

Preferential hydration and the exclusion of cosolvents from protein surfaces

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Protein stability is enhanced by the addition of osmolytes, such as sugars and polyols and inert crowders, such as polyethylene glycols. This stability enhancement has been quantified by the preferential hydration parameter which can be determined by experiments. To understand the mechanism of protein stability enhancement, we present a statistical mechanical analysis of the preferential hydration parameter based upon Kirkwood–Buff theory. Previously, the preferential hydration parameter was interpreted in terms of the number of hydration waters, as well as the cosolvent exclusion volume. It was not clear how accurate these interpretations were, nor what the relationship is between the two. By using the Kirkwood–Buff theory and experimental data, we conclude that the contribution from the cosolvent exclusion dominantly determines the preferential hydration parameters for crowders. For osmolytes, although the cosolvent exclusion largely determines the preferential hydration parameters, the contribution from hydration may not be negligible. © 2004 American Institute of Physics. [DOI: 10.1063/1.1759615]

I. INTRODUCTION

The stability of proteins is modulated by the addition of cosolvents.^{1,2} Some cosolvents, such as sugars and polyols act as protein stabilisers, whereas others, including urea and guanidine hydrochloride denature proteins.^{1,2} Organisms living under extreme conditions, in order to protect proteins from denaturation, accumulate protein stabilizers in the cell.^{2–5} Furthermore, it has been realised that the effect of other biomolecules present in the cell affects the folding, stability and function of the proteins (molecular crowding).^{6,7} Inert cosolvents, such as polyethylene glycol (PEG), have been used to model such crowding effects.^{6,7}

How do cosolvents stabilize proteins? Measurement of the preferential hydration parameter is indispensable in providing an answer to this question. The addition of cosolvent molecules to a protein solution is accompanied by a change of water activity. The preferential hydration parameter, ν_{21}^m , expresses the influence of water's chemical potential μ_1 upon the chemical potential of the protein μ_2 as:^{8,9}

$$\nu_{21}^m = - \left(\frac{\partial \mu_2}{\partial \mu_1} \right)_{P,T,m_2}, \quad (1)$$

where m_i is the molality of species i , and P and T are the pressure and temperature of the system.¹⁰

The preferential hydration parameter can be measured by densitometry, involving dialysis equilibrium,⁸ as well as by analytical ultracentrifugation.¹¹ This parameter, when rewritten using Wyman's relationship¹²

$$\nu_{21}^m = \left(\frac{\partial m_1}{\partial m_2} \right)_{P,T,\mu_1}, \quad (2)$$

signifies the number of water molecules which accompany the addition of a protein molecule under constant μ_1 .^{8,9} This suggests that the preferential hydration parameter is related to the structure of the aqueous solution which surrounds the protein.^{8,9,11} Indeed, assuming that water and cosolvent molecules bind competitively to the protein surface, Tanford,¹³ Timasheff,⁸ Eisenberg,¹¹ and Schellman¹⁴ have shown that the preferential hydration parameter is expressed as

$$\nu_{21}^m = N_{21}^m - \frac{m_1}{m_3} N_{23}^m, \quad (3)$$

where N_{21}^m and N_{23}^m are the numbers of water and cosolvent molecules bound to the protein surface. Upon biochemical reaction, the preferential hydration change

$$\Delta \nu_{21}^m = \Delta N_{21}^m - \frac{m_1}{m_3} \Delta N_{23}^m, \quad (4)$$

is related to the change of solvent binding, ΔN_{21}^m and ΔN_{23}^m .

