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The hydrophobic effect: a reappraisal

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Abstract - What is the reason for the low solubility of non-polar substances in water? Is it true that it is water which expels them? Are the oil drop and the globular state of protein formed by water that avoids contact with non-polar groups or is it the interactions between the non-polar groups that are responsible for their compact packing in aqueous media?

INTRODUCTION

In studies of the reason for the low solubility of non-polar substances in water initiated at the beginning of the century, there have been several waves (see e.g. ref. 1), but the largest, which is still at its extreme, has been induced by the surprizing discovery that the solubility (X) of all these substances is a minimum at about room temperature (Fig. 1). This immediately led to the puzzling conclusion that although the transfer of an insoluble solute to water requires expenditure of a large work (i.e. the Gibbs energy of transfer is large and positive as $\Delta G=-RT \ln X$ and X is small), the enthalpy of transfer, ΔH, is zero at this temperature, T_H. This follows from the mathematical condition of the extremum of the solubility constant:

$$(\partial \ln X)/\partial T = -(\partial \Delta G/RT)/\partial T = \Delta H/RT^2 = 0$$

As $\Delta G=\Delta H-T\Delta S$, the zero enthalpy, ΔH, where the Gibbs energy, G, is large and positive might only mean that the entropy of transfer of a non-polar substance from the pure liquid state to water, ΔS, is large and negative (Table 1). Thus, it appears as if the low solubility of the non-polar solute in an aqueous solution is caused by an unfavourable entropy decrease resulting from transfer of this solute to water (refs. 4-6).

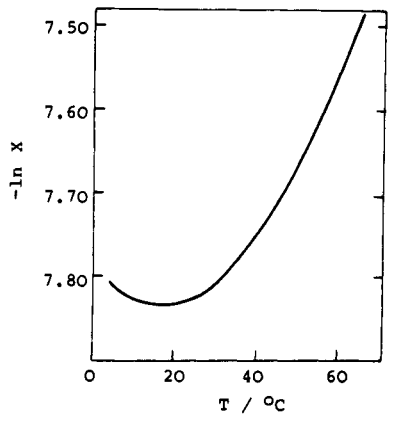

Fig. 1. Typical temperature dependence of the solubility of a non-polar substance (benzene) in water. The example is taken from ref. 2, omitting experimental details.

TABLE 1. Solubility, Gibbs energy, enthalpy, entropy and heat capacity increment of transfer of some typical non-polar substances from the pure liquid phase to water at 25°C

Substance and its surface area in Å^2	Solubility in mole fractions x 10^4	ΔG	ΔH	ΔS	ΔC_p
		($kJ \cdot mol^{-1}$)		($J \cdot K^{-1} \cdot mol^{-1}$)	
Benzene 240	4.01	19.4	2.08	-58.06	225
Toluene 275	1.01	22.8	1.73	-70.7	263
Ethylbenzene 291	0.258	26.2	2.02	-81.0	318
Cyclohexane 273	0.117	28.2	-0.10	-94.8	360
Pentane 272	0.095	28.7	-2.00	-102.8	400
Hexane 282	0.020	32.5	0	-109.1	440

See refs. 2,3.

WATER ORDERING BY NON-POLAR SOLUTES

The entropy decrease accompanying transfer of a non-polar substance to water was explained by the ordering of water in the presence of this substance. One of the arguments in favour of such an assumption arose from the observation that the entropy decrease associated with the transfer of a non-polar molecule to water is proportional to the surface area of this molecule (Table 1). It agreed also with the finding that dissolution of non-polar substances in water results in a considerable heat capacity increase proportional to the surface area of the solute molecules (refs. 7,8). The heat capacity increment is just that which one would expect if an increase in temperature leads to the gradual melting of water ordered by the presence of non-polar molecules. In this case, the heat capacity increment should decrease with increasing temperature, as the influence of a non-polar substance on the state of water should vanish at elevated temperatures (ref. 9). This was indeed found to be the case (Fig. 2). Calorimetric studies showed that the heat capacity increment associated with the transfer of various non-polar substances from the pure liquid phase to water decreases with increasing temperature, asymptotically approaching zero (ref. 10).

