Liel Sapir and Daniel Harries

WISDOM OF THE CROWD

On a hot summer's day you decide to cool off in the neighborhood swimming pool. The pool is jam-packed with others trying to do the same, but you venture to go in, anyway. The stress and crowded feeling is (more or less) what a macromolecule would feel inside a living cell. The third or so dry mass of an average cell translates in the pool analogy to you easily being able to hold hands with your nearest neighbors. Some four decades ago it was realized that this kind of environment must have implications to the way cellular macromolecules interact. To get from one side of the pool to the other, for example, would require a collective movement of you and your neighbors to allow passage. The mere excluded volume of your neighbors (and some people in the pool seem to have much more of that than others) makes it difficult to find specific partners by simple diffusion. But crowding may have its pros, too. By driving macromolecules such as proteins towards the more compact state, even steric interactions alone may suffice to stabilize their structure. Additional effective forces between neighboring molecules in solution (that can be repulsive or attractive by nature) further modulate, or even dominate, this stabilization. Importantly, effective forces in solution can be exerted not only by large macromolecules, but also by molecularly small solutes (perhaps sardines were added to the pool?), but their action often shows distinct differences from larger crowders in the temperature dependence of crowding. Can we devise a common language to describe the rich variety of possible solvation effects on macromolecules in simple yet thermodynamically descriptive terms? We review the thermodynamic implications of solvation in dense solutions, comment on the importance of molecular interactions beyond excluded volume to this effect, and outline the implications for small versus large solutes. Finally, we discuss how the interactions between all components in a dense milieu modulate the temperature response of macromolecular stability.

FROM *IN VITRO* TO *IN VIVO* (AND BACK TO THE GLASS)

Many biochemical experiments *in vitro* are performed in very dilute aqueous solutions, so that the biological macromolecules of interest, e.g. proteins, DNA, polysaccharides, or membranes, are solvated in essentially pure water. Structural transitions are determined by the interactions between the macromolecule and solvent in these binary mixtures. For example, protein folding in pure water is largely driven by the hydrophobic effect, whereby protein-water interactions stabilize the native state of proteins. Moreover, the unique thermodynamic nature of these solvent-solute interactions determines the thermal stability of proteins witnessed, for example, in the process of cold-denaturation.[1]

Biologically relevant environments are anything but simple: proteins do not fold in a "vacant swimming pool". In fact, cells are usually highly dense, see Figure 1, containing about 30-40% by volume of excluding dry matter: proteins, nucleic acids, plasma membranes, metabolites, and many other molecules.[2, 3] In this highly packed environment the structural transitions of proteins as well as other biomacromolecules can be, and usually are, affected by the presence of other molecules. The study of protein structural properties and folding in vivo is thus increasingly being addressed experimentally.[4–7]

In a reduced picture that still retains something of the cellular complexity, macromolecular transitions in highly concentrated environments can be modeled by thinking about ternary mixtures, containing in addition to the solvent and macromolecule of interest another component termed a "cosolute". The properties of macromolecules in these solutions are then naturally affected by both the changes in solvent (typically water) activity, as well as by the interaction between solution components and the macromolecule itself.[8–12] These cosolutes can be very different in size, chemical composition, and physical characteristics; ranging from ions, through small metabolites, to very large polymers. The important effect of the solution was acknowledged, in some cases, already over a century ago. For example, the quest to resolve how ions impact proteins in aqueous solution goes back to Hofmeister's work in the late 19th century.[13–16]

Throughout evolution organisms have developed various strategies to adapt to their environment. A ubiquitous mechanism is the reversible accumulation of high (sometimes even molar) concentrations of molecularly small cosolutes in response to osmotic stress.[17, 18] These cosolutes, termed "osmolytes", are used by organisms from all kingdoms to adjust the inner-cell osmolality to match that of the surrounding media. Many osmolytes are considered compatible with the cellular milieu, in the sense that they allow the proper activity of cellular macromolecules. They include urea and methylated ammonium or sulfonium compounds (e.g., TMAO), derivatives of amino acids (e.g., glycine betaine), and sugars or other polyhydroxy compounds, such as the polyols inositol[19] and mannitol.[17, 20] Often, protective osmolytes can form hydrogen bonds in solution, and can as well be zwitterionic. But in addition they can possess considerable non-polar moieties, and this amphipathic

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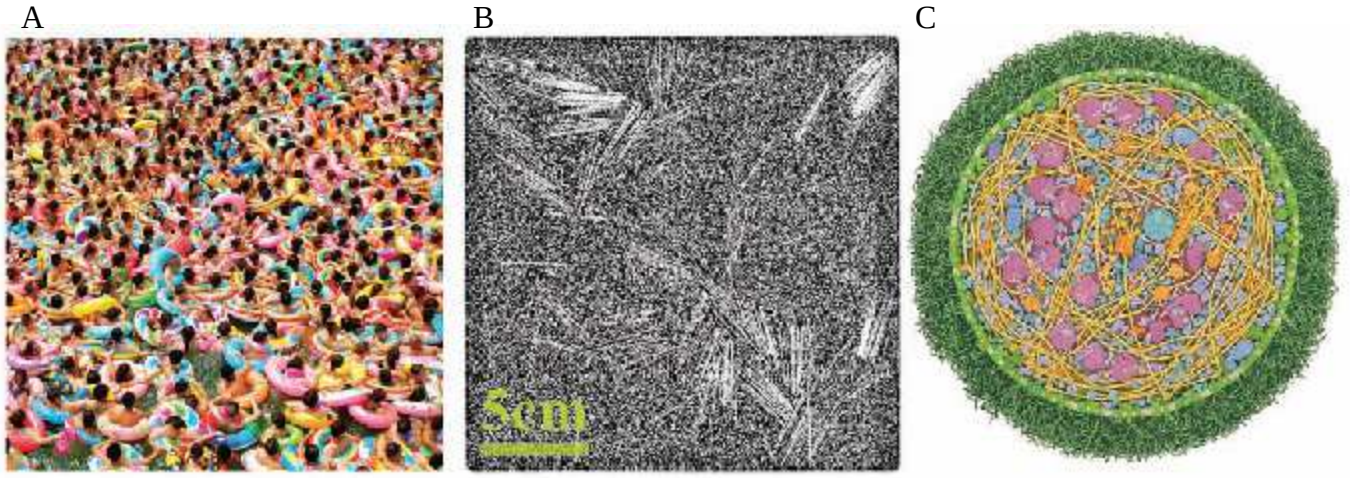