What is the dominant contribution to the preferential hydration parameter? Timasheff and co-workers^{15,16} have shown that the preferential hydration parameters of proteins in aqueous polyethylene glycol (PEG) solutions can be explained from the steric exclusion of PEGs from protein surfaces. This analysis agrees with the molecular crowding analysis which asserts that the large volume from which the crowders are excluded was shown to be the dominant factor of the modulation of biochemical equilibria by crowders, such as PEGs.^{6,7} Furthermore, the exclusion of cosolvents was demonstrated to account for the preferential hydration behavior of osmolytes, such as sugars and polyols.^{17,18}

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Others interpreted the preferential hydration parameters via protein hydration.^{19–22} Parsegian and co-workers, in their “osmotic stress” analysis,^{19–21} argued that the dominant factor of the preferential hydration parameter change upon biochemical reaction is the change in hydration.^{19–22} The underlying assumption is: Osmolytes and crowders do not bind proteins, because they are excluded from protein surfaces.^{19–22} Therefore, it follows that $\Delta N_{23}^m = 0$ and $\Delta \nu_{21}^m = \Delta N_{21}^m$.^{19–25} Record and co-workers²² developed a sophisticated thermodynamic model called the local-bulk partitioning model (LBPM) to clarify the condition upon which $\Delta \nu_{21}^m = \Delta N_{21}^m$ is accurate. They have shown that $\nu_{21}^m = N_{21}^m$ applies for cosolvents which are completely excluded from the hydration shell of proteins. However, only very strongly excluded osmolytes, such as betaine, are close to satisfying this condition.²²

Are preferential hydration parameters determined by cosolvent exclusion or protein hydration? Steric exclusion^{15,16} and molecular crowding analyses^{6,7,17,18} support the former, whereas osmotic stress^{19–21} and LBPM²² support the latter. Are these interpretations equivalent? Osmotic stress analysis^{19–21} and LBPM²² suggest the equivalence of the two, whereas molecular crowding analysis suggests that the former interpretation is erroneous.^{6,7} It is imperative, therefore, to clarify the true origin of the preferential hydration parameters.

Recent papers by one of us^{25,26} have developed a theoretical foundation of preferential hydration and cosolvent-induced modulation of biochemical equilibria. This is based upon a rigorous statistical thermodynamic theory developed by Kirkwood and Buff (KB).^{27–31} It was clarified that the exclusion of cosolvents is not equivalent to zero binding: The zero binding approach to cosolvent exclusion was shown to lead to an overestimation of hydration changes.²⁵ Furthermore, it was also shown that excess solvation numbers can be calculated from experiments by the combination of preferential hydration and volumetric measurements.^{25,26}

However, the mechanism of preferential hydration is far from being clarified. Can the preferential hydration parameter be interpreted by cosolvent exclusion or hydration? Are they equivalent? Does LBPM give an accurate description of preferential hydration? These questions remain unanswered. In this paper, we will answer the above questions by applying KB theory to preferential hydration and volumetric data taken from the literature.

II. THEORY AND METHODS

A. Kirkwood–Buff theory

Consider a solution consisting of water ($i = 1$), protein ($i = 2$), and cosolvent ($i = 3$) molecules, where the protein is infinitely dilute. Kirkwood–Buff theory^{27–31} gives the following rigorous relationship on the preferential hydration parameter, ν_{21} :

$$\nu_{21} = - \left(\frac{\partial \mu_2}{\partial \mu_1} \right)_{T,P,n_2} = N_{21} - \frac{n_1}{n_3} N_{23}, \quad (5)$$

and a rigorous relationship on partial molar volume of protein V_2

$$V_2 = -V_1 N_{21} - V_3 N_{23} + kT \kappa_T, \quad (6)$$

where ν_i and n_i are the chemical potential and density (molarity) of the species i , and N_{2i} are the excess solvation number of species i around the protein. N_{2i} is defined in terms of the correlation function $g_{2i}(\mathbf{r})$ between the components 2 and i as:

$$N_{2i} = n_i N_A \int d\mathbf{r} [g_{2i}(\mathbf{r}) - 1], \quad (7)$$

where N_A is Avogadro's number.