The concept, according to which the hydrophobic effect results from the undesirable entropy decrease of water (i.e. its ordering) in the vicinity of a non-polar solute and, thus, could be regarded as the expelling action of water on the solute molecules, has gained wide popularity. It has become conventional to regard the tendency of hydrophobic solutes to associate as an effect caused by the new independent type of molecular interaction in aqueous solutions (refs. 11,12), in spite of some feeling of discomfort induced by the fundamental difference of this hydrophobic interaction from all other types of known interactions. The situation where the entropy effect dominates the enthalpy one is indeed rather unusual, the more so that in the cases connected with water rearrangement these two effects are expected to compensate each other completely (ref. 13). Therefore, many attempts were undertaken to find some other source of the unfavourable entropy decrease upon mixing the molecules which differ in their size (refs. 14-18).

HYDROPHOBIC INTERACTIONS IN PROTEINS

Hydrophobic interactions have become especially popular among protein chemists who have actually coined this concept to explain the compactness of protein molecules (ref. 6). There are indeed many non-polar groups in protein macromolecules and many of them are clustered together as if avoiding contact with water. Therefore it was tempting to regard a globular protein in aqueous solution as an oil drop in water stabilized by hydrophobic interactions.

This point of view was supported by the finding that the unfolding of a compact protein structure, i.e. the process which can be regarded as a transfer of internal non-polar groups of a protein to water, proceeds with a rather small enthalpy at room temperature (ref. 19), suggesting that the entropy factor might play some role in the stabilization of the compact state of a protein.

No less impressive was the finding that the unfolding of a protein structure results in a significant heat capacity increase which cannot be explained by the gain of configurational freedom upon disruption of the rigid native structure (refs. 20-22). This heat capacity increment does not depend on the way by which the compact protein structure is disrupted when the protein is denatured (by the presence of high concentrations of denaturants, extreme pH's or temperature) but is very specific for a given protein and is proportional to the number of contacts between its non-polar groups (ref. 23). Calorimetric studies over a broad temperature range showed that the heat capacity difference between the native and denatured states of a protein is likely to decrease as the temperature is increased (Fig. 3). Extrapolating this tendency, one can assume that the difference drops to zero at about 140°C (Fig. 4).

Therefore the analogy between protein denaturation and dissolution of non-polar solutes in water is sufficiently close to suppose that the unfavourable entropy decrease resulting from the hydration of non-polar groups might be a factor determining the compact state of protein molecules. Thus, water ordering by non-polar groups appeared in proteins as a means of stabilizing the non-polar core of molecule, i.e. as a force responsible for the compactness and integrity of protein structures.

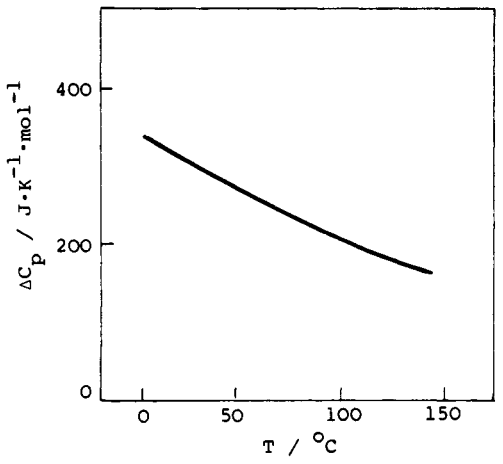

Fig. 2. Temperature dependence of the heat capacity increment of benzene at transfer from the pure liquid phase to water according to ref. 11.

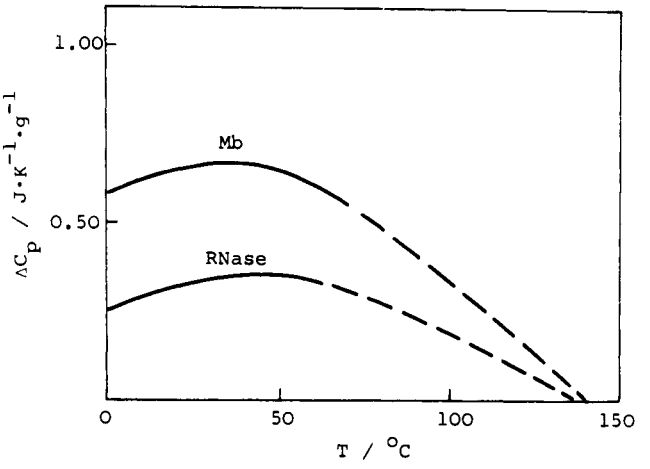

Fig. 4. Denaturational increment of the partial specific heat capacity of pancreatic ribonuclease A (RNase) and sperm whale myoglobin (Mb).

