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Forty Years of Research on Osmolyte-Induced Protein Folding and Stability

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Most organisms that have adapted to environmental stresses have done so by production and accumulation of certain small organic molecules, known as osmolytes that arose by natural selection and have the ability to stabilize intracellular proteins against the environmental stress. It is well known that osmolytes stabilize proteins and induce folding of aberrant proteins and therefore, it is of therapeutic use for a large number of protein misfolding diseases. Thus, it is very important that the present knowledge of the ability and mechanism of osmolyte-induced protein folding and structural stabilization should reach to researchers working in different avenues. In around 40 years of research, we have gained great advances in various aspects of protein folding and structural stabilization induced by osmolytes. To summarize and discuss the original findings, many short review articles and few long reviews have also been available but almost all have focuses on specific aspects. To get a clear picture of the effect of osmolytes on protein folding and structural stabilization, it is necessary for the benefits of the general readers, to combine and discuss all findings made during its 40 years of life. This review article is therefore, designed to give a collective knowledge on almost all facets of the progresses made on osmolyte-protein interaction to-date.

Keywords: Compatible osmolytes, Preferential hydration, Gibbs free energy, Protein stabilization, Stress condition, Protein folding

INTRODUCTION

Plants, animals and microorganisms need to adapt to environments in the biosphere that would ordinarily denature proteins and enzymes or otherwise cause disruption of life-giving cellular processes. These hostile environments involve such stresses as extremes of temperature, pH, cellular dehydration, desiccation, high extracellular salt, and even the presence of denaturing concentrations of urea inside cells [1-3]. A mechanism of adaptation that protects the cellular components against these denaturing stresses involves the accumulation of small organic molecules known as osmolytes [1-3]. These organisms typically contain osmolytes at several millimolar concentrations [4-6]. Two defining characteristics of protecting osmolytes are that they stabilize proteins against denaturing stresses, and their presence in the cell does not largely alter protein functional activity [1,7-21]. The basic premise is that natural selection of protecting osmolytes is based upon selection for a particular molecular-level that confers generic stabilization to all proteins without altering

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their functional activity [2,3]. A number of cellular osmolytes have been proposed to act as chemical chaperones to rescue defective proteins and to protect native proteins from proteotoxic intracellular environments [22]. The term "chemical chaperone" was chosen to reflect that though these osmolytes possess properties akin to molecular chaperones, they are not protein molecules that facilitate protein folding and contribute to acquire thermo tolerance.

A list of almost all the osmolytes used by organisms by various organisms is given in Table 1. Chemically, these osmolytes can be grouped into three major classes (as seen in Table 1): polyols (mannitol, glycerol, sorbitol, inositol, pinitol, sugar and sugar derivatives), free amino acids (glycine, alanine, proline) and their derivatives (taurine, octopine, β-alanine), and methyl ammonium compounds including trimethylamine-N-oxide (TMAO), glycerophosphocholine (GPC), glycine betaine (betaine) and sarcosine. Often, they are classified as compatible or counteracting based on their effects on the functional activity of proteins. Compatible osmolytes increase protein stability against denaturation with little or no effect on their function under native conditions [1,23-27]. Representatives of this class include certain amino acids (*e.g.*, proline and glycine) and polyols (*e.g.*, trehalose, sucrose and sorbitol). Counteracting osmolytes consist of the methylamine class of osmolytes, which are believed to have the special ability to protect intracellular proteins against the inactivating and destabilizing effects of urea [28,29]. In contrast to compatible osmolytes, counteracting osmolytes are believed to cause changes in protein function that are opposite of the effects that urea has on protein function [28,30-33]. Examples of organs and even whole animals that are rich in urea-containing cells are mammalian kidney which contains betaine and GPC as counteracting osmolytes, and cartilaginous fishes and coelacanth which use TMAO as the principal counteracting osmolyte [34-38]. Very recently, we are also able to make another systematic classification of osmolytes based on the structure-function relation [13] (see Fig. 1). Class I includes polyhydric alcohols (sorbitol, glycerol, xylitol, adonitol, mannitol) and amino acids and derivatives (glycine, alanine, proline, serine, lysine, β-alanine and taurine) that have no significant effects on both protein stability in terms of Gibbs free energy change at 25 °C ($\Delta G_D°$) and k_{cat}. Class II represents methylamines (sarcosine, dimethylglycine, betaine, trimethylamine N-oxide) that increase both $\Delta G_D°$ and k_{cat}, but decrease K_m. Sugars (glucose, fructose, galactose, sucrose, raffinose, stachyose) that increase $\Delta G_D°$, but decrease both K_m and k_{cat} belong to class III.

Except for urea (used only by a comparatively few groups of animals), osmolytes are widespread in occurrence; for example, betaine is used in every kingdom of life, and most marine invertebrates, numerous prokaryotes, and many mammalian cell types use amino-acid osmolytes. Taurine is widespread among marine animals and some mammalian organs [1]. Carbohydrate osmolytes occur in most variety in photosynthesisers, but are not exclusive to plants and algae; for instance, sorbitol is an osmolyte in some marine algae and in mammalian kidneys [2,39]. Sugars and polyols are also the dominant solutes accumulated in organisms adapting to freezing, such as terrestrial plants, insects, reptiles and some polar fishes [2,39]. Many organisms use mixtures of osmolyte types; *e.g.*, the mammalian kidney along with urea, contains the polyols, myo-inositol and sorbitol, the methylamines, GPC, glycine betaine, and taurine [34,35].

Around 4 decades (1972-2010) have been invested to understand the effect of these compounds on protein structure, folding and function. We have gained tremendous knowledge

Table 1. Three Classes of Naturally Occurring Osmolytes Used by Organisms under Various Stress Conditions

Amino acids and derivatives	Polyols and sugars	Methylammonium salt
Proline, phenylalanine, valine, leucine, isoleucine, serine, glutamine, arginine, lysine, glycine, aspartate, β-alanine, ectonine, taurine, hypotaurine, thiotaurine	Glycerol, sorbitol, manitol, pinitol, inositol, glucose, fructose, sucrose, raffinose, stachyose, trehalose, mammosylglycerate, glucosylglycerate	Glycine betaine, L-carnitine, glycerophosphorylcholine, choline, creatine, trimethylamine N-oxide, N-methyltaurine, sarcosine

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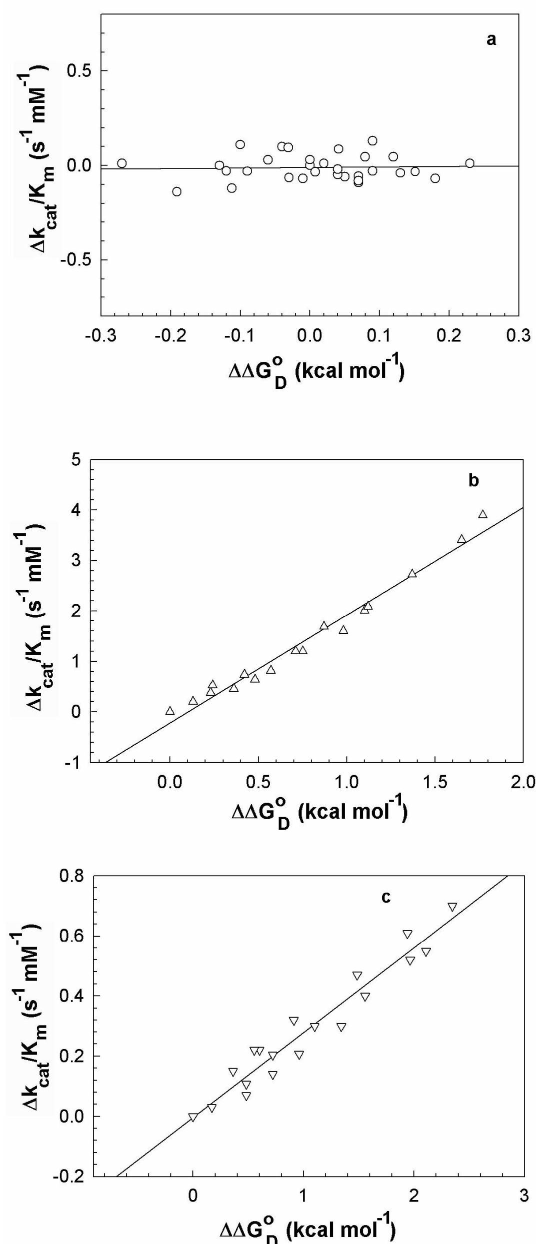

Fig. 1. Relationship between protein stability and catalytic efficiency. Plot of ($\Delta k_{cat}/K_m$) *vs.* $\Delta\Delta G_D{}^o$ of RNase-A obtained in the presence of various osmolytes as class I (polyols and amino acids), class II (methylamines), and class III (sugars). Symbols (o), (Δ), and (∇) represent classes I, II and III, respectively. The plots are redrawn using data published earlier from our laboratory [13].

on many facets of osmolyte-protein interaction including, its effect on protein folding landscape, enzymatic kinetic parameters, thermodynamic mechanism and various applications in health and industry. A large number of short review articles have appeared in the literature that focus on specific avenues of the osmolyte-protein interaction. However, to date there appeared no review article that contains collective data on the progresses made in around 40 years. This review article is therefore, designed to give a collective knowledge on almost all facets of the progresses made on osmolyte-protein interaction to date. We have incorporated all aspects of osmolyte-protein interaction and discussed in depth. We have also pointed out upcoming avenues that might be important as future direction.