By solving Eqs. (5) and (7), N_{21} and N_{23} can be determined from experimental values. In so doing, experimental values of ν_{21} and V_i at a given set of n_1 and n_3 are necessary, as was shown previously by one of us.^{25,26} Prior to these papers, there was no way to determine N_{21} and N_{23} directly and unambiguously from experiments.^{25,26}

N_{2i} consists of two contributions. The first contribution is due to the solvents' inaccessibility to the protein core, and the second is due to solvent reorganization.^{25,32–35} To extract the latter, the excluded volume, V_E , i.e., the sum of intrinsic (core) volume V_I and thermal volume V_T ^{32,33} should be calculated from protein structure data. Therefore,

$$N'_{2i} = N_{2i} + n_i V_E, \quad (8)$$

gives the statistical mechanical generalisation of “the number of bound solvent molecules.”^{25,26} N'_{2i} does not signify the number of solvent molecules coordinating the protein. N'_{2i} expresses the contribution of excess solvation number from the solvation shell.²⁵ In this theory, no assumption upon the range of solvation shell is necessary.

B. Processing of the experimental data

As outlined in the previous subsection, the calculation of N'_{21} and N'_{23} requires thermodynamic and structural data, in particular, partial molar volumes (V_1 , V_2 , and V_3) preferential hydration parameter ν_{21} in molarity scale, densities n_1 and n_3 , and excluded volume V_E . Here we explain how these values were calculated from data available in literature.

The partial molar volumes V_1 , V_2 , and V_3 were obtained as follows. First, V_2 is taken from the apparent molar volume of proteins in the limit of infinite protein dilution ($V_2^{\infty, \text{app}}$). Although, at finite protein concentrations, partial molar and apparent molar volumes are different, these two volumes become identical at the limit of infinite protein dilution.³⁶ The rest V_1 and V_3 were calculated from density data measured as a function of the concentration of species 3, by using a well-established method.^{28,37,38}

ν_{21} s are usually found in the experimental literature in the molality–molality unit. This is unsuitable for Eq. (5) which requires a molarity–molarity unit. The conversion was carried out by a well-known equation derived at infinite protein dilution:³⁹

$$\nu_{21} = \frac{n_1}{m_1} \nu_{21}^m + n_1 V_2. \quad (9)$$

The estimation of V_E requires detailed information on protein structure in the presence of cosolvents. We have used crystallographic data for the calculation of V_E . Slight shrink-

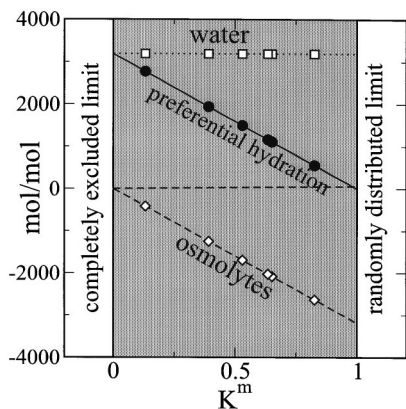


FIG. 1. Preferential hydration of bovine serum albumin (BSA) analyzed by LBPM. ν_{21}^m (filled circle), N_{21}^m (square), $-(m_1/m_3)N_{23}^m$ (diamond), and N_{23}^m (dashed line) are plotted in the presence of 6 cosolvents, which are, from the left to right, betaine, proline, trimethylamine N-oxide, trehalose, potassium glutamate, and glycerol. The experimental data are taken from Ref. 22, in which K^m and ν_{21}^m are tabulated.

ing of native structures in the presence of protein stabilizing cosolvents,^{40,41} have not been taking into account in this paper, due to the unavailability of precise structural information of such structures.

V_E for Ribonuclease A was taken from Chalikian *et al.*³² V_E for Bovine serum albumin (BSA) was calculated by the method outlined by Chalikian *et al.*³² from the structure calculated by homology modeling, due to the unavailability of structural data.⁴²

III. PREFERENTIAL HYDRATION IN THE PRESENCE OF OSMOLYTES AND CROWDERS

A. Local-bulk partitioning model

Local-bulk partitioning model (LBPM) of Record and co-workers²² is the most sophisticated thermodynamic model proposed thus far for the interpretation of the effect of cosolvents on protein stability.