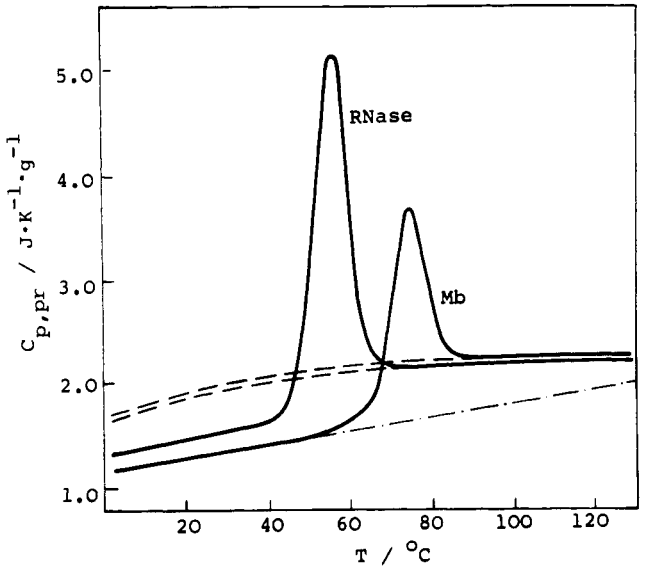

Fig. 3. Temperature dependence of the partial specific heat capacity of pancreatic ribonuclease A (RNase) and sperm whale metmyoglobin (Mb) in solution with pH 4.4. The dashed line corresponds to the function for a completely unfolded protein: RNase without S-S crosslinks and Apoform of Mb in acidic solution (pH 2.2) (for details see ref. 27).

This force was expected to be a long range one as the hydration effects should manifest at a distance of at least two water layers. One could then imagine that this feature might play an important role in directing the folding of a polypeptide chain towards a unique compact structure. In particular, one might suppose that such a long range force could maintain the protein in a rather compact but disordered (non-rigid) state in which the amino acid residues are not tightly packed and can move searching for a proper adjustment, i.e. in liquid-like "molten globule" state (refs. 24-26).

THE HYDROPHOBIC EFFECT AT ELEVATED TEMPERATURES

The conclusion on the role of hydration in the hydrophobicity of non-polar solutes and in the stability of the compact protein state was based mainly on information obtained at room temperature. This is understandable since most of the available experimental data on the solubility of slightly soluble solutes and on the denaturation of proteins were obtained at room temperature, as this was the easiest to do experimentally and at this temperature the hydrophobicity of non-polar solutes is a maximum. Variation of temperature was used only to determine heat capacity increments upon transfer of non-polar solutes to water, the values of which were considered as an important argument in favour of the conception of water ordering by non-polar solutes.

However, the heat capacity increment provides evidence not only for the existence of additional ordering of water in the presence of a non-polar solute, but also that the extent of this ordering decreases as the temperature is increased, i.e. that the solvated water melts gradually on heating the solution. Correspondingly, one would expect that a temperature increase should result in a decrease of the absolute value of the negative entropy of transfer of the non-polar solute to water, and that at some sufficiently high temperature T_S it should become zero (Fig. 5).

It is interesting that this temperature T_S, at which the entropy of transfer of a non-polar substance from the pure liquid phase to water becomes zero, is universal for all the substances studied and is about 140°C, if the heat capacity increment of their transfer to water decreases with increasing temperature in the same way as for benzene (refs. 3 and 27). However, this in itself is not as surprising as it seems to be, because the entropy and the heat capacity of transfer of a non-polar solute to water are both proportional to the surface area of the solute, i.e. their ratio, $\Delta S/\Delta C_p$, which actually determines the temperature at which the entropy becomes zero, is a universal constant for all non-polar substances studied (ref. 22).