DISCOVERY OF COMPATIBILITY PARADIGM

Osmolytes Are Compatible with Protein Stability, Enzyme and Cellular Functions

The compatibility hypothesis [40], later extended by Clark, Somero, Wyn Jones and others [1,41], recognizes that inorganic salts (especially NaCl) at high cellular concentrations disrupt protein function *in vitro,* but the major osmolyte salts usually do not, even at several milli-molar concentrations. Use of organic osmolytes, in fact, should maintain enzyme functions without significant disruptions over a wide range of external salinities. General compatibility has been tested *in vivo* by manipulating cell osmolytes, hypothesizing that cells under hyperosmotic stress suffer if osmolyte levels are reduced, whereas in the presence of a osmoprotectant, cells grow better (exogenous compatible osmolytes or precursors). The eubacterium *E. coli*, for which growth slows with increasing external osmolality, uses K^+ and glutamate as major osmotic agent [42]. Nevertheless, cell growth is inhibited by high osmolality of K^+ and glutamate, and is greatly improved by extracellular betaine, which through uptake replaces cellular K^+ and glutamate [43]. Similarly, one line of mammalian renal medullary cells (PAP-HT25) in hyperosmotic culture uses primarily sorbitol as an osmolyte. In another observation renal cells suffered when deprived of myo-inositol, but improved when betaine was provided [44].

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Osmolyte compatibility is believed to result from the absence of osmolyte interactions with substrates and co-factors, and the non-perturbing or favorable effects of osmolytes on macromolecular-solvent interactions. Among all the compatible osmolytes, polyols are the most prevalent molecules used by nature to protect organisms against the stresses of high osmotic pressure and freezing [1,45]. For the last several years we have been investigating the compatibility paradigm of osmolytes in the face of enzyme kinetic parameters (K_m and k_{cat}) and protein stability, T_m or C_m, (the midpoint of heat or chemical-induced denaturation) and $\Delta G_D°$ and their relation thereof. We have shown that almost all polyol osmolytes do not significantly alter protein stability ($\Delta G_D°$) and hence enzymatic kinetic parameters (K_m and k_{cat}) of RNase-A and lysozyme thereby making these osmolytes ideally compatible with the enzyme functions and protein stability [9].

However, it is important to note that compatibility does not hold true in general, and may depend on the nature of enzymes and osmolytes used. For instance, although glycerol has been shown to be compatible with functions of many enzymes [9,46], it is found to be non-compatible with several other enzymes [46-48]. Observation on the compatibility of osmolyte with functional activity of enzymes is explained in the light of the finding that osmolytes are preferentially excluded from the protein domain [49]. It has been argued that since these co-solvents are excluded from the vicinity of the protein surface, *i.e.*, there is no direct interaction between the osmolyte and the protein, they are expected to have no effect on K_m and k_{cat} [1,24]. However, the possibility that these osmolytes may have minor effects on the association of substrate with enzyme through solvation effects on substrates or enzyme active sites by means of effects on the thermodynamic activity of substrates or enzyme, cannot be ruled out [24,48].

Another significant discovery that the protein stability ($\Delta G_D°$) at physiological pH and temperature is also not affected by polyols, indicates that the protein turnover may not be affected by the presence of these osmolytes [10]. The compatibility of osmolytes with protein stability ($\Delta G_D°$) holds true for many compatible osmolytes, namely amino acids and their derivatives [8-11]. It is argued that the main reason for not perturbing the $\Delta G_D°$ of proteins by osmolytes at physiological pH and temperature is due to the fact that there is perfect enthalpy-entropy compensation in the presence of osmolytes or, in other words, there is perfect balance of preferential exclusion (stabilizing force) and preferential binding (destabilizing force) of the osmolytes to the proteins [9-11]. In another development, recently molecular dynamic simulation study have uncovered that the osmolytes have almost no effect either on the thermodynamics of hydration of small non-polar solutes or on the hydrophobic interactions at the pair and many-body level [50]. Therefore, this neutrality of osmolytes toward hydrophobic interaction (one of the primary driving forces in protein folding) is at least partially responsible for making osmolytes compatible with protein stability in terms of $\Delta G_D°$ [50].

Osmolytes Do Not Alter the Native Protein Structure (Structural Compatibility)

Thermodynamic equilibrium study merely yields the free energy difference between folded and unfolded structures with no indication as to the extent that the structure of the individual states has been perturbed. Folded structures of most proteins are sensitive to changes in environmental conditions such as temperature, pressure, moisture content, and the presence of salts and other solutes. Significant perturbations in thermodynamic conditions can cause changes in secondary and tertiary structures, leading to a partial or complete loss of their activity. Organisms are known to adapt to such perturbations in different ways, including evolutionary adaptations that endow stability/activity under extreme conditions (*e.g.*, as in extremophiles) or through accumulation of osmolytes [50]. The first evidence for the non-perturbing nature of osmolytes on the folded native structures comes from spectroscopy measurements. Measurement of the far- and near-UV CD spectra and near-UV absorption spectra of many native proteins in the absence and presence of osmolytes are (within experimental errors) identical [10-12,51], indicating that both secondary and tertiary structures are not perturbed by the presence of osmolytes. The second evidence comes from the size exclusion chromatography results that revealed that osmolytes have no effect on the dimensions of the native fold [32,52,53]. Most convincingly, X-ray result on co-crystallization of RNase-A fragment with TMAO revealed that the native structure of protein is unperturbed by TMAO

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[54]. Thus, measurements of optical properties, dimensional properties, and X-ray data clearly indicate that the native structure of a protein is the same in the presence or absence of polyols. This conclusion is also supported by the 2-dimentional NMR studies that measures exchange rates of individual labile protons. It has been observed that glycine (up to 2 M concentration) has negligible effect on the intrinsic quality and nature of the NMR spectra of proteins [18]. Interestingly, simulation study [50] also proposed that the neutrality of osmolyte towards hydrophobic interactions is at least partially responsible for making the "structure non-perturbing" osmolyte.

Effects of Osmolytes on Internal Dynamics/Native State Flexibility

Native state flexibility information in the presence of osmolytes has been obtained primarily from amide H-D exchange (HX) rates, and all available data concur that osmolyte attenuates structural fluctuations in proteins [19,55,56]. But the observations on HX have a number of technical issues [57,58]. HX rates suggest that the mobility of deeply buried, rigid segments of the polypeptide is more affected than superficial domains. For example, studies employing site-specific NMR-detected HX rates [18,27,57] have concluded that the osmolyte inhibits slow, large scale unfolding like transitions but has no detectable effects on small-scale fluctuations. On the contrary, studies employing FTIR to follow the overall fraction of exchanged protons have indicated that both slow and fast exchange rates are affected by the presence of osmolyte [19,56,59,60]. Recently, Kim *et al*. [19] suggested that the adoption of different exchange conditions might account for the discrepancy. The analysis of exchange rates in terms of structural fluctuations also presumes knowledge of the prevailing exchange regime (EX1 or EX2). Alternative exchange routes, such as solvent penetration, which are difficult to distinguish from EX2, would exhibit pH dependence, are generally dismissed as unimportant, more from the impossibility to determine their relative magnitude rather than on experimental grounds [19]. Furthermore, questions have been raised on the sensitivity of HX rates for reporting on the flexibility of native state of the protein. Recently, Qu and Bolen [57] and Wooll *et al*. [58] emphasized that HX rates are sensitive to large amplitude unfolding like transitions but are intrinsically insensitive with respect to fluctuations of the native fold. It is argued that even if the osmolyte did inhibit the internal fluctuations, they would be hard to detect in EX2 rates as open and closed states involved in the exchange are similar in surface area with a negligible shift in the closed to open equilibrium.

