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DENATURATION OF PROTEINS IN METHANOL/WATER MIXTURES

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The solvophobic theory developed earlier by Sinanoğlu introducing the use of molecular surface areas and microthermodynamic surface and interfacial tensions at molecular dimensions is applied to the interpretation of calorimetric data on denaturation of lysozyme in a wide range of methanol/water mixtures. The experimental values of standard unitary free energies of denaturation correlate well with our predictions. The molecular surface area change of the protein upon denaturation is evaluated using the solvophobic theory. The maximum in the stability of the native form of the protein is predicted to occur at 8% (v/v) methanol. This is found to be in agreement with the experimental results.

1. Unitary standard free energies of denaturation of proteins in methanol/water mixtures

The 'solvophobic theory' [6a] for the solvent denaturation of biopolymers as well as other molecular association (or its reverse) processes was developed quantitatively and has been tested experimentally in the interim in diverse fields [1–3,8–12]. Two key notions introduced by the solvophobic theory [4,6a] were: (a) the molecular surface area of a molecule, or rather that of the inner surface of a molecular solvent cavity enveloping the solute, and (b) the 'microthermodynamic surface tension' applicable down to single-molecule dimensions of cavity inner surfaces. These notions which occurred in the detailed working equations of the solvophobic theory have been found rather fruitful in biopolymer tertiary and higher structure studies, drug-biomolecule associations [1–3], HPLC [8], in quantum chemistry [9], in environmental chemistry [10] and others [11,12].

Recently, the native form of globular proteins has been reported to be stabilized by a minimization of the surface of contact between the protein and sucrose solutions as the solvent (see refs. 1–3) providing important confirmation of the notions of solvophobic theory in such a complex solvent system. A detailed analysis of the denaturation of a protein in sucrose solution shows that its stabilizing action depends only on the surface energy of the solvent system [2,3]. This was tested by calculating the surface tension at the melting temperature of the protein in different sucrose/water systems. The surface tension is constant at the melting temperature for any sucrose concentration, thus indicating that this is the only cohesive force that stabilizes the folded form of the protein.

Analogous results were obtained when measuring the interaction between tubulin and other proteins with structure-stabilizing solvents (water, sucrose, water/glycerol) [2,3]. The present paper provides new evidence supporting the predictions by Sinanoğlu in the solvophobic theory (for a recent survey see ref. 4a and references therein) in the sense that it is the cavity free energy part of the solvophobic potential that provides the driving force for molecular associations and that a linear correlation exists between the microthermodynamic surface tension ($\kappa\gamma$) [4b] and the standard unitary free energy of denaturation of a protein in

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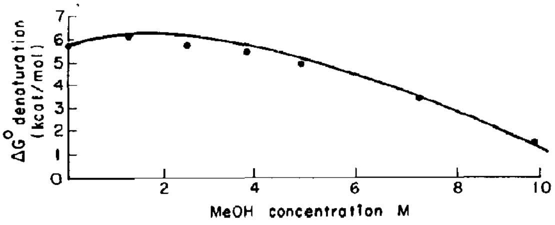

Fig. 1. ΔG^0 denaturation (kcal/mol) plotted vs. MeOH concentration (M).

different methanol/water mixtures.

We shall concentrate here on a series of calorimetric measurements performed by Veliçelebi and Sturtevant [5] of the standard free energies of denaturation of lysozyme in methanol/water mixtures. Fig. 1 summarizes the calorimetric data [5].

The secondary effect of adsorption of methanol in the surface layer of interaction of the solvent with the protein can be explained by means of the Gibbs adsorption isotherm:

$$\frac{n}{s} = -(\partial\gamma/\partial\mu)_T = -\frac{a}{RT}\left(\frac{\partial\gamma}{\partial a}\right)_T$$

