Biophysical Chemistry 25 (1986) 243–251
Elsevier

BPC 01108

EFFECTS OF THERMODYNAMIC NONIDEALITY ON PROTEIN INTERACTIONS

EQUIVALENCE OF INTERPRETATIONS BASED ON EXCLUDED VOLUME AND PREFERENTIAL SOLVATION

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Received 20th June 1986
Accepted 29th September 1986

Key words: Thermodynamic nonideality; Excluded volume; Preferential solvation

Published results on the stabilization of proteins by sucrose (J.C. Lee and S.N. Timasheff, J. Biol. Chem. 256 (1981) 7193) have been reexamined and interpreted in terms of thermodynamic nonideality. The composition dependence of activity coefficients may be accounted for on a statistical-mechanical basis using the concept of excluded volume. An expression is derived in which the effect of sucrose on determination of the partial specific volume of a protein, previously interpreted in terms of preferential protein solvation, is also seen to be attributable to excluded volume. Gel chromatographic studies of the reversible unfolding of α-chymotrypsin are presented which demonstrate temperature- and sucrose-mediated changes in the effective volume of the enzyme. These measurements support the quantitative interpretation of the stabilization in terms of thermodynamic nonideality arising from the difference between covolumes for sucrose and the two isomeric states of α-chymotrypsin. By establishing the equivalence of the two approaches that have been used to account for the effects of inert solutes on protein transitions, the present investigation eliminates the need for any distinction between such solutes on the basis of molecular size; and also enhances greatly the potential sensitivity of thermodynamic nonideality as a means of probing protein isomerizations, since greater displacement of the equilibrium position may be effected by small rather than by macromolecular solutes present at the same weight concentrations

1. Introduction

The effects of inert solutes on proteins and their mutual interactions have attracted considerable attention in recent years, during which period these consequences of thermodynamic nonideality have been examined for large [1–10] and small [11–18] inert compounds. Whereas the effects of added macromolecular solutes have been considered in relation to composition-dependent activity coefficients based on a statistical-mechanical application of the excluded volume concept [19–22], results obtained by including small inert solutes in protein solutions have been interpreted in terms of preferential solvation of the protein [23,24]. Both interpretations concur in attributing the thermodynamic effects to entropic changes caused by addition of the inert solute. Indeed, there is experimental support for this disregard of enthalpic changes [14,16]. It becomes a matter of interest and importance, therefore, to establish the equivalence of these two approaches, and thereby to obviate any need for decisions about the size of inert solute at which the changeover from interpretation in terms of preferential solvation to interpretation in terms of excluded volume should occur. The purpose of this investigation is to consider published results pertaining to the effects of small solutes on protein solutions in terms of statistical-mechanical concepts and hence il-

lustrate the correspondence between the two treatments of thermodynamic nonideality.

Since measurements of partial specific volume have provided the major experimental evidence for considering the effects of small solutes such as sucrose and glycerol in terms of preferential solvation, the first point requiring attention is the development of a procedure for analyzing those results on the alternative basis of excluded volume. Experimentally, the difference between two estimates of the partial specific volume provides the means for quantitative evaluation of the extent to which a protein is preferentially solvated in the presence of a small solute, M. One estimate of the apparent partial specific volume of the protein (ϕ_A^*) is obtained by comparing the densities of protein solutions with the density of solvent containing the same molal concentration of small solute that is present in the protein solutions. The other estimate (ϕ'_A) is obtained from the difference between the densities of protein solutions and those of small solute solutions with which they are in dialysis equilibrium (i.e., under conditions of constant chemical potential of small solute). In the sense that partial specific volume ($\bar{v}_A$) is defined as the volume increase per g protein in the absence of any other changes in chemical compositions [i.e., $\bar{v}_A = (\partial V/\partial g_A)_{T,P,m_j \neq m_A}$], ϕ_A^* is clearly the estimate of the true thermodynamic quantity. Previous practice [13–18] has been to interpret the difference between ϕ'_A and ϕ_A^* in terms of a preferential interaction parameter ξ_M, the number of grams of small solute bound per g protein [25]; and the fact the ξ_M was consistently negative implied a preferential enrichment of solvent (water) at the protein domain (or, in other words, an exclusion of small solute from the protein domain). An alternative approach is to employ the statistical-mechanical concept of excluded volume to relate the difference between ϕ'_A and ϕ_A^* to the nonidentity of inert solute concentrations in the two phases of the experiments conducted at constant chemical potential of M.

