possible to say on the basis of optical rotatory dispersion measurements just what the underlying structural change is. However, since the optical rotatory dispersion changes occur gradually over a wide range of surface tension, we suppose that the structural modification is a minor one. Our present hypothesis is that in solvents of low surface tension the two peptide rings are held less tightly together than in solvents of high surface tension, where the requirement of minimum cavity surface area would be more demanding.

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Thermodynamic Studies of a Model System for Hydrophobic Bonding*

Donald M. Crothers† and David I. Ratner‡

ABSTRACT: The free energy, heat, and entropy of complex formation between actinomycin and deoxyguanosine are measured in a series of solvent mixtures with various percentages of methanol and water. The complex is destabilized in the presence of increasing methanol concentration, due to an increasingly negative entropy of complex formation. The results favor the standard view of hydrophobic effects as arising from an ordering of solvent molecules around the solute over the description in terms of varying solvent surface tension (Sinanoğlu, O., and Abdulnur, S. (1965), *Federation Proc. 24*, 5).

It has long been recognized that water has special qualities as a solvent which cause it to encourage certain kinds of interactions between the particles of a dissolved substance. Of particular importance for biochemistry is the tendency of water to promote association between uncharged, and often nonpolar, molecules or parts of molecules in solution. These interactions are usually classed as "hydrophobic" (see Kauzmann (1959) for a general discussion) because of the preference for self-interaction rather than exposure to the solvent. Our understanding of the thermodynamic basis for hydrophobic interactions comes primarily from experiments on the solubility of small nonpolar substances in water (Frank and Evans, 1945), which indicate that exposure of these molecules to the solvent leads to an entropy loss resulting from ordering of water molecules in the neighborhood of the solute. This effect is accompanied by a small evolution of heat (analogous to ice formation) in the case of hydrocarbons, and by practically no heat change when aromatic substances are studied. Such interactions are clearly of importance in biochemical systems, as ex-

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† Alfred P. Sloan fellow.

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emplified by the destabilization of many biopolymers by nonaqueous solvents in a manner not obviously correlated with dielectric constant or hydrogen-bonding properties.

It is difficult to achieve unmabiguous interpretations of thermodynamic measurements on solvent effects in systems of biochemical significance. The molecules involved are necessarily larger and more complicated than the simple hydrocarbons which serve as models for hydrophobic interactions. Reactions of proteins can include a contribution to the thermodynamic parameters from conformational changes of the polymer or from charge neutralization, in which case one cannot isolate those effects due primarily to the influence of the solvent. Yet it would seem important to provide examples of the thermodynamic characteristics of hydrophobic bonding in molecules of the size encountered in biochemical reactions, if for no other reason than to demonstrate that the arguments on the simple model compounds can be extended to large systems.

Two characteristics seemed to us to be of particular importance in searching for an appropriate model system. It is necessary that electrostatic effects and charge neutralization be avoided, so that if an association reaction is studied neither partner should contain an ionic group. Furthermore, to isolate solvent effects the thermodynamic parameters should be studied in several solvents, which requires solubility under a range of conditions. Appropriate systems do not seem plentiful. One which presented itself is the complex formation between actinomycin, a chromopeptide containing two five-membered cyclic peptide rings attached to an aminophenoxazone chromophore (Brockmann, 1960), and deoxyguanosine. The structure of this complex is not certain, but by analogy with the structure of the DNA complex (Müller and Crothers, 1968) it seems likely that the deoxyguanosine purine ring forms a π complex with the actinomycin chromophore. It is possible, again by analogy with the DNA complex, that there is a hydrogen bond from the actinomycin carboxamide group to the deoxyribose ring oxygen. Hydrophobic effects are clearly important in stabilizing the complex, since binding is much weaker in organic solvents, whether hydrogen bonding or not. The rate of complex formation (Müller and Spatz, 1965) is nearly diffusion limited, so that no drastic change in actinomycin conformation can be involved. (This contrasts with the behavior of the DNA complex.)