It employs Eq. (3) as its foundation, which is rewritten in the following form:²²

$$\nu_{21}^m = N_{21}^m \left\{ 1 - \frac{N_{23}^m/N_{21}^m}{m_3/m_1} \right\}. \quad (10)$$

The local-bulk partitioning coefficient K^m is defined as²²

$$K^m = \frac{N_{23}^m/N_{21}^m}{m_3/m_1}, \quad (11)$$

which leads to

$$\nu_{21}^m = N_{21}^m (1 - K^m). \quad (12)$$

Record and co-workers assumed that N_{21}^m is not affected by osmolytes excluded from protein surfaces. Assuming further that N_{21}^m is proportional to solvent-accessible surface area (SASA) of the protein, K^m was shown to be calculated from experimental ν_{21}^m .²² It is noteworthy that $K^m \geq 0$, since all the quantities on the right-hand side of Eq. (11) are positive.

Figure 1 shows the preferential hydration of bovine serum albumin (BSA) in the presence of various osmolytes at 1 molal concentration. LBPM shows that the more excluded

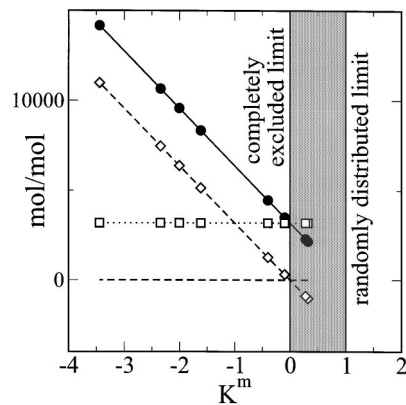


FIG. 2. Preferential hydration of BSA in aqueous PEG solutions analyzed by LBPM. ν_{21}^m (filled circle), N_{21}^m (square), $-(m_1/m_3)N_{23}^m$ (diamond), and N_{23}^m (dashed line) are plotted from the right to left for PEG 200, 400, 600, 1000, 2000, 4000, and 6000. PEG concentration was at 10% v/w for 200–1000, and at 4% v/w for 2000–6000. Experimental data for ν_{21}^m (20C, pH = 7.0) were taken from Ref. 16.

($K^m \approx 0$) the osmolyte is, the closer the preferential hydration parameter ν_{21}^m becomes to the number of water molecules in the shell, N_{21}^m .²² Can this model be applied to crowders, such as PEGs, which are known also to be excluded from protein surfaces? As shown in Fig. 2, the larger the PEG's molecular weight becomes, the more negative the local-bulk partitioning coefficient K^m becomes. However, negative K^m is unphysical. This shows that the basic assumptions of LBPM are not realistic for crowders.

LBPM is based upon the assumption that the hydration shell, i.e., the domain of the solution whose structure is different from the bulk, is confined within the first hydration layer.²² However, mounting evidence suggests that the range of this alteration is beyond the first hydration layer.^{28,34,35} Taken together, the unphysical interpretation of LBPM for crowders suggests that the long-range nature of the hydration shell must be taken into account.

B. Kirkwood–Buff approach to crowders

Here we apply KB theory to elucidate the origin of preferential hydration in the presence of crowders.

