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Review article

Protein Folding in the Presence of Osmolytes - a Complex Interplay of Multiple Forces

Vandhana Srinivasan, Ajirni Rajendran and Sheeza Khan*

School of Life Sciences, B. S. Abdur Rahman Crescent Institute of Science and Technology, Vandalur, Chennai, Tamil Nadu, India

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Abstract

Living organisms employ various approaches to evade stressful environmental conditions such as high and low temperatures, salinity, and drought. The most adapted strategy to circumvent such stress conditions is the use of osmolytes, which are low molecular weight organic compounds. A large amount of evidence clearly demonstrates the role of osmolytes in conferring stability to proteins. Much is now known about the interaction mechanisms that exists between osmolytes and proteins. Osmolytes exert their effect on protein stability by acting on the thermodynamic equilibrium, 'native conformation ↔ denatured conformation' in the reverse direction. There are various forces that osmolytes interact with proteins to make such an effect on this equilibrium. The preferential hydration phenomenon is most accepted for the explanation of protein folding in the presence of osmolytes. The unfavorable interaction between the peptide backbone and osmolyte molecules has been understood to be the driving force for the preferential hydration effect. Contrary to this, the stabilization of proteins induced by polyols is solvophobic in nature. Numerous other models have been devised to explain the interactions between proteins and osmolytes at the atomic level. In this review, we systematically reviewed all major forces involved in osmolyte-protein interactions.

Keywords: osmophobic effects; solvophobic effect; protein stability; osmolytes

1. Introduction

One of the hallmark features of proteins is their ability to self-assemble into their functionally stable three-dimensional conformations. The self-assembling attribute of proteins helps to generate the vast variety and selectivity in all the biological processes carried out by them. But all these processes require the compact functional conformation of the protein to be stable. It is a well-known fact that the sequence of amino acids (Pierotti, 1965) coupled with the property of the solution in which the protein is placed play a huge role in determining the stability of the protein (Dill & Stigter, 1995; Yancey, 2004). Usually, the solution components comprise of different ions, small organic compounds, salts, and

*Corresponding author: E-mail: sheezakhan.sls@crescent.education
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chaperones (both chemical and molecular). It is now known that modifying the properties of a protein solution—such as by adding osmolytes, adjusting pH, or varying ion or salt concentration—can often correct the folding of proteins prone to misfolding (Yang et al., 1999; Tanaka et al., 2005; Leandro & Gomes, 2008). Many human genetic diseases occur as a result of the incorrect folding of proteins either to their non-functional forms or conformationally less stable forms, for example, Phenylketonuria (PKU), Parkison's disease, Alzheimer's disease, and familial amyloid polyneuropathy (Herczenik & Gebbink, 2008; Leandro & Gomes, 2008). Missense mutations are also believed to be one of the causes of these diseases. Considerable effort and time has been devoted to understand the mechanisms and causes of protein aggregation (Dong et al., 1995; Chi et al., 2003; Yan et al., 2004), to develop strategies to protect proteins from undergoing aggregation and to remodel protein aggregation pathways (Meng et al. 2001; Chi et al., 2003; Borwankar et al. 2011), as well as if possible to refold conformationally correct proteins from their aggregate forms (Meersman & Heremans, 2003). Additionally, compounds that can alter the properties of a protein solution can drive the protein folding into a conformationally correct and functional pathway. A variety of low molecular weight compounds have been identified to be accumulated in the diverse life forms existing in the extremes of environmental conditions throughout the world (Yancey et al., 1982; Yancey, 2003; Yancey et al., 2004). The fluctuations in the environmental conditions disturb the osmotic balance inside the cell which severely impairs protein structure, stability, and function (Diamant et al., 2001). Under these conditions it has been reported that organisms accumulate osmolytes (low molecular weight compounds) and regulate cell osmolarity, and thus protecting protein stability and function (Yancey, 2005; Bolen & Rose, 2008; Hoffmann et al., 2009).

For each different extreme environmental condition, a different osmolyte is accumulated by the living cells. For example, disaccharide osmolytes serve as a rescue mechanism under freezing temperatures, while methylamines are specifically accumulated to mitigate high urea stress conditions, among other examples (Crowe et al., 1992; Storey, 1997; Yancey, 2004). Apart from these, there are certain osmolytes which may perturb protein stability or may sometimes refold the misfolded proteins into their correct conformation (Chang et al., 1996; Jacob et al., 1997; Uversky et al., 2001; Leandro & Gomes, 2008). Therefore, it is crucial to delineate the effects of each individual osmolyte on different proteins, determining whether their impact is universal or protein-specific. This approach may prove to be a promising and effective strategy for correcting or treating various proteopathies.

1.1 Classification and distribution of osmolytes

The characteristic properties that differentiate osmolytes from other organic compounds are twofold: (1) they never bind to proteins and therefore universally stabilize them (Timasheff, 2002). Since osmolytes solely alter the structure of water, they are compatible with enzyme function, preserving enzymatic activity (Myers & Jakoby, 1975; Wang & Bolen, 1996; Yancey et al., 1982), and stabilizing proteins against denaturing stress conditions (Santoro & Bolen, 1992; Taneja, & Ahmad, 1994; Xie & Timasheff, 1997a; Anjum et al., 2000). (2) Osmolytes are neutral molecules under nearly all conditions (Harries & Rösgen, 2008).