To suppress the controversies arising out of interpreting HX rates, Strambini and co-workers [61-63] using Trp phosphorescence spectroscopy (a remarkably sensitive technique for probing the flexibility of globular proteins) measured phosphorescence lifetime, which is a direct probe of the local flexibility of the protein matrix around the chromophore ($_{T}$) and the bimolecular rate constant (k_q) for the quenching of phosphorescence by acrylamide in solution. They discovered that there is a sharp distinction between proteins with a compact globular fold and internally hydrated proteins in terms of perturbation by internal fluctuations by osmolytes. From the modulation of $_{T}$ and k_q of azurin in the presence of sucrose, xylitol, and trehalose across a wide temperature range, it was concluded that the sugar osmolyte attenuates structural fluctuations principally when macromolecules are internally hydrated and thermally expanded or loose [61]. However, in another study they demonstrated that for apoazurin, alcohol dehydrogenase, alkaline phosphatase and glyceraldehydes-3-phosphate dehydrogenase, 1.8 M TMAO does not perturb the flexibility of these macromolecules in a temperature range between -10 °C and up to near the melting temperature [64]. Attenuation of structural fluctuation in the native state, therefore, on the other hand, is not the general response to the osmolyte. The sharp distinction between proteins with a compact globular fold and internally hydrated proteins, together with the positive role of temperature seem to indicate that important effects of osmolyte on protein dynamics are linked to the possibility of dehydrating-compacting the native state. Thus, from the observations obtained from Trp spectroscopy, osmolytes compact the native structure or influence the internal dynamics only when proteins undergo thermal expansion or are structurally loose.

In another development, contrary to the above observations obtained from phosphorescence spectroscopy, studies using NMR spin-relaxation measurements on RNase-A [65], demonstrated that osmolyte, TMAO restricts the increase in

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conformational space sampled by the N-H bond vectors in the presence of guanidine hydrochloride alone. Thus, osmolyte causes more restricted, native-like protein fluctuations, possibly limiting access to higher energy conformational sub-states that would otherwise, ultimately lead to protein denaturation. Several of the protein sites experiencing this TMAO-induced reversal of dynamics reflect those identified by hydrogen exchange experiments [66], which occur on a much slower time scale. Indeed the result is in agreement with other experimental and theoretical studies, indicating that TMAO reverses the effects of chemical denaturant, urea by decreasing the fluctuations of the native state [57,67-69]. These observations, therefore, point to a relation between stability and dynamics of enzymes. In agreement, several studies have shown that the native state of a protein consists of inter converting high (most compact) and low (less compact) activity state ensembles [70]. It has also been demonstrated that the presence of osmolytes shifts the native conformational equilibria toward the most compact protein species within native-state ensembles [70]. Taken together, despite of their differences in the interpretation of the results obtained from many techniques, the effect of the osmolytes on the internal dynamics cannot be discounted as there is no direct relationship between activity and thermodynamic stability ($\Delta G_D°$) of enzymes in the presence of osmolytes suggesting that osmolytes have direct consequences on native structure ensemble [13]. Different osmolyte classes may have different consequences on the ensemble nature of the native state [13].

NATURALLY OCCURING DESTABILIZING OSMOLYTE SYSTEM

Although compatible osmolytes are largely accumulated to stabilize protein and enzyme systems, nature has not ignored the use of protein destabilizing osmolyte system to act as efficient osmoprotectants. The metabolic waste, urea and many other osmolytes (arginine, histidine, and lysine), referred as non-compatible are also very good osmoprotectant [71]. It is well-known that urea is a chaotropic agent that disrupts non-covalent responsible for the globular structure of proteins [29,38,72,73]. This loss of structure influences enzyme kinetic properties such as maximal velocity (V_{max}) and K_m [29,38] and alters the midpoint of denaturation curves of proteins [72,74].

To protect from the deleterious effects of urea on protein stability and function, organisms accumulate other stabilizing osmolytes such as TMAO, sarcosine, glycine betaine and GPC. When the molar ratio of the urea to methylamine is appropriate (often 2:1), counteraction works the best [2,38,75]. The two effects are shown to be algebraically additive [28,38,76] amidst some conflicting reports [77-80]. Based on activity measurements, urea increases K_m and decreases k_{cat} of enzymes but methylamines just have opposite effects [32,39]. The urea-methylamine counteraction system is mainly confined to at least in two independent systems, namely, mammalian kidney, and many marine elasmobranch fishes. Interestingly, urea concentration in marine elasmobranch fishes reaches up to (300-500·mM) [1,81,82] and 400-600 mM in mammalian kidney. It increases up to 3-4 M in xeric rodents under antidiuretic conditions [83].

Other common non-compatible osmolytes that organisms use are arginine, lysine, and histidine [2]. Based on thermodynamic measurements, many investigators reported that arginine, histidine, and lysine destabilize proteins at physiological pH. All these osmolytes lower both T_m and $\Delta G_D°$, the Gibbs free energy change on denaturation of proteins at 25 °C [7,24,84]. It is also well known that the destabilizing effect of arginine is due to its preferential binding to proteins [85]. However, the effect of lysine is unusual. Low concentration of lysine destabilizes proteins, while its high concentrations stabilize proteins [84]. Intracellular role of non-compatible osmolytes are not properly understood but it is speculated that these osmolytes may perhaps be involved in maintaining protein quality control or act as ligands to many proteins [86]. Thus these non-compatible osmolytes may be directly involved in the modulation of functional activity of proteins.

UNDERSTANDING THERMODYNAMIC BASIS OF PROTEIN STABILITY

Effect of Osmolytes on Melting Temperature (T_m) and Gibbs Free Energy Change at 25 °C ($\Delta G_D°$) of Proteins Upon Denaturation

A number of compatible osmolytes common to bacteria and eukaryotes have been shown to increase T_m (melting temperature) and C_m (melting concentration) of proteins (see

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Fig. 2). For instance, one molar of trehalose increases the stability of lysozyme by 8 °C and sucrose by 4 °C for RNase-A [19,87]. Extensive investigation of the effect of polyol osmolytes on T_m indicated that their effect on T_m increases in a osmolyte concentration dependent manner [9-11,16,19]. Interestingly, stabilizing effect of polyols increases as a function of the length and number of OH groups of the polyhydric alcohol [88]. On the other hand, stabilizing effect of sugar osmolytes is found to depend not only on their concentration but also on their size; larger the size of the sugar molecules, the more is the increase in T_m [12]. For example, the stabilizing effect of sugar osmolytes in terms of T_m goes in the order of stachyose > raffinose > sucrose > glucose ~ fructose ~ galactose, which is, in fact, the increasing order of the sugars size. Similar to compatible osmolytes, all counteracting osmolytes also offer big increase in T_m in a concentration dependent manner [51,74]. For instance, glycine-based osmolytes (glycine, sarcosine and betaine) increased the T_m values for RNase and lysozyme (*e.g.*, 8.2 M sarcosine increased the T_m for RNase by 22 °C [89]. However,

