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THE DENATURATION MAXIMA OF PROTEINS AND OF DRUG-BIOMOLECULE COMPLEX FORMATION IN A WIDE RANGE OF METHANOL/WATER MIXTURES

SOLVOPHOBIC THEORY PREDICTIONS AS COMPARED TO EXPERIMENTS

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Independent experiments have shown that both protein folding (G. Velicelebi and J.M. Sturtevant, Biochemistry 18 (1979) 1180) and drug-biomolecule complexation (D.M. Crothers and D.I. Ratner, Biochemistry 7 (1968) 1823) in a wide range of compositions of methanol/water mixed solvents exhibit a maximum at 8% (v/v) MeOH. This hitherto unexplained phenomenon is shown to be given a priori by the 'solvophobic theory' developed earlier by Sinanoğlu which had related the solvent effects including water in biochemistry to the then introduced 'molecular surface areas' and to 'microthermodynamic cavity inner surface tensions' and in a different version to interfacial microtensions between side chains and the solvent. Both analyses carried out in the present paper in detail for MeOH/water mixtures show how the denaturation or complexation free energies are predicted for the entire range of MeOH/water compositions from only data at one point. The molecular surface area changes for the conformational processes are obtained as well as the free energies in the hypothetical but theoretically important in vacuo limits with no solvent present.

1. Introduction

The effect of solvents on associations between molecular groups as in protein folding, some isomerizations, complex formation, etc., was treated in detail by Sinanoğlu [1] quantitatively and by Sinanoğlu and Abdulnur [2] qualitatively. The theoretical framework of the 'solvophobic theory' in terms of macroscopic properties of the solutes and solvent as well as the geometric molecular surface areas of solutes was put forward by Sinanoğlu in a series of papers (see, for example, refs. 3–5).

In considering a generic association of the form: $A + B \rightleftharpoons AB$, the standard unitary solvophobic effect of molecular interactions within liquids can be separated into two kinds of forces:

(a) Those of a statistical-mechanical nature that arise from local rearrangements of the solvent. These are: (1) The difference in free energy of cavity formation [4] between the complex AB and the isolated groups A and B. This term is denoted $\Delta G_C = G_C^{AB} - G_C^A - G_C^B$, which is an association-driving force. (2) The difference in the free energy of interaction of these solutes with the discrete solvation layers and the continuum of solvent beyond. This force is ΔG_{int}. The combined effect $\Delta G_{int} + \Delta G_C$ constitutes the major part of $\Delta G^0_{solv.\,eff.(a)}$ [3–5].

ΔG_{int} may be a dissociation- or association-driving force, so that depending on the system we may have [1] $\Delta G^0_{solv.\,eff.(a)} > 0$ or < 0 ([4]).

It will be shown in this paper that for methanol/water mixtures, the association-driving force reaches its largest value not at pure water but at 8% (v/v) MeOH. This property certainly cannot be inferred if one does not consider the microscopic corrective factor [5] to the bulk surface tension of the solvent when calculating the free

energy of formation of a cavity of molecular dimensions. The solvophobic theory [3–5] including this corrective factor is found below to agree with the experimental evidence that the biggest solute squeezing effect is obtained at 8% (v/v) MeOH.

(b) Dissociation-driving forces arising from the reduction of the gas phase A-B interaction by the dielectric medium. These forces are built into the 'reduction' term $\Delta G_{\text{red.}}$ [3–5].

In this paper we shall be concerned with a generic calculation of the separate effects $\Delta G^0_{\text{solv. eff.(a)}}$ and $\Delta G_{\text{red.}}$ for alcohol/water mixtures. The full statistical mechanical driving force was proven by Sinanoğlu ([4]) to be proportional to the 'microthermodynamic molecular surface area change' $\Delta\sigma$ of the association (or conformational change). That is

$$\Delta G^0_{\text{solv. eff.(a)}} = \delta\Delta\sigma \tag{1}$$

δ is a negative constant which will be called the (microscopic) differential surface tension. It depends on the eluent and on the nature of the hydrocarbonaceous phase of the solute. Experimental confirmation of eq. 1 was provided by high-pressure liquid chromatography experiments (HPLC) performed by Horváth et al. [6]. The logarithm of the capacity factor of the chromatography column was plotted vs. the hydrocarbonaceous surface area (HCSA) for different kinds of solutes (acids, amino acids, amines) using a buffer as eluent. Linear plots for each kind of solute were found.