Various schemes have been adopted recently to put osmolytes in various categories so as to understand their mode of action in protein stabilization (Yancey et al. 1982; Yancey, 2001). Based on their chemical structure, osmolytes have been put into

three categories: (1) polyols and sugars, (2) amino acids and their derivatives, and (3) methylamine compounds. Another method of categorization is based on their effects on protein function: those that have no effect are termed compatible osmolytes, while those that do affect protein function belong to the counteracting osmolytes category (Borowitzka & Brown, 1974; Pollard & Wyn Jones, 1979; Bowlus & Somero, 1979; Yancey et al., 1982; Wang et al., 1995; Wang & Bolen, 1996; Haque et al., 2005a,b). A third way of categorizing osmolytes is based on their ΔG_D^o (Gibbs energy of stabilization of the protein at 25°C) and the enzyme kinetic parameters (K_m and k_{cat}) of protein taken together: (1) Class I including polyhydric alcohols and amino acids and their derivatives that have no significant effects on both ΔG_D^o and k_{cat}; (2) Class II includes the methylamines, which increase both ΔG_D^o and k_{cat}, and decreases K_m; and (3) sugars that increase ΔG_D^o, but decrease both K_m and k_{cat} that belong to class III (Jamal et al., 2009) (see Figure 1).

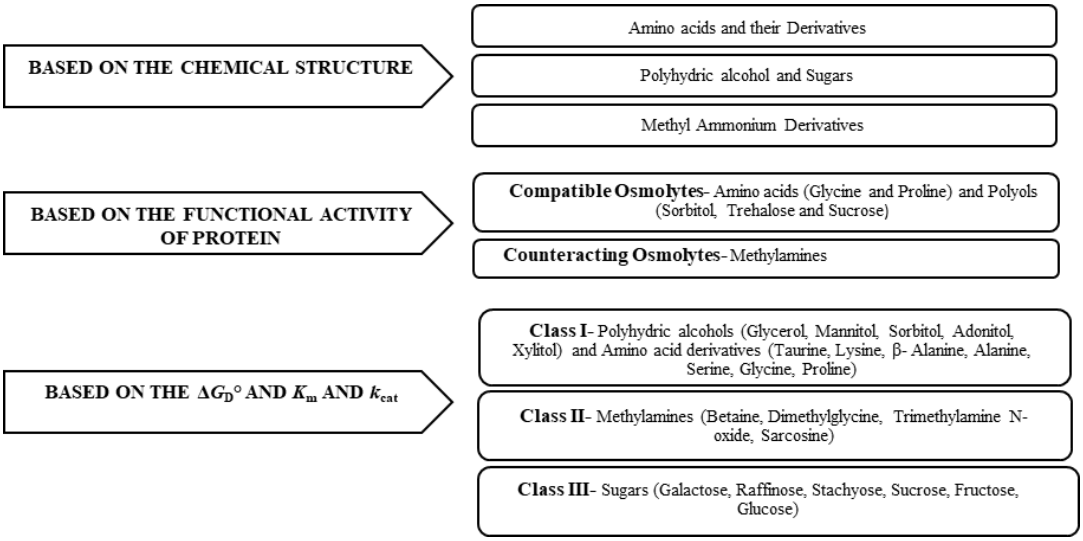

Figure 1. Classification scheme for osmolytes

Osmolytes are utilized by all the living organisms: prokaryotes, plant and animal kingdom. For example, betaine is found in all forms of life. Taurine, on the other hand, is utilized as an osmolyte in both marine invertebrates and mammals (Yancey et al., 1982). Sorbitol is found in certain marine algae, fishes, and in the mammalian kidney (Yancey et al., 2004; Yancey, 2005). Apart from using individual osmolytes, many organisms accumulate a mixture of osmolytes; e.g., the mammalian kidney contains urea along with the polyols (myo-inositol and sorbitol), the methylamines (GPC, glycine betaine) and taurine (Bagnasco et al., 1986; Garcia-Perez & Burg, 1990).

1.2 Consequences of protein-osmolyte interaction

1.2.1 Native protein-osmolyte interactions

As discussed in detail by Timasheff (1992, 1998, 2002a,b), osmolytes do not have a direct interaction with protein: therefore, they are expected not to cause significant alteration on the native state of the protein. This characteristic of osmolytes of not altering the native

conformation of proteins has been well demonstrated by the spectroscopic measurements (Mojtabavi et al., 2019; Kushwah et al., 2020; McParland et al., 2021; Song et al., 2021; Khan et al., 2023). Earlier studies showed that the spectral properties of proteins remained unchanged when studied using both circular dichroism (CD) spectroscopy and UV-visible spectroscopy (Haque et al., 2005a,b; Singh et al., 2005; Haque et al., 2006; Hagedorn et al., 2010). This indicates that osmolytes do not interact with the proteins directly. Similar results were obtained when the dimensions of a native protein were measured in the presence of osmolytes using size exclusion chromatography (Qu et al., 1998; Baskakov & Bolen, 1998). Xie and Timasheff (1997a,b,c) also came to the same conclusion based on their investigations on RNase-A preferential interaction measurements. Most convincing of all were the studies of the co-crystallization of RNase-S fragments with TMAO, sarcosine, betaine, and taurine which showed that the osmolytes did not perturb the folded structure of the protein (Ratnaparkhi & Varadarajan, 2001).