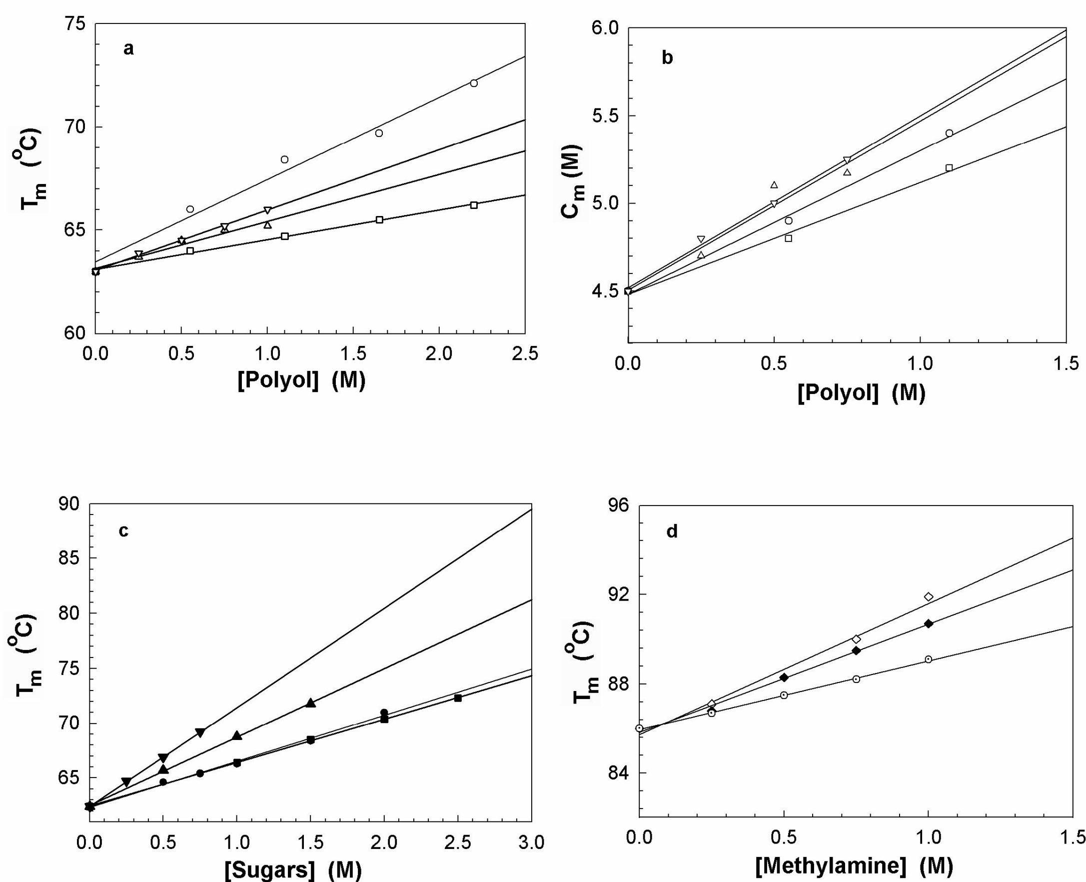

Fig. 2. Effect of various osmolytes on the heat-induced and GdmCl-induced denaturation of RNase-A at pH 7.0, measured in terms of T_m and C_m, respectively. Osmolytes used are: sorbitol (o), xylitol (Δ), adonitol (▽), glycerol (□), glucose (●), fructose(■), sucrose (▲), stachyose (▼), TMAO (◊), sarocsine (◆) and glycine betaine (⊙). The plots are redrawn using the data published earlier from our laboratory [9-11,16,19,87].

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some glycine-based methylated osmolytes including sarcosine and glycine betaine are found to have a tendency to destabilize proteins (or decrease T_m) at high concentrations due to its propensity to bind to the exposed hydrophobic groups in the denatured state [89]. There is another class of perturbing osmolyte that includes arginine, lysine and histidine. This class of osmolyte is found to decrease T_m due to binding to the denatured state of a protein as opposed to the compatible osmolytes that increase T_m and $\Delta G_D°$. Detailed studies demonstrated that the increase in T_m of proteins upon osmolyte addition is due to a shift in the protein denaturation equilibrium, N state ↔ D state, toward the right. It is believed that the preferential exclusive effect exhibited by the osmolytes is the main driving force for making a shift in the denaturation equilibrium leading to the stabilization of proteins by osmolytes.

Similar to the effect of osmolytes on T_m all counteracting osmolytes also increase $\Delta G_D°$ of proteins in a concentration dependent manner. However, unlike counteracting osmolytes, there are ambiguous reports on the effect of compatible osmolytes on $\Delta G_D°$ at physiological pH and temperature. Systematic studies show that compatible osmolytes do not have significant effect on $\Delta G_D°$ of proteins at physiological temperature and pH while there is a big increase in T_m in their presence [8,10,11]. Therefore, we argue that the change in T_m of proteins by compatible osmolytes cannot be regarded the real yardstick of the extent of protein stabilization by osmolytes. However, two other laboratories (Timasheff and co-workers and Bhat and co-workers) report that $\Delta G_D°$ of proteins are increased by compatible osmolytes at physiological pH and temperature [14-16,87,90]. This discrepancy may be due to the methods employed in the estimation of $\Delta G_D°$. For instance, Timasheff and co-workers analyzed thermal denaturation curves by assuming a value of zero for ΔC_p. In fact, ΔC_p values of proteins are zero neither in the absence nor in the presence of compatible osmolytes. Bhat and co-workers estimated $\Delta G_D°$ of proteins in the presence and absence of compatible osmolytes at the T_m. Therefore, ΔG_D of a protein in the presence of osmolytes estimated at the T_m of control will not be same as that expected at 25 °C for ΔC_p of proteins in the absence and presence of osmolytes is not zero.

The protein stabilization in terms of $\Delta G_D°$ depends on two factors namely, $\Delta H_D°$ (ΔH_D, the denaturational enthalpy change at 25 °C) and $\Delta S_D°$ (ΔS_D, the denaturational entropy change at 25 °C). Estimation of enthalpy and entropy contributions to $\Delta G_D°$ in a given solvent condition using the values of T_m, ΔH_m and ΔC_p, of RNase-A and lysozyme obtained in the presence of polyol osmolytes using appropriate thermodynamic relations, revealed that there is a perfect enthalpy-entropy compensation of proteins in the presence of all polyol osmolytes at pH 7.0, *i.e.*, $\Delta G_D°$ is unperturbed [10,11]. Therefore, thermodynamically the non-perturbing effect of compatible osmolytes on $\Delta G_D°$ is due to the perfect balance between enthalpy and entropy. On the other hand, it has been shown earlier that preferential binding of the osmolytes destabilizes proteins while preferential exclusion stabilizes proteins [49]. Therefore, unperturbed $\Delta G_D°$ of proteins in the presence of compatible osmolytes indicate that there is perfect balance of preferential binding and exclusion at physiological pH and temperature in the presence of compatible osmolytes.

Effect of Osmolytes on Protein Stability Depends on pH

Although osmolytes stabilize proteins, the stabilizing effect appears to depend on the type of solvent conditions used. Effect of polyol osmolytes is generally pH-dependent [10,11,14,15]; lower the pH, higher the stabilizing effect. For example, almost all polyol osmolytes (sorbitol, xylitol, mannitol, glycerol, adonitol) are found to have no significant effect on protein stability (in trems of $\Delta G_D°$) at physiological conditions (neutral pH and 25 °C) [10] but are found to strongly enhance $\Delta G_D°$ at lower pH values [9,11,14,15]. Similarly, the sugar osmolyte, trehalose has also been reported from Bhat’s laboratory [87] to have the same trend of pH-dependent stabilizing effects. Other sugars (glucose, fructose, galactose, sucrose, raffinose and stachyose) are also observed to have more stabilizing power (in terms of $\Delta G_D°$) on proteins at lower pH values [12]. Chemical potential measurement of RNase-A in the presence of sorbitol at pH 5.5 and 2.0 also suggests that sorbitol stabilizes RNase-A more at pH 2.0 than at 5.5 [15]. The current notion of this pH-dependence of $\Delta G_D°$ of proteins is explained in the light of the report that different charge states of a protein affect its hydrophobicity [91]. In fact, protein hydrophobicity increases with a decrease in pH

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due to the protonation of COO^- groups [91]. Interestingly, polyol and sugar osmolytes are more preferentially excluded from the hydrophobic surfaces by solvophobic interaction between the hydrophobic groups and the -OH groups present in polyol osmolytes [87].

Another interesting observation that has been made is that amino acid osmolytes (glycine, proline, isoleucine, leucine, phenylalanine, valine) and amino acid derivatives (taurine, β-alanine) do not show significant pH-dependent stabilization effects on proteins in terms of $\Delta G_D°$ [7,8]. In fact, these osmolytes are found to have no significant effects on $\Delta G_D°$ of proteins at all pH values. Although they do not alter $\Delta G_D°$ of proteins at all pH values, the extent of stabilization of proteins in terms of T_m is more at lower pH than at physiological pH. Thus, the effect of amino acids and derivatives is somewhat different from that of polyols and sugars.

All osmolytes are not universal protein stabilizing compounds. Rather the effect of some osmolytes are, in fact, denaturing at some solvent conditions. For example, TMAO, contrary to overwhelming stabilization effect near neutral pH, strongly destabilize proteins at lower pH values (pH 5.0 and lower) [51]. In fact, TMAO is positively charged at pH below 4.5 as it has a pK_a in the pH range 4.56-4.75 [28,57]. Interestingly, another methylamine osmolyte, glycine betaine was also found to destabilize or lose its stabilization power at lower pH values (pH 5.0 and below) while it still stands as a strong protein stabilizer near neutral pH [92].