2. Theory

Consider a series of experiments in which protein solutions are brought into dialysis equilibrium with solvent containing a small (diffusible) solute, M. If α and β superscripts are used to designate the protein and diffusate phases, respectively, ϕ_A^* and ϕ_A^* are determined as limiting values ($c_A^\alpha \to 0$) from the expressions,

$$(\rho - \rho_o^\alpha)/c_A^\alpha = (1 - \phi_A^* \rho_o^\alpha) \tag{1a}$$

$$(\rho - \rho_o^\beta)/c_A^\alpha = (1 - \phi'_A \rho_o^\beta) \tag{1b}$$

in which ρ is the density of the dialyzed protein solution with concentration c_A^α, ρ_o^α that of a solution of the small solute M with the same molal concentration as that present in the protein-containing phase (α), and ρ_o^β that of the diffusate. It should be noted that use of eq. 1a is consonant with the conventional assumption [13–15] that there is no change in the partial molar volume of the protein when it is dissolved in solvent which contains small solute M – an assumption for which there is support from experimental studies with sucrose [14] and glycerol [13,15] as M. The aim is to relate the difference between ϕ'_A and ϕ_A^* to the nonidentity of small solute concentrations in the α and β phases that results from the condition for constant thermodynamic activity of M.

In statistical-mechanical terms the activity coefficient, y_i, of species i may be expressed [19] as,

$$\ln y_i = \sum \alpha_{ij} m_j + \text{higher terms} \tag{2a}$$

in which j is allowed to span all species, including i, and

$$\alpha_{ij} = U_{ij} - M_j \bar{v}_j + \frac{z_i z_j (1 + \kappa r_i + \kappa r_j)}{2I(1 + \kappa r_i)(1 + \kappa r_j)} \tag{2b}$$

where U_{ij} is the covolume of species i and j, the second term denotes the partial molar volume of j, and the third describes charge-charge interactions with conventional nomenclature [20]. At low concentrations m_j it is customary to neglect the higher terms and consider only those corresponding to the second virial coefficient.

Combination of the condition for constant thermodynamic activity of small solute, i.e.,

$$y_M^\alpha m_M^\alpha = y_M^\beta m_M^\beta \tag{3a}$$

with the truncated form of eq. 2a for the activity

coefficients y_M^α and y_M^β gives,

$$m_M^\alpha/m_M^\beta = \exp\left[\alpha_{MM}\left(m_M^\beta - m_M^\alpha\right) - \alpha_{AM}m_A^\alpha\right] \quad (3b)$$

In making use of eqs. 3a and 3b we are referring all measurements of the thermodynamic activity of small solute to a single standard state in which $m_M \to 0$ and the protein concentration, c_A, is zero. On expansion of the exponential and neglecting terms higher than first order, eq. 3b may be rearranged to the form,

$$\left(m_M^\beta - m_M^\alpha\right)/m_A^\alpha = \alpha_{AM}m_M^\beta/\left(1 - \alpha_{MM}m_M^\beta\right) \quad (4)$$

where the concentrations are all defined on a molar basis. For interpreting density measurements it proves more convenient to express the left-hand side of eq. 4 as the ratio of weight concentrations (g/ml), in which case

$$\left(c_M^\beta - c_M^\alpha\right)/c_A^\alpha = M_M\alpha_{AM}m_M^\beta/\left[M_A\left(1 - \alpha_{MM}m_M^\beta\right)\right] \quad (5)$$

where M_M and M_A denote the respective molecular weights of M and A, while m_M^β continues to refer to the molar concentration of M in the diffusate.