Fig. 1: Structural transitions in crowded systems of various length scales. A) A crowded pool on a hot summer day in Suining, China. Exclusion between bathers is mediated by hard steric interactions as well as softer ones. (Credit: Top Photo Corporation/Alamy.) B) Granular system of rods associating in the presence of small ball bearings. Figure reproduced from ref. 105. C) An entire Mycoplasma mycoides cell, about 300 nanometers in size, as depicted by D.S. Goodsell.[106] The illustration shows only the larger cellular components: proteins, DNA, and the plasma membrane.

nature may be important for their biological role.[21] Even inorganic ions can serve as osmolytes (for example in some halophilic organisms), although the charge they carry adds even more complexity to their interactions with macromolecules. In certain cases, osmolytes bestow unique capabilities on organisms under the most extreme conditions. A prominent example is the remarkable adaptation of Tardigrades, also known as "water bears", to dehydration, which is made possible at least in part by the accumulation of trehalose, a disaccharide.[22] At very low water content, the sugar can form a glassy matrix inside the cells[23] that helps to protect and stabilize the biological macromolecules, even for years, Figure 2.[24]

Protective osmolytes, sometimes referred to as "chemical chaperones", stabilize proteins in their native, folded, state.[25] Their action has been suggested to be analogous to "macromolecular crowding", that is known to be exerted by much larger, polymeric, macromolecules by virtue of their volume exclusion.[26, 27] Osmolytes along with other cellular components (including "crowding" macromolecules), act to shift protein folding equilibrium towards the compact folded state. The extent of stabilization can be quantified by

$$\Delta\Delta G = \Delta G_C - \Delta G_W \qquad (1)$$

where we denote the free energy changes associated with pure water and cosolute solution as ΔG_W and ΔG_C, respectively. Note that although the double Δ is seemingly redundant, it is a common biochemical notation used to stress that it describes a difference that is due to some modification of the original system. Specifically here, $\Delta\Delta G$ for protective osmolytes is typically $\Delta\Delta G < 0$, Figure 3, while denaturants such as urea lead to $\Delta\Delta G > 0$.

Fig. 2: A colored scanning electron micrograph of a Tardigrade ("water bear") in its active state (top) and the "tun" state (bottom). In the tun state this sub-millimeter creature can survive long periods of dehydration. (Credit: Eye Of Science/SPL.)

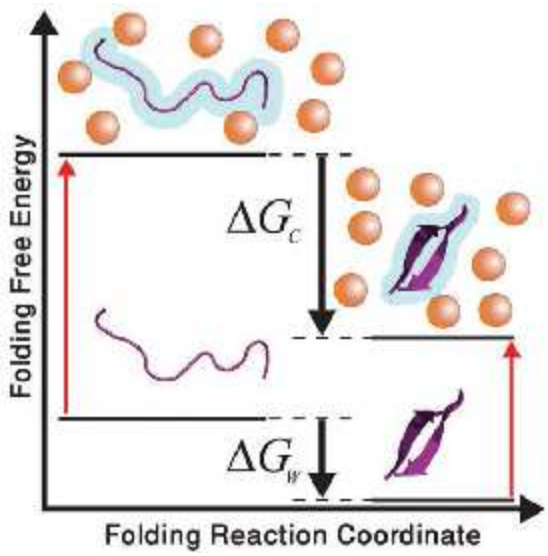


Fig. 3: Free energy scheme of protein folding in water and in solution with an added excluded cosolute. In pure water the free energy change upon folding is ΔG_W. The addition of excluded cosolute (tan spheres) destabilizes the folded state (right), but destabilizes the unfolded state (left) to an even greater extent, because the unfolded ensemble exposes a larger "unfavorable" interface to the solvent. Subsequently, the folding free energy change in the presence of cosolute, ΔG_C, is more negative than ΔG_W. The shaded cyan area around the protein represents the "preferentially hydrated" volume from which the cosolutes are excluded.

FROM GIBBS TO KIRKWOOD AND BUFF

The thermodynamic analysis of stabilization of macromolecules by added cosolutes goes back to Gibbs in his work "*On the Equilibrium of Heterogeneous Substances*".[28] Discussing the free energy associated with the formation of an extended interface between two phases, Gibbs made the necessary link between the excess (or accumulation) of cosolute at that surface and the ensuing free energy of the interface (or "surface tension").

An analogous analysis can be applied to macromolecular solvation in a binary water-cosolute mixture.[29, 30] A convenient and instructive physical realization considers a dialysis setup, where a semipermeable membrane separates a ternary macromolecule-water-cosolute mixture from a binary water-cosolute mixture. The membrane allows only the diffusion of water from one compartment to the other. The Gibbs-Duhem relation for the binary mixture at constant pressure and temperature requires that

$$n_w d\mu_w + n_c d\mu_c = 0 \qquad (2)$$

where n_w and n_c are the numbers of solvent (water) and cosolute molecules in the binary mixture, respectively, and μ_w and μ_c are the corresponding chemical potentials. Applying a similar relation to the ternary mixture yields the so called "Gibbs adsorption isotherm",[31]

$$dG_M = -N_w d\mu_w - N_c d\mu_c \qquad (3)$$

where dG_M is the macromolecule (or interface) free energy change, and N_w and N_c are the numbers of water and cosolute molecules, respectively, that bathe the macromolecule in the ternary mixture. Although eq. 3 contains two unknown variables (water and cosolute numbers in the ternary mixture), the necessary equality of chemical potentials in both compartments imposes an additional constraint introduced by the binary mixture, eq. 2, which together with eq. 3 yields

$$dG_M = -N_w\left(1 - \frac{n_w/n_c}{N_w/N_c}\right)d\mu_w = -N_c\left(1 - \frac{n_c/n_w}{N_c/N_w}\right)d\mu_c \qquad (4)$$