There are no mechanistic data on why osmolytes behave differently at both physiological and lower pH values. However, on thermodynamic grounds, as mentioned above, there is perfect compensation between the enthalpy and entropy in the presence of polyol osmolytes leading to no change in $\Delta G_D°$. At lower pH values (below 7.0) where $\Delta G_D°$ is increased in the presence of polyols, protein stabilization is dominated either by enthalpic or entropic factors depending on the type of protein in consideration. For example, protein stabilization by all the polyol osmolytes is under enthalpic control for lysozyme but under entropic control for RNase-A [9,10,93]. Additionally, in the case of sugar osmolytes, protein folding is under enthalpic control for RNase-A [12,94] while entropic in case of α-lactalbumin [94]. For TMAO, at pH ≥ 5.0, protein stabilization is enthalpically unfavorable and entropically favorable for many proteins. However, the unfavorable enthalpy change outweighs the favorable entropy change to yield an unfavorable free energy change ($\Delta\Delta G_D° > 0$) [51,95]. At pH values < 5.0 enthalpic contribution to protein destabilization outweighs the entropic contribution leading to a $\Delta\Delta G_D° < 0$; hence, destabilization is under enthalpic control for two proteins, lysozyme and α-lactalbumin while for RNase-A, protein destabilization is under entropic control. For amino acid osmolytes and their derivatives, similar to polyols, there is perfect compensation between enthalpy and entropy at both physiological and lower pH values leading to no significant change in $\Delta G_D°$ at all pH [8].

Osmolytes Counter Denaturing Effects of Urea, Salt, Pressure on Proteins and Induce Refolding

Some osmolytes, especially, methylamines, in addition to imparting stabilization, are found to counteract specifically the deleterious effects that denaturant urea may have on protein stability in terms of T_m and $\Delta G_D°$ [34,38,77]. The urea-methylamine counteraction does not work at any concentration ratio between urea and methylamine but is found most appropriate at 2:1 urea to methylamine (for detail see subheading "NATURALLY OCCURING DESTABILIZING OSMOLYTE SYSTEM". Among the methylamines, betaine is known for its ability to counter the effect of high concentration of salts on protein stability in many higher plants, bacteria, and in some mammalian cells [23,96,97]. TMAO has also been demonstrated to have the ability to counter the denaturing effects of pressure on proteins [98]. This is why these osmolytes are found predominantly in the sea bottom dwellers [99,100]. Interestingly some osmolytes are found to prevent cold denaturation of proteins [101]. Disaccharides, most notably trehalose, are built up in anhydrobiotic dormant organisms (*e.g.*, baker's yeast). However these sugars do not follow the mechanisms of non-interactive compatibility. Rather they bind to macromolecules and membranes, effectively replacing water [102]. TMAO has been demonstrated to have the ability to induce refolding of intrinsically or thermodynamically unfolded proteins [53]. Some osmolytes behave as "chemical chaperones" by promoting the correct refolding of unfolded proteins *in vitro* and in cell [103-107]. Many osmolytes are also reported to reverse protein aggregation [103,108-110].

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PROGRESSES ON OSMOLYTE-PROTEIN INTERACTIONS

Mechanism of Osmolyte-Induced Protein Stabilization

In the equilibrium protein folding reaction, D state ↔ N state, protecting osmolytes push the equilibrium toward N, whereas denaturing osmolytes push the equilibrium toward D. As yet, there is no universal molecular theory that can explain the mechanism by which osmolytes interact with the protein to affect the thermodynamic equilibrium. However, the most convincing evidence comes from the work of Timasheff and co-workers who have shown that stabilizing osmolytes are preferentially excluded from the immediate vicinity of the protein domain and this preferential exclusiveness is the main driving force for osmolyte-induced protein stabilization [90,111]. This preferential exclusion is brought about by three general means [112]. One being the solvophobic interaction between osmolyte and N and D states of proteins; a second means draws from surface tension effect, in that osmolytes that increase surface tension should also be excluded preferentially from the protein surface [16,87,113,114]. A third means of achieving preferential exclusion arises from excluded volume considerations [115-117]. Later on, Bolen and co-workers, on the basis of transfer free energy measurements of amino acid side chains and peptide backbone from water to osmolyte solutions, demonstrated that osmolyte stabilization of proteins originates from the predominant unfavourable interaction of the osmolytes with the peptide backbone [31,52,118] while side chain interactions contribute weakly. Unfavorable interactions between a solvent component (osmolyte solution) and a protein functional group (peptide backbone) are traditionally classified as solvophobic, and this unfavorable interaction has been termed as the osmophobic effect [119]. Transferring native proteins to osmolyte solution increases the chemical potential, *i.e.*, destabilizing the native state. The reason why osmolyte stabilizes the proteins against denaturation is that they destabilize the unfolded state much more than they destabilize the native state [119]. This same conclusion was also drawn by Timasheff [49], based on thermodynamic measurements that permit evaluation of change in chemical potentials of the native and denatured proteins on transfer to osmolyte solutions [49]. In another development, it has also been reported that these osmolytes have different preferences to interact with the various surface groups of proteins and thus affect the thermodynamic transition between protein states that expose different kinds and amounts of surface area [120].

Native and Denatured States are Differently Preferentially Hydrated

On the basis of preferential interaction measurements between protein and osmolytes, Timasheff and co-workers [14,15] pointed out that there is no requirement that a co-solvent be preferentially excluded from the native protein in order to be a structure stabilizer. But the strong preferential exclusion of osmolytes from the denatured state is the driving force of the osmolyte-induced protein stabilization. In a systematic study by Timasheff and co-workers, the preferential hydration of the native RNase-A was found to slightly decrease with increasing temperature. Between 20 and 35 °C, there is no change in preferential interaction parameters of the native protein in the presence of sorbitol. Its magnitude (0.37-0.46 g of water/g of protein) is similar to the generally observed hydration values of most globular proteins [121-123]. There is no significant increase in the preferential hydration of RNase-A even at 48 °C (and pH 5.5) where the protein still exists in the native conformation. However, denatured state is overwhelmingly preferentially hydrated at 48 °C and pH 5.5 [14,15]. Interestingly, in the denaturation transition region, the preferential hydration remains at higher values for the denatured protein but not for the native one. Many pieces of evidence also suggest that there is no requirement that co-solvent be preferentially excluded from the native protein in order to be a structure stabilizer or destabilizer. What is required is that the preferential binding to the denatured state must be negative or smaller [18,124,125]. Therefore, the source of protein stabilization is due to increased preferential hydration of the denatured state, not the native state.

THEORETICAL MODELS TO EXPLAIN PROTEIN-OSMOLYTE INTERACTIONS

Several models are currently used to obtain molecular-level interpretations of the data [126-128]. These models can

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be divided into two broad classes. One class of explanation focuses on changes in concentrations of the solvent and solute, and interprets osmolyte-induced effects in terms of binding equilibria of the solute and solvent to the biological macromolecules [126,129-131]. A second class focuses on excluded-volume effects that result from the increase in steric repulsions in solute-solvent mixtures relative to pure solvent [132-134]. The fact that equivalent data are interpreted in fundamentally different ways highlights the controversy surrounding the driving forces responsible for solute-induced changes in conformational equilibria. Supports for steric repulsions as a major stabilizing force comes from the observations that the native state of proteins is not maximally compact [135,136] and osmolytes decrease the apparent specific volume and adiabatic compressibility of several native proteins, proving that they are more compact [135,137]. Simulation studies also indicate that steric repulsions can be a major driving force in osmolyte-induced folding. First, Monte Carlo simulations that treat the denatured state as a string of small hard spheres show that small solutes could drive folding [138]. Second, lattice model simulations that restrict the configurational space available to the protein chain (*i.e.*, constrain the chain to be compact) predict that compactness will cause secondary structure formation [139] which is true for many proteins [140-142].