In regard to the consideration of density measurements, eqs. 1a and 1b are readily rearranged to give

$$\left(\phi_A' - \phi_A^*\right) = \left(\rho_o^\beta - \rho_o^\alpha\right)\left(1 - \phi_A^*c_A^\alpha\right)/c_A^\alpha\rho_o^\beta \quad (6)$$

Furthermore, the density difference may be expressed in terms of the solute-free solvent density, ρ_o^o, as

$$\left(\rho_o^\beta - \rho_o^\alpha\right) = \left(1 - \bar{v}_M\rho_o^o\right)\left(c_M^\beta - c_M^\alpha\right) \quad (7)$$

where $\bar{v}_M$ is the partial specific volume of M. Again we note that this expression presumes validity of the conventional assumption that no volume change accompanies dissolution of inert solute M in solvent. Combination of eqs. 4–7 and subsequent rearrangement leads to the expression,

$$\left(\phi_A' - \phi_A^*\right) = \frac{\left(1 - \bar{v}_M\rho_o^o\right)\left(1 - \phi_A^*c_A^\alpha\right)M_M\alpha_{AM}m_M^\beta}{\rho_o^\beta M_A\left(1 - \alpha_{MM}m_M^\beta\right)} \quad (8)$$

Since reported values of ϕ_A' and ϕ_A^* are obtained by extrapolating $d\rho/dc_A^\alpha$ to zero protein concentration, the second term in the numerator of eq. 8 is unity and hence a linear relationship is predicted between $(\phi_A' - \phi_A^*)$ and $m_M^\beta/\rho_o^\beta(1 - \alpha_{MM}m_M^\beta)$. The slope of this linear relationship defines the quantity $(1 - \bar{v}_M\rho_o^o)\alpha_{AM}M_M/M_A$, which in turn allows the estimation, via eq. 2b, of U_{AM}, the covolume of protein and small solute.

3. Experimental

Bovine pancreatic α-chymotrypsin (crystallized, three times, lyophilized and salt-free) was obtained from Sigma and used without further purification. The concentrations of enzyme solutions, prepared by direct dissolution of the crystals in 0.01 M HCl, or in 0.01 M HCl containing either sucrose (0.5 M) or glycerol (10%, v/v), were determined spectrophotometrically at 278–280 nm on the basis of the absorption coefficients ($A_{1\,cm}^{1\%}$) tabulated in refs. 14 and 15.

In experiments designed to examine the effect of temperature on the hydrodynamic size of α-chymotrypsin, which is monomeric under these acidic conditions [14,26], enzyme solution in 0.01 M HCl (1 ml, 3 mg/ml) was subjected to zonal gel chromatography on a column of Sephadex G-100 (1.5 × 36.8 cm) equilibrated at the appropriate temperature (24, 32, or 40°C) with 0.01 M HCl and maintained at a flow rate of 24 ml/h. The eluate was divided into 1.35-ml fractions, the concentrations of each fraction being monitored at 280 nm.

Difference gel chromatography [27] has been used to examine the effects of sucrose (0.5 M) and glycerol (10%, v/v) on the isomeric transition between native and unfolded states of α-chymotrypsin in 0.01 M HCl. Solutions of α-chymotrypsin (20 ml, approx. 1 mg/ml) in 0.01 M HCl containing the appropriate small solute were applied to a column of Sephadex G-100 (1.3 × 12.5 cm) equilibrated at 32°C with 0.01 M HCl, after which the column was eluted with 0.01 M HCl containing the appropriate small solute. Throughout the experiment the flow rate of the column was maintained at 13.8 ml/h, and the

eluate was collected in 0.57-ml fractions, which were monitored at either 278 or 280 nm after 3-fold dilution.

