

Original Research Article

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Osmolyte Effect on Denaturation on Proteins

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ABSTRACT

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Plants, animals, and microorganisms have adapted to such environmental stress by evolving means to protect their proteins and other cell components against such denaturing stress. Denaturants, such as urea and guanidinium chloride (GdmCl), destabilize proteins. In contrast, osmolytes that protect cells against environmental stresses such as high temperature, desiccation, and pressure can stabilize proteins. Thus, a complete understanding of the stability of proteins and a description of the structures in the diverse DSEs requires experimental and theoretical studies that provide a quantitative description of the effects of both osmolytes and denaturants. The diffusion constant does not show any change in presence of increasing concentration of BSA. It is also found from the experiment that the number of bound urea molecule to the GlnRS surface reduced markedly in the presence of 0.25 M TMAO as osmolytes. So it may be concluded that preferential exclusion, due to steric repulsion is best fitted to explain the protective action of osmolytes. Osmolytes leads to the compaction of the protein molecule, due to the steric repulsion eventually ending up with expulsion of urea from the protein core

Introduction

Generally, isolated proteins are subjected to the severe environmental stress conditions encountered in the biosphere, many of them are unable to cope with the stress and will denature. Plants, animals, and microorganisms have adapted to such environmental stress by evolving means to protect their proteins and other cell components against such denaturing stress (Yancey *et al.*, 1982). A common mechanism evolved by these organisms is synthesis and intracellular accumulation of certain small organic solutes known as organic osmolytes.

These naturally occurring solutes include specific amino acids, certain polyols, and particular methyl amine species. That is an organic osmolyte solution is expected to provide general protection against denaturation in any proteins, even if that protein did not evolve in the presence of the organic osmolyte.

Any mechanism offering generalized protection of proteins against denaturation is of fundamental importance to issues of protein folding, stability and function. The assumption is that osmolytes were derived through natural selection and this implies

that particular physiochemical properties of stabilizing organic osmolyte solutions were selected for their ability to protect macromolecular and other components of the organism (Somero, 1986). In vitro these solutes typically stabilize the native state of globular proteins and favor the formation of protein assemblies. Increase in the concentration of such solutes generally drives protein processes in the direction that reduces the amount of water accessible surface area (ASA). Solute that function as osmoprotectants in vivo have been shown excluded from biopolymer surface. Preferential exclusion of osmolytes from biopolymer surface has been proposed to be the physical basis of their evolutionary selection.

Thermodynamic Overview

Sucrose and glycerol were used to stabilize biological systems, whereas urea and guanidium hydrochloride were used to solubilize coagulated systems and to unfold (denature) proteins. The aim in the use of these cosolvent was to displace the to the right or left the chemical equilibrium,

reactant $\rightleftharpoons$ product

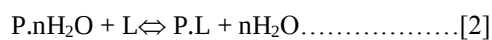
The basic Wyman linkage equation states that at any ligand concentration, m_L , the gradient of the equilibrium constant with respect to the ligand activity is equal to the change in the binding of the ligand to the biological system during the course of the reaction (constant T and constant P).

$$(\delta \log K / \delta \log a_L)_{mp} = v_L^{\text{prod}} - v_L^{\text{react}} = \Delta v \dots \dots \dots [1]$$

where k is the equilibrium constant of the reaction, a_L is the activity of the ligand, and the v_L^{prod} and v_L^{react} are the bindings of the ligand to the two end states of the reaction. W, L and P refer to the water, ligand (cosolvent) and protein (macromolecule).

Interaction of Solvent Component with Protein Loci

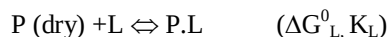
In solution any locus on the surface of the protein molecule must be in contact with a solvent component, because a vacuum cannot be tolerated in an aqueous medium. The reference state is the protein dissolved in water, in which it is fully hydrated. Therefore, in a binary solvent, the binding of the nonaqueous solvent component to any locus must displace water i.e. binding is an exchange reaction.