1.2.2 Osmolyte compatibility with protein function and stability

Polyols (such as sorbitol and myo-inositol), amino acids and their derivatives do not alter the protein function and thus can be amassed in cells over a wide range of concentrations. This concept of osmolyte compatibility was introduced for the first time for the single celled algae, *Dunalliela*, that inhabits the Dead Sea where it subjected to high osmotic pressure to which it responds by accumulating glycerol. Osmolyte compatibility was further demonstrated by studies on isolated mammalian renal enzymes (arginosuccinate and uricase). No change in the enzyme kinetic properties were seen in the presence of polyols, in contrast to the highly perturbing effect of urea and NaCl (Brown & Simpson, 1972; Yancey et al., 1982; Clark, 1985). The compatibility of osmolytes with protein structure and function is due to the absence of any direct interactions between the proteins and the osmolytes. Osmolytes only restructure the solution around the proteins. Polyols are the most prevalent molecules among the compatible osmolytes, and they circumvent stress like high osmotic pressure and freezing (Yancey et al., 1982; Carpenter et al., 1997). It was experimentally shown that protein stability (ΔG_D^o) and the enzymatic kinetic parameters (K_m and k_{cat}) of proteins remain unaltered in the presence of polyols, thereby making these compounds ideally compatible with enzyme function and protein stability (Haque et al., 2005a; Jamal et al., 2009). The explanation for this compatible behavior in terms of ΔG_D^o and the enzymatic kinetic parameters at physiological pH and temperature is that the osmolytes bring about an adequate enthalpy-entropy balance, or that the osmolyte to the protein preferential exclusion (stabilizing force) and preferential binding (destabilizing force) cancel out each other (Haque et al. 2005a,b; Haque et al., 2006). Hydrogen exchange (HX) rate studies of N-H protons of amides with intermediate rates in the presence of sucrose clearly demonstrate that osmolytes do not affect protein function but they stabilize proteins (Wang et al., 1995).

Numerous examples of osmolytes affecting enzyme function are documented in the literature. TMAO was found to counteract the elevation of the Michaelis constant (K_m) by urea [e.g., K_m for ADP of pyruvate kinase (Burg et al. 1996; Yancey & Somero, 1980) and creatine kinase (Yancey & Somero, 1980)], the K_m for NADH of A4-lactate dehydrogenase (Yancey & Somero, 1980) and K_m for glutamate of glutamate dehydrogenase (Yancey & Somero, 1980). TMAO counteracts urea-induced decrease in the V_{max} of creatine kinase and arginosuccinate lyase (Yancey & Somero, 1980). KCl was found to increase the K_m of muscle type lactate-dehydrogenase and TMAO was able to reverse the effect of KCl (Desmond & Siebenaller, 2006). Urea, which decreases the V_{max} of porcine arginosuccinate was counteracted by betaine which increased V_{max} (Yancey,

1992). For enzymes from a wide variety of organisms, Yancey and Somero (1980) found that urea alone generally increased K_m and decreased k_{cat}, whereas TMAO alone counteracted the effect of urea by decreasing K_m while increasing k_{cat}. Methylamines have been proposed to be activators of functional properties such as V_{max} (Yancey et al., 1990). However, Burg et al. (1999) showed that urea and methylamines (glycerophosphorylcholine, TMAO and betaine) had the similar effect of reducing both the K_m and V_{max} of aldose reductase. Myers and Jacoby (1973) tested the effects of glycerol and other polyhydric alcohols on the kinetic parameters of sixteen enzymes, and found that each of the sixteen enzymes tested underwent a change in either K_m or turnover number or both. In the skeletal muscle myosin, complete inhibition of the K^+ EDTA ATPase by urea (2.0 M) was observed; TMAO increased myosin activity, while betaine had no effect. TMAO or betaine (1.0 M) when combined with urea (2.0 M), effectively protected the ATPase activity of myosin against inhibition (Ortiz-Costa et al., 2002).

1.2.3 Osmolytes and its effect on stability-function relationship of proteins

A series of equilibria exist for protein between the most compact globular structure to fully expanded random coil and a protein can assume many configurations between these two extreme structures. Urea shifts this series of equilibria towards the random coil structure, either by increasing binding favored in the random coil form, or by altering the solvent environment of the protein. TMAO, shifts this equilibrium towards the compact structure by preferentially stabilizing the more compact forms (Mashino & Fridovich, 1987). The effects of urea and TMAO were found to differ on xanthine oxidase, alcohol dehydrogenase, lactic dehydrogenase, arginosuccinate lyase, and catalase. Urea hindered the activity of all the enzymes, and reduced the stability of catalase, according to the hypothesis that urea decreases the compactness of globular structure of proteins. TMAO, on the other hand, favored compact globular structures over more expanded structures which was demonstrated by activation of arginosuccinate lyase, alcohol dehydrogenase, and lactic dehydrogenase and by the increased thermal stability of catalase in its presence. However, inhibition of both xanthine oxidase and catalase in the presence of TMAO demonstrates that the compact globular structure may not be the most active conformer. Therefore, one can conclude that the most stable conformer of a particular enzyme may not be the most active state of the protein (Mashino & Fridovich, 1987).

Myo-inositol, sorbitol and betaine were found to inhibit Na^+ /K^+-ATPase, Ca^{2+}-ATPase, and calmodulin-stimulated Ca^{2+}-ATPase (CaM) pumps in isolated RBC membranes (Moeckel et al., 2002). However, these osmolytes in low concentrations (50 µmol/L) were found to increase the activity of these pumps. The exact mechanism of osmolyte-induced ATPase inhibition is unknown but it is speculated that osmolytes either binds to the exposed surface of the pump resulting in dose-dependent inhibition of the pump activity, or the osmolytes change the physical structure of the membrane providing less favorable conditions for the pump to operate, resulting in lowering of ATPase activity (Moeckel et al., 2002). TMAO enhances both the V_{max} and k_{cat}/K_m of trypsin, but for chymotrypsin, under similar conditions, these enzyme kinetic parameters remain unaffected (Kumar et al., 2005). Furthermore, TMAO was found to induce conformational changes in trypsin but not in chymotrypsin, indicating that osmolyte-induced conformational change may lead to change in enzyme activity (Kumar et al., 2005). TMAO decreased the catalytic activity of myoglobin but it affected neither the secondary and tertiary structure nor the stability of the protein (Bellezza et al., 2009).