Figure 3 shows the KB interpretation of the preferential

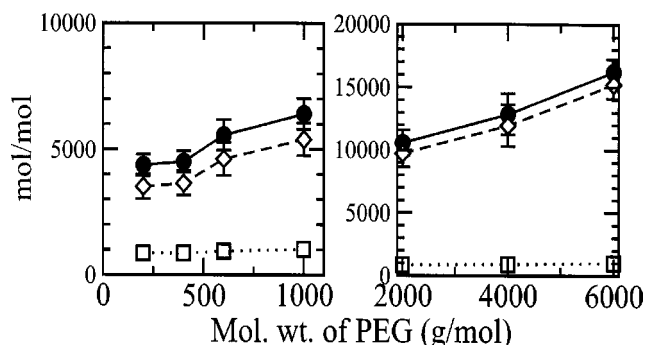


FIG. 3. Preferential hydration of BSA in aqueous PEG solutions. The same data as Fig. 2 were interpreted by KB theory. ν_{21} (filled circle), N_{21} (square), and $-(n_1/n_3)N_{23}$ (diamond). ν_{21} and V_2 were taken from Ref. 16, V_1 and V_3 were calculated from Refs. 50 and 51.

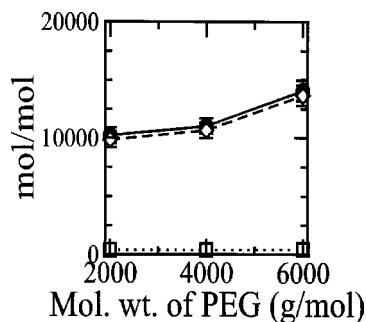


FIG. 4. Preferential hydration of ribonuclease A (pH 3.0, 20 C) in aqueous PEG solutions interpreted by KB theory. ν_{21} (filled circle), N'_{21} (square), and $-(n_1/n_3)N'_{23}$ (diamond). ν_{21} and V_2 were taken from Ref. 16, V_1 and V_3 were calculated from Refs. 50 and 51.

hydration of BSA in aqueous PEG solutions, which was interpreted by LBPM in Fig. 2. It is observed that the majority of the preferential hydration parameter, ν_{21} , comes from the $-(n_1/n_3)N'_{23}$ term. This suggests that (a) N'_{23} is negative and (b) the contribution of N'_{21} is secondary in ν_{21} . These features are observed also for other proteins, in particular for ribonuclease A, as shown in Fig. 4.

A further investigation into the features (a) and (b) observed in Figs. 3 and 4 is necessary to further our understanding of the mechanism of crowder action. With regards to (a), the negative N'_{23} indicates that PEGs are strongly excluded from protein surfaces. This is rationalized by combining Eqs. (7) and (8), yielding $N'_{23} = n_3(N_A \int d\mathbf{r} [g_{23}(\mathbf{r}) - 1] + V_E)$. Therefore, the negative N'_{23} should arise from the contributions of regions where $g_{23}(\mathbf{r}) < 1$. Since the exclusion of PEGs from protein surfaces lowers g_{23} at the surface, the cosolvent exclusion may cause the negative N'_{23} .

With regards to (b), osmotic stress analysis assumed that the preferential hydration parameter is determined by protein hydration.^{19–21} However, as shown in Figs. 3 and 4, the contribution from hydration is secondary for preferential hydration in PEG. The contribution from hydration becomes increasingly small as the molecular weight of PEG increases. Therefore, Figs. 3 and 4 support the steric exclusion^{15,16} and molecular crowding analyses.^{6,7,17,18} Preferential hydration parameters for crowders are mainly determined by the exclusion of crowders from protein surfaces. Figures 3 and 4 have also demonstrated clearly that there is no correlation between hydration and cosolvent exclusion.

Now we compare our KB approach to LBPM. Although LBPM obtained unphysical K s in Fig. 2, the observed dominance of $-(m_1/m_3)N^m_{23}$ in the preferential hydration parameter for high molecular weight PEGs is qualitatively consistent with the KB analysis. In LBPM, N^m_{21} and N^m_{23} were assumed to be coordination numbers of water and cosolvent molecules in the hydration shell. However, prior to the application of KB theory,^{25,26,28} the real physical meaning of N^m_{21} and N^m_{23} was unknown, in fact, Timasheff^{8,23} suggested they have no real physical meaning. At the time, N^m_{21} and N^m_{23} were assumed to conform to a single equation [Eq. (3)] and were thus considered to be indeterminants.^{8,23} However, N^m_{21} and N^m_{23} were replaced by well-defined physical quantities N'_{21} and N'_{23} when KB theory was applied [see Eq. (5)]. N'_{21}