Since there is a linear relation between the logarithm of the capacity factor and $\Delta G^0_{\text{solv. eff. (a)}}$ assuming that $\Delta G^{0,\text{in vacuo}}_{\text{assoc.}} + \Delta G_{\text{red.}}$ is a constant for each kind of solute and since the HCSA is proportional to the thermodynamic quantity $\Delta\sigma$ as shown in ref. 6 ($\Delta\sigma \cong -0.7$HCSA), eq. 1 is satisfactorily confirmed.

The availability of HPLC data makes it possible to evaluate the proportionality constant δ for $\Delta G^0_{\text{solv. eff. (a)}}$ for methanol/water mixtures used as eluents in the range of compositions 0–40% (v/v) MeOH, and solutes with a hydrocarbonaceous phase similar to that of the *o*-toluic acid which was used in the HPLC experiment hereby considered.

For this range of the solvent composition, it will be demonstrated that the term $\Delta G_{\text{red.}}$ is rather insensitive to solvent composition in the case of a denaturation (or association) of a complex and the folding of a protein, therefore, since the constant δ is independent of the class of solute, it suffices to know the standard unitary $\Delta G^\circ_{\text{assoc.}}$ at a particular solvent composition to be able to predict $\Delta G^\circ_{\text{assoc}}$ for the whole range 0–40% (v/v) MeOH in H_2O.

2. Generic calculation of the microscopic differential surface tension

The aim of this section is to provide a formula which allows the evaluation of δ for MeOH/H_2O mixtures and for solutes having a hydrocarbonaceous phase equivalent (this term is rigorously defined below) to that of the solutes used in the HPLC experiment, i.e., a Partisil 1025 ODS chromatographic column and *o*-toluic acid.

Each term in the part $\Delta G^0_{\text{solv. eff.(a)}}$ will be now examined separately in order to prove eq. 1 and to provide a means of calculating δ. The cavity free energy G_{cJ} corresponding to the creation of a cavity of molecular dimensions for placing a molecule of solute J in the solvent is:

$$G_{cJ} = k_1\gamma_1\sigma \tag{2}$$

where k_1 represents the extent to which the macroscopic surface tension differs from the 'molecular or micro-surface tension'. k_1 depends on the class of solvent (polar, nonpolar) and on the size of the cavity relative to the average radius of the solvent molecule. For quasi-spherical molecules $k_1 = k_1((v_1/v_J)^{-1/3})$ where v_1/v_J is the molecular volume ratio. For comparable radii of curvature of solute and solvent molecules, $k_1 \cong k_1(1)$. $k_1(1)$ depends on bulk thermodynamic properties of the pure solvent itself and is given by the expression derived by Sinanoğlu ([3]).

$$k_1(1) \cong \frac{N^{1/3}\Delta E^0_{\text{vap}}}{V^{2/3}\gamma\left(1 - \frac{\mathrm{d}\ln\gamma}{\mathrm{d}\ln T} - \frac{2}{3}\alpha T\right)} \tag{3}$$

where ΔE^0_{vap} is the standard heat of vaporization of 1 mol of solvent which will be regarded as independent of pressure [6]. V is the mole volume,

N, Avogadro's number and $\mathscr{A}$ the thermal expansion coefficient ($\mathscr{A} = (\partial \ln v_1/\partial T)_p$). Clearly, from eq. 2, ΔG_c is an association-driving force ($\Delta\sigma < 0$ while the microthermodynamic surface tension is positive) but, as we shall prove later, the overall effect $\Delta G_{\text{solv. eff.(a)}}$ is a dissociation-driving force.