The heat and entropy of complex formation have been measured (Gellert *et al.*, 1965; Müller and Spatz, 1965) with the conclusion that approximately 9 kcal/mole of heat is evolved in the process and that ΔS° is negative. The decrease in energy upon association is clearly what makes the reaction favorable. This may at first seem surprising for a reaction which is supposed to be mediated by hydrophobic effects, since association should lead to the "melting" of some of the ordered water around the two reaction partners, and should be accompanied by absorption of heat (or little heat change as for aromatic systems). A number of other reactions supposedly promoted by hydrophobic interactions also show a large negative ΔH of association; a recent example is the dimerization of actinomycin (Crothers *et al.*, 1968), along with such reactions as the stacking of bases in single-stranded nucleic acids (Brahms *et al.*, 1966; Leng and Felsenfeld, 1966; Poland *et al.*, 1966). One would ordinarily argue that the source of the energy which leads to an evolution of heat on association is something other than the solvent effects related to hydrophobic bonding, with dispersion forces, dipole–dipole interactions, etc., between the two partners being likely candidates.

A view of hydrophobic bonding divergent in some respects from the picture which emphasizes the ordering of water around the solute is contained in the calculation of Sinanoğlu and Abdulnur (1965) on the solvent dependence of the free energy of denaturation of DNA. They found that one term among many effects was responsible for the major part of the variation of the stability of DNA from solvent to solvent, this being the free energy required to form a cavity in the solvent in order to accommodate the solute. This free energy was taken proportional to the surface tension of the solvent. An association reaction leads to combination of two cavities (around the two reaction partners) into one of roughly equivalent volume but smaller surface area round the complex. Hence a solvent with a large surface tension effectively "squeezes" the two reactants together to form a complex. Water has a uniquely high value of the surface tension, which would explain its highly solvophobic properties in comparison with other solvents (which may have considerable ordering analogous to that observed in water). A correlation with solvent surface tension of observed properties related to solvophobic effects has been noted for the photochemical dimerization of thymidine (Wacker and Lodemann, 1965), the formation of a complex between quinone and hydroquinone (Moser and Cassidy, 1965), and the optical rotatory dispersion spectrum of actinomycin (Crothers *et al.*, 1968).

Another interesting feature of the surface tension approach to solvophobic effects is that it provides an alternate source for the heat evolved on complex formation. The surface tension of water is about 74 ergs/cm^2; the temperature dependence of this free energy allows it to be resolved into a positive ΔH (which is the same as ΔE since volume changes are negligible in surface phenomena) of 115 ergs/deg cm^2. The combination of two cavities to one of smaller area therefore should release a substantial amount of surface energy and result in an entropy decrease. If the same reaction is carried out in another solvent of lower surface tension the amount of energy released should be smaller.

It should be clear that these two different ways of looking at hydrophobic bonding yield rather different predictions concerning the thermodynamic consequences of changing the solvent in an association reaction. If ordering of the solvent is the major effect, going from water to a nonaqueous solvent should give a *more* negative ΔS of association, with

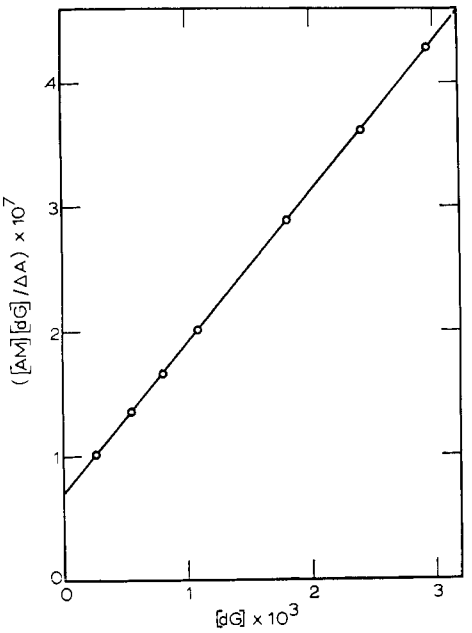

FIGURE 1: Typical plot of spectrophotometric data according to Benesi–Hildebrand equation. [AM] and [dG] = input concentration of actinomycin and deoxyguanosine, respectively. ΔA = absorbance change (at 425 mμ) due to presence of deoxyguanosine. The solvent is phosphate buffer at 25.14°.