Thus, eq. 4 links the free energy of macromolecule solvation within a binary cosolute-water mixture with quantities termed the "preferential interaction coefficients", Γ_i, defined as

$$\Gamma_w = N_w\left(1 - \frac{n_w/n_c}{N_w/N_c}\right) = -\left(\frac{\partial G_M}{\partial \mu_w}\right)_{T,\,P,\,m_M} \qquad (5)$$

$$\Gamma_c = N_c\left(1 - \frac{n_c/n_w}{N_c/N_w}\right) = -\left(\frac{\partial G_M}{\partial \mu_c}\right)_{T,\,P,\,m_M} \qquad (6)$$

where m_M is the macromolecular molality. Here, the *preferential hydration* coefficient, Γ_w, reflects the relative excess or deficit of water molecules around the macromolecule with respect to the binary (bulk) solution. Similarly, the preferential *solvation* (or if you prefer – "osmolation") by cosolute, Γ_c, represents the relative excess of cosolute molecules surrounding the macromolecule. The two preferential interaction coefficients are related through $\Gamma_w = -\Gamma_c(n_w/n_c)$, indicating that a surface deficit of one component necessarily requires a corresponding excess of the other.

Wyman,[32] Tanford,[33] and others[34–40] developed different strategies, both experimental and theoretical, in order to measure and interpret preferential interaction coefficients. Although different studies sometimes use a mindboggling variety of definitions for these coefficients, specific thermodynamic relations link them with one another. For example, a convenient measure for preferential solvation by cosolute, Γ_c, is given by[37, 38] $\Gamma_{\mu_w,\mu_c} \equiv (\partial m_c/\partial m_M)_{T,\mu_w,\mu_c}$, where m_i is the molality of component i. This definition is naturally related to the above dialysis experiment, if we imagine the ternary mixture to be composed of two volumes: the water-cosolute subsystem/domain and a ternary protein-water-cosolute subsystem/domain. The chemical potentials of water and cosolute are kept constant throughout the binary (water-cosolute) mixture subsystem as long as it is very large. Then, in the ternary mixture subsystem the *bulk* cosolute molality (far away from the macromolecular interface) would necessarily be equal to the molality in the binary mixture, $m_c = n_c/M_w n_w$, with M_w the molar weight of water. However, the relative exclusion/inclusion of cosolute around the macromolecule will modify the molality in the macromolecular domain subsystem (close to the macromolecular interface), so that $m_c = (n_c + n_M\Gamma_c)/M_w n_w$, hence yielding (after taking the derivative) $\Gamma_c = \Gamma_{\mu_w,\mu_c}$.

It now becomes evident from eqs. 5-6 that preferentially excluded cosolutes, for which $\Gamma_c < 0$ and $\Gamma_w > 0$, increase the chemical potential of the macromolecule:

$$dG_M = -\Gamma_w d\mu_w = -\Gamma_c d\mu_c \qquad (7)$$

The extent of destabilization is thus directly related to excess and deficit of solvent and cosolute at the interface formed between the macromolecule and the solvent. Different macromolecular conformations would be destabilized to various extents depending on their solvent accessible surface areas and on their interactions with solution components. The solvent accessible surface area of the denatured (unfolded, extended) state of the macromolecule is larger, so that these conformations are destabilized to a larger extent than the compact (folded) conformations. In terms of preferential hydration, in the presence of excluded cosolute Γ_w for the unfolded state (e.g. of a protein) is more positive than that of the folded state. Upon folding, therefore, the change in preferential hydration is negative, $\Delta\Gamma_w < 0$, which directly translates into folding stabilization, $\Delta\Delta G < 0$, Figure 3. Thus, the Gibbs adsorption isotherm, eq. 7, can essentially describe the full gamut of cosolute action on proteins, ranging from preferentially excluded to preferentially included cosolutes (see illustrative image in Figure 4).

Experimentally, the preferential interaction coefficients can be measured, for example, through the variation in solvation free energy with solution osmotic pressure. The folding free energy is related to the change in preferential hydration when protein concentration is low by[41, 42]

$$\Delta\Gamma_w = \nu_w^{-1}\frac{\partial\Delta\Delta G}{\partial\Pi} \qquad (8)$$

where ν_w is the partial molar volume of pure water and Π is the osmotic pressure. Using eq. 8, the preferential interaction coefficient can be readily related to a common biochemical mea-

Fig. 4: Preferential interactions in the wild. Photograph of fish avoiding sharks taken at the Meeru island in the Maldives. The fish are "preferentially excluded" from the sharks. Consequently, the sharks can be said to be "preferentially hydrated". (Credit: Karl Robertson/Alamy.)

sure in protein folding: the so called *m*-value, typically defined as the slope of $\Delta\Delta G$ with cosolute *molar* concentration, C_c.[43] Hence, one can relate $\Delta\Gamma_w$ with the *m*-value through[44, 45]

$$m = \Delta\Gamma_w \frac{RT}{C_w} \frac{\partial \ln a_c}{\partial \ln C_c} = \nu_w \Phi_{c_c} RT \Delta\Gamma_w \qquad (9)$$

where R is the gas constant, C_w is water molarity, a_c is cosolute activity, and Φ_{c_c} is the molar osmotic coefficient. An alternative, and perhaps a more practically pleasing measure, is the *molal*-scale equivalent:

$$\tilde{m} = \frac{\partial \Delta\Delta G}{\partial m_c} = \nu_w \Phi RT \Delta\Gamma_w \qquad (10)$$

where Φ is the molal osmotic coefficient. See the Box "Osmotic Pressure and the van 't Hoff Equation" for further details on osmotic pressure, concentration scales, and osmotic coefficients.