4. Results and discussion

4.1. Concentration dependence of activity coefficients for small solutes

In the application of eq. 2 to macromolecular solutes there has been a general tendency (but see refs. 21 and 22) to consider species to be rigid impenetrable spheres (radius r) or selected other regular shapes [28]. Although this geometrical requirement is unlikely to be an unduly restrictive condition for small solutes, the validity of describing the composition dependence of activity coefficients in terms of excluded volumes, i.e., eq. 2, must be closely examined. For example, the conformity of the activity coefficients of small charged solutes with Debye-Hückel theory clearly precludes their description in such a manner because of the dominance of the charge-charge contribution to the activity coefficient. However, the two most commonly used small inert solutes, sucrose and glycerol, are both uncharged and hence the concentration dependence of activity coefficient for the pure solute may be defined operationally in terms of covolume (U_{MM}) and partial molar volume ($M_M\bar{v}_M$) contributions provided that ln y_M increases linearly with molar concentration, m_M.

Fig. 1 summarizes the concentration dependence of activity coefficients for sucrose (fig. 1a) and glycerol (fig 1b), the results [29] being plotted in the format indicated by eq. 2. The present requirement for description of the results in terms of linear relationships is eminently reasonable in both cases, despite the extension of the concentration range beyond that for which activity coefficients may be expressed with confidence in terms of second virial coefficients only. Combination of the value of 0.19 l/mol for the second virial coefficient (α_{MM}) of sucrose obtained from fig. 1a with the partial molar volume ($M_M\bar{v}_M$) of 0.21 l/mol [14] leads to an estimate of 0.40 l/mol for U_{MM}, the self-covolume of this solute. On the basis of spherical geometry ($U_{MM} = 32\pi N r_M^3/3$) this corresponds to an effective radius, r_M, of 0.27 nm. Corresponding calculations with glycerol yield values of 0.029 l/mol for α_{MM} (fig. 1b), 0.071 l/mol for $M_M\bar{v}_M$ [15], 0.100 l/mol for U_{MM}, and 0.17 nm for r_M. It is therefore concluded that the statistical-mechanical interpretation of activity coefficients in terms of excluded volume does appear to be valid for these particular solutes. However, it must be stressed that these effective radii are considerably smaller than the respective hydrodynamic radii of 0.48 and 0.33 nm [30] obtained by using the Stokes-Einstein relationship, a comparison which serves to emphasize the operational nature of these effective radii for particular covolume calculations.

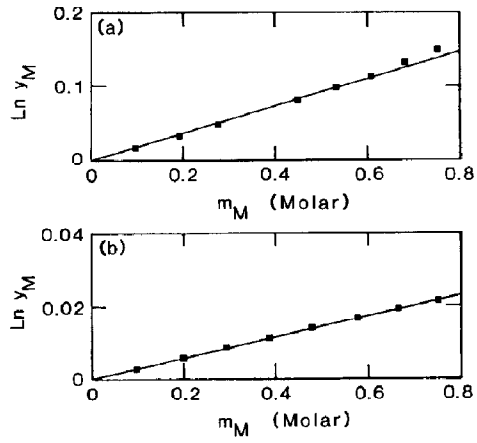

Fig. 1. Concentration dependence of the activity coefficient, y_M, of (a) sucrose and (b) glycerol: the results, taken from ref. 29, are plotted according to eq. 2 for evaluation of the second virial coefficient, α_{MM}.

From the viewpoint of calculating covolumes for proteins and these small solutes, the important point to emerge from fig. 1 is not so much the value of r_M but rather the demonstration that the activity coefficients of sucrose and glycerol are amenable to interpretation in excluded volume terms. Because of the smallness of r_M and its proximity to the notional radius of water molecules, 0.14 nm [30], it seems reasonable to assume that the summed radii of protein and small solute for calculations of covolume ($U_{AM} = 4\pi N(r_A + r_M)^3/3$) approximate r_A, whereupon the hydrated

molar volume of the protein (V_A^h) should provide a reasonable estimate of U_{AM}.

4.2. Interpretation of partial specific volume measurements in terms of excluded volume

Having established the adequacy of the statistical-mechanical approach to describe the activity coefficients of pure sucrose and glycerol solutions, the next step is to test whether the excluded volume concept continues to describe the composition dependence of the activity coefficient of a small solute in protein solute mixtures. This aspect is examined by applying eq. 8 to published results [14] for the effect of sucrose on the apparent partial specific volume of proteins from measurements obtained at constant chemical potential of small solute (ϕ'_A).