Schellman presents the most sophisticated binding analysis [127,129,130,143] with protein solvation in solvent-solute mixtures modeled as competitive binding of the solvent and solute to sites on the protein. His analysis leads to a new form of the binding polynomial in which all terms depend on solute-solvent exchange instead of on solute addition. This analysis differs from that of Timasheff in the treatment of the solvent component, which is held constant in Timasheff's analysis. Schellman's analysis, although limited, [129] is the only binding model that faithfully portrays many features of solute-induced protein stabilization and destabilization. The most commonly employed binding model, osmotic stress analysis (OSA), attributes the osmolyte-induced changes in protein conformations to water-protein binding and interprets the data in terms of differences in the number of water molecules bound to each state [126,144]. OSA analysis quantifies the change in the number of water molecules bound to each state by assuming that the solute makes no contribution to the free energy change and that all water molecules bind the protein with the same binding constant. However, Timasheff [145], on the other hand, showed that OSA violates the laws of thermodynamics. Even if these theoretical objections could be overcome, interpretation of data with OSA leads to the unrealistic conclusion that the number of bound water molecules depends on which osmolyte is used.

In another development, Saunders *et al.*, [146] was able to dissect the contributions arising out of steric exclusion and binding effect of polyol osmolytes on cytochrome-c using scale particle theory. They demonstrated that that while steric repulsions (hard interaction) are a major driving force for the stabilization of the native and an intermediate state (A state), binding interactions (soft interaction) between the protein and water, and between protein and osmolyte can enhance or attenuate this stabilization. Interestingly, binding interaction leads to stabilization of the A state but destabilizes the native cytochrome-c. They argued that the native-state destabilization from soft interactions could result from more favorable osmolyte-protein interactions or less favorable water-protein interactions [146]. Similarly, the A-state stabilization from soft interactions could result from less favorable osmolyte-protein interactions or more favorable water-protein interactions. Therefore, the actual mechanism must be a combination of the two classes, and models based on this combination lead to valuable insight. In a similar observation, Weatherly and Pielak [147] also concluded that steric conclusion alone cannot explain the effects of osmolytes on the thermodynamic equilibria. Thus, in a modified revised model of osmolyte-induced protein stabilization is described as a balance of hard interaction (steric exclusion) and soft interaction (preferential binding). Weatherly and Pielak [147] further show that simple models are not sufficient to understand osmolyte-protein interactions because osmolytes interact differently with proteins. For instance, betaine is most excluded from bovine serum albumin, whereas TMAO is most excluded from cytochrome-c [147,148].

Kirkwood-Buff Theory in Understanding Osmolyte-Protein Interaction

It is assumed that protein stability and solubility in the presence of osmolytes depend on protein solvation by water or osmolytes (osmolation). Traditionally, only the relative

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preference can be measured, and that the individual solvation contributions of water and osmolyte are inaccessible. Recently, using Kirkwood-Buff theory, Rosgen and co-workers [149,150] determined hydration and osmolyte solvation (osmolation) of peptide backbone for all the classes of osmolytes. They demonstrated that the major solvation effects on protein side-chains originate from the osmolytes, and that the hydration mostly depends on the size of the side-chain. The peptide backbone unit displays a much more variable hydration in the different osmolyte solutions. Indeed, osmolytes can be grouped according to their peptide unit solvation behavior. Although methylamines, TMAO, sarcosine and betaine show very little change in the hydration of the peptide unit upon transfer from water to 1 M osmolyte, the osmolation is large and negative. This indicates that the methylamines are strongly excluded from the peptide unit. The amino acid proline and the polyols, glycerol, and sorbitol are also strongly excluded from the peptide unit, but in the presence of these osmolytes a large amount of water is also excluded from the peptide unit. The saccharides, sucrose, and trehalose, exert their effects differently than do the other protecting osmolytes. These osmolytes favorably interact with the peptide unit. Concomitantly, the peptide unit becomes excessively hydrated. The net effect of trehalose is a large hydration of the peptide unit with a net-zero solvation by trehalose. Sucrose is even more enriched around peptide groups. Only the denaturing osmolyte, urea obeys the classical solvent exchange mechanism in which the preferential interaction with the peptide unit excludes water [151].

Molecular Dynamic Simulation (MDS) Studies of Protein-Osmolyte Interaction

From a thermodynamic standpoint, the stabilization of proteins (higher ΔG_D° for unfolding) is directly related to the osmolyte's preferential interaction with the protein [49,152-154]. Simply stated, osmolytes stabilize proteins in their native state if they are more strongly excluded from the unfolded state than from the native state of a protein. Therefore, the osmolytes' effect depends on the extent to which bulk water serves as a better solvent to osmolytes than water in the vicinity of the protein. To further evaluate the molecular details of osmolyte effect on water structure and the consequent effect of the structure of water molecules on resisting thermal unfolding, various simulation studies have been carried out. At present MDS studies of only two stabilizing osmolyte classes (methylamine and polyols) are available in the literature. The simulations show enhancement of water structure by TMAO in the form of a slight increase in the number of hydrogen bonds per water molecule, stronger water hydrogen bonds, and long-range spatial ordering of the solvent. These findings suggest that TMAO stabilizes proteins via enhancement of water structure, such that interactions with the amide unit are discouraged [95]. In agreement, several other investigators also demonstrated that TMAO prevents protein unfolding by ordering and strengthening water structure while also preferentially excluded from the protein's surface [69,155-157]. Therefore, this structure-making action of TMAO enhances the penalty associated with protein unfolding and exposure to solution (an increased hydrophobic effect) while at the same time diminishing the ability of water molecules to compete with protein intramolecular hydrogen (H) bonds. Contrary to TMAO, other MDS studies [158] of concentrated binary mixtures of different polyols (glycerol, xylitol, adonitol, sorbitol, myo-inositol and scylloinositol) in water indicate that water ordering decreases in the presence of high concentrations of polyols, in agreement with other experimental results [159,160] and simulations [161,162]. The extent of water disordering and H bond loss in the presence of polyol solute generally grows with proportion to the number of polyol hydroxyl groups but also depends sensitively on the number of osmolyte internal H bonds, an isomer specific property. Interestingly, the formation of more distorted H bonds between water and osmolyte correlate well with a shift toward more linear water-water H bonds in the polyol's vicinity. This suggests that water binding to osmolyte is less compatible with the water H bond network and that in the presence of osmolytes; the remaining water-water H bonds are optimized while, in concert, the overall network's tetrahedral structure is diminished. In a similar study many carbohydrates and polyols are shown to interfere with water structure [160]. Taken together, results on TMAO and polyol osmolytes indicate that effects on water structure are completely opposite. Therefore, water structure perturbation by osmolytes cannot be regarded as the generic cause of the origin of the native protein structural stabilization or preferential exclusiveness of the osmolytes from the protein surface.

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Interestingly, recent experiments convincingly show little correlation between osmolytes' impact on water structuring in the bulk and their action as protein stabilizers [159]. In another observation, it has also been demonstrated that osmolytes do not always need to be excluded from the surface of proteins in order to stand as structural stabilizer [151]. For example, trehalose exceptionally stabilizes proteins by binding to the native protein [151].

OSMOLYTE-MEDIATED PROTEIN FOLDING

Effect of Osmolytes on Protein Folding Intermediates/Folding Pathway

During the course of protein folding, in addition to correct folding which leads to the production of functional protein, incorrect folding may also occur, leading to the formation of misfolded protein or aggregation. In fact, misfolding or aggregation occurs frequently [163-166] inside cells. Polyols such as sucrose, glucose, and sorbitol, are commonly used as protein refolding assistants [167] to suppress aggregation. There are no significant mechanistic data on how these osmolyte chaperones refold or relieve aggregated/misfolded proteins. One mechanism of protein folding is that it proceeds via hydrophobic collapse wherein a hydrophobic core is first formed followed by secondary and tertiary structural rearrangements to yield the native protein [168]. The hydrophobic core so formed is prone to aggregation because it is largely exposed to the solvent. Polyols, such as sucrose, glucose, glycerol and trehalose are believed to bind to the hydrophobic core, causing a decrease in its hydrophobic character [167]. At the same time, the presence of polyols also increases the viscosity of the refolding buffer, and thus reducing the probability of collision among the hydrophobic core [110,169]. Recent studies of the products of the submillisecond folding reactions of several proteins, including barstar [170], ribonuclease A [171,172], lysozyme [173], cytochrome c [174] and apomyoglobin [175], suggest that these proteins fold *via* accumulation of early intermediates which are structurally highly heterogeneous. In the presence of TMAO or sarcosine, these became more homogeneous and structured [176]. It has also been proved that different osmolytes induce different structures in this early intermediate [176], which indicates that inside the cell one protein may undergo different folding pathways depending on the types of osmolyte present.