In the proof that the part of the standard unitary solvophobic potential that arises from rearrangements of the solvent molecules (hereby denoted $\Delta G^0_{\text{solv. eff.(a)}}$), i.e., the statistical-mechanical part, is proportional to the thermodynamic microscopic cavity surface area change of the process, Sinanoğlu [4] demonstrated that not only G_c [3], but also G^J_{int} for any solute species J is proportional to the thermodynamic microscopic area σ_J. For comparable size of solute and solvent [4]

$$G^J_{\text{int}} = \sigma_J\left(-k_{\text{He(l)}}\gamma_{\text{He}} - k_1\gamma_1 + k_{1\text{He}}\gamma_{1\text{He}}\right) \tag{4}$$

where $k_{\text{He(l)}}\gamma_{\text{He}}$ is the microscopic surface tension of a hydrocarbonaceous liquid phase similar to the nonpolar groups of species J. $k_{1\text{He}}\gamma_{1\text{He}}$ is the interfacial microscopic surface tension [4] between a polar solvent (in the HPLC case, the eluents are MeOH/H_2O mixtures) and the hydrocarbonaceous phase. The interfacial bulk surface tension between a polar solvent and the polar groups of J is approx. 0. Therefore, we can now write:

$$\begin{aligned}\Delta G^0_{\text{solv.eff.(a)}} &= \Delta G_{\text{int}} + \Delta G_c \\ &= \Delta\sigma\left(k_{1\text{He}}\gamma_{1\text{He}} - k_{\text{He(l)}}\gamma_{\text{He}}\right)\end{aligned} \tag{5}$$

Thus, the differential microsurface tension is:

$$\delta = k_{1\text{He}}\gamma_{1\text{He}} - k_{\text{He(l)}}\gamma_{\text{He}} \tag{6}$$

Since the interfacial microscopic surface tension will be later shown to be smaller than $k_{\text{He}}\gamma_{\text{He}}$ (the same relation holds at the macroscopic level), $\Delta G^0_{\text{solv. eff.(a)}}$ is positive, hence it is a dissociation-driving effect. The unitary standard free energy of association of two species A and B to give the complex AB is [3]:

$$\begin{aligned}\Delta G^0_{\text{assoc.}} = \Delta G^{0,\text{in vacuo}}_{\text{assoc.}} + \Delta G_c + \Delta G_{\text{int.}} \\ - RT \ln RT/P_0V + \Delta G_{\text{red.}}\end{aligned} \tag{7}$$

The term $-RT \ln RT/P_0V$ arises from the difference in the positional entropy of each species J (J = A, B, AB) when a J molecule goes from the molecular gas volume (RT/P_0 per mol) to a free volume V (mole volume) in the cavity. From eq. 5 the standard unitary free energy of association can also be given in the form:

$$\begin{aligned}\Delta G^0_{\text{assoc.}} = \delta\Delta\sigma + \Delta G^{\text{in vacuo}}_{\text{assoc.}} + \Delta G_{\text{red.}} \\ - RT \ln(RT/P_0V)\end{aligned} \tag{7'}$$

In the case of a conformational change, the entropic term $-RT \ln(RT/P_0V)$ cancels out. The capacity factor [6] k of the chromatographic column is given by:

$$\ln k = \frac{-\Delta G^0_{\text{assoc.}}}{RT} + \varphi \tag{8}$$

where φ is the so-called phase ratio characteristic for a given column.

A plot of $\ln k$ vs. $\Delta\sigma$ is linear for any given set of solutes (acids, amino acids, amines) and a fixed column ([6]). Since all the plots have the same slope it can be concluded that the difference in the intercepts is due to differences in the quantity $\Delta G^{0,\text{in vacuo}}_{\text{assoc.}} + \Delta G_{\text{red.}}$ which is fixed for each set of solutes and that the slope is $-\delta/RT$. Since $\ln k$ was calculated in the HPLC experiments from the separate contributions corresponding to the terms of eq. 6, we can now obtain the differential microsurface tension from

$$\delta = \frac{\Delta G_{\text{int}} + \Delta G_c}{\Delta\sigma} \tag{9}$$

For polar eluents, δ would be the same for two solutes with equivalent hydrocarbonaceous parts. For pure water δ could be obtained from the slope of the linear plot of $\ln k$ vs. the hydrocarbonaceous surface area of the solute in the fixed column (as given in ref. 6) but the assumption that this area is proportional to the contact area, although not completely ad hoc, cannot be fully justified. We have decided instead to evaluate δ from eq. 9 for the whole range 0–100% (v/v) MeOH in H_2O and to compare the result with that of pure water.