For α-chymotrypsin in the presence of the osmolytes TMAO, betaine, sarcosine, proline, and sucrose, showed linear increase in enthalpy (ΔH) and Gibbs free energy

(ΔG_D^o) with osmolyte concentration, while these parameters showed a sharp decrease in the presence of denaturants such as urea and GdmCl. Apart from these, the activity of α-chymotrypsin was not significantly enhanced in the presence of protecting osmolytes, though GdmCl and urea inhibited the activity of α-chymotrypsin (Attri et al., 2010). From this, one can conclude that though a good correlation is found to exist between enzyme stability and function, the relation shows variation with osmolyte type.

1.2.4 Three-dimensional model for protein-osmolyte interactions

Numerous studies on protein structure have reported on the intrinsically disordered domains present in one-third of the known proteins. Such domains are kwon as IDRs (intrinsically disordered domains) (Darling & Uversky, 2018). These domains have certain unique properties, for example, these domains are polar in nature, and have long stretches of glutamic acid residues, hence, they will not form alpha-helices at physiological pH. They also have few amin acid residues with hydrophobic aromatic-base side chains. Moreover, they are rich in proline. All these unique features lead to the formation of localized disordered structures. This disordered structure confers proteins with greater conformational flexibility. The proteins containing extensive IDRs are called intrinsically disordered proteins, or IDPs (Berlow et al., 2018). Furthermore, these properties make IDPs the hub of all biological pathways (Haynes et al., 2006; Das et al., 2012; Wright & Dyson, 2015; Uversky, 2016; Nussinov et al., 2017; Staby et al., 2017). In cells, newly translated proteins undergo three-dimensional folding in the endoplasmic reticulum in the presence of proteins known as molecular chaperones, such as hsp70 and hsp90 (Radli & Rüdiger, 2018). Additionally, osmolytes also help the proteins to fold into the correct three-dimensional conformation. Studies showed that trimethylamine N-oxide caused a loss of function of IDPs by inducing changes in the conformation (Bhat et al., 2017). Trehalose demonstrated a gain in structure of a disordered alpha-synuclein protein (Naik et al., 2016). Osmolytes may be accumulated inside cells by two pathways- either by de novo synthesis or by transport into the cell through specific transport molecules and proteins. Figure 2 represents the hypothesis given by Rumjanek (2018), explaining the effect of osmolytes on the conformation of IDPs. It can be seen from Figure that in the presence of a stressful environment, osmolytes are accumulated according to the mechanisms described above. Increased intracellular levels of osmolytes influence the conformation of the IDRs and change their properties and functions. The refolded IDRs display new conformations that may lead to performing new functions in the cell (Blose et al., 2011; Holmstrom et al., 2015).

2. Factors affecting protein stability

2.1 Covalent force

2.1.2 Disulfide bonds

Disulfide bonds are the covalent bonds between two sulfur residues of two cysteines. These can be present as inter- or intra-molecular bridges in a protein molecule. This bond, which is present in both native and unfolded protein states, has high enthalpic value and hence stabilizes the protein to a great extent (Klink et al., 2000). The disulfide bond contributes to protein stability by decreasing the conformational entropy of the unfolded polypeptide chain. Approximately 2.5-3.5 kcal/mol of stabilization is conferred by this bond

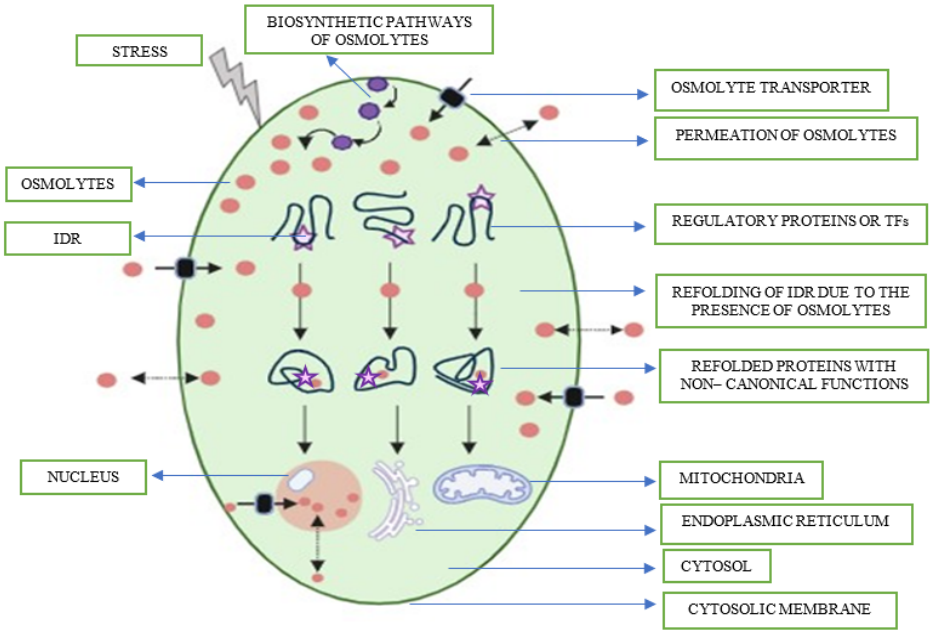

Figure 2. Model for protein osmolyte interaction

to the proteins (Braxton, 1996). Usually, it is extracellular proteins that harbor disulfide bonds as the interior of the cell has a reducing environment due to which the sulfhydryl groups remain in a reduced state (Fahey et al., 1977). In a few cases, it was observed that native S-S bridges were formed only when proteins were folded into their secondary or sometimes tertiary structure (Creighton, 1993). The sulfhydryl moieties oxidize readily in the extracellular space to form disulfides. Hence, if a cysteine residue in any protein is present in the extracellular environment that can lead to a complex protein folding pathway with no beneficial consequences.