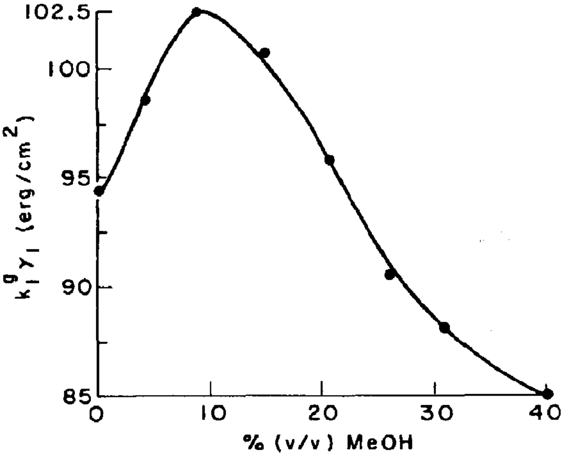

Fig. 2. $k_1^g\gamma_1$ (erg/cm^2) plotted vs. % (v/v) MeOH. The $k_1^g \simeq k_1^e$ values are as evaluated by HPLC by Horvath et al. [8].

where a is the mixing activity of methanol, γ the bulk surface tension of the mixture and n/s the excess moles of methanol per unit surface area (a positive quantity, since $(\partial\gamma/\partial a)_T < 0$; see however fig. 2). However, this is not the appropriate equation to use here as it involves the bulk macroscopic surface tension γ. γ is not applicable to the microscopic protein/solvent interface. The isotherm describing the adsorption down to an interface of molecular dimensions should not be the one of Gibbs but a corrected version considering the microthermodynamic surface tension $k^g\gamma$ introduced earlier for solvent forces by one of us [4]. The isotherm for molecular dimensions becomes:

$$\frac{n}{s} = -\left(\frac{\partial k^g\gamma}{\partial\mu}\right) = -\left(\frac{\partial k^g}{\partial\mu}\right)\gamma + k^g\left(\frac{\partial\gamma}{\partial\mu}\right)$$
$$= -\frac{a}{RT}\left(\frac{\partial k^g}{\partial a}\right)\gamma + k^g\left(\frac{\partial\gamma}{\partial a}\right)$$

According to the solvophobic theory [4,6a] the standard unitary free energy of 'association' (actually folding of the protein) is given by:

$$\Delta G^0_{\text{assoc.}} = \Delta G^{0,\text{in vacuo}}_{\text{assoc.}} + \Delta G_{\text{red.}} + (\Delta G_{\text{e.s}} + \Delta G_{\text{v.d w}}) + \Delta G_{\text{c}} \qquad (1)$$

The notation is that of ref. 6a. Δ accounts for the difference between the folded and unfolded conformer. The first term on the right-hand side represents the free energy for the process:

$$\begin{pmatrix}\text{unfolded}\\ \text{conformer}\end{pmatrix} \rightleftharpoons \begin{pmatrix}\text{folded}\\ \text{conformer}\end{pmatrix} \text{ in the absence of solvent.}$$

The second term is positive (therefore, it represents a dissociation-driving effect) which corre-

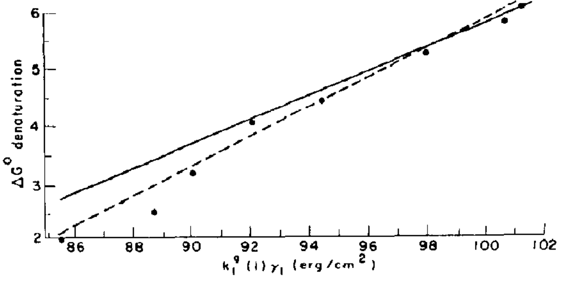

Fig. 3. ΔG^0 denaturation of lysozyme in MeOH/H$_2$O mixtures plotted vs. the microthermodynamic surface tension of the mixture (the experimental thermodynamic data are from ref. 5). The microthermodynamic solvophobic theory is that of ref. 6a. (— — — —) Experimental, (———) predicted from the microthermodynamic solvophobic theory data. MeOH range 8–40% (v/v); see fig. 2.

sponds to the weakening of the electrostatic interaction by the dielectric solvent as well as the weakening of the van der Waals forces between associating groups by solvation layers [6b]. The third and fourth terms correspond to the direct electrostatic and van der Waals interaction of the solute with the discrete layers of solvent and the continuum of solvent beyond [4,6a]. In the range of solvent compositions where the calorimetric measurements were made $\Delta G_{\mathrm{int.}} = \Delta G_{\mathrm{e.s}} + \Delta G_{\mathrm{vdw}}$ is assumed to be quite constant as observed earlier (cf. refs. 7a and 7b) For conformational changes, the 'free volume contribution' ($-RT \ln(RT/P_0 V_1)$, $P_0 = 1$ atm, $T \equiv$ working temperature, V_1 mole volume of the solvent), i.e., a term entropic in its nature, cancels out in fig. 3 because the process is of the type A $\rightleftharpoons$ B.