If A ≡ octadecylsilica column at 25°C and B ≡ *o*-toluic acid

$$\delta_{AB} - k_1(1)\gamma_1 = \frac{\Delta G_{\text{int,e.s.}} + \Delta G_{\text{int,v.d.w.}}}{\Delta\sigma} \tag{10}$$

The electrostatic (e.s.) and van der Waals part of ΔG_{int} were calculated in ref. 6 by Horvath et al. as in ref. 3 (see also ref. 7 or ref. 5). Using the definition of δ, eq. 10 can be written:

$$k_{1Hc}(1)\gamma_{1Hc} - k_{Hc}^{(1)}\gamma_{Hc} - k_1(1)\gamma_1 = \frac{\Delta G_{int,e.s.} + \Delta G_{int,v.d.w.}}{\Delta\sigma} \quad (11)$$

Where HC represents a hydrocarbonaceous liquid phase equivalent to the octadecylsilica column. Since $G^{A}_{int,v.d.w.} \cong G^{AB}_{int,v.d.w.}$, eq. 11 could be written:

$$k_{1Hc}\gamma_{1Hc} - k_{HC}\gamma_{Hc} - k_1\gamma_1 = \frac{\Delta G_{int,e.s.} - G^{B}_{v,d.w.}}{\Delta\sigma} \quad (12)$$

The quantity $\Delta\sigma$ was taken from ref. 6 as 20% of the surface area of the solute that is used with a fixed column. For *o*-toluic acid, this gives $\Delta\sigma \cong -32$ Å^2. $\Delta G_{e.s.}$ was directly calculated from the formula:

$$\Delta G_{e.s.} = \frac{N}{2}\mathscr{P}\mathscr{D}\Delta(\mu^2/\nu) \quad (13)$$

The notation being fully consistent with ref. 5. μ represents the dipole moment of each solute species (A, B, AB). $\mathscr{P}$ is the molar polarizability and $\mathscr{D}$ is related to the dielectric constant ϵ of the solvent:

$$\mathscr{D} = \frac{2(\epsilon - 1)}{2\epsilon + 1} \quad (14)$$

The term $\Delta G_{e.s.}$ is fairly insensitive to the changes in MeOH composition and it will be taken as constant for the range 0–100% (v/v) MeOH ($\Delta G_{e.s.} \cong 0.5$ reduced (RT) units), since $\mathscr{P}$ and $\mathscr{D}$ are quite insensitive to changes in the eluent composition.

$\Delta G_{int,v.d.w.}$ was evaluated according to ref. 7 directly from handbook properties of *o*-toluic acid and MeOH/H_2O mixtures by Horváth et al. [6]. The microthermodynamic surface tension of the eluent mixtures was calculated using eq. 3. We can see that in the range 0–40% the dissociation-driving statistical-mechanical part of the solvophobic effect reaches its smallest value not at pure H_2O ('hydrophobic bonding') but at 8% (v/v) MeOH. This is so, since while the bulk surface tension of the eluent is a monotonically decreasing function of MeOH composition, the microthermodynamic surface tension is not, in fact it reaches a maximum at 8% MeOH. It is at this maximum (due to drastic local changes in the eluent composition as MeOH is added to pure water) where the biggest squeezing effect should be observed, since it is then when the dissociation of the complex is less enhanced by the statistical-mechanical solvophobic effect while the $\Delta G_{red.}$ term remains quite insensitive in the range 0–40%. This crucial prediction was confirmed using denaturation free energy data from ref. 6 as well as from refs. 8 and 9 in ref. 10. The value obtained from the slope of ln k vs. hydrocarbonaceous area of the solute (fixed column) is $\delta = -0.267RT$ units per Å^2 for the pure water buffer. From fig. 1 we calculate $\delta = -0.27$. This good agreement indicates that the assumption $\Delta\sigma = -0.7$ hydrocarbonaceous area is correct.