Studies in which few novel disulfide bonds were introduced into proteins demonstrated mixed results. For example, in the case of T4 lysozyme, out of the five disulfide bonds introduced into the said protein, two reduced the protein stability, but three stabilized the protein. The net result after the introduction of five disulfide bonds to the T4 lysozyme was the reduction in stability of the protein as compared to the wild type protein (Betz, 1993). However, in the case of RNase Hn and ribonuclease barnase, Kanaya et al. (1991) and Clarke et al. (1995) found that the introduction of disulfide bond increased the stability of protein by 2.8 kcal/mol and 1.2 and 4.1 kcal/mol, respectively. Therefore, we are still far from understanding the role of disulfides in protein structure.

2.2 Non-covalent forces

2.2.1 Hydrophobic interactions

One of the major non-covalent forces stabilizing a protein is hydrophobic interaction. This leads to the burial of almost all the hydrophobic groups present in a protein to the interior core of a globular protein (Kauzmann, 1959). According to the calculations, if one mole of hydrophobic residue is removed from protein surface, it leads to an approximately 4 kcal

gain in energy (Alonso et al., 1991). Proteins have a strong tendency to bury all their hydrophobic groups in the cores since the presence of such groups only tends to increase the total energy of the system. Whenever hydrophobic groups are present on a protein surface, they are only to perform some special function. For example, various multimeric enzymes bring together their subunits by the hydrophobic interactions between their exposed hydrophobic residues.

The thermodynamic factors that are responsible for the hydrophobic force are still not completely understood. When a non-polar compound is transferred from an organic solution (reference state) to water, the free energy of transfer (ΔG_{tr}), is given by

$$\Delta G_{tr} = \Delta H_{tr} - T\Delta S_{tr}$$

Where, ΔH (enthalpy), T is the temperature (298.15), and ΔS (entropy).

When the temperature of water is low, the solubility of hydrophobic amino acids residues is lower, which causes the mutually attracted water molecules to structure around these residues. The negative unfavorable entropy causes the all the hydrophobic groups to come together. On the contrary, at high temperature the entropy of water molecules increases, and there is enthalpy driven hydrophobic force (Alonso et al., 1991).

2.2.2 Van der Waals forces

These interactions result from the London forces with distance dependence of $1/r^6$ and on extremely short-range repulsive interactions resulting from the overlap of electron orbitals with a distance dependence of $1/r^{12}$. If by any means the packing of a hydrophobic core can be optimized, then these forces can be optimized. Inside the protein, van der Waals interactions are observed between the protein side chains. The packing density of proteins, which is in the range of 0.72-0.77 g mL^{-1} (Harpaz et al., 1994), suggests that very large London-forces exist in a native protein.

A large number of mutational analysis studies (Alber, 1989; Kellis et al., 1989; Matthews, 1993; Matthews, 1995, 1996) also indicates that van der Waals interactions contributed significantly to protein stability; however, Fersht (1997) noted that it was difficult to quantify those forces due to changes in packing and the presence of other interactions at the same time. Griko et al. (1994) observed only a small contribution of van der Waals interaction (around 0.24 kcal mol^{-1}) to the conformational stability of barnase. However, the destabilizing effect of cavities formed in protein cores is mainly due to the loss of van der Waals interactions (Kellis, et al., 1989; Harpaz et al., 1994).

2.2.3 Aromatic-aromatic interactions

Weakly polar aromatic interactions can occur due to the asymmetric arrangement of electrons in the aromatic amino acid aromatic rings (Makhatadze & Privalov, 1992). Approximately, sixty percent of the aromatic amino acids in proteins participate in such interactions (Makhatadze & Privalov, 1992). The partial positive charge of ring hydrogen atoms and the partial negative charge of planar ring faces are important in such interactions. Three specific orientations with respect to inter-planar angles are responsible for these interactions. These are: (1) 0-30^o (near parallel face to face interactions), (2) 30-60^o (tilted geometry), and (3) 60-90^o (perpendicular T-shaped packing geometry). The 60-90^o orientation is often found in protein. Aromatic-aromatic interactions are formed when the distance between centers of aromatic rings is less than 7Å. Aromatic groups also have

the tendency to interact with other atoms and groups such as water, sulfur, and amino-groups (Makhatadze & Privalov, 1992).

From the investigations of surface solvent-exposed Tyr13/Tyr17 pairs indicated that the interaction energy between the two aromatic groups contributed only1.3 kcal/mol to protein stability (Hagedorn et al., 2010), which was only slightly higher than the stabilization expected from the hydrophobic contribution of burying the surface area between them. Therefore, it can be said that there was little apparent extra stabilization due to the presence of the aromatic pair.

2.2.4 Hydrogen bonding

The concept of the hydrogen bond was first introduced by Huggins around 1920 (Latimer & Rodebush, 1920). These bonds result from the interaction between an electronegative atom, covalently bound to hydrogen, and another electronegative atom. Hydrogen bonds are the bonds which have a distance of less than 3 Å between the H-donor and the H-acceptor and have donor hydrogen-acceptor angle below 90^{o}. Electrostatic interactions drive the formation of the hydrogen bond. These bonds play a very important role in holding the secondary structural elements together, binding with substrates, water molecules, etc. The strength of a hydrogen bond is 2-10 kcal/mol (Creighton, 1993). When a protein is unfolded, all the hydrogen bonding partners (potential) form bonds with water. But when the protein folds, these hydrogen bonds with water molecules are broken and the same bonding is formed with hydrogen bonding partners in protein chain. It is generally accepted that H-bonds make a positive contribution to protein stabilization (Pace et al., 1996).