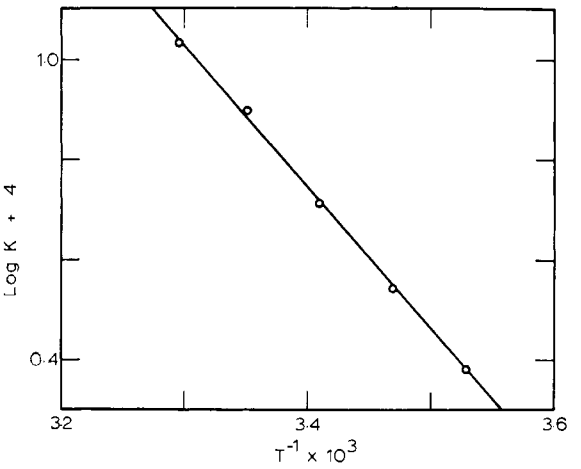

FIGURE 2: Plot of the log of the dissociation constant of the actinomycin–deoxyguanosine complex *vs.* reciprocal temperature. The solvent is 10% methanol and 90% phosphate buffer.

ΔH also becoming *more* negative due to the absence of heat absorption in melting solvent clusters. If the surface energy terms are dominant, ΔH should become *less* negative in going from a solvent of high surface tension to one of lower surface tension. The effects due to surface entropy should lead to a *less* negative ΔS of association in going from water to a nonaqueous solvent. These predictions are subject to experimental test, and it is this comparison which was one of the prime motives for the measurements described here.

It was originally intended to carry out thermodynamic measurements on complex formation between actinomycin and deoxyguanosine in a series of pure solvents, but it did not prove feasible to detect sufficient complex formation in any pure solvent but water. We therefore switched to mixed solvent systems, specifically mixtures of methanol and water. The surface tension arguments cannot be applied quantitatively to mixed solvents since the local surface tension may depend on preferential solvation effects, but this should not vitiate the qualitative comparison outlined above.

Materials and Methods

Actinomycin samples, prepared as described previously (Crothers *et al.*, 1968), were the gift of Dr. W. Müller. Deoxyguanosine (Cyclo Chemical Co., Grade I) was used without further purification. Thermodynamic quantities were determined in mixtures of 0.01 M phosphate buffer (sodium salt) (pH 6.95) and methanol (Fisher, Spectranalyzed). The mixed solvents were adjusted to an apparent pH of 7.5 as determined with a glass electrode. Solutions of deoxyguanosine were centrifuged to clarify them; all solutions were filtered before measurements were made. Concentrations of actinomycin and dG were determined spectrophotometrically, using extinction coefficients measured in these solvents. Final concentrations were made up by volumetric addition of dG and actinomycin solutions.

Equilibrium constants were determined using a Beckman DU-2 spectrophotometer, equipped with thermospacers and jacketed cuvets. Temperatures within the cells were maintained within ±0.03°. The difference in absorption in the presence of deoxyguanosine, ΔA, was read directly using an actinomycin solution as reference. Problems were encountered with the adsorption of actinomycin to the walls of glass flasks and spectrophotometer cells. Solutions were uniformly allowed to equilibrate with the flask for at least 24 hr prior to measurement and were brought to the temperature of measurement before introduction into the spectrophotometer cuvets. These later were soaked with the actinomycin reference solution for 1 hr at the appropriate temperature prior to use.