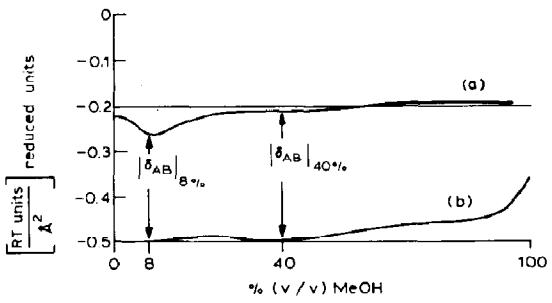

Fig. 1. Comparison between the microthermodynamic interfacial surface tension and the against-vacuo-surface tensions of the solvent and of the hydrocarbonaceous liquid phase plotted vs. MeOH concentration. $\Delta G_{int.}$ was directly calculated by Horváth et al. [6]. (a) Microthermodynamic surface tension of MeOH eluent ($k_1\gamma_1$). (b) $k^{(1)}_{1Hc}\gamma_{1Hc} - (k_{Hc}\gamma_{Hc} + k_1\gamma_1)$ (from eq. 11).

The δ values obtained in fig. 1 for the adsorptive association of *o*-toluic acid to the column apply to any pair of solutes provided the hydrocarbonaceous phase has: (i) the same interfacial microsurface tension; (ii) the same against-vacuo-microthermodynamic surface tension of the liquid hydrocarbonaceous phase.

3. Prediction of standard unitary free energies of denaturation of the native form of a protein and of a complex in methanol/water mixtures knowing only the $\Delta G^0_{\text{assoc.}}$ at one solvent composition

The purpose of this section is two-fold: on the one hand, to prove that the MeOH/H_2O mixtures do not change their microdielectric properties much, as far as the solvent effect is concerned, in the range 0–40% (v/v) MeOH (this is precisely the range where ΔG_{int} remains insensitive to solvent composition, see fig. 1). On the other, to evaluate for this range the terms which are not of a statistical-mechanical nature: $\Delta G^{0,\text{in vacuo}}_{\text{assoc.}} + \Delta G_{\text{red.}}$. Given this constant for a protein-folding process and for a complex formation, using the δ values of fig. 1, one can evaluate $\Delta G^0_{\text{assoc.}}$ for any solvent composition. For a protein denaturation process: folded conformer $\rightleftharpoons$ unfolded conformer, we obtain

$$\Delta G^{\text{in vacuo}}_{\text{assoc.}} + \Delta G_{\text{red.}} = \Delta G^0_{\text{assoc.}} - \delta\Delta\sigma_{\text{assoc.}} \qquad (15)$$

in reduced (RT) units we obtain from fig. 1:

$$\Delta G^{0,\text{in vacuo}}_{\text{assoc.}} + \Delta G_{\text{red.}} = \Delta G^0_{\text{assoc.}} - (-0.5 + k_1\gamma_1)\Delta\sigma_{\text{assoc.}} \qquad (16)$$

This equation is valid near pure water composition. $\Delta G^0_{\text{assoc.}}$ corresponds to the standard free enthalpy of the process: unfolded conformer $\rightleftharpoons$ folded conformer in an arbitrary solvent mixture. $\Delta\sigma_{\text{assoc.}}$ for a protein folding can be obtained from the slope of the linear correlation: $\Delta G^0_{\text{denat.}} \leftrightarrow k_1\gamma_1$ (cf. ref. 10) This linear correlation holds as long as ΔG_{int} remains insensitive to solvent composition (0–40% MeOH). $\Delta\sigma_{\text{assoc.}} = -172$ Å for the lysozyme protein. The general equation [10] for lysozyme folding [8] in MeOH/H_2O mixtures is:

$$\Delta G^{0,\text{in vacuo}}_{\text{assoc.}} + \Delta G_{\text{red.}} = \Delta G^0_{\text{assoc.}} + \delta 172\ \text{Å}^2 \qquad (17)$$