Fig. 2 summarizes, in the format mentioned in relation to eq. 8, the effect of sucrose on partial specific volume measurements for α-chymotrypsin (■), chymotrypsinogen A (□), and ribonuclease (●), a value of 0.19 l/mol for α_{MM} (fig. 1a) having been used for the construction of this plot from experimental results (ϕ'_A, ϕ^*_A, m^β_M) reported in table III of Lee and Timasheff [14]. The first point to note is the curvilinear nature of these plots, which contrasts with the linearity predicted by eq. 8; but this disparity must be viewed in light of the adequacy of a truncated form of eq. 2 for the activity coefficient of small solute. On the basis, therefore, that eq. 8 refers to limiting tangents (as $m^\beta_M \to 0$), the results have been interpreted in terms of the slopes of the broken lines shown in fig. 2. Further details of such analyses are provided in table 1, from which it is evident that for each of the three proteins the deduced covolume for sucrose and protein (U_{AM}) matches fairly closely the hydrodynamic volume (V_A^h) inferred from the Stokes radius. In view of the smallness of sucrose in relation to the size of the proteins, the essential identity of U_{AM} and V_A^h is considered to support the present interpretation of the effect of sucrose on partial specific volume measurements in statistical-mechanical terms of excluded volume.

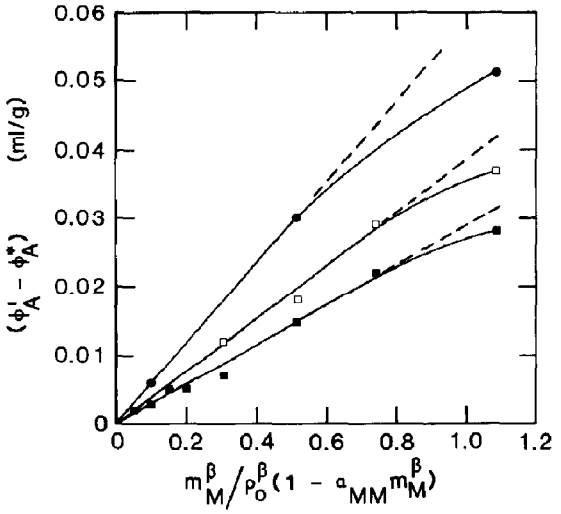

Fig. 2. Statistical-mechanical interpretation of published results [14] for the effect of sucrose concentration (m^β_M) on the partial specific volumes of proteins measured under conditions of constant composition (ϕ^*_A) and constant chemical potential (ϕ'_A) of sucrose. Results for ribonuclease (●), chymotrypsinogen A (□) and α-chymotrypsin (■) are plotted in accordance with eq. 8, the broken lines representing estimates of the limiting tangents as $m^\beta_M \to 0$.

Although the concept of excluded volume has been shown to account for the effect of sucrose on the partial specific volume measurements conducted at constant chemical potential of small solute, it must be emphasized that this finding is merely confirming the earlier explanation of the results in terms of preferential solvation [14]. Indeed, that analysis of the extent to which sucrose penetrates the three-dimensional protein structure leads to the conclusion that sucrose is effectively excluded from the hydrated protein domain, whereas the present treatment signifies compliance of the results with the assumption that sucrose is so excluded. The major importance of the current treatment is thus not so much the recognition of the sucrose-mediated phenomenon as an excluded volume effect, but rather its expression in statistical-mechanical terms that provide quantitative analysis of the phenomenon in the framework of thermodynamic activity coefficients. This aspect is amplified further in section 4.3, which considers the effect of sucrose on the reversible denaturation of α-chymotrypsin.