Why is this temperature T_S so important? Because the entropy is a temperature derivative of the Gibbs energy, $\partial \Delta G/\Delta T = -\Delta S$, and, if the entropy of transfer is zero, this means that the Gibbs energy of transfer has an extremum at this temperature. Thus, the Gibbs energy of transfer of a non-polar substance from the pure liquid state to water is maximum at T_S which is the universal temperature for all known non-polar substances (Fig. 6). As is clear, this maximum value of ΔG is provided only by the enthalpy of transfer. At the temperature T_S the enthalpy of transfer of a non-polar molecule from the pure liquid phase to water, $\Delta H(T_S)$, is not zero as it is at T_H, but is large and positive (Table 2). Unfortunately not in all cases this enthalpy can be determined with good enough accuracy. Being obtained by extrapolation of the value obtained at room temperature its accuracy depends essentially on the precision of the heat capacity determination which decreases with decrease of the solubility of studied solute. It is especially low for the aliphatic hydrocarbons as are pentane and hexane with their too low solubility. But even from the very preliminary data which are presented in Table 2 one can see the close correspondence between the enthalpy of transfer and the enthalpy of vaporization of the non-polar substances at 140°C. This shows that the maximal hydrophobic interaction between non-polar molecules which takes place at T_S is mainly due to van der Waals interaction between the non-polar molecules in the liquid phase (ref.27).

It follows then that at this temperature T_S water does not solvate the non-polar solute, i.e. the solute molecules are not hydrated. At all temperatures below T_S there are clear signs of hydration of the non-polar solute (the negative entropy and the lower enthalpy of transfer and the significant heat capacity increment), and all these signs are more evident as the temperature decreases. It becomes clear that the decrease of the Gibbs energy of transfer which occurs at temperatures below T_S, is caused by the hydration of the non-polar solute. Without hydration, and without the heat capacity increment caused by hydration, the Gibbs energy of transfer would not change as the temperature is decreased below T_S. This means that the Gibbs energy of hydration of a non-polar solute is negative: it equals zero at T_S and increases in magnitude as the temperature decreases (for details see ref. 27).

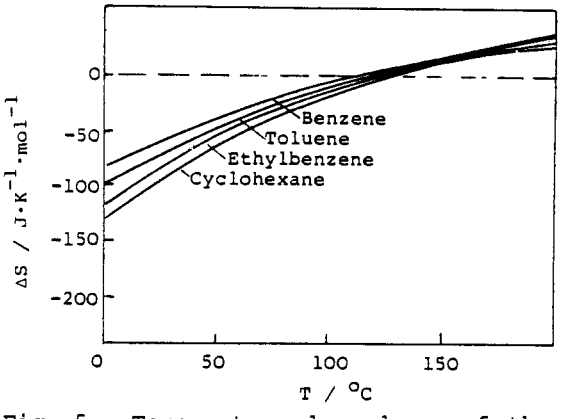

Fig. 5. Temperature dependence of the entropy of transfer of various non-polar substances from the pure liquid phase to water according to ref. 27.

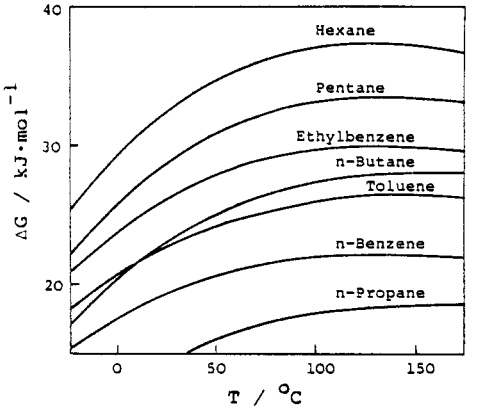

Fig. 6. Temperature dependence of the Gibbs energy of transfer for various liquid hydrocarbons to water according to ref.27.

TABLE 2. Enthalpy of transfer from the pure liquid phase to water and enthalpy of vaporization from this phase for some non-polar substances at 140°C

Substance	Benzene	Toluene	Ethyl-benzene	Cyclo-hexane	Pentane	Hexane
$\Delta H^{trans}/kJmol^{-1}$	25	28	35	36	39	46
$\Delta H^{vap}/kJmol^{-1}$	24	32	36	29	21	26

The Gibbs energy of transfer of non-polar molecules from the pure liquid phase to water is in fact the only measure of the expulsion of these molecules from aqueous solution, i.e. a measure of the hydrophobic interaction between the non-polar molecules. It follows from the above, that the hydrophobic interaction results from the van der Waals interactions between the non-polar molecules, while the hydration of these molecules has only a negative effect - it increases the solubility of these solutes in water (refs. 27-29).