In spite of these general and robust relations, the Gibbs adsorption isotherm and its consequences are, as Guggenheim stated, "*as far as pure thermodynamics can take us*".[46] Experiments that derive preferential interaction coefficients from thermodynamic data using, for example, eq. 8 only testify to the value of $\Delta\Gamma_w$ and not to its underlying values of N_w and N_c. Gibbs[28] and others[46] introduced the concept of a "dividing surface" in order to circumvent the problem and gain physical insight to these numbers. However, exact determination of these quantities requires additional information on solution structure at the molecular level.

The Kirkwood-Buff (KB) solution theory[50] provides the exact, statistical thermodynamic, framework that links between the microscopic molecular-level quantities, as embodied in the pair correlation functions between solution components, with the macroscopic quantities, i.e., free energies and preferential interaction coefficients. A fundamental quantity in the KB theory is the Kirkwood-Buff integral (KBI): the total correlation between two species given by the spatial integration

$$G_{ij} = \int_v (g_{ij} - 1) dv \qquad (11)$$

In eq. 11, g_{ij} is the pair distribution function that describes the spatial correlation between components *i* and *j*, and is a measure of "solution structure". This correlation function is frequently given in terms of the radial and azimuthal components of the vector from one of the components to the other, or even more simply in terms of the radial distribution function, $g_{ij}(r)$. In models, this correlation described by $g_{ij}(r)$ can be determined, e.g., through molecular dynamics (MD) simulations, or calculated from a potential of mean force of intermolecular interactions.[44, 51, 52] The KBI's can be related to the excess number of component *i* around a macromolecule, $G_{iM} = (N_i - n_i)/n_i n_M$.[39, 40] For example, G_{WM}, quantifies the total hydration of the protein, while also stemming from the molecular property g_{WM}.[53, 54]

The KBI can be directly used to calculate various thermodynamic quantities. Specifically, the preferential hydration is given by[40,54]

$$\Gamma_w = C_w (G_{WM} - G_{CM}) \qquad (12)$$

This illustrates how valuable the KB theory can be for the analysis of MD simulations of proteins, since the molecular-level pair distribution functions allow us to determine preferential hydration of different protein conformations, which in turn is related to thermodynamic stability.[52,55] Eq. 12 therefore reflects that solvation by cosolute and water determines the preferential interaction as well as the ensuing thermodynamic manifestations, including the Gibbs adsorption isotherm expressed in eq. 8. Moreover, the KB theory allows to individually determine in molecular models and simulations the two quantities, G_{WM} and G_{CM}. Interestingly, through the "inverse KB theory" originally prescribed by Ben-Naim,[56] these KBIs can also be directly experimentally measured.[45,53,57]

FROM STERIC REPULSION TO SOFT INTERACTIONS

The first attempt at formulating a molecular-level mechanism for the action of excluded cosolutes on macromolecules is due to Asakura and Oosawa. Their seminal work on "depletion interations"[58, 59] explained the cosolute-induced coagulation of a colloidal suspension in terms of molecular interactions. The major assumption in the Asakura-Oosawa model (AOM) is that the cosolutes, which in their case were in fact polymeric macromolecules, interact with the colloids (which served as the macromolecules of interest in this scheme) through a completely steric, i.e. hard core, repulsion interaction. This interaction is conveniently described by the potential of mean force between cosolute and macromolecule (PMF_{CM}), Figure 5A. This PMF_{CM} is an *effective* potential, since it implicitly integrates the contributions of solvent into a single cosolute-macromolecule interaction.

BOX

Osmotic Pressure and the van 't Hoff Equation

The schematic shows an experimental setup often used to describe how chemical potential differences can translate into hydrostatic pressures, leading to a colligative property termed "osmotic pressure". The apparatus consists of two compartments separated by a semipermeable membrane that allows only solvent to pass. To the left of the membrane is pure solvent (water), with chemical potential μ_W^{pure}. To the right is a mixture of solvent with an additional solute. In the mixture, water has a mole fraction $X_W < 1$, and chemical potential $\mu_W^{mixture}$. For an ideal solution at pressure P and temperature T, the chemical potentials necessarily satisfy the inequality

$$\mu_W^{mixture}(P, T, X_W) < \mu_W^{pure}(P, T).$$

This simply reflects the added mixing entropy that lowers the chemical potential in the mixture so that $\mu_W^{mixture}(P, T, X_W) = \mu_W^{pure}(P, T) + RT \ln X_W$, where R is the gas constant. Hence, solvent will flow from the left and into the right compartment, elevating the solution level and raising the pressure exerted on the mixture by Π. Added pressure elevates the chemical potential, so that the net flow of solvent will continue until thermodynamic equilibrium is finally reached when

$$\mu_W^{mixture}(P + \Pi, T, X_W) = \mu_W^{pure}(P, T).$$

At this point Π defines the osmotic pressure of the mixture. This expression implies further that

$$\mu_W^{pure}(P, T) + \Pi \left(\frac{\partial \mu_W^{pure}}{\partial P}\right)_T + RT \ln X_W = \mu_W^{pure}(P, T)$$

where we have assumed that the partial derivative, $(\partial \mu_W^{pure}/\partial P)_T = v_W^{pure}$, is pressure independent. It follows that $\Pi v_W^{pure} = -RT \ln X_W = -RT \ln(1 - X_C)$, where X_C is the solute (or cosolute) mole fraction. Since for dilute solutions $\ln(1 - X_C) \cong -X_C$, we find

$$\Pi v_W^{pure} = X_C RT$$

Under the same dilute solution conditions, the osmotic pressure can also be quantified in terms of cosolute molar concentration (defined as the number of moles of cosolute per liter of solution) $C_C \approx X_C / v_W^{pure}$, so that

$$\Pi = C_C RT$$

This is known as the van 't Hoff equation for the osmotic pressure, which because of its entropic origins is reminiscent of the ideal gas law.[47] For his "[...] *discovery of the laws of chemical dynamics and osmotic pressure in solutions*",[48] Jacobus H. van 't Hoff received the first Nobel prize in chemistry in 1901.[49]