Let us consider thermodynamically a protein molecule in its fully hydrated reference state. It is possible to define formally a free energy of hydration, $\square G_w^0$. As the sum of the free energies of interaction of all water molecules with all the interacting loci. Physically, the consequence of these interactions can be described as the mass of water that, any instant, travels nonrandomly in the same direction as the protein in a transport process. Division by the molecular weight of water gives the effective number of water molecules that hydrate the protein, WH. Formally it can be described by a hypothetical equilibrium in an aqueous medium between empty (dry) protein loci and the same loci in a hydrated state.



The exchange of eq.2 requires the departure of water molecules with a free energy change $-G_w^0$ (which can be zero if a particular water molecule was not effected by the protein) $\square G_L^0$ and the occupancy of the vacated sites by ligand molecules with a free energy change, for the hypothetical equilibrium between empty (dry) protein loci in an aqueous medium and loci occupied by the ligand;



The net free energy change, measured experimentally as the free energy of binding, ΔG_b^0 is therefore an exchange free energy, ΔG_{ex}^0

$$\Delta G_L^0 = \Delta G_{ex}^0 = \Delta G_L^0 - \Delta G_W^0 \dots\dots\dots [3]$$

Schellman has treated the exchange reaction in terms of the binding equilibrium constant, K_b , which for an exchange reaction is the exchange constant K_{ex}

$$K_b = K_{ex} = ([P.L][H_2O]/[P.H_2O][L]) = K_L / K_W \dots\dots\dots [4]$$

Where K_L and K_W are the hypothetical equilibrium constant for binding of ligand and water to a dry site on the protein.

The weakly interacting ligands used to modulate the reactions are used at high concentrations, and may occupy as much as 40% of the solvent volume. These ligands are so referred as cosolvents. The requirement of high concentrations means that the interactions of the cosolvent with the protein proceeds with low free energy changes. As a consequence, the measured binding and the change in binding defined by Wyman linkage equation are a preferential binding. The epithet "preferential" refers to the relative affinities of the interacting loci on the protein for ligand and water. If the affinity is greater for ligand than for water, there is an excess of ligand in the protein domain relative to bulk solvent composition. This defines preferential binding of ligand. If there is a deficiency of ligand i.e., a greater affinity of water, the measured binding is negative, and there is preferential exclusion of the ligand and an excess of water in the protein domain. This defines preferential hydration. Therefore, binding and exclusion are symmetrical phenomena on the two sides of

a point of neutrality, defined by whether for the ligand $\Delta G_L^0 > \Delta G_W^0$ or $\Delta G_W^0 > \Delta G_L^0$.

Thermodynamic Binding : Preferential Hydration

When a cosolvent is added to the aqueous protein solution firstly the colligative decrease in water activity is observed. The second consequence will be examined by carrying out the equivalent operation of introducing a hydrated protein molecule into the aqueous cosolvent solution. The immediate effect is the perturbation of chemical potential of the cosolvent by the protein,

$$(\delta\mu_L/\delta m_p)m_L = (\delta\mu_p/\delta m_L)m_p$$

This perturbs the chemical equilibrium in the domain of protein. To restore the chemical equilibrium, the chemical potential of the cosolvent in the protein domain must be changed by an identical amount but with a sign opposite to that of the perturbation. This can be accomplished by adjusting the concentration of the cosolvent, m_L in the domain of the protein, in the domain of the protein ($\mu_L = \mu_L^0 + RT \ln m_L \gamma_L$) by the increment.