This conclusion concerning the mechanism of the hydrophobic interaction disagrees with the conventional point of view, which was considered above. The widely spread misinterpretation of the role of hydration of non-polar solutes seems to arise from the confusion between the hydrophobicity and the hydrophobic interaction. Intuitively, these two notions seem almost similar. In fact, they differ qualitatively as the first one is measured in solubility units, i.e. in mole fractions, and the second one is measured in Gibbs energy units. Correspondingly, the maximum hydrophobicity is achieved at a temperature T_H, i.e. at about 20°C, while the maximum hydrophobic interaction is achieved at T_S, which is about 140°C. A proper choice between these two very different temperatures as a reference temperature for a thermodynamic analysis of the dissolution of non-polar substances in water is essential, because it actually determines the efficiency of the analysis. The choice of T_H as a reference temperature does not permit the evaluation of the thermodynamic parameters of the components of this complex process, namely the dissociation of non-polar molecules in the liquid phase and their association with water, i.e. their hydration. As has been shown above, this can be done successfully only using T_S as a reference temperature and, what is important, this does not require any assumptions about the molecular mechanism of the process considered (for details see ref. 27).

It should be noted that the Gibbs energy of hydration of non-polar solutes is in itself an integral quantity consisting, as one can imagine, of many components, e.g. the work associated with cavity formation in the solvent, the energy of the solute-solvent interaction, the work involved in the rearrangement of the solvent molecules (water ordering) around the cavity, etc. One can evaluate these components only by using some molecular model. Thus, according to the scaled particle theory for hard spherical molecules, the Gibbs energy of cavity formation in the solvent is positive, depends on the relative size of the solvent and solute molecules and increases with increasing temperature (refs. 15,18). This component is expected to be especially large in water which has the smallest molecules of any solvent. Nevertheless, as we have seen, in aqueous solution the total Gibbs energy of hydration of a non-polar solute is negative. This means that upon dissolution of a non-polar solute in water, the other components of the hydration process more than compensate for the work required for cavity formation.

STABILIZATION OF PROTEIN STRUCTURE

Let us consider now the temperature dependence of the thermodynamic functions describing the transition of a protein from the native to the denatured state.

The main consequence of the denaturational heat capacity increase is that the enthalpy and entropy of protein denaturation increase as the temperature increases, approaching some constant level at about 140°C (Fig. 7). These levels have been found to be the same for all compact globular proteins studied (refs. 23 and 27).

One can notice a clear similarity and some distinct differences between the enthalpy and entropy functions of protein denaturation and these for the transfer of a non-polar substance to water. In both cases these functions are increasing and in both cases figures the same temperature 140°C which, as

discussed above, is the temperature at which water does not solvate the non-polar groups. Thus, the enthalpy and entropy of protein denaturation at 140°C do not include the effects of hydration but they correspond to the conformational transition of the polypeptide chain involving disruption of intermolecular bonds (primarily hydrogen and van der Waals) maintaining the compact protein structure. The principal difference between these two processes is in the entropy value: while the entropy of transfer of a non-polar solute to water is zero at 140°C, the entropy of protein denaturation at this temperature is large and positive (refs. 23 and 27).

The difference in the entropy value, at a temperature when hydration effects are absent, might only mean that the protein interior is not a liquid-like non-polar phase but is a crystal-like phase, which is specified by a definite positive melting entropy. The main thermodynamic consequence of this specificity of a protein molecule is that the entropy of protein transition from the native to the denatured state becomes zero at a much lower temperature than the entropy of transfer of non-polar substances from the liquid phase to water. Usually the temperature T_S^d for protein denaturation is only few degrees higher than the temperature T_H^d, at which the enthalpy of protein denaturation becomes zero. For most of the known proteins, this temperature is between 0°C and 30°C, and at this temperature the compact native protein structure is most stable (Fig. 8). This follows from the fact that the Gibbs energy difference between the native and denatured states of a protein is a maximum at the temperature at which the entropy difference of these states is zero, since, as has been stated, it is the condition for the extremum of the ΔG function.