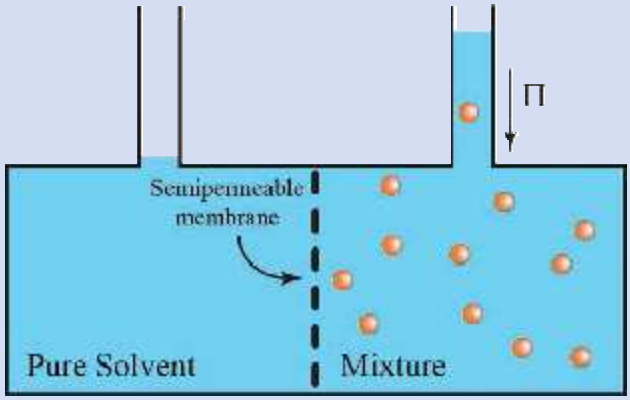
The above relation can be readily extended to non-ideal solutions by repeating the derivation but substituting the mole fraction with the activity $a_i = \gamma_i X_i$, where γ_i is the activity coefficient of component i. This non-ideality then results in a modified van 't Hoff equation

$$\Pi = \Phi_{C_C} C_C RT$$

where Φ_{C_C} is the molar osmotic coefficient that describes the deviations from ideal behavior (with analogy to the compressibility factor of gases). The activity, and hence the osmotic pressure, can be alternatively quantified using other concentration scales, as long as the appropriate reference concentration is taken into account, e.g. $a_i = \gamma_{C,i} C_i / C_i^0 = \gamma_{m,i} m_i / m_i^0$. In the last equality, we introduced another convenient concentration scale: the molal (mole of solute per kilogram of solvent), so that for a cosolute with molality m_C,

$$\Pi = \Phi m_C RT$$

and Φ is the osmotic coefficient on the molal scale. This concentration scale has gained popularity in reporting osmotic pressures, possibly because it is easily related to mole fractions. Correspondingly, if the cosolute dissociates into n moles of particles for every mole of molecular formula (for sodium chloride, e.g., $n = 2$), then $\Pi = n \Phi m_C RT$. Instead of pressure units, frequently the osmotic pressure is described in terms of solution osmolality, defined as $n \Phi m_C$.



Steric repulsion in AOM creates a layer around each of the colloids into which the center of mass of the cosolutes cannot penetrate. This is the so called "depleted" or "excluded volume". Upon colloid dimerization, the excluded volume of the two colloids overlap, thereby reducing the total excluded volume in solution by $\Delta V_{ex} < 0$, Figure 5B. The gain in the volume available to cosolutes, or rather the gain in released excluded volume, increases the cosolutes' translational entropy, thereby lowering the free energy in favor of the more compact dimer relative to the monomers. The ensuing depletion force[60] is therefore an *effective* force that drives colloidal (or more generally macromolecular) association. Think of the depletion force as an added attractive interaction between macromolecules relative to a solution with no excluded cosolutes.

Although the above molecular mechanism was proposed to specifically explain the action of excluded cosolutes in a colloidal suspension, and has been applied in that specific context

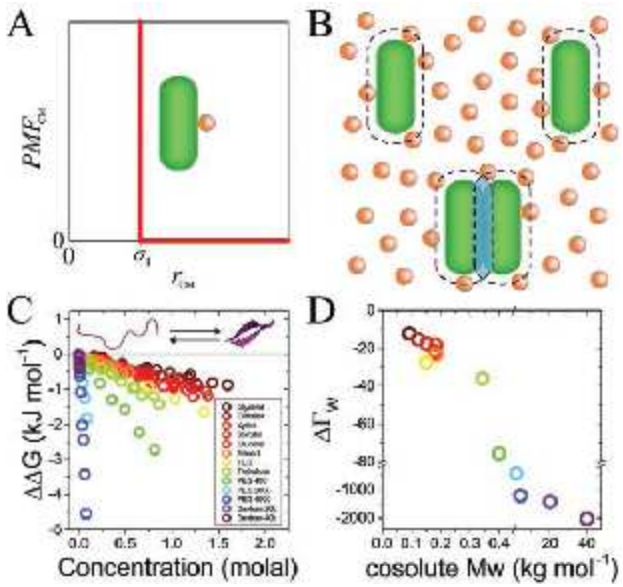


Fig. 5: Steric cosolute-macromolecule repulsion and the Asakura and Oosawa model. A) The steric (hard core) potential of mean force for the cosolute-macromolecule interaction, PMF_{CM}. B) Scheme of colloidal (macromolecular) dimerization in the presence of cosolutes that interact sterically with the colloids. Upon dimerization, the exclusion layers around each colloid (dashed black lines) overlap with one another (shaded blue area) thereby driving the dimerization. C) Experimental cosolute induced stabilization, $\Delta\Delta G$, of a folding peptide (top, schematic) in the presence of many excluded cosolutes, is linear in cosolute concentration. D) The change in preferential hydration upon folding, $\Delta\Gamma_w$, for the same model peptide, derived from the slope of the curves in panel (C), grows with cosolute molecular weight. Panels A and B reproduced from ref. 51. Panels C and D reproduced from ref. 66.

ever since,[58–61] its broader scope and importance are eminent. This did not escape Asakura and Oosawa themselves; writing on the emergent effective depletion force they stated:[58] *"Such a force perhaps plays an important role in biophysical problems, because the medium in biological systems may be regarded as solutions of macromolecules, i.e., of various proteins."* Indeed, about two decades later, an analogous approach was developed in order to explain macromolecular structural transitions (binding, folding, etc.) in the crowded environment of biological cells.[26, 62] Using scaled particle theory,[63, 64] the "crowding" paradigm underlined the fundamental role that steric interactions play in these systems.