Osmolyte-induced stabilization has also been observed due to increased folding rates [177,178], presumably by facilitating condensation of polypeptides into the semi-compact transition state: the rate-limiting step in protein folding. However, some authors argued that osmolytes have an opposing effect on the protein folding rate, since they are viscogenic and retard diffusion of polypeptides through the solvent [177-183]. Therefore, the ability of osmolyte is most likely a direct consequence of its stability promoting property. Studies on a cold-shock protein, calpastatin B, show that there is a distinct retardation of the folding rate at increased viscosity by osmolytes or other viscogenic agents, indicating that displacement of solvent is an important determinant of the kinetics of this rapidly folding protein [177-183]. A contrasting example is provided by chymotrypsin inhibitor 2 (CI2), which is largely unaffected by solvent viscosity. CI2 shows a steady increase in folding rate upon addition of sucrose [184]. The lack of diffusive character indicates that CI2 crosses the diffusive barrier without significant displacement of osmolyte molecules [184].

In another observation, Chang *et al*. [185] have shown that osmolyte (sugar) forces protein to fold by collapsing the unfolded protein chain into a compact structure, and thereby enhanceing misfolding and aggregation. Because such collapsed structure may be too compact and rigid, rendering the misfolded structure being unable to reorganize into the native state. Thus, osmolyte may be useful when the unfolded or folding intermediates are too soluble and cannot be readily converted into a more compact structure. Interestingly, it has been shown that α-synuclein is highly soluble even in the unfolded state, but attains a folded structure in the presence of TMAO [186]. Addition of sugars to acid-denatured cytochrome-c also gives species that are nearly identical to the molten globule state [115], a common intermediate in protein folding [187]. Moreover, the stabilizing effect of sucrose on the molten globule state increases with both the size and concentration of sugars. This intermediate structure is found to have structural properties similar to that of molten globule states of cytochrome-c formed by addition of salts [115].

Although some efforts have been directed towards

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understanding the effects of osmolytes on protein folding, little is known about their effect on folding intermediates and partially unfolded states of proteins. It is known that osmolytes have a profound influence on protein folding either by binding, acting on the folding intermediates, or by enhancing the folding rate. Interestingly, it has been reported recently that glutamate has more effect on the molten globule states than the more extended denatured state in glutaminyl-tRNA synthetase [188]. Therefore, systematic investigation on the effect of osmolytes on the partially denatured or intermediate states formed during the protein folding may change our current notion on how osmolytes induce protein folding. The effect of osmolyte-induced protein folding inside the cell and *in vitro* may not be completely *via* its effect either on the native or denatured states. Rather the folding intermediate states may play a crucial role in interaction with the osmolytes. Partially unfolded states are important not only in understanding the folding processes but may also in many diseases that involve extracellular protein aggregation and amyloid fibril formation, such as Alzheimer's disease and Scrapie [189,190]. Similar intracellular protein aggregates (Lewy bodies) are also known to play an important role in other neurodegenerative diseases, such as Parkinson's disease [191]. Very little is known about the role of osmolytes on such processes, although it is generally recognized that cells in the brain accumulate high concentrations of several osmolytes [192,193]. Therefore, investigating the effect of osmolytes on the protein folding intermediates may yield valuable insights on how osmolytes regulate these cellular processes. This may eventually help to develop osmolytes as therapeutic tools for disease associated with protein misfolding/aggregation. In fact, in a mouse model, trehalose has been shown to be effective in attenuating protein aggregation and subsequent life span for Huntington's disease.

APPLICATIONS IN HEALTH AND BIO-TECHNOLOGY

Possibility of Osmolytes for Treatment of Birth Defects

The complex and interrelated reactions, which make up living processes, are dependent upon the presence of proteins, not only as catalysts, but also in their role as structural molecules, as storage and carrier molecules, and as molecular motors. All of these functions require that nascent polypeptide chains correctly fold into the biologically active, three-dimensional structure of the native state. It is well known fact that cellular environment is crowded [128], and molecular crowding promotes protein aggregation, and thus enhances the need for chaperone action [128]. Besides, in many inborn errors of metabolism, mutant gene products are structurally altered and may not fold correctly while the function of the proteins is only partially impaired. This may signal the quality control system to retain and degrade the mutant proteins, resulting in its deficient functional activity. Evidence is accumulating that many human diseases are caused by improper folding of nascent polypeptides as they achieve a final three-dimensional structure [194-198]. This is so, in part, because such proteins partially/fully lose their activities and/or result into trafficking defects [199]. This group includes Alzheimer's disease, transmissible spongiform encephalities, serpin deficient disorders, haemolytic anaemia, Huntington's diseases, cystic fibrosis, diabetes type II, amylotropic lateral sclerosis, Parkinson's disease, dialysis related amyloidosis and more than 15 other less known diseases [197]. Recently, it has been shown that specific chemical chaperones are able to correct such misfolded conformations to prevent the excessive degradation various mutant proteins and consequently promoting the intracellular functional activity of the mutant proteins. For instance, osmolytes have been shown to reverse the intracellular retention of many of misfolded proteins such as CFTR [200,201], α-chymotrypsin [202], aquaporin-2 [201], p-53 and β-glycoprotein [203] in the endoplasmic reticulum. Recently, several osmolytes (glycerol, betaine, taurine and TMAO among others) have also been shown in cell culture models to correct protein folding and trafficking defects in ΔF508 CFTR in cystic fibrosis [204], the prion protein PrP [105], and temperature sensitive mutants of the tumor suppressor protein p53, the viral oncogene protein pp60 and the ubiquitin activating enzyme E1 [205]. Glycerol, TMAO and DMSO have been used to reverse the ΔF508 mutated cells of cystic fibrosis [206,207]. Osmolytes can also inhibit the conversion of cellular prion protein (PrP^{c}) to the protease-resistant and amyloid-forming PrP^{sc} associated with transmissible encephalopathies [208]. Powell and Zeitlin [206] also showed that TMAO and glycerol control fibril assembly

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of amyloid-β (Aβ) which is a defining characteristic of Alzheimer's disease [206]. TMAO also inhibited formation of fibrillar structures from β-amyloid peptides [106]. Recently, certain osmolytes, especially trehalose, were found to be effective in slowing the aggregation of several aggregation prone proteins including Aβ (1-40) [209], insulin [210], W7FW14F [211] and Huntingdon protein [212]. More importantly, trehalose increases human neuroblastoma cell (SH-SY5Y) viability in the presence of Aβ aggregates [209], and alleviates the extended polyglutamine chain induced symptoms in a mouse model of Huntington's disease [212]. Another osmolyte, trimethylamine-N-oxide (TMAO) protectes MC65 cells under conditional expression of amyloid protein precursor carboxyterminal fragments [213]. In an exciting experiment, dietary trehalose enhanced survival of mice with a disease similar to that of human Huntington's chorea. The brains of these animals had fewer of the protein clumps that characterize this neurodegenerative disease [212,214]. The addition of glycerol to prokaryotic cells expressing mutant forms of (phenylalanine hydroxylase) PAH induced the synthesis of the recombinant proteins at higher levels and led to an increase in its catalytic activity [215]. This effect was also observed after addition of TMAO (5 mM) and taurin (10 mM) to the culture medium. Gain of function of destabilized variants of human proteins by chemical chaperones is quite evident in the case of the cytosolic cystathionine synthase (CBS) deficiency of which develops into classic homocystinuria. The expression of mutant forms of CBS in a yeast expression model, in the presence of a range of chemical chaperones (TMAO, glycerol, sorbitol, praline and DMSO) induced an increase in activity and in the tetrameric formation of the protein [216]. However, the rescue of enzyme activity was only detected when the mutant variants were expressed in the presence of the studied compounds, and not when these were added to the purified mutant protein. Interestingly, a synergistic effect resulting from the combination of these compounds was observed. This led to the suggestion that in this case, the osmolytes exert their effects by favoring productive folding pathways, possibly by minimizing destabilized intermediate conformations, as observed for barstar [176]. Several chemical chaperones have also been reported to promote protein refolding into a native state and suppress aggregation that accompanies protein refolding for a large number of disease causing mutant proteins [22,105,204,205,217,218]. A list of disease causing mutant proteins and their rescuability by specific chemical chaperones is described by Leandro and Gomes [167]. Therefore, a well-designed specific chemical chaperone should lead to a powerful drug specifically for treating an inherited disorder. Understanding of how these chemical chaperones interact with proteins and enzymes will provide the fundamental knowledge on the molecular basis of specific chemical chaperone action, and designing and translating them into clinical applications. Researches how to use osmolytes as pharmacological compounds to specifically stabilize a mutant enzyme are also underway.