$$\begin{aligned} (\delta m_L/\delta m_p)\mu_L &= -(\delta\mu_L/\delta m_p)m_L/(\delta\mu_L/\delta m_L)m_p \\ &= -(\delta\mu_p/\delta\mu_L)m_p = \end{aligned}$$

$$\Gamma_{PL} \dots\dots\dots [7]$$

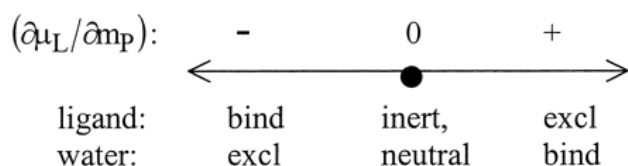
The quantity which is the preferential binding defined above, is the binding measured experimentally in dialysis equilibrium or vapor pressure osmometry and which appears in the Wyman linkage equation. Equation shows that $(\partial m_L/\partial m_p)\mu_L$ is a purely thermodynamic quantity., it is the mutual perturbation of the chemical potentials of cosolvent and protein and hence its identification as thermodynamic binding. This also means

that a molecule does not have to be in contact with protein to be bound to it.

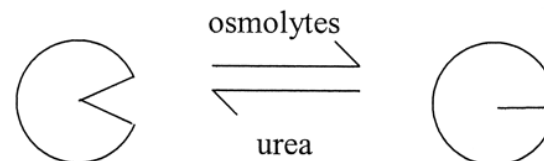
The perturbation of chemical potential $(\delta\mu_L/\delta m_P)m_L$ can be positive if the interaction between the cosolvent and the is unfavorable (most sugars, polyols or methylamines), or it can be negative if the interaction is favorable (urea, guanidium hydrochloride). Therefore Γ_{PL} can also be positive or negative. Negative Γ_{PL} means preferential exclusion of cosolvent, which means preferential hydration.

$$\begin{aligned}\Gamma_{PW} &= - (m_W/m_L) \Gamma_{PL} \\ &= (m_W/m_L) (\delta\mu_L/\delta m_P)m_L / (\delta\mu_L/\delta m_L)m_P \\ &\dots\dots\dots[8]\end{aligned}$$

Eq. 8 shows, like preferential binding, like preferential hydration is a measure of the perturbation of the chemical potential of the cosolvent by the protein. The two binding parameters Γ_{PL} and Γ_{PW} are equivalent ($m_W \Gamma_{PL} = -m_L \Gamma_{PW}$). From Eq. 7 and 8 it is evident that for any given protein the value of preferential hydration may be different for various cosolvents at identical concentration (positive for trehalose and negative for urea). Similarly for any water-cosolvent system the value of Γ_{PW} and Γ_{PL} will be defined by the protein added (urea is preferentially bound to native b-lactoglobulin and excluded from myoglobin). The fact is that the preferentially excluded stabilizing osmolytes interact with proteins just as strongly as, say urea but with an opposite sign of free energy change.



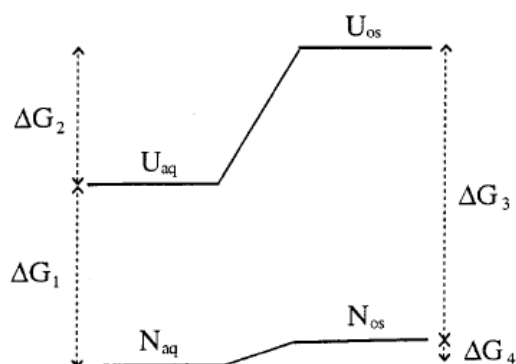
Thermodynamic effects of the addition of preferentially excluded and preferentially bound cosolvents on a reaction can be equal magnitude but will drive the reaction in opposite directions, as has been demonstrated by qu. et al. for the effects of urea and naturally protecting osmolytes on the stokes radius of reduced and carboxymethylated ribonuclease A.



There is one situation depicted in this picture, in which the lowering of the activity of water by addition of a cosolvent can modulate a reaction. This necessarily involves a change in water of hydration. This situation exists when the reacting cavity is totally impenetrable to osmolyte molecules. This would be true of a narrow channel, a narrow interstice or a pocket internal to protein molecule with an opening that only permits water molecules to cross it.