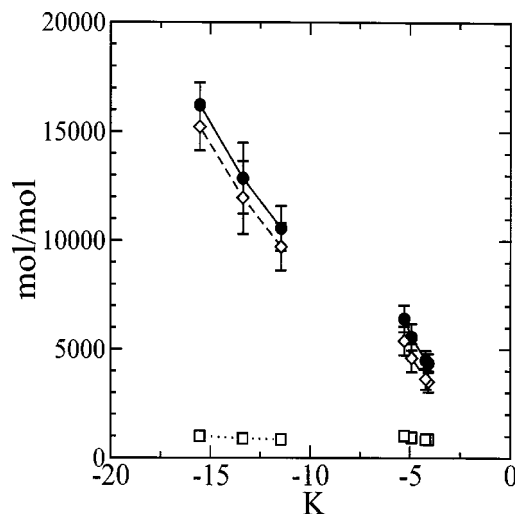


FIG. 5. Preferential hydration of BSA in aqueous PEG solutions. The same data as Figs. 2 and 3 were interpreted by KB theory and plotted against the partition coefficient K as defined by Eq. (13). ν_{21} (filled circle), N'_{21} (square), and $-(n_1/n_3)N'_{23}$ (diamond).

and N'_{23} now signify excess solvation numbers in the shell, which can be positive or negative. If one defines the “partition coefficient” K by,

$$K = \frac{N'_{23}/N'_{21}}{n_3/n_1}, \quad (13)$$

negative values for K will be allowed in this definition. (It is noteworthy that K is introduced here only to understand the relationship between N'_{21} and N'_{23} and that K does not relate to the conventional concept of partitioning. By thus defining K , Fig. 5 presents the redrawing of Fig. 2 based upon KB theory. By comparison, LBPM captures the feature that for large crowders, the exclusion of crowders is the dominant contribution to the preferential hydration. In Fig. 5, N'_{21} , an excess hydration number in the shell, depends little on the molecular weight of PEGs. This agrees with LBPM, which assumed that N^m_{21} is constant over cosolvent concentrations. However, there are fundamental difference between the two theories: The former is the result of an analysis using a rigorous KB theory, whereas the latter involves heuristic assumptions. N'_{21} and N'_{23} in the former are the excess solvation numbers in the shell, whereas N^m_{21} and N^m_{23} in the latter represents the coordination number of the solvents. Note that the KB treatment is free from the arbitrariness of a boundary between the hydration shell and the bulk, which is necessary for the definition of the coordination numbers.

Osmotic stress analysis proposed that $\Delta \nu_{21} \approx \Delta N'_{21}$.^{19–21} However, for crowders used often in this analysis, the exclusion of crowders is dominant. In this case, $\nu_{21} \approx -(n_1/n_3)N'_{23}$. This suggests that by using strongly excluded cosolvents, osmotic stress analysis estimates the change of crowder exclusion rather than the change of hydration.^{25,48}

C. Osmolytes

Here we turn to low molecular weight cosolvents known commonly as osmolytes.

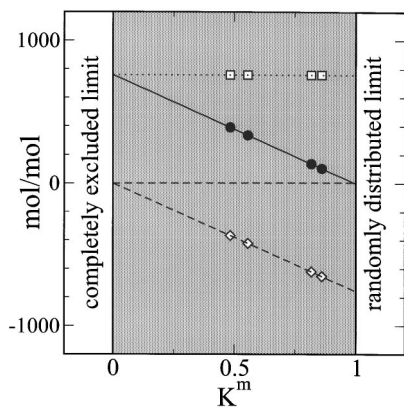


FIG. 6. Preferential hydration of ribonuclease A in aqueous osmolyte solutions interpreted by LBPM. ν_{21}^m (filled circle), N_{21}^m (square), $-(m_1/m_3)N_{23}^m$ (diamond), and N_{23}^m (dashed line) are plotted from the left to the right for trehalose, glycerol, sucrose, glucose. ν_{21}^m at $m_3=1$ molal were calculated from experimental data (Refs. 52–55).