From an investigation conducted on tRNA synthetase with its substrates, it was concluded that hydrogen bonds give 1kcal mol^{-1} per hydrogen bond to the energetics of substrate binding. Various other investigations were in agreement with this conclusion (Pace et al., 1996).

Studies involving the comparison of glyceraldehyde-3-phosphate dehydrogenase from four different organisms differing in their thermostability and with 50% identical in their sequences demonstrated a clear correlation between thermostability and the number of buried charged residues H-bonded to buried neutral residues (Tanner et al., 1996).

2.2.5 Electrostatic interactions

An ion-pair is formed when an interaction is established between the charged amino acids. The energy associated with this electrostatic interaction of an ion-pair is calculated using the Coulomb law:

$$E = \frac{Q_1 Q_2}{\varepsilon R_{12}}$$

In the equation, Q_1 and Q_2 refer to the two charges, ε represents the dielectric constant associated with the medium, and R_{12} refers to the distance between the two charges. From the equation, it can be understood that the dielectric constant and distance components determine the magnitude of the effect of an ion-pair on the stability of the protein. The typical distance that exists between the two charges of an ion-pair is 4 Å. But it has been suggested that ion-pairs having distances between their charges of greater than 4 Å also significantly contribute to protein stability. Since entropy decreases and

desolvation is affected when opposite charges are brought close together, investigators believe that these ion-pairs do not contribute to protein stability (Horovitz & Fersht, 1990). Various other studies carried out at high temperature concluded that the desolvation penalty decreases due to decrease in the dielectric constant of water. Hence, there is a decrease in the desolvation penalty if the dielectric difference is reduced between the protein and the solution (Horovitz & Fersht, 1990). From the studies it can be concluded that these interactions play a small role in protein stability.

3. Models to Explain Osmolyte Protein Interactions

3.1 Experimental models

3.1.1 Preferential interactions

The arrangement of solutes or solvent molecules around a protein present in solution using thermodynamic measurements can be explained by the excluded volume or preferential interaction models developed by Timasheff (1998). The molar ratios of the water and solute determine the arrangement of these two components in the solution. However, when a protein is also added into the solution, the arrangement of the water and solute molecules changes around the protein molecule and is different from the arrangement existing in the bulk solution. This happens because water and solute interact with the protein molecule differently. If the solute interacts more with the protein, the phenomenon is called “solute binding”. In this case the number of water molecules near the protein surface will be decreased, so it is said there is not “preferential hydration” of protein. But in the opposite scenario, if more water interacts with protein compared to solute, the phenomenon is called “preferential hydration” of protein molecule or “preferential exclusion” of the solute (Timasheff, 1998) (see Figure 3).

The preferential interaction parameter $(\partial m_3/\partial m_2)_{T,\,\mu 1,\,\mu 3}$ is an important thermodynamic quantity that can be measured using equilibrium dialysis (Lee et al., 1979). Here, m_2 and m_3 represent the molal concentrations of protein (component 2) and solute (component 3), respectively, and the partial derivative represents the change in concentration of solute that takes place in the vicinity of the protein when a small increment of protein is added to solution. Component 1 refers to water, and the subscripts on the partial derivative mean the experimental measurement is conducted such that temperature (T) and chemical potentials of water ($\mu 1$) and solute ($\mu 3$) inside and outside the dialysis bag are constant. From the above description, it can be stated that the preferential binding and preferential exclusion of solute near the surface of the protein can either be less or greater when compared to the solute concentration in the bulk solution. If the value of this parameter is positive, it means “solute binding”. If it is negative, then it means “preferential exclusion” of solute (Bolen, 2004). Bolen (2004) demonstrated that all osmolytes have a negative value for this parameter. The negative value of this parameter for osmolytes is due to huge unfavorable interaction between the osmolytes and the peptide backbone (Hamaguchi & Kurono, 1963; Liu & Bolen, 1995), which causes stabilization of the native state of protein compared to unfolded state (Xie & Timasheff, 1997b; Timasheff, 2002a).

3.1.2 Osmophobic effect

Bolen and Baskakov (2001) proposed the osmophobic theory. The theory is based on the transfer free energy measurements of amino acid side chains and the protein backbone

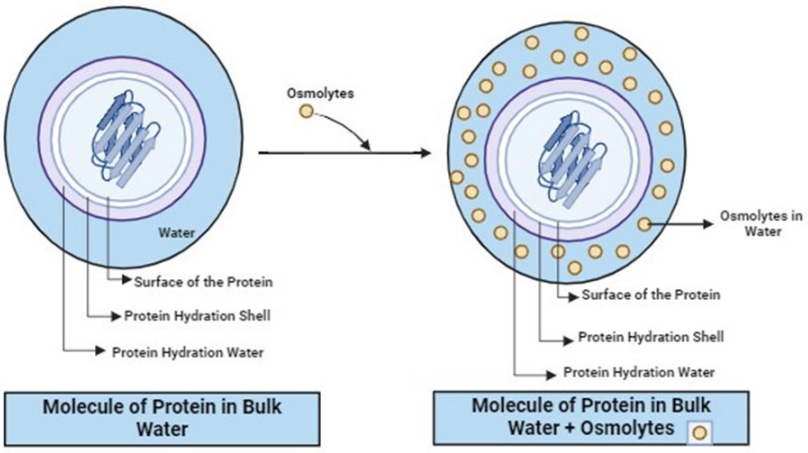

Figure 3. The arrangement of solute or solvent molecules around the protein present in solution

from water to osmolyte solution and asserts that the effect of osmolytes on the protein stability is due to a solvophobic thermodynamic force called the osmophobic effect. The osmophobic effect is a result of the unfavorable interaction of the osmolyte with the peptide backbone (Bolen & Baskakov, 2001).