Table 1

Evaluation of covolumes for sucrose (M) and proteins (A) from partial specific volume measurements

Protein	M_A	$\bar{v}_A$ (ml/g) [a]	α_{AM} (l/mol) [b]	U_{AM} (l/mol) [c]	Hydrodynamic parameters		
					$s_{20,w}$ (S) [d]	r_A (nm) [e]	(l/mol) [f]
α-Chymotrypsin	25000	0.738	5.4	23.9	2.75 (26)	2.11	23.7
Chymotrypsinogen A	25500	0.734	7.4	26.1	2.75 (26)	2.18	26.1
Ribonuclease	13700	0.693	6.3	15.8	2.02 (31)	1.84	15.7

[a] Taken from table III of ref. 14.
[b] Inferred from the slope of the limiting tangent in fig. 2.
[c] From eq. 2b, $U_{AM} = \alpha_{AM} + M_A\bar{v}_A$ since $z_M = 0$.
[d] Numbers of parentheses denote the sources of $s_{20,w}$.
[e] Stokes radius of the protein deduced from $s_{20,w}$.
[f] Hydrated volume of the protein based on the Stokes radius and spherical geometry.

4.3. *Effect of small solutes on isomeric transitions*

The use of thermodynamic nonideality arising from the space-filling effects of added inert solutes has been recommended recently [9] as a means of establishing the equilibrium coexistence of isomeric protein states. Specifically, the suggested procedure entails examination of the effect of an uncharged solute, M, on the apparent isomerization constant under conditions such that the protein concentration is sufficiently small in relation to that of M for terms in m_M to be effectively the sole source of thermodynamic nonideality. Under those conditions the equilibrium position of a protein isomerization A $\rightleftarrows$ B is governed by,

$$X' = m_B/m_A = X \exp[(U_{AM} - U_{BM})m_M] \qquad (9)$$

where X is the thermodynamic isomerization constant and X' the apparent isomerization constant measured in the presence of a concentration m_M of inert solute [9]. From the logarithmic form of this expression it is evident that a plot of (ln $X' -$ ln X) vs. m_M is linear with a slope of $(U_{AM} - U_{BM})$. Although M was envisaged originally to be macromolecular, figs. 1 and 2 indicate that the concept of excluded volume should extend to solutes of all sizes.

The feasibility of using this approach with small solutes is examined by considering a spectrophotometric study [14] of the reversible unfolding of α-chymotrypsin in millimolar HCl. Equilibrium constants, or rather their logarithms, have been taken from fig. 4a of Lee and Timasheff [14], which establishes the parallel nature of Van't Hoff plots for the thermal denaturation of α-chymotrypsin in the presence of various sucrose concentrations. Such identity of slopes signifies the absence of any enthalpic contribution to the change in X, an assumption inherent in the excluded volume treatment. It also permits values of (ln $X' -$ ln X) to be obtained from the vertical spacing between the Van't Hoff plots. From fig. 3, which presents an analysis of these results in accordance with the logarithmic form of eq. 9, the covolume U_{BM} for sucrose and the unfolded form of the enzyme (form B in the terminology of Biltonen and Lumry [32]) is 4.2 l/mol greater than

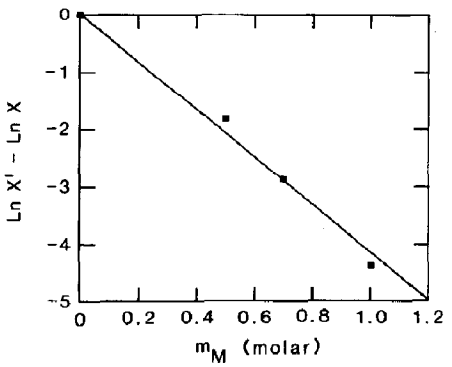

Fig. 3. Evaluation of the apparent molar covolume difference, $(U_{AM} - U_{BM})$, from the effect of sucrose concentration (m_M) on the isomerization constant for the reversible unfolding of α-chymotrypsin in millimolar HCl. The experimental points, calculated from fig. 4a of Lee and Timasheff [14], are plotted in accordance with the logarithmic form of eq. 9.