It is notable that if protein denaturation were to proceed without a heat capacity increase, i.e. without hydration of non-polar groups, the enthalpy and entropy of protein denaturation would not depend on temperature, being ΔH^o and ΔS^o, respectively, and the Gibbs energy difference between the native and denatured states ($\Delta G=\Delta H^o-T\Delta S^o$) would be a linearly decreasing function of temperature. This is because the dissipative force of the thermal motion ($T\Delta S^o$) is proportional to temperature (Fig. 9). However, when protein denaturation proceeds with a heat capacity increase, i.e. with hydration of the non-polar groups exposed upon unfolding, then the Gibbs energy difference between the native and denatured states deviates from linearity. The correction of the Gibbs energy function resulting from hydration, ΔG^{hyd}, is always negative and increases in magnitude with decreasing temperature (ref. 27). As a result, at some sufficiently low temperature the stability of the native state of a protein can decrease to zero and the protein can denature. This cold denaturation process has been demonstrated for a number of globular proteins (see e.g. refs. 31,32). In contrast to heat denaturation, cold denaturation of proteins proceeds with a release of heat, i.e. with a decrease in the enthalpy and entropy, showing that it is accompanied by extensive hydration of the non-polar groups exposed upon denaturation (Fig. 10).

CONCLUDING REMARKS

It turns out, therefore, that the hydration of non-polar groups is not the means by which the compact state of a protein molecule is stabilized and is not the reason that the solubility of non-polar solutes in water is low. Just the opposite: the hydration of non-polar groups increases the solvation tendency of these groups in water and destabilizes the compact protein structure which is in fact stabilized by the van der Waals and hydrogen bonding of the tightly packed amino acid residues. This destabilizing action of water solvation is zero at about 140°C and increases as the temperature decreases.

Assuming under the "hydrophobic interaction" the cumulative effect of the van der Waals interactions between non-polar groups and of the hydration of these groups, one can see that this integral effect should increase with increasing temperature. However, the hydrophobic interaction increases due to the decrease in the hydration contribution at increasing temperature (and not to its increase as was believed earlier) because these two contributions differ in their sign.

An important consequence of the fact that the van der Waals and hydration effects are contributing to the hydrophobic effect with the opposite sign is the biphasic character of this integral effect. Indeed, since the van der Waals interaction is a short range one and the hydration effect is a long

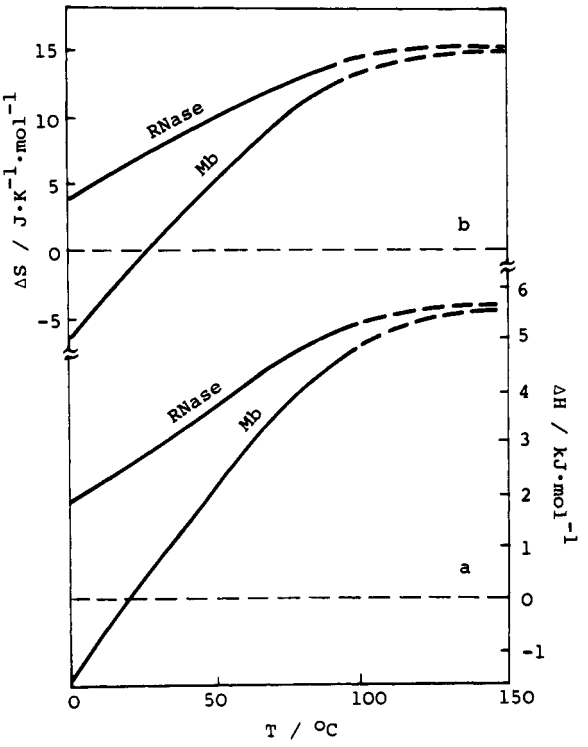

Fig. 7. Temperature dependences of the molar enthalpy (a) and entropy (b) of denaturation of myoglobin and ribonuclease A (per mole of amino acid residues) in solutions providing maximal stability of these proteins.

