water but stabilizes proteins (Arakawa and Timasheff, 1983). Within this context, Eq. 8 would quantitatively represent the competition between the nonspecific surface-tension effect and all other specific mechanisms that affect protein stability.

To conclude, as long as $\Delta g_{i,3}^{adh}$, and hence $\gamma_{i,R}$, can be considered solvent-independent, a linear dependence of T_m on γ_3 should be observed. Departures from linearity indicate more or less significant perturbations of the free energy of adhesion between the protein and the solvent induced by the additive. In this respect, the $T_m-\gamma_3$ plot could represent a simple means of diaganosing whether the surface tension effect provides an overwhelming contribution to stabilization.

Conclusions

The experimental evidence provided by the present investigation strongly suggests the central role played by interfacial phenomena in protein unfolding. It has been shown that the influence of the hydroxylic additives examined on the conformational stability of lysozyme can be fairly well described by a linear relationship between the melting temperature of the protein and the bulk surface tension of the mixed solvent. The molecular thermodynamic model developed provides a physical validation of the preceding observation and allows us to outline the conditions under which the Gibbs equilibrium criterion applied to the structural transition between the folded and the unfolded states leads to a linear dependence of melting temperature on surface tension. It seems worth noting that both quantities are easily measurable, and this supports the possibility of using the model for the interpretation and correlation of thermal unfolding data. In addition, useful suggestions can be gained for devising strategies for reinforcing the three-dimensional structure of proteins.

Concerning the problem of stabilization, it should be remembered that one can try to stabilize proteins either intrin-

sically, by chemical or genetic modifications, or by changing the properties of the environment surrounding the protein. The solvent engineering approach attempts to achieve an acceptable degree of stabilization by modifying the nature and concentration of the protein environment. To reach this goal, a greater knowledge of the molecular mechanisms involved in solvent-mediated stabilization is required. The experimental results reported in the present study clearly demonstrate that the action of most common stabilizers relies on their ability to increase the surface tension of water. A complete understanding of how this is accomplished and application of these results to the design of an optimal aqueous medium are areas of active research.

Notation

A = radiation absorbance
h = molar enthalpy ($J \cdot mol^{-1}$)
n = number of moles
R = gas constant ($8.314\ J \cdot mol^{-1} \cdot K^{-1}$)
s = molar entropy ($J \cdot mol^{-1} \cdot K^{-1}$)
x = mole fraction
ϵ = molar extinction coefficient ($mol^{-1} \cdot m^2$)
$\vartheta(w)$ = sensitivity function defined by Eq. 27

Subscripts and superscript

b = pure buffer
max = maximum
mix = mixture property
N = normalized value
conf = conformational property

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