U_{AM}, the corresponding quantity for sucrose and native α-chymotrypsin (form A). On the basis of the value of U_{AM} reported in table 1 (23.9 l/mol, and considered equal to V_A^h), the covolume difference deduced from fig. 3 signifies that an 18% increase in volume (or a 6% increase in Stokes radius) accompanies the reversible unfolding of α-chymotrypsin under these conditions. In terms of excluded volume, therefore, the observed stabilization of α-chymotrypsin by sucrose finds rational explanation by invoking the denatured isomer (hydrated) to be larger and/or more asymmetric than the native hydrated enzyme, whereupon the mere occupancy of space by the inert solute (sucrose) brings about displacement of the isomerization equilibrium towards the native, more compact, hydrated enzymic form – the isomer that is clearly favored form the viewpoint of entropic considerations.

It is obviously of interest and importance to determine whether the spectrophotometrically detected unfolding of α-chymotrypsin [14,32] is, indeed, accompanied by an observable increase in effective size of the enzyme. For this purpose

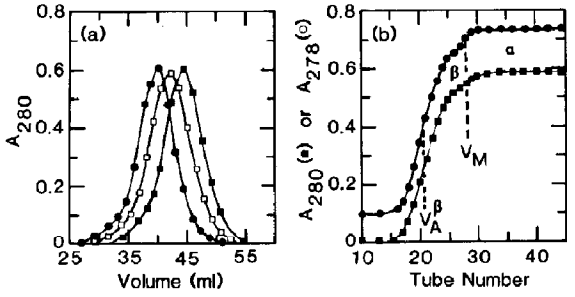

Fig. 4. Gel chromatographic demonstrations of the reversible unfolding of α-chymotrypsin in 0.01 M HCl. (a) Elution profiles obtained in zonal experiments of a column of Sephadex G-100 (1.5×36.8 cm) maintained at 24°C (■), 32°C (□), and 40°C (●).(b) Elution profiles obtained in differential gel chromatography [27] experiments in which enzyme in 0.01 M HCl containing either 0.5 M sucrose (●) or 10% (v/v) glycerol (■) was applied to a column of Sephadex G-100 (1.3×12.5 cm) preequilibrated at 32°C with 0.01 M HCl, the ordinate scale for the sucrose experiment being displaced by 0.1 absorbance units to separate the two patterns: V_A^β denotes the elution volume of α-chymotrypsin in 0.01 M HCl; and V_M that of small solute (sucrose or glycerol), which marks the delineation between β-phase and the plateau of original composition (α-phase).

α-chymotrypsin has been subjected to gel chromatography on a column of Sephadex G-100 equilibrated at various temperatures with 0.01 M HCl, this more acidic environment (pH 2 cf. pH 3) being used to displace the isomeric transition into a more convenient temperature range [32]. From fig. 4a it is evident that an increase in temperature from 24 to 40°C gives rise to a decrease of 4 ml in elution volume; and that at 32°C, the reported midpoint of the transition under these conditions [32], the elution volume is approximately halfway between the two extreme values. Consideration of the difference between the native (24°C) and unfolded (40°C) forms in terms of the usual calibration plot [33] signifies a 25% change in apparent molecular weight (volume), or an 8% difference between the effective hydrodynamic radii of the two isomeric states: this agrees reasonably well with the 6% difference inferred from fig. 3. Such demonstration of the existence of a change in effective Stokes radius clearly strengthens the case for considering the stabilization of α-chymotrypsin by sucrose in terms of excluded volume. Moreover, additional evidence for this concept comes from differential gel chromatography experiments designed to detect the displacement of the A ⇄ B isomeric transition by small solutes.

Fig. 4b presents elution profiles obtained by applying α-chymotrypsin (approx. 1 mg/ml) in 0.01 M HCl containing sucrose (0.5 M) or glycerol (10%, v/v) to a 17 ml column of Sephadex G-100 equilibrated with 0.01 M HCl at 32°C, the midpoint of the isomeric transition (where $X' = 1$). In this design of experiment the existence of an initial plateau region (β) in which the concentration of enzyme differs from that in the plateau of original composition (α) implies different migration rates (elution volumes) of α-chymotrypsin in the presence and absence of small solute. From fig. 4b it is clear that the concentration of enzyme in the β region is less than that in the α-phase in experiments with either sucrose (●) or glycerol (■) included in the applied solution. The consequent conclusion that α-chymotrypsin migrates more slowly in the presence of small solute thus provides further support for attributing the enhancement of enzyme stability to effects of excluded volume on thermodynamic nonideality; i.e., to

nothing more specific than the ability of these small inert solutes (which cannot penetrate the hydration zone of proteins) to occupy space and hence displace equilibria in the direction of the more compact forms of hydrated enzyme species.