$\Delta G^0_{\text{denat.}} = -\Delta G^0_{\text{assoc.}}$ for the lysozyme denaturation in MeOH/H_2O mixtures was obtained experimentally by Velicelebi and Sturtevant [8]. Therefore, averaging the quantity $(\Delta G_{\text{red.}} + \Delta G^{\text{in vacuo}}_{\text{assoc.}}) \simeq -55RT$ units for the range 0–40% we obtain the approximate formula in reduced units:

$$\Delta G^0_{\text{assoc.}} = -55 + \delta(-172) \qquad (18)$$

Consider now a complex formation process:

Actinomycin (A) + deoxyguanosine (B) $\rightleftharpoons$ AB (19)

in MeOH/H_2O mixtures (cf. ref. 9).

The microthermodynamic quantity or rather the geometric molecular surface area change $\Delta\sigma$ can again be obtained from the linear correlation $\Delta G^0_{\text{assoc.}} \leftrightarrow k_1\gamma_1$ holding in the range where $\Delta G_{\text{int.}}$ is

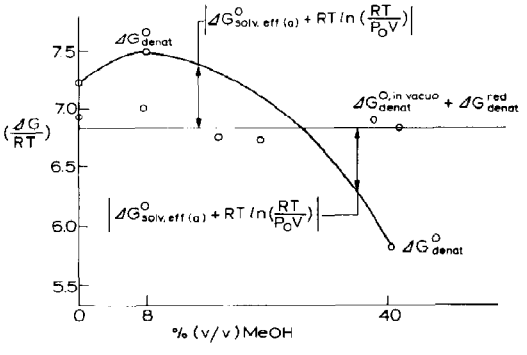

Fig. 2. ($\Delta G/RT$) plotted vs. % (v/v) MeOH. $T = 25$°C.

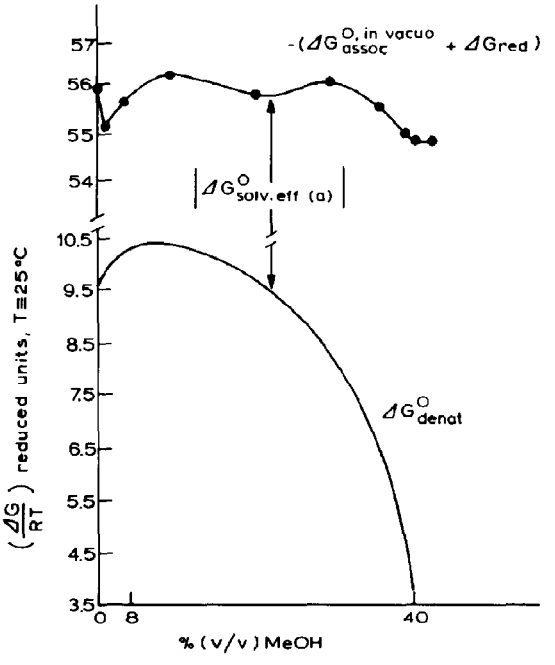

Fig. 3. The unitary free energy of dissociation of the actinomycin-deoxyguanosine complex. The experimental values of ΔG^0 were obtained by Crothers and Ratner [9].

insensitive to solvent composition, i.e.: 0–40% $\Delta\sigma_{\text{assoc.}} = -36$ Å^2. In this case $\Delta G^0_{\text{assoc.}}$ also has the entropic contribution: $-RT\ln(RT/P_0V) = -10$ reduced units. Therefore, always in reduced units: $\Delta G_{\text{red.}} + \Delta G^{0,\text{in vacuo}}_{\text{assoc.}} = 10 + \Delta G^0_{\text{assoc.}} + \delta$ (36 Å^2). Figs. 2 and 3 clearly demonstrate that $[\Delta G^{0,\text{in vacuo}}_{\text{assoc.}} + \Delta G_{\text{red}}]$ can be averaged to a near constant value for the range 0–40% (v/v) MeOH and also by subtracting the ordinates corresponding to both plots we obtain the statistical-mechanical part of the solvophobic effect.

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