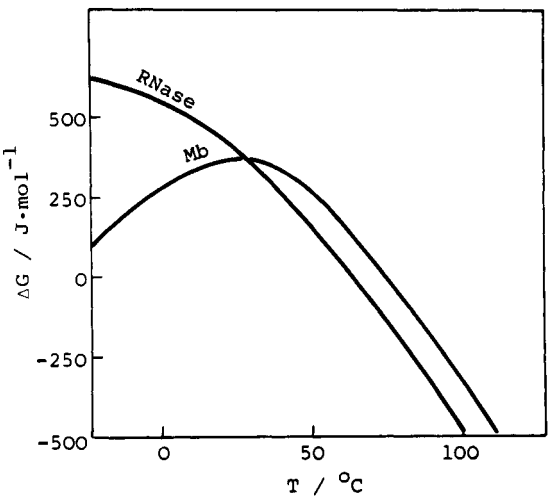

Fig. 8. The Gibbs energy difference of the denatured and native states of myoglobin and ribonuclease A under the same conditions as indicated in Fig. 7.

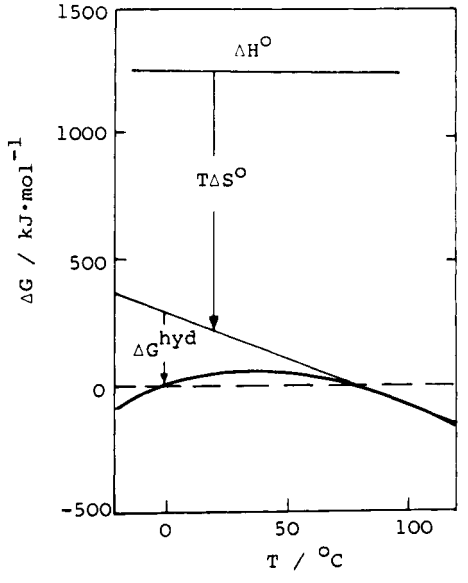

Fig. 9. Contributions of the dissipative force, $T\Delta S^{o}$, and water solvation effect, ΔG^{hyd}, to the stabilization of an abstract globular protein consisting of about 200 amino acid residues (for details see ref. 30).

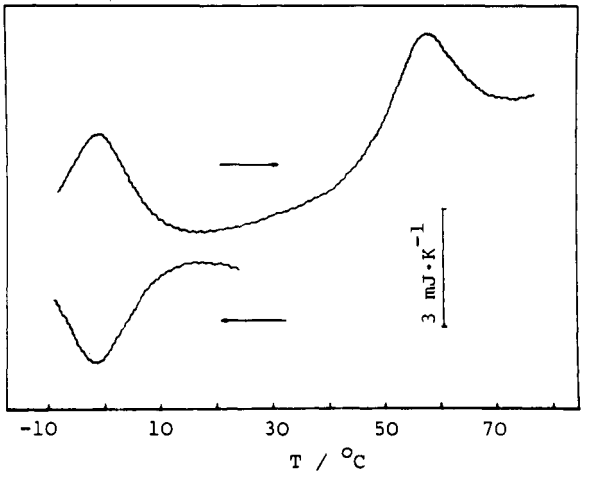

Fig. 10. Microcalorimetric recording of the heat effect upon cooling and subsequent heating of an aqueous solution of apomyoglobin (pH 4.8). The low temperature peaks correspond to the release of heat upon cold denaturation and to the heat absorption upon renaturation of the protein at subsequent heating; the high temperature peak corresponds to heat absorption upon heat denaturation (for details see ref. 32).

range one, it is evident that the "hydrophobic interaction" should be attractive at short distances and repulsive at long distances (exceeding the size of a water molecule). This might be one of the reasons for the extreme cooperativity of a tightly packed native protein domain which is disrupted in an all-or-none way and always involves penetration of water inside the protein structure (ref. 23). This would also explain the failure of all attempts to discover the hypothetical liquid-like "molten globule" state of proteins in which the amino acid residues are proposed to be held together only by the long range interactions which were supposed to be the hydrophobic interactions (see e.g. ref. 33).

One can notice that this alternative to the conventional concept of the hydrophobic effect brings us back to the twenties when the hydrophobicity of non-polar solutes was explained by their "like to like" attraction (ref. 34). But as cyclicity is a general principle in the evolution of science, one can consider the presented above as the next wave in our comprehension of the hydrophobic effect.

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