This demonstration that the effects of small solutes on protein interactions may also be interpreted in terms of the statistical-mechanical idea of excluded volume suggests greater potential for observations of thermodynamic nonideality as a means of detecting isomeric transitions. Initially, the use of macromolecular inert solutes was recommended [9] for this purpose, since at that time the effects of small solutes were being considered to reflect preferential solvation [13–18]: the present investigation has removed the need for any such distinction between inert solutes on the basis of molecular size. Furthermore, much greater sensitivity results from the use of small solutes in studies employing thermodynamic nonideality as a probe of protein isomerizations, a factor clearly evident by comparing the effectiveness of sucrose and Dextran T10 (a polysaccharide with M_r 10000) as inert solutes for the stabilization of α-chymotrypsin. On the basis of fig. 3, the effect of sucrose on the isomeric transition is described by the relationship $X' = X \exp(-4.2\ m_M)$, which predicts that a 20% sucrose solution (0.58 M) should effect an 11-fold decrease in X'. If Dextran T10 were used as inert solute, the corresponding expression based on values of 2.11 and 2.23 nm for r_A and r_B (table 1) and 2.6 nm for r_M [9] would be $X' = X \exp(-21\ m_M)$: a 20% solution of Dextran T10 (0.02 M) would thus cause considerably less change ($X' = 0.66X$) in the equilibrium position. Clearly, the use of small inert solutes that are excluded from hydrated protein domains is advantageous from the sensitivity viewpoint.

5. Concluding remarks

The most important factor to emerge from this investigation is the reconciliation of the two approaches that have been used to account for the effects of inert solutes on protein interactions. Specifically, it has been shown that results for small solutes, previously interpreted in terms of preferential solvation [13–18], also find an explanation in terms of the statistical-mechanical idea of excluded volume. A corollary also applies: the effects of inert macromolecular solutes could be interpreted in terms of preferential solvation rather than of excluded volume, although this would lead to too general a view of what the term 'solvation' accounted for. From the viewpoint of predicting quantitatively the effects of inert solutes on protein interactions, the statistical-mechanical interpretation based on the idea of excluded volume clearly affords greater potential by virtue of its ability to account for displacements of protein transitions in terms of thermodynamic nonideality.

A point of experimental interest is the use of gel chromatography (fig. 4) to obtain evidence of an isomeric transition (the reversible unfolding of α-chymotrypsin) by small solutes (sucrose and glycerol). Previous attempts (e.g., refs. 6, 14, and 15) to obtain evidence for the equilibrium coexistence of compact and unfolded isomeric protein states have been relatively unsuccessful, but indirect methods such as circular dichroism were used in those investigations. Since a difference between the molecular volumes or shapes of the two isomeric states provides the logical explanation of the observed thermodynamic nonideality, the effect of inert solute on a hydrodynamic parameter is clearly the most direct means of establishing the existence of such isomeric transitions.

Finally, the present analysis of the effects of small inert solutes on protein interactions in statistical-mechanical terms has enhanced greatly the potential sensitivity of thermodynamic nonideality as a means for probing protein isomerizations, since greater displacement of the equilibrium position is effected by a small inert solute which is excluded from the hydration domain of a protein than is achieved by including the same weight concentration of a macromolecular solute.

Acknowledgements

We are extremely grateful to Dr. S.N. Timasheff of Brandeis University for the stimulating discus-

sion (with D.J.W.) of thermodynamic nonideality that led to this investigation. Financial support by the Australian Research Grants Scheme is also gratefully acknowledged.

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