It was also demonstrated in ref. 7a that the quantity $(\Delta G^{0\ \mathrm{in\ vacuo}}_{\mathrm{assoc.}} + \Delta G_{\mathrm{red.}})$ can be averaged for the range 0–40% and can be regarded as insensitive to the solvent composition for this range. The only term strongly dependent on the solvent composition is $\Delta G_{\mathrm{c}} = k^{\mathrm{g}}(1)\gamma_1 \Delta\sigma$, where k^{g} (1) γ_1 is the against-vacuo-microthermodynamic surface tension of the solvent mixtures [6]; γ_1, the bulk (planar) surface tension of the solvent, a monotonically decreasing function of methanol concentration (cf. ref. 8); k^{g}_1, the microscopic surface tension factor [4,6a]; and $\Delta\sigma$, the molecular surface area change of the protein upon folding [6a].

As displayed in fig. 2, the microthermodynamic surface tension is not a monotonically decreasing function of the methanol concentration, unlike the bulk surface tension, but it has a maximum at 8% (v/v) MeOH.

Two facts strongly support the predictions of the solvophobic theory [6a] that the cavity term provides the main driving force for the association here:

(a) The maximum stability of the native form of the lysozyme is obtained at approx. 8% (v/v) MeOH. That is precisely the composition at which the maximum in the microthermodynamic surface tension [6a] occurs.

(b) In the range 8–40%, the correspondence % (v/v) MeOH–$k^{\mathrm{g}}_1\gamma_1$ is one-to-one and there exists a linear correlation between $k^{\mathrm{g}}_1\gamma_1$ and $\Delta G^0_{\mathrm{denat.}}$ as displayed in fig. 3. The slope gives an estimation of the microthermodynamic molecular surface area [6a] change: $\Delta\sigma_{\mathrm{denat.}} = 172$ Å^2/molecule.

2. The second type of derivation of the solvophobic theory

The statistical-mechanical part of the full unitary standard solvophobic effect (i.e., the combined interaction and cavity terms) has been proven recently by Sinanoğlu [4a] to be proportional to the microthermodynamic surface area change $\Delta\sigma$.

$$\Delta G^0_{\mathrm{assoc.}} = \left(\Delta G^{0,\mathrm{in\ vacuo}}_{\mathrm{assoc.}} + \Delta G_{\mathrm{red.}}\right) + \delta\Delta\sigma \tag{2}$$

The proportionality constant δ has been calculated recently by the present authors [7a] for methanol water/mixtures. It depends on the hydrocarbonaceous liquid phase equivalent (cf. ref. 7a) to the hydrocarbonaceous part of the solute and on the solvent.

δ is called the 'microscopic differential surface tension' and is defined as the difference between the microscopic interfacial microthermodynamic surface tension [4a] (between the hydrocarbonaceous liquid phase and the solvent phase) and the microthermodynamic surface tension (against vacuo) of a hydrocarbonaceous phase. The values plotted vs. methanol concentration are displayed in fig. 4.

Using the unitary standard free energies of denaturation of lysozyme from ref. 5 and the δ values, we observe [7a] that $(\Delta G^{0,\mathrm{in\ vacuo}}_{\mathrm{assoc.}} + \Delta G_{\mathrm{red.}})$

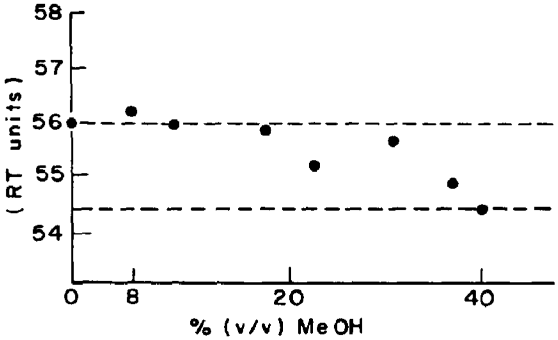

Fig. 4. $\Delta G^{\mathrm{in\ vacuo}}_{\mathrm{v.dw}}$ (denaturation of lysozyme) + ΔG_{red} (RT units).