The osmophobic effect is best explained on the grounds that osmolytes do not bind to the proteins (both native and unfolded forms) (Lee & Timasheff, 1981; Arakawa et al., 1990a). This implies to solvophobic interactions between the protein surface and the osmolyte leading to an increase in the Gibbs energy of the protein species (Gekko & Timasheff, 1981; Liu & Bolen, 1995; Wang & Bolen, 1997; Qu et al., 1998). In comparison to the native protein, the unfolded protein is more solvophobic for the osmolytes, demonstrating that proteins are more stable in the presence of osmolytes as compared to water alone (Liu & Bolen, 1995).

3.1.3 Surface tension

The surface tension phenomenon arises due to the different concentration ratios of small molecules near the surface of a protein and in the bulk solution. Gibbs analysis of surface phenomenon showed that (i) solutes which increase the surface tension of water are concentrated more in the bulk solution as compared to their concentration at the air/water interface and (ii) solutes that decrease the surface tension of water are concentrated more at the air/water interface than the bulk solution. Therefore, if a cavity which is of the size of a protein is introduced into the bulk solution, the solute that increases the surface tension of water will be preferentially excluded from near the surface of the cavity and the solutes that decrease the surface tension of water will be preferentially bound at the surface of cavity.

The studies showing correlation between surface tension increments and the stability of different proteins led to the concept that different solutes affect proteins by causing the formation of empty spaces in the solute containing solution for accommodating native and unfolded proteins in those empty spaces, and this depended on the surface tension of the solution (Kita et al., 1994; Lin & Timasheff, 1996; Kaushik & Bhat, 1998; Kaushik & Bhat, 2003). Sharp et al. (1991) and Nicholls et al. (1991) hypothesized that this

phenomenon could be used to quantify the hydrophobic protein stability. These reports were in sync with reports showing that the surface tension of water is increased by the presence of sucrose and this increases thermal protein stability (Lee & Timasheff, 1981). But this is not the case with the other osmolytes (Kita et al., 1994). Urea also increases the surface tension of water and hence, proteins should be preferentially hydrated in the presence of urea, forcing them to fold according to surface tension theory. However, experiments show that urea preferentially binds to the native protein (Prakash et al., 1981), giving a result that is exactly opposite to what surface tension theory predicts. The failure of surface tension theory to correctly predict protein behavior in the presence of urea is similarly mirrored by its incorrect predictions of protein behavior in the presence of TMAO. TMAO was experimentally found to decrease the surface tension of water (Kita et al., 1994) and thus it is predicted that it should bind to proteins leading to destabilization of proteins. However, TMAO was found to force proteins to fold (Baskakov et al., 1998; Baskakov et al., 1999). Therefore, Auton et al. (2006) concluded that surface tension could not be the force solely responsible for these osmolyte-induced effects on proteins as its effects are greatly attenuated by one or more additional forces. It was also found that the measured preferential interactions of ArgHCl and LysHCl (Kita et al., 1994) with bovine serum albumin did not give a good correlation with the increase in surface tension. A similar lack of correlation was also found for $MgCl_2$ (Arakawa et al., 1990b).

3.2 Theoretical models

3.2.1 Excluded volume model

Excluded volume is the volume occupied by a solute molecule in a solvent and this space cannot be occupied by another molecule in the same solvent. It depends on two parameters of the solute molecule: shape and conformation. This concept was first designed by Paul John Flory, and Werner Kuhn coined this term in 1934. Suppose there were two identical non-flexible spheres placed in dilute solution, it was observed that the mid-point of these two spheres can only be placed close enough so that the distance remains twice as their radius. In simple words, a spherical molecule will exclude molecules like it to a distance eight times the space it occupies. But, during calculations, the distance is counted only twice, which become only four times greater than their physical volumes (Schachman & Lauffer, 1949; Timasheff, 1998; Schellman, 2003).

This model explains well the preferential exclusion of PEGs from proteins (Arakawa & Timasheff, 1985; Bhat & Timasheff, 1992). Urea and guanidinium chloride have larger radii than a water molecule, and according to this model, they are expected to preferentially hydrate proteins for excluded volume reasons. But it is known that urea and guanidinium chloride bind with the proteins in the native state and destabilize them (Arakawa & Timasheff, 1984; Timasheff, 1992).

3.2.2 Scaled particle theory

This is a convenient method for expression of the solvation (hydration) free energy of the rigid-sphere solute (Pierotti, 1965; Pierotti, 1976). This theory does not explain in detail the interactions among the solute particles in the solution or structure component of the solution. The free energy of solvation of a rigid-sphere solute is dependent on the solvent density ρ, pressure P, and temperature T of the system. A solvent molecule is modeled as a hard sphere with a properly chosen diameter (Reiss et al., 1959; Lebowitz et al., 1965;

Minton, 1983). Scaled particle theory predicts the free energy of solvation of a protein in terms of the work of forming a cavity in the solution large enough to accommodate the protein.