Figure 6 shows the preferential hydration of ribonuclease A in the presence of trehalose, sucrose, glucose and glycerol interpreted by LBPM. The preferential hydration parameter ν_{21}^m (in the molality scale) is the sum of a large positive contribution from N_{21}^m and a large negative contribution from $-(m_1/m_3)N_{23}^m$. In Fig. 7, the same set of systems is analyzed by KB theory, where the preferential hydration parameter (in the molarity scale) is shown to be a sum of positive contributions from N'_{21} and $-(n_1/n_3)N'_{23}$. The positive sign of the latter, again, suggests that the osmolytes are excluded from protein surfaces. However, in contrast to the case of crowders (PEGs), the contribution from osmolyte exclusion is not dominant. The contribution from hydration cannot be ignored in the preferential hydration parameter for low molecular weight osmolytes as seen in Fig. 7. Therefore, molecular crowding, steric exclusion, osmotic stress and LBPM approaches do not give an accurate description of the preferential hydration in the presence of osmolytes.

Previously, Timasheff and co-workers^{52–55} attributed the preferential hydration parameters in the presence of sugars and polyols to the change in binding of these osmolytes to proteins. This analysis assumed that protein hydration does not change when the osmolytes are added. The validity of this assumption is examined in Fig. 7 which suggests that hydration has a minor dependence on the chemical nature of the osmolytes. More importantly, Fig. 7 illustrates that the correlation between K and the preferential hydration is no

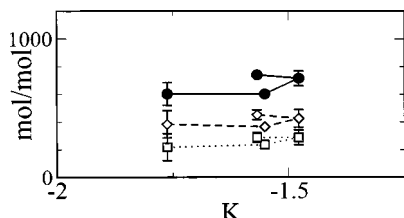


FIG. 7. Preferential hydration of ribonuclease A (cf. Fig. 6) interpreted via KB theory, plotted against the partition coefficient K defined by Eq. (13). ν_{21} (filled circle), N'_{21} (square), and $-(n_1/n_3)N'_{23}$ (diamond). V_E were taken from Ref. 32.

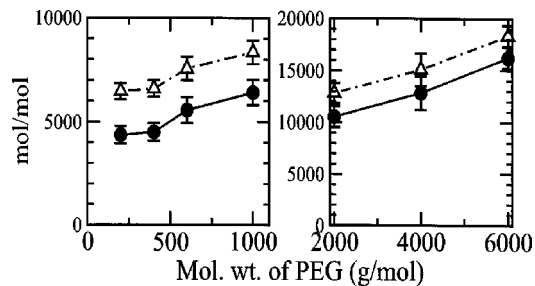


FIG. 8. Comparison between preferential hydration parameter ν_{21} (filled circle) and contribution from $-n_1 G_{23}$ (triangle) via KB theory, for the same system as Figs. 2, 3, and 5.

longer straightforward, in contrast to the simple correlation in Fig. 6. This suggests that there is no simple relationship between N'_{21} and N'_{23} as was assumed in previous thermodynamic models.

D. Interpretation via covolumes

The role of cosolvents on preferential hydration has been analyzed by molecular crowding approaches.^{6,7,17,18} Here I present a comparison between molecular crowding and the KB approaches.