In various work it is established that unfavorable interaction between the particular osmolytes and the peptide backbone is responsible for the ability of these osmolytes to protect against denaturation (Liu and Bolen, 1995). Compatible osmolytes (polyols, amino acids) protect against extreme of temperature, dehydration and high salt environment while counteracting osmolytes like TMAO, Lglu protect cellular proteins against urea inactivation (Yancey *et al.*, 1982). Given that these denaturing stresses are very different from one another, there must be different mechanisms for compatible and counteracting osmolytes to act. The transfer model has been a fixture in biophysical chemistry since at least the 1930s (McMeekin *et al.*, 1935) and it has

contributed prominently to the concept of hydrophobic interactions as well as to the understanding of protein folding. For the purpose of discussion a thermodynamic cycle can be considered that provides an experimental means of using transfer free energies to understand solute-induced protein stability.



It is clear with both native and unfolded protein (Bolen D. W, Biochemistry, 1997, 36) that the unfavorable interaction of the peptide backbone with TMAO dominates the free energy contributions of the side chains. Hydrophobic side chains do not contribute energetically either to the native or to the unfolded state of Rnase T1, rather it is the interactions of TMAO with the polar and charged groups that make up the most of the side chain contributions. It was shown that that it is unfavorable to transfer the native state from water to 1M TMAO by 1.7kcal/mol but it is much more unfavorable (5.9 kcal/mol) to transfer the unfolded state. Thus, in the TMAO the native to unfolded conversion is 4.2kcal/mol less favorable than it is in water, and it is the unfavorable interaction of TMAO with the backbone that is responsible for the stabilization. The side-chains play no role in stabilization, in fact, the small contributions from the side chains actually promotes unfolding. An unfavorable transfer of native or denatured state from water to TMAO means that, on

the whole, the native or denatured state of the protein prefers to interact more with water than with TMAO, that is, the transfer model predicts preferential hydration of both the native and the denatured states of Rnase A in the presence of TMAO and provides a molecular rationale for the origin of preferential hydration while identifying the major chemical moiety (backbone) responsible for the ability of TMAO to stabilize proteins.

The problem with the transfer model is that there is some point at which the model could breakdown. Some of these include the validity of subtracting the transfer free energy of glycine from other aminoacids to obtain side chain transfer free energy, the validity of assuming group additivity, the validity of assuming full exposure of all groups in the unfolded state, the approximation of ignoring electrostatic effects and of ignoring activity coefficients in evaluating amino acid transfer free energy. But despite these problems it can be concluded that the major factor responsible for stabilization by osmolytes can be identified from transfer data.

Results and Discussion

Most interestingly, none of the counteracting osmolytes are universally effective. The counteraction ability depends on the nature of the protein and the denatured state. The mechanism of this powerful counteraction ability of some osmolytes is not known at this point. But we can make some tentative conclusions. The TMAO counteraction of urea effect has been studied in detail. It was concluded by several investigators that TMAO counteraction can be entirely accounted for by unfavorable free energy of transfer of peptide backbone from water to an osmolyte-water mixture. Side chains

contribute little. Recent work by Murphy and coworkers suggests that some of this unfavorable transfer free energy may originate from the ordering of the water structure by TMAO.

Polyols do not show any significant effect in many study. A number of other studies have previously noted this differential effect of osmolytes. In many situations, limited proteolysis is thought to take place from denatured states that are at equilibrium with the native state. If the denatured states are destabilized, it is expected that proteolysis under limiting condition will decrease. It has been noted previously that although TMAO decreased the limited proteolysis rate of Lactate dehydrogenase by Trypsin no significant effect is seen with glycine-betaine as an osmolyte. Clearly the nature of the protein and the osmolyte pair determines the outcome. Although the osmophobic force is likely to be of general importance, specific effects of different osmolytes may be superimposed to yield the differential effect of different osmolytes.

