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Osmolyte depletion viewed in terms of the dividing membrane and its work of expansion against osmotic pressure

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ABSTRACT

How osmolytes enhance the folding, binding, and self-assembly of biological macromolecules at a microscopic scale has long been a matter of debate. Ambiguities persist on the key interpretive concepts, such as the "effective membrane" (which marks the boundary of the volume from which osmolytes are excluded) and the "free energy of exclusion" of osmolytes from biomolecular surfaces. In this paper, we formulate these elusive concepts based upon chemical thermodynamics and rigorous statistical thermodynamics (the Kirkwood-Buff theory). Positioning of the membrane at the osmotic dividing surface is crucial in order not to affect the thermodynamics of solvation. The notion of the free energy (work) of excluding osmolytes is refined to the expansion work against the osmotic pressure, which indeed describes the change of solvation free energy at dilute osmolyte concentrations.

1. Introduction

Self-assembly is important in a wide range of scientific disciplines, from biochemistry (protein assembly and self-aggregation) [1–8] to nanoscience and colloid and interfacial science [9–14]. Self-assembly is caused by the attractive forces between the constituent macromolecules, which cannot be understood quantitatively without a consideration of the surrounding water and cosolvent molecules that mediate these forces [1–16].

When considering how cosolvents work to enhance self-assembly, we are faced with a variety of synonyms for cosolvents (e.g. solutes, cosolutes, hydrotropes, solubilisers, additives, denaturants, stabilisers, kosmotropes, chaotropes, or osmolytes) which has brought further complications [1–7,15]; here we define "cosolvents" as the third component in general, and "osmolytes" for a particular class of cosolvents, which are involved in the stabilisation of the native protein structure, protein-ligand interaction, as well as self-assembly [1–8,15,16]. A clear, molecular-level understanding of the role of osmolytes will have a fundamental and far-reaching significance not only in a wide variety of scientific disciplines but also in applications (such as in formulation science), where the right choice of cosolvents makes a drastic difference in solvent-related processes [17–24].

So how, at a molecular level, do osmolytes enhance the folding, binding and self-assembly of macromolecules? The following two hypotheses have coexisted for decades [1–10]:

1. the **depletion forces** hypothesis, which attributes the enhancement of association to the exclusion of osmolytes from biomolecules, colloids, or surfaces [3,4,6–8,13,14,17]; and
2. the **hydration forces** hypothesis, which attributes the enhancement of association to the change of hydration induced by the presence of osmolytes [2,5,9,10,15].

Are these two hypotheses equivalent or contradictory? This has indeed been a very difficult question to answer from a molecular basis [1–5,25–28]. The reason is twofold: (i) unlike protein-ligand binding or the self-assembly of biomolecules, protein-osmolyte interactions are weak and non-specific [1–7,25–28]; and (ii) osmolytes act on proteins not by binding but by depletion, i.e., being preferentially excluded from proteins [1–7,25–28]. Yet the early development of biomolecular thermodynamics focused mainly on specific interactions; this has made it challenging to describe weak, non-specific, depletion interactions based upon the stoichiometric binding models of specific interactions [25–28]. These seminal earlier theories [2–5,25–28] remained purely phenomenological and approximate, until only recently the rigorous statistical thermodynamic re-derivation, based upon the Kirkwood-Buff (KB) theory, has finally brought a clear description of weak, non-specific interactions [6,7,19,20].

The assumed equivalence between the two hypotheses has led to the osmotic stress technique (OST), i.e., the use of the osmolytes for

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probing hydration changes that accompany protein-ligand interactions, allosteric effects, and ion channel openings [2,5,9,10,15]. The basic assumption of OST is that the addition of osmolytes somehow exerts osmotic pressure on hydration water molecules that are located in the vicinity of biomolecules [5]. This approach, despite its widespread use, has also been a cause of debate over the last few decades [1–7]. Using a rigorous KB theory, we have shown that the exclusion of osmolytes from biomolecular surfaces is the origin of the osmolyte-induced equilibrium shift [6,7]. This conclusion, which supports the depletion hypothesis, is the rigorous theoretical endorsement of the macromolecular crowding theory [29,30], yet is at odds with the hydration hypothesis [2,5,9,10,15] which assumes the equivalence between osmolyte exclusion and biomolecular hydration.

However, we believe that the theoretical basis for the presumed equivalence between hydration and depletion should be revisited from a rigorous statistical thermodynamic perspective, as it can provide an alternative interpretive approach on preferential solvation. The previous controversy over the equivalence of depletion and hydration can be summarised into following four main points [1–7]:

(I) **Introduction of an effective or hypothetical semi-permeable membrane**, which separates the biomolecular vicinity from the bulk solution [3–5].
(II) **Free-energy change of the system due to the osmotic pressure** arising from the inaccessibility of the osmolytes [5,9], which persists even in the absence of the semi-permeable membrane, due to osmolytes' steric inaccessibility [5,9].
(III) **"Free energy of exclusion" of osmolytes from biomolecules** as the driving force of macromolecule-macromolecule and surface-surface association, because association reduces the work required to exclude osmolytes [3,4].
(IV) **Pressure-volume work of excluding osmolytes** can be attributed solely to the change of biomolecular hydration [5].