Biotechnological Applications of Osmolytes

Osmolytes are becoming increasingly useful in molecular biology and biotechnology, such as stabilization of laboratory and pharmaceutical reagents [219]. Stabilizing osmolytes improve reconstitution of functioning prokaryotic and eukaryotic protein-membrane complexes [220] and improve crystallization of proteins [221]. Betaine improves cell-free transcription/translation and also polymerase chain reaction by improving either the efficiency of DNA-polymerase or the affinity between primer and template DNA [222,223]. Interestingly, it also maximizes monoclonal antibody production by hybridoma cells [224]. Hyperglycemia in diabetes mellitus leads to build up of the osmolyte sorbitol, with implications for treatment [225]. Betaine being present in pathogenic bacteria helps them to grow well in the presence of salts and urea, so betaine perhaps play a role in urinary tract infections and in drug design for treatment [226]. Protein misfolding *in vitro* may lead to a decrease in the production yield of the native functional protein. Therefore, with the development of recombinant DNA techniques, strategies for improving the rate of correct protein refolding relative to incorrect folding have become a major focus of biotechnology [227-229]. One of the successful strategies in improving protein yield is to incorporate some osmolyte in the bacterial growth media during protein expression to suppress inclusion body formation [230]. Solution containing osmolyte combo is also currently used to resolubilize and refold the inclusion body [231].

In agriculture, transfer of genes for osmolyte production

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from salt-tolerant into salt-intolerant species is being used to adapt plants for saline and drought conditions [232]. Crop plants are also being engineered to accumulate a variety of so-called compatible solutes against various stress conditions so as to increase the crop yields [233-235]; several reviews have recently discussed osmoprotection in plants and its potential application in improving drought and salt stress tolerance [236-238].

Some osmolytes have unique non-osmotic roles other than counteraction. Taurine, for example, high in mammalian heart and brain and many marine invertebrates may be an antioxidant (among other properties) [239]. Betaine is a ligand for many proteins [240] and is involved in many biological methylation reactions [241]. Proline accumulation in water-stressed plants may be primarily for maintaining redox states than for compatibility or stabilizing aspects [234]. Cyclitols that aid plants in water retention may also scavenge free radicals generated during drought, cold and other stresses [242]. But in many cases the selective rationales for certain osmolyte patterns and types in certain organisms are not known. For example, for reasons not clear, contents of osmolyte-type differ and change dramatically in different ways among mammalian tissues during development [243]. Therefore, further studies on unique properties of osmolytes need to be conducted.

UPCOMING AVENUES

Osmolytes Modulate Molecular Chaperones

In a systematic study of refolding of heat-denatured malate dehydrogenase at 47 °C in the presence of each of the molecular chaperones (GroEL, DnaK and ClpB) was measured after supplementation of osmolytes, betaine, trehalose, proline, or glycerol [244]. Surprisingly, all osmolytes were found to have a regulatory effect on the folding of thermally denatured malate dehydrogenase by individual and combinations of chaperones GroEL, DnaK and ClpB. With the exception of trehalose, low physiological concentrations of proline, glycerol, and especially glycine betaine activated the molecular chaperones, likely by assisting local folding in chaperone-bound polypeptides and stabilizing the native end product of the reaction. High osmolyte concentrations, especially trehalose, strongly inhibited DnaK-dependent chaperone networks, such as DnaK+GroEL and DnaK+ClpB, probably because high viscosity affects dynamic interactions between chaperones and folding substrates and stabilizes protein aggregates. Thus, during combined salt and heat stresses, cells can specifically control protein stability and chaperone-mediated disaggregation and refolding by modulating the intracellular levels of different osmolytes. In other observations, trehalose accumulation in yeast not only suppresses protein aggregation during heat shock but also interferes with chaperone-assisted protein refolding *in vivo* and *in vitro* [104]. Osmolytes promote the *in vitro* refolding by GroEL of a mutant enzyme, which cannot be refolded either with the chaperone or osmolytes alone [245], Taken together, all observations indicate that in addition to their activity as chemical chaperones that directly controls protein stability, osmolytes may also indirectly regulate protein homeostasis in cells by controlling the activity of molecular chaperones.

Modulation of Protein Stability by Osmolyte Mixtures

Mixtures of organic osmolytes occur in cells of many organisms, raising the question of whether their actions on protein stability are independent or synergistic. Although there are some reports on their additive effects [12,246], several studies indicate that mixtures are more beneficial than the individual osmolytes at equimolar concentrations in stabilizing or refolding proteins. For instance, an equimolar mixture of arginine and glutamic acid has been found to show better solubilization than the sum effect of each component individually [108]. Furthermore, mixtures of TMAO and trehalose can be more efficient in refolding proteins than expected from simple additive effect [247]. Simultaneous addition of the charged L-amino acids, Arg and Glu (Arg+Glu) to the dilute solution of proteins (Ref2NM, MAGOH, and WW34) significantly reduces aggregation during the process of concentration by increasing the solubility limit while the individual equimolar osmolytes do not have significant effect on the aggregation [108]. In another observation, TMAO and sucrose enhanced thioflavin T detected aggregation of 1SS-α-lac amyloid fibril formation, but a mixture of trehalose and TMAO substantially inhibited the aggregation. Furthermore, certain osmolyte mixtures are more beneficial than the individual osmolytes in rescuing

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misfolded phenotype of mutant protein, cystathionine beta synthase [216]. Taken together, the mixture of differing osmolytes may be helpful in different types of applications in biological systems. Further progresses in understanding the synergism of osmolyte mixtures in many biological processes will yield insights on the application of osmolytes and in the development of suitable therapeutics in which one osmolyte does not work but an osmolyte combo serves as a magic solution. Also, a mixture of osmolytes may significantly reduce the concentrations of each osmolyte used, making cells/tissues to easily adapt to these conditions.

SUMMARY AND PERSPECTIVES

It is understood from this review that during the 40 years of dynamic research in the area of protein-osmolyte science, we have made various landmark discoveries which helped us to answer the question: How do living organisms survive under the denaturing stress, be it high or low temperature stress, salt stress, water stress, urea stress or pressure stress. Intracellular osmolytes are accumulated to give generic stabilization of proteins. The unfavorable interaction of the osmolytes with the peptide backbone (osmophobic effect) is the cause of generic stabilization. However, the effect of many osmolytes may be protein specific. Interestingly, many osmolytes are potential denaturants at specific solution conditions. The refolding experiments carried out on many different wild type and mutant proteins in the presence of various osmolytes indicate that osmolytes ability to induce protein refolding is not true in general but in case by case basis. On thermodynamic grounds, protein folding in the presence of osmolytes is either under enthalpic or entropic control depending on the type of the protein chosen. Furthermore, an osmolyte preferentially binds to some proteins while it is preferentially excluded from the surface of another protein to yield stability. In the light of these observations, it is highly essential to understand the origin of specific interactions of an osmolyte with proteins of different chemical and physical properties keeping in mind that unfavorable peptide backbone-osmolyte interaction is the general cause of generic protein stabilization. It appears to be very unlikely that osmolytes' major preference is the peptide backbone. Rather the solution condition wherein the osmolyte and proteins are dissolved or the nature of the exposed side chains and peptide backbone of a protein will dictate whether a given osmolyte will be exclude or bind to the protein surface. Interestingly, protein folding intermediate states of some proteins have profound interaction with osmolytes than the denatured states. Most importantly, therefore, caution must be taken in interpreting protein folding data using the osmophobic model as effect of osmolyte.

Recent progresses in molecular dynamic simulations and computational studies have greatly magnified our molecular level understanding on protein-osmolyte interaction and the mechanism of osmolyte-induced protein folding. Furthermore, the upcoming avenues including osmolytes interaction with the molecular chaperones and multiple osmolyte system are areas of great intellectual curiosity as these systems are meant to circumvent multiple stresses. The changing need of human health due to various stresses including, global change in environment, in addition psychological and hormonal imbalances are of great concern. Incorporation of some dose of stress specific osmolyte in many pharmaceutical formulations might be of great utility to increasing stress tolerance of the cells or individuals while improving the efficacy of the drug. Osmolytes promising implications on biotechnology, health and industry makes the osmolyte-protein science a potential research area for many researchers working in different avenues. Thus researchers working in translational and clinical aspects must aptly utilize this wealth of information on mechanistic and potential applications to gear up for use as therapeutic for many human diseases.

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