In AOM the extent of cosolute stabilization is given by $\Delta\Delta G = \Pi\Delta V_{ex}$, hence predicting a linear scaling between cosolute molality and the induced macromolecular stabilization, since for the low concentration (van 't Hoff) regime, $\Pi \propto m_c$ (See Box). This is consistent with the Gibbs adsorption isotherm, eq. 8, as long as Γ_w is independent of concentration. Furthermore, in AOM $\Delta\Delta G$ scales with the gain in excluded volume, hence anticipating a scaling with cosolute size through ΔV_{ex}. Both these predictions were corroborated by many experimental investigations of excluded cosolute action on protein folding and association processes. For example, we have studied the folding process of a model β-hairpin peptide in water and in solutions containing many excluded cosolutes, including osmolytes and polymeric crowders, and the results demonstrate the AOM predicted scaling relationship, Figure 5C-D.[42, 65, 66]

The AOM holds another major prediction regarding depletion forces: since the underlying PMF_{CM} is athermal, the effect is necessarily *completely* entropic. This is an example showing that the temperature dependence of $\Delta\Delta G$, or alternatively its entropic, $T\Delta\Delta S$, and enthalpic, $\Delta\Delta H$, contributions, where $\Delta\Delta G = \Delta\Delta H - T\Delta\Delta S$, can be used as a constraint on any proposed molecular mechanism.[51, 67] The entropic and enthalpic contributions can be conveniently mapped out in an entropy-enthalpy plot, Figure 6A. For an AOM-like mechanism, the induced stabilization will be completely entropic and should reside along the orange line in Figure 6A. Other possible relative contributions of entropy and enthalpy delineate several regions in this stability plot.

Interestingly, only over the past several years have experiments begun to be scrutinized for this third prediction of AOM. Such experiments require measurements of the entropic and enthalpic contributions to the free energy. This can be done, for example, by measuring biomolecular stability as a function of temperature, i.e. van 't Hoff analysis of thermal melting curves. The emerging picture from such experiments is not always consistent with AOM. In fact, most cosolutes do not induce a completely entropic effect, but rather have in addition some enthalpic contribution. For example, in many cases polymeric crowders, such as polyethylene glycol and dextran, induce stabilization which is entropically favorable, as AOM predicts, but is mitigated by an unfavorable enthalpy, $\Delta\Delta H > 0$, sector IIb in Figure 6A.[65, 66] More surprisingly, osmolytes, and in certain cases even polymeric crowders, can induce a depletion force that is enthalpically dominated and incurs an entropic penalty, $\Delta\Delta H < 0$ and $T\Delta\Delta S < 0$, sector Ia.[42, 65, 66, 68] For example, the model peptide discussed above exhibits these distinct thermodynamic fingerprints in the presence of a variety of excluded cosolutes, Figure 6B.

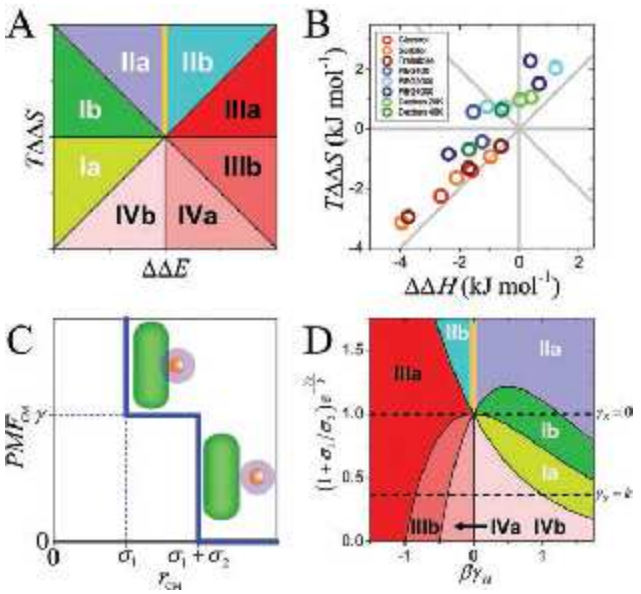


Fig. 6: Enthalpic depletion forces and "soft" macromolecule-cosolute interactions. A) Schematic entropy-enthalpy plot delineating different possible characteristic thermodynamic regimes. The orange line represents the Asakura-Oosawa model result. B) Entropy-enthalpy plot for cosolute effects on a β-hairpin peptide folding (described in Figure 5C-D). C) To consider cosolute action beyond AOM, the hard core repulsion can be augmented by a "soft" interacting layer shown in the potential of mean force for the cosolute-macromolecule interaction, PMF_{CM}, defined in eq. 13. D) Mapping of the thermodynamic regimes in panel (A) onto the parameter space of the PMF_{CM} in panel (C). Different parameter profiles of the effective interaction yield distinct thermodynamic fingerprints. Panels A, C, and D are reproduced from ref. 51, and panel B is reproduced from ref. 65.

The discrepancy between AOM and the entropic and enthalpic contributions to osmolyte-driven depletion forces can be resolved by reconsidering the underlying PMF_{CM}. Motivated by simple Monte-Carlo simulations of a coarse-grained system,[69] we may consider a PMF_{CM} that in addition to the hard core repulsion is augmented by another longer-ranged component that can be either repulsive or attractive. This type of interaction can be simplified by using a step-like (or in the attractive case – a square-well) interaction potential as a function of distance from the surface:

$$PMF_{CM}(r_{CM}) = \begin{cases} \infty & r_{CM} \leq \sigma_1 \\ \gamma & \sigma_1 < r_{CM} \leq \sigma_1 + \sigma_2 \\ 0 & \sigma_1 + \sigma_2 < r_{CM} \end{cases} \quad (13)$$

where σ_1 and σ_2 are the widths of the two "interaction layers" (hard core and soft interaction, respectively), and γ is the height of the soft shell interaction component, Figure 6C. We take γ to be a free energy barrier (or well) height (or depth), i.e. consisting of entropic (γ_S) and an energetic (γ_H) components, $\gamma = \gamma_H - T\gamma_S$, where T is the absolute temperature.