The scaled particle theory (SPT) can be used to calculate the entropic component of the effect of carbohydrates on protein stability (Saunders et al., 2000; Davis-Searles et al., 2001). By subtracting the entropic contribution from the measured change in stability, the enthalpic contribution can be calculated (Saunders et al., 2000; Davis-Searles et al., 2001). SPT theory can explain the stabilizing effect of various polyols on the A-state of ferricytochrome c at low pH (Davis-Searles et al., 1998). The effect of sucrose and fructose on the thermal stability of RNase-A and α-lactalbumin are entropic in nature and can be accurately accounted for by SPT (O'Connor et al., 2004). The stability prediction by SPT changes because of the contribution that the osmolytes make to the entropy of solvation of the native and denatured states of proteins. SPT calculates preferential binding parameters in those cases where enthalpic interactions between the protein and the co-solute are not important like in case of the effect of sucrose and fructose on the stability of RNase-A and α-lactalbumin (O'Connor et al, 2007).

3.2.3 Kirkwood–Buff approach

The solvation of the protein side chains in the presence of osmolytes was first demonstrated by Rosgen and coworkers (Harries & Rösgen, 2008; Rösgen, 2009). This approach was named Kirkwood-Buff theory, in which they calculated the osmolation of osmolyte and hydration of protein for all osmolytes. It was also determined that the hydration of side chains depends mostly on their size. The peptide backbone unit is hydrated to a variable extent in different osmolyte solutions. According to this approach, the osmolytes were categorized based on their solvation of the peptide unit. It was observed though that certain osmolytes (methylamines) demonstrated very little peptide unit hydration when the protein was transferred from water to 1 M methylamine solution, but the magnitude of osmolation was large with negative value. This indicated that methylamines were excluded from the peptide chain. A similar effect was found in the case of proline and polyol osmolytes with slight variation that water also was excluded from peptide vicinity. However, contrary to methylamines, amino acids and polyols, the saccharide osmolytes were found to interact with the peptide unit, causing the hydration of the peptide unit with no solvation of the saccharide osmolytes. This very aptly describes the weak interaction of denaturants, as exhibited by X-ray crystallography and NMR (Dunbar et al., 1997; Mattos & Ringe, 2001; Pierce et al., 2008). Urea displays the classical solvent exchange mechanism where its preferential interaction with the protein peptide chain excluded water (Auton et al., 2008).

3.2.4 Molecular crowding approach

The primary feature of macromolecular crowding is that all osmolytes occupy the same space at the same time with other molecules (Joshi & Kishore, 2022). This basically is the excluded volume model (Figure 4). The repulsion between different molecules exists with disregard to any other type of interactions. This phenomenon is everlasting, and the organisms have adapted to it (Ellis, 2001; Minton, 2001). The hypothesis in which proteins as seen as hard spheres greatly emphasizes the effect of excluded volume on their thermodynamic and hydrodynamic properties (Zimmerman & Minton, 1993).

This phenomenon happens when we consider that a significant solution volume is filled with various other components, other than water alone. All the processes that occur at high macromolecule concentrations (50-400 mg/mL), occupying up to 40 percent of medium volume, causes a decrease in the intracellular component volume in the cell (Fulton, 1982; Zimmerman & Trach, 1991; Ellis & Minton, 2003). This phenomenon is found in all living systems. The protein folding process involves passing through an unstable intermediate which can aggregate (Hartl & Hayer-Hartl, 2002). The crowding phenomenon in these cases leads to aggregate formation (Hartl & Hayer-Hartl, 2002; Hatters et al., 2002). Crowding compounds are currently being employed in *in vitro* biochemical studies to mimic *in vivo* conditions (Hartl & Hayer-Hartl, 2002). This can help to understand how this phenomenon can influence a biochemical process.

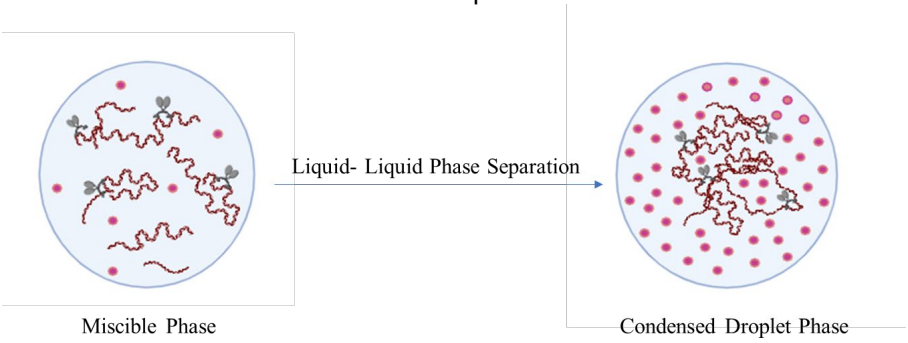

Figure 4. The effect of macromolecular crowding on the protein stability

4. Conclusions

From the above discussions we conclude that protein-osmolyte interactions involve the interplay of various forces. Among all the models discussed in the text above, the most convincing mechanism shows the preferential exclusion of osmolytes from the immediate vicinity of the protein domain and this preferential exclusion of osmolyte serves as the major driving force for osmolyte-induced stabilization of protein. This preferential exclusion of the osmolyte from the protein domain is the result of (i) solvophobic/osmophobic interaction between the osmolyte and the native (N) and denature (D) states of proteins, (ii) osmolytes that increase surface tension being preferentially excluded from the protein surface- the surface tension effect and (iii) excluded volume considerations.

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6. Conflicts of Interest

There is no conflict of interest.

ORCID
Vandhana Srinivasan https://orcid.org/0009-0000-7070-5733
Sheeza Khan https://orcid.org/0000-0002-0654-4901

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