Ambiguity persists in (I)–(IV), especially when the membrane was employed explicitly in the experimental setup, sometimes it was deemed superfluous [5] yet was nevertheless employed conceptually in the interpretation [3–7]. What is even more puzzling is that the osmotic pressure is assumed to arise from osmolytes' inaccessibility to the hydration shell, even when there is no real semi-permeable membrane separating the hydration shell (vicinity) from the bulk (this is how the hypothetical "effective membrane" has been introduced to the system [3–5]). Therefore, the first aim of our paper is to develop a rigorous statistical thermodynamic theory, in order to clarify what the "effective" membrane really does to the osmolyte-induced shift of biomolecular equilibria, thereby clarifying (I) and (II) as summarised above.

By introducing the "effective" semi-permeable membrane explicitly into our theory, we will be able to address our second aim, i.e., is to examine the validity of another elusive concept, the "free energy of exclusion" of osmolytes from biomolecules, as summarised by (III) and (IV) [3,4]. Is the work of osmolyte exclusion really the change of biomolecular solvation free energy? The fact that this question has been addressed only phenomenologically and intuitively has perpetuated confusion in the study of the osmolyte effect.

Addressing the above two aims will lead to a novel and alternative approach to the preferential solvation theory, which provides a clearer physical picture on the roles of water and osmolytes on biomolecular equilibria.

2. Preferential solvation in the presence of a semi-permeable membrane

Here we formulate the theory of biomolecular solvation in the presence of a semi-permeable membrane. Let us consider N_1^L water molecules and N_u^L solute molecules (denoted by u throughout) in a volume V^L enclosed by a semi-permeable membrane, which allows only water molecules to pass through. This local region (denoted by L) enclosed by the membrane is surrounded by the bulk solution (whose volume is V), which consists of N_1 water molecules and N_2 cosolvent molecules. The entire system, composed of the bulk and the local regions, are under constant pressure (P) and temperature (T). Keeping P as a variable for the sake of generality for the moment, the Gibbs-Duhem equations for the exterior (which contains water and osmolyte molecules) and interior (which contains water and solute molecules) can be written in the following manner [6,7,20]:

$$n_1 d\mu_1 + n_2 d\mu_2 - dP = 0 \tag{1}$$

$$n_1^L d\mu_1^L + n_u^L d\mu_u^L - d\Pi = 0 \tag{2}$$

where n_i and n_i^L respectively represent the concentration of the species i in the bulk and the local regions, μ_i and μ_i^L are the corresponding chemical potentials, and Π is the osmotic pressure due to the inaccessibility of the cosolvent into the local region L and of the solute to the outside of L. (Note that, just as in the classical chemical thermodynamic theories of osmotic pressure, the sole function of the membrane is the selective permeation of molecular species; accordingly, no membrane/surface term appears in the theory). Now we consider the equilibrium conditions. Subtracting Eq. (1) from Eq. (2), and applying the equilibrium condition $\mu_i = \mu_i^L$ for water and cosolvent, we obtain

$$-d\mu_u^L = \frac{n_1^L - n_1}{n_u^L} d\mu_1 - \frac{n_2}{n_u^L} d\mu_2 - \frac{1}{n_u^L}(d\Pi - dP) \tag{3}$$

Using Eq. (1) to eliminate $d\mu_2$, Eq. (3) can be rewritten as

$$-d\mu_u^L = \left(\frac{n_1^L - n_1}{n_u^L} + \frac{n_1}{n_u^L}\right) d\mu_1 - \frac{1}{n_u^L} d\Pi \tag{4}$$

From Eq. (4) we obtain

$$\begin{aligned}\left(\frac{\partial \mu_u^L}{\partial \mu_1}\right)_{T,\Pi} &= -\left(n_1 G_{u1} + \frac{n_1}{n_u^L}\right) = -n_1\left(G_{u1} + \frac{1}{n_u^L}\right) \\ &= -n_1\left(G_{u1} + \frac{V^L}{N_u^L}\right)\end{aligned} \tag{5}$$

where

$$G_{ui} = \frac{n_i^L - n_i}{n_i n_u^L} \tag{6}$$

is the KB integral, as has been defined in our previous papers and shown to be equivalent to the statistical thermodynamic definition [6,7,20,31]. We will use Eq. (5) later to establish the connection between the KB integrals and volumetric properties derived from the dependence of μ_u^L on Π. From Eq. (2) we obtain

$$\left(\frac{\partial \mu_u^L}{\partial \Pi}\right)_{T,\mu_1} = \frac{1}{n_u^L} = \frac{V^L}{N_u^L} \tag{7}$$

where $\frac{V^L}{N_u^L}$ signifies the volume enclosed by the membrane per solute.

Let us now focus on V^L, the volume enclosed by the membrane. The interpretation of Eq. (7) can be facilitated by the following variable

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