Using the Kirkwood-Buff solution theory results, eqs. 11-12, we can resolve the thermodynamic effects that such a PMF_{CM} has on the macromolecular process of interest. It should be reemphasized that the PMF_{CM} represents an *effective* interaction since it includes (implicitly) the contributions from all macromolecule-solvent interactions.[51] Using the relation $\beta\, PMF_{CM} = -\ln g_{CM}$, where $\beta = (kT)^{-1}$ and k is Boltzmann's constant, and assuming independence of PMF_{CM} from cosolute concentrations (a reasonable assumption for low concentrations) the extent of stabilization and its entropic and enthalpic/energetic components can be directly determined. For the burial of the surface of the macromolecule of interest, representing e.g. protein folding or association, these contributions (per unit surface area) emerge as:

$$\frac{\Delta\Delta S}{RN_{AV}C_C} = \sigma_1 + \sigma_2\left[1 - e^{-\beta\gamma}(1 + \beta\gamma_H)\right] \quad (14)$$

$$\frac{\Delta\Delta E}{RTN_{AV}C_C} = -v_2\beta\gamma_H e^{-\beta\gamma} \quad (15)$$

where N_{AV} is Avogadro's number. The leading term in $\Delta\Delta S$ is, not surprisingly, the AOM component stemming from the hard core repulsion interaction represented by σ_1. The next term in the entropic component, as well as the exclusive contribution to $\Delta\Delta E$, stem from the soft interaction layer. We note in passing that since solutions are usually hardly compressible in the biological regimes, we can often interchange between energy and enthalpy. Nonetheless, the combined effects of pressure and cosolute addition on protein folding have also been extensively studied.[70–72]

We can now map the various thermodynamic regimes in Figure 6A onto the parameter space that defines the PMF_{CM}, Figure 6D. The AOM prediction for completely entropic depletion forces is recovered when the second (soft) interacting layer vanishes, $\gamma = 0$. However, for any other value of γ, there will also be an energetic contribution to $\Delta\Delta G$. For example, entropic depletion forces that are enthalpically mitigated, such as those seen with many polymeric crowders, emerge when the soft interaction is attractive, Figure 6A, sector IIb. Remarkably, in order to induce enthalpic depletion forces that incur an entropic penalty, as was shown experimentally for many protective osmolytes, the PMF_{CM} has to be composed of a soft interaction that is both entropically attractive, $\gamma_S > 0$, and energetically repulsive, $\gamma_H > 0$. This can be regarded as a minimal requirement for the emergence of enthalpic depletion forces, which is also supported by our mean field theory for macromolecules in cosolute solutions.[73]

The range of possible thermodynamic fingerprints that involve different entropic and enthalpic contributions underscore that the temperature dependence of the molecular level PMF_{CM} is crucial for understanding cosolute-induced macromolecular structural transitions, along with their macroscopic temperature dependence. Another convenient, and analogous, approach to account for the effect of temperature is to consider a temperature-dependent preferential hydration coefficient, $\Delta\Gamma_W(T)$. Through the Gibbs adsorption isotherm, eq. 8, this dependence necessarily implies that $(\partial\Delta\Delta H/\partial m_C) = -\nu_W\Phi RT^2(\partial\Delta\Gamma_W/\partial T)$, where the molal osmotic coefficient Φ is assumed to be temperature independent. This relation can perhaps most easily be understood as a *temperature-dependent effective excluded volume*. Unlike AOM, this excluded volume is not entirely related to steric interactions (i.e. molecular sizes, volumes, or shapes), but rather integrates in addition all other underlying interactions in the solution. Thus, we can speak of an excluded volume related to a depletion interaction, but this should not be imagined to result from steric interactions (or molecular volumes) alone.

At the molecular level, the effective PMF_{CM} is determined by the interactions of both the solvent and the cosolute with the macromolecule. These interactions are directly related to the underlying pair distribution functions and reflect the intricate interplay between all components in solution. Naturally, the soft repulsion discussed above emerges from these interactions, along with their temperature dependence. Studies of the properties of water-osmolyte solutions suggest that osmolytes strengthen the hydrogen bonds between neighboring water molecules.[74–76] These modifications then translate to differences in interaction energies of cosolute with bulk water versus cosolute with water molecules in the protein's hydration shell.[55] It would be interesting in future studies to explore the specific mechanism that generates the augmented form of the PMF_{CM} required in enthalpic depletion forces.

The solvent accessible surface area of proteins that interact with all solution components is a complex mosaic of different moieties with distinct, and sometimes completely opposite interactions with components of the bathing solution. This was well appreciated by the transfer free energy model and related methodologies, where the free energy of protein solvation is decomposed into contributions from the different surface types.[77–85] Since these transfer free energies can be calculated for different protein conformations, the method allows to derive the *m*-value, and thereby the preferential interaction coefficients. The above scheme outlined by eqs. 13-14 can be extended to incorporate these local specific surface considerations by accounting for a moiety-specific PMF_{CM}. In this more elaborate realization, the different contributing interfaces can also have distinct temperature-dependences.

FROM DEPLETION THROUGH EXCLUSION TO BRIDGING BY INCLUSION

So far we have considered the central paradigm of excluded cosolute effects on macromolecules: preferentially excluded cosolutes drive macromolecular compaction through the "depletion force". Conversely, preferentially *included* cosolutes act to stabilize the different macromolecule conformations, but states with larger solvent exposed interfaces are stabilized more, hence shifting the structural equilibrium towards the extended state of the macromolecule. Urea, for example, denatures proteins through preferential inclusion.[86–89] But over the last few years a new regime of cosolute effects has been explored, whereby preferentially included cosolutes *stabilize* compact macromolecular states. How is that possible?