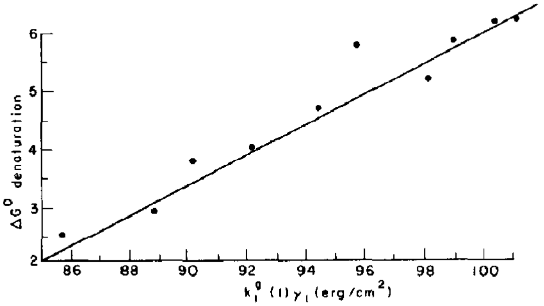

Fig. 5. Predicted $\Delta G^0_{\text{denaturation}}$ from eq. 4 plotted vs. $k_1^{g}(1)\gamma_1$ for the range of concentrations 8–40% (v/v) MeOH in water.

can be taken as its average value for the range 0–40% (cf. fig. 4). We use the formula:

$$\Delta G^0_{\text{assoc.}} = \left(\Delta G^{0,\text{in vacuo}}_{\text{assoc.}} + \Delta G_{\text{red.}}\right) + \delta(-172\ \text{Å}^2) \quad (3)$$

In reduced (RT) units: $\Delta G^{0,\text{in vacuo}}_{\text{assoc.}} + \Delta G_{\text{red}} = -55$ so we can write in reduced units:

$$\Delta G^0_{\text{assoc.}} = -55 + \delta(-172\ \text{Å}^2) \quad (4)$$

When the unitary standard free energy values from eq. 4 are plotted vs. $k_1^{g}(1)\gamma_1$ on the other hand, we obtain from the slope (cf. fig. 5):

$$\Delta\sigma_{\text{predicted}} \cong -166\ \text{Å}^2$$

Thus, the two types of derivations of the solvophobic effect, both given by Sinanoğlu [4a,6a], equivalent in principle, but differing in their implementations, give two independent evaluations from experiment of the molecular surface area change $\Delta\sigma$ of denaturation. The two values for lysozyme, $\Delta\sigma_{\text{denat.}} = 271$ Å^2 and $\Delta\sigma'_{\text{denat.}} = 166$ Å^2 are indeed only ±2% apart from their average of 169 Å^2, adding validity to the method.

The above demonstrates thereby how denaturation studies in varying solvents may be used to extract molecular information about tertiary (and higher) structural changes in proteins as well as to predict the solvent forces in other solvent compositions.

References

1 K. Gekko and S. Timasheff, Biochemistry 20 (1981) 4667.
2 S. Timasheff, J. Lee, E. Pitz and N. Tweedy, J. Colloid Interface Sci. 55 (1976) 658.
3 J. Lee and S. Timasheff, J. Biol. Chem. 256 (1981) 7193.
4 a. O. Sinanoğlu, in: Molecular Interactions, vol. 3, eds. H. Ratajzack and W. Orville-Thomas (Wiley, New York, 1982) p. 283.
b. O. Sinanoğlu, J. Chem. Phys. 75 (1981) 463.
5 G. Veliçelebi and J. Sturtevant, Biochemistry 18 (1979) 1180.
6 a. O. Sinanoğlu, in: Molecular associations in biology, ed. B. Pullman (Academic Press, New York, 1968) p. 427.
b. O. Sinanoğlu, Adv. Chem. Phys. 12 (1967) 283.
7 a. O. Sinanoğlu and A. Fernández, Biophys. Chem. 21 (1985) 157.
b. O. Sinanoğlu and S. Abdulnur, Fed. Proc. 24 (1964) part III, S-12.
8 C. Horvath, W. Melander and I. Molnar, J. Chromatogr. 125 (1976) 123.
9 F. Birnstock, H.-J. Hofmann and H.J. Köhler, Theor. Chim. Acta 42 (1976) 311.
10 G. Belfort, as discussed in Environ. Sci. Technol. 14 (1980) 1037.
11 V.I. Danilov, in the press.
12 O. Sinanoğlu; Int. J. Quantum Chem. 18 (1980) 381.