Equilibrium constants for dissociation of the complex, K_{dissocn}, were determined from a Benesi–Hildebrand (1949) plot of the spectrophotometric data

$$\frac{[\text{AM}][\text{dG}]}{\Delta A_{425}} = \frac{K_{\text{dissocn}}}{\Delta E_{425}} + \frac{[\text{dG}]}{\Delta E_{425}}$$

where [AM][1] and [dG] are the total (initial) concentrations of actinomycin and deoxyguanosine, respectively, and ΔE_{425} is the difference in the molar extinction coefficients for free and complexed actinomycin at 425 mμ. For each solvent, thermodynamic constants were obtained from the van't Hoff relation, using five values of K_{dissocn} spanning 20°. Straight lines were fitted to the Benesi–Hildebrand and van't Hoff plots by a

[1] Abbreviation used that is not listed in *Biochemistry* 5, 1445 (1966), is: AM, actinomycin.

TABLE I: Thermodynamic Quantities for the Reaction of Actinomycin with Deoxyguanosine to Form Complex.

Solvent	$\Delta H°$ (kcal/mole)[a]	$\Delta G°$ (kcal/mole)[a]	$\Delta S°$ (cal/mol deg)[a]
Phosphate buffer (PB)	-10.3 ± 0.8	-4.44 ± 0.006	-19.8 ± 2.5
10% methanol–PB	-12.99 ± 0.2	-4.26 ± 0.02	-29.3 ± 0.7
25% methanol–PB	-14.05 ± 0.4	-4.00 ± 0.02	-33.7 ± 1.5
40% methanol–PB	-13.40 ± 0.3	-3.50 ± 0.02	-33.2 ± 1.1

[a] Standard heat, free energy and entropy of complex formation, referred to a standard state of 1 mole/l. at 25°. Volume per cent of methanol mixed with phosphate buffer.

least-squares method; the uncertainty limits reported were determined from the least-squares analysis.

Results

Figure 1 shows a typical Benesi–Hildebrand plot of the spectrophotometric data, and Figure 2 shows the temperature dependence of the dissociation constants determined in this manner.

Table I summarizes the thermodynamic data for the association of actinomycin with deoxyguanosine in several solvent systems, and Figure 3 shows the thermodynamic parameters plotted against the volume per cent of methanol in the mixture.

Discussion

Increasing the percentage of methanol in a mixed methanol–water solvent results in a gradual destabilization of the actinomycin–deoxyguanosine complex. The destabilization results from an increasingly negative entropy of association, which is partly compensated by an increasingly negative enthalpy of association. Following the discussion previously mentioned, this result is consistent with the view that the major influence in hydrophobic bonding is the ordering of the solvent around the solute. In this regard, one can note that the change in ΔH from water to 10% methanol divided by the analogous change in ΔS is 280°K and the similar ratio for changing from 10 to 25% methanol is 240°K, both numbers within experimental error of the melting temperature of water. Hence it would seem reasonable to assign the changes in ΔH and ΔS to the reduced amount of ordering of water around the solute in the higher concentrations of methanol. No evidence is found for the thermodynamic changes expected on the basis of the simple surface tension model of solvophobic effects.

It should not be assumed as a result of these experiments that the energy required for cavity formation in the solvent plays no role in the thermodynamics of association reactions in solution. The very fact that a correlation has been found between solvophobic effects and bulk surface tension in some systems indicates that these effects may be present. However, these experiments do indicate that even for molecules the size of actinomycin and deoxyguanosine the influence of surface effects is masked by the thermodynamic consequences of ordering water molecules around the dissolved particles.

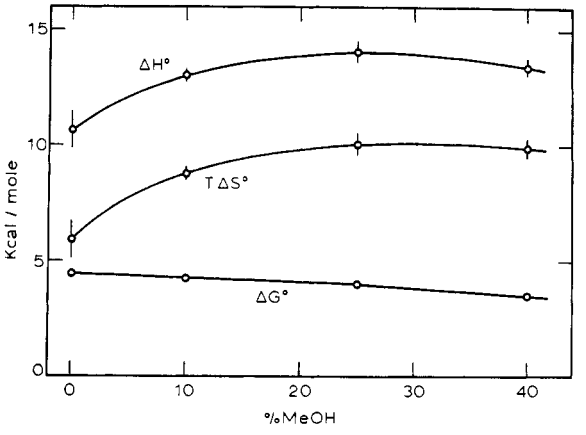

FIGURE 3: Variation of the standard thermodynamic quantities with volume per cent methanol mixed with phosphate buffer. $\Delta H°$, $T\Delta S°$, and $\Delta G°$ refer to complex dissociation in a standard state of 1 mole/l. at 25°. The fact that heat and entropy of complex formation become more negative as the methanol concentration increases supports the view that the major solvent effect is an ordering of water molecules around the solute.