If cosolute attraction to the macromolecular surface is strong enough, the preferentially included cosolutes can simultaneously "bind" to two or more distant macromolecular moieties or surfaces. This spatially correlated binding forms "bridges" that stabilize the macromolecular compact state. The resulting "bridging interaction" shifts the equilibrium state of the macromolecule towards a compact state, which is structurally and energetically different from the compact state stabilized by depletion forces, Figure 7. Similar mechanisms driven by correlations have been long known to be induced by ions that stabilize compact polyelectrolyte structures, as seen, e.g., in DNA precipitation by multivalent ions.[90]

Although the importance of cosolute-induced bridging has not yet been directly demonstrated for proteins, it has been suggested as a mechanism for collapse or coagulation of many other polymers[91–94] and colloidal[95, 96] systems. For example, bridging resides at the heart of the phenomenon of cononsolvency, where a mixture of two "good" solvents (towards a polymer macromolecule) become a bad one.[97, 98] Interestingly, this mechanism by which cosolutes act on macromolecules can depend sensitively on temperature, too. A prominent example is the temperature-dependent structural transition of a colloidal suspension that demonstrates re-entrant solidification.[96] At low temperatures, the colloids bathed in a solution of excluded polymer are in a crystal-like state stabilized by depletion forces. As the temperature increases, the colloids form a homogenous dispersion, but at still higher temperature the bridging interaction stabilizes a more compact, aggregative structure. This kind of re-entrant behavior can be rationalized by considering a temperature dependent PMF_{CM}.[99]

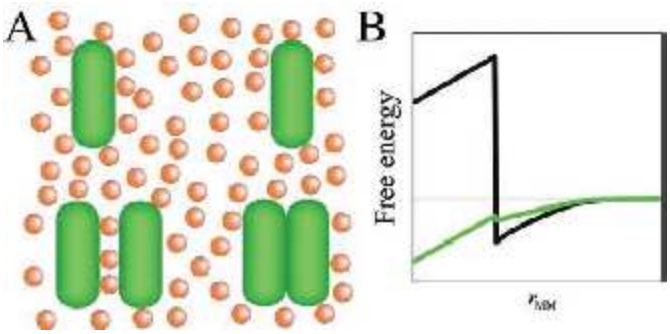


Fig. 7: The bridging interaction stabilizes a compact macromolecular state through preferential cosolute inclusion. A) Preferential inclusion usually destabilizes the compact state (bottom right) in favor of the extended state (top). However, sufficiently strong attraction of cosolute to the macromolecule can stabilize a different compact, "bridged", state (bottom left). B) The effective interaction between macromolecular moieties as a function of distance between them. A preferentially excluded cosolute stabilizes the most compact state at full contact (green curve). A preferentially included cosolute, whose cosolute-macromolecule interaction is strong enough, destabilizes the most compact state, but instead stabilizes a different compact, "bridged", state (black curve). Figure shows results of a mean field model, reproduced from ref. 99.

SUMMARY

Biological macromolecules are usually solvated in dense and crowded cellular media, far removed from the dilute solutions usually studied *in vitro*. Careful tuning of the cellular milieu allows organisms to adapt to challenges set by their environment, by employing the surrounding "cosolutes" to modify the structural stability of macromolecules. "Protective osmolytes" are known to stabilize proteins in their folded, native, state, while other cosolutes, like urea, can destabilize the folded state. Thermodynamically, the action of cosolutes on protein stability is related to their preferential interactions with protein surfaces through the "Gibbs adsorption isotherm". Preferentially excluded cosolutes stabilize the folded states, while preferentially included cosolutes destabilize them. In order to gain mechanistic insight into the origins of exclusion or inclusion, it is possible to use structural solution data, e.g., pair distribution functions, and link these to the thermodynamic properties through the Kirkwood-Buff solution theory.

The first mechanistic explanation for the action of preferentially excluded cosolutes was proposed by Asakura and Oosawa. Their model highlights the steric cosolute-macromolecule interactions and predicts entropic "depletion forces" that drive macromolecular compaction. Similar arguments were suggested to explain the action of cellular crowding on protein folding. However, many experimental results cannot be rationalized by this mechanism, because the thermal stability of proteins in water versus crowded solutions suggests that energetic contributions are nonnegligible. Moreover, in certain cases cosolutes induce depletion forces dominated by enthalpy where in fact entropy is decreasing in the process. These enthalpic depletion forces can be explained by temperature dependent cosolute-macromolecular interactions that include a "soft" repulsion component in addition to the steric, hard-core, interactions. This temperature dependence propagates also to the thermal stability and structure of macromolecules.

If the "soft component" of cosolute-macromolecule interaction is attractive, the cosolute can be preferentially included around the macromolecule, and thereby destabilizes the compact state. If it is attractive enough, though, it can stabilize another compact macromolecular state through the "bridging interaction", due to correlated binding. This type of effective interaction between and within macromolecules and colloids also exhibits a temperature dependence that traces back to the underlying molecular contributions to the cosolute-macromolecule interaction.

The thermodynamic principles underlying the role of cosolutes in biological structural transitions, and specifically their impact

on protein folding and association, are accretively being elucidated. These developments should serve as constraints that direct future research towards a comprehensive molecular mechanism of cosolute action. In this endeavor, both theoretical and computational techniques, such as molecular dynamics simulations,[21, 55, 100–104] and temperature-dependent experiments, should play pivotal roles. Foreseeable developments should allow, for example, the well-established transfer free energy models to be extended to distinguish the different mechanistic role played by different protein moieties in the complex process of folding in the presence of added cosolutes. Important strides have been made, but the search continues for a fully predictive theory of macromolecular stability and interactions in solution.

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