What may be the effect of destabilization of the partially denatured state? Increasing concentration of proteins does not perturb the diffusion constant of either glycerol or glycine-betaine, two well established osmolytes. This proves that glycerol or glycine-betaine do not bind to the native state of the protein. So it clearly proves that number of denaturant molecules surrounding the protein reduces in presence of osmolyte, which ultimately leads to the protection of protein from unfolding. And also it is supported that denaturation process of urea on protein is

accompanied by a binding interaction between urea and protein.

There are several models, which have been used to describe the interaction between osmolytes and protein. The best known two theories are the binding theory and the excluded volume theory or steric repulsion theory. The protein osmolyte binding theory is largely redundant on the emerging evidences provided by Record *et al.*. To interpret the responses of proteins in vivo and in vitro to changes in osmolyte/solute concentration and to coupled changes to water activity (osmotic stress) and for a quantitative understanding of the thermodynamic consequences of interaction of osmolytes and water with BSA surface a isoosmolal preferential interaction coefficient is introduced (ΔG_{int}) (23). For several osmolytes including L-glu, trehalose, proline, glycine-betaine, glycerol, and TMAO the value of this coefficient for BSA surface is reported.

All the values are negative. The negative value of preferential interaction coefficient indicate preferential exclusion of the solute from the BSA surface and obviously the local concentration of the solute in the vicinity of the protein surface is lower than their bulk concentration. For BSA, betaine is the most excluded and glycerol is the least excluded osmolyte. Between these two extremes, the magnitude of preferential interaction coefficient decreases in the order glycine-betaine>>proline>TMAO>trehalose~Lglu> glycerol. The order of their exclusion from protein surface correlates with their effectiveness as osmoprotectants.

Fig.1 Meaning of the slope, PW . The departing species consists of the n stoichiometric waters of hydration (○), additional preferential hydration water (□), and cosolvent molecules (●). Pattern of distribution of water and cosolvent molecules over the rest of the protein surface remains unchanged during the course of the reaction.

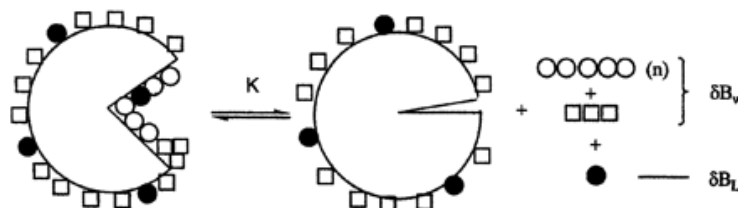
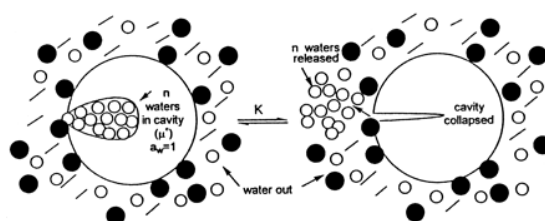


Fig.2 Release of water molecules from a cavity impenetrable to all cosolvents: $a_w(\text{in}) > a_w(\text{out})$, water; ●, cosolvent.



This exclusion may be due to the increase of steric repulsion in the osmolyte solvent mixture relative to the protein core (Knoll *et al.*, 1983). Support for the conclusion that steric repulsion is a major stabilizing force can be obtained from the diffusional studies conducted on the glycerol and betaine. The diffusion constant does not show any change in presence of increasing concentration of BSA. It is also found from the experiment that the number of bound urea molecule to the GlnRS surface reduced markedly in the presence of 0.25 M TMAO as osmolytes. So it may be concluded that preferential exclusion, due to steric repulsion is best fitted to explain the protective action of osmolytes. Osmolytes leads to the compaction of the protein molecule, due to the steric repulsion eventually ending up with expulsion of urea from the protein core.

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