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Polymerization–Depolymerization of Tobacco Mosaic Virus Protein. XI. Osmotic Pressure Studies of Solutions in Water and in Deuterium*

Severo Paglini† and Max A. Lauffer

ABSTRACT: Polymerization of tobacco mosaic virus (TMV) protein as a function of concentration has been studied by the method of osmometry at various temperatures between 4 and 25°. The solvents were usually tenth ionic strength phosphate or barbital buffer in water at pH values of 6.0, 6.5, and 7.5, and tenth ionic strength phosphate buffer in D_2O at pD values of 7.0 and 8.0. The theory of linear condensation polymerization was extended to take into account departures from ideality at high concentration. It is assumed that such departures were the sum of a calculated excluded volume effect and a calculated Donnan effect. The theory could be fitted reasonably satisfactorily to the data obtained in aqueous solvents up to concentrations of 30 mg/ml. The theory permits evaluation of M_0, the molecular weight of the smallest polymerizing unit and K, the equilibrium constant for polymerization. The values of M_0 usually turned out to be close to 50,000. Since the molecular weight of a stable trimer of the basic chemical unit of TMV protein is 52,500, all of the data were analyzed on the assumption that M_0 actually is 52,500. The values of K thus obtained indicate that in aqueous solutions the polymerization at a temperature of 4° is not appreciably affected by pH or specific buffer ions. Values of $\Delta H°$ and $\Delta S°$ were evaluated for polymerization at pH 6.5 and at 7.5 in tenth ionic strength phosphate buffer and were found to be of the same order of magnitude as those previously reported by Banerjee and Lauffer (Banerjee, K., and Lauffer, M. A. (1966), *Biochemistry 5*, 1957), namely, +30,000 cal/mole for $\Delta H°$ and +124 entropy units for $\Delta S°$. Polymerization in D_2O was more complex. Only the data at 4° at pD 7.0 and 8.0 could be analyzed in terms of the theory presented here. In contrast with the results obtained in aqueous solvent, the equilibrium constant was much higher at pD 7.0 than at pD 8.0. This indicates that the reaction in D_2O, particularly at pD 7.0, resembles more the high-temperature than the low-temperature polymerization process. At pD 8.0 and 20°, π/c increased with protein concentration. This effect can be interpreted quantitatively as being due to the Donnan term, with the protein in the double-disk state over a wide range of concentrations. The measured values of π/c at pD 7.0 at 8 and 11.7° were very much lower than the calculated Donnan contribution. This is evidence that the polymerization process at pD 7.0 is different from that at pD 8.0 and that it leads to a product which does not exhibit the Donnan effect. Previous studies have shown that TMV does not exhibit the Donnan effect in the osmometer and that high-temperature polymerization leads to a polymer with a structure resembling the arrangement of protein in TMV. Experiments designed to correlate light scatter and osmotic measures of polymerization in terms of condensation polymerization theory yielded the correct value of M_0 but a value of H 70% higher than that calculated from the appropriate constants of the solutions.

Tobacco mosaic virus protein (TMV) is one of the best investigated proteins and many of its biophysical properties are known. Of these, its polymerization–depolymerization (Lauffer *et al.*, 1958) has been studied in detail (Ansevin and Lauffer, 1963; Lauffer, 1964, 1966a,b; Ansevin *et al.*, 1964; Stevens and Lauffer, 1965; Banerjee and Lauffer, 1966; Smith and Lauffer, 1967; Shalaby and Lauffer, 1967; Khalil and Lauffer, 1967).

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† Member and External Fellow of the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina. Present address: Instituto de Virología de Córdoba, Córdoba, R. Argentina.