At low cosolvent concentrations⁵⁶

$$\left(-\frac{n_1}{n_3}N_{23}\right)^0 = n_1 N_A \int d\mathbf{r} [1 - g_{23}^0(\mathbf{r})] \quad (14)$$

derived from Eq. (7). This quantity is proportional to the second virial cross coefficient

$$B_{23} = N_A \int d\mathbf{r} [1 - g_{23}^0(\mathbf{r})]. \quad (15)$$

B_{23} is determined mainly by protein-cosolvent covolume defined in molecular crowding analysis.^{6,7,17,18,49} Protein-cosolvent covolume is the volume around the protein in which the cosolvents are not accessible. Molecular crowding analysis shows that this covolume determines B_{23} dominantly.^{6,7,17,18,49} This B_{23} in molecular crowding analysis is the dominant contribution to preferential hydration parameter.⁶ This means

$$\nu_{21}^0 \approx n_1 B_{23}, \quad (16)$$

which, in the framework of KB theory, can be generalized into finite cosolvent concentration as

$$\nu_{21} \approx -n_1 G_{23}, \quad (17)$$

where $G_{23} = N_{23}/n_3$ is called the KB parameter, which at $n_3 \rightarrow 0$ satisfies $G_{23}^0 = -B_{23}$.

Figures 8 and 9 compare the preferential hydration parameter with the contribution from the G_{23} in the presence of PEGs. These figures show that the larger the PEG is at a given w/v concentration, the more dominant $-n_1 G_{23}$ becomes in the preferential hydration parameter. It is not clear, however, why this approximation [Eq. (17)] works better for ribonuclease A than BSA. Further studies involving simulations will be necessary to address this question. For low molecular weight osmolytes, Fig. 10 shows that this approximation [Eq. (17)] grossly overestimates the preferential

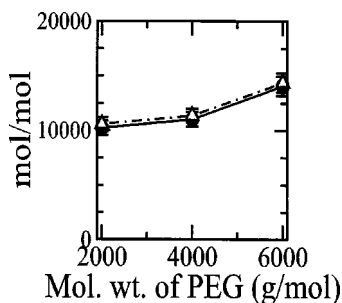


FIG. 9. Comparison between preferential hydration parameter ν_{21} (filled circle) and contribution from $-n_1 G_{23}$ (triangle) via KB theory, for the same system as Fig. 4.

hydration parameter. This tendency becomes more prominent for osmolytes whose preferential hydration parameter is smaller.

The above observation applies for lower cosolvent concentrations (data not shown). It therefore suggests that the covolume (second virial) approximation [Eq. (16)] used in molecular crowding analysis is an excellent approximation for large crowders. For the small osmolytes, although the covolume is the dominant contribution, the consideration of hydration may not be negligible for some osmolytes.

IV. CONCLUSION

In this paper, we demonstrated that the mechanism of cosolvent-induced modulation of protein stability can be clarified by employing KB theory. We have used KB theory to extract the excess solvation numbers from the shell by the combination of preferential hydration and volumetric data available in the literature.

We have shown that for large crowders (such as PEGs), the steric exclusion is the dominant contribution to the preferential hydration parameter, a result in line with the tradition from Kauzmann⁵⁷ and Ogston.⁵⁸ However, for low molecular weight osmolytes, both osmolyte exclusion and hydration contribute to the preferential hydration parameter.

This analysis is compared to previous thermodynamic and statistical mechanical models. Our analysis showed that the molecular crowding^{6,7,17,18} and steric exclusion^{15,16} analyses for large crowders is accurate, whereas hydration may contribute unnegligibly for small molecular weight osmolytes. The observation is considered robust against the

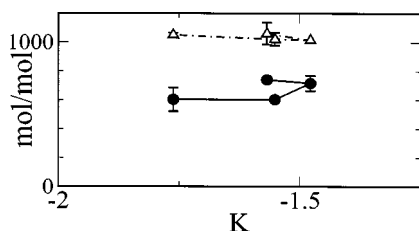


FIG. 10. Comparison between preferential hydration parameter ν_{21} (filled circle) and contribution from $-n_1 G_{23}$ (triangle) via KB theory, plotted against partition coefficient K for the same system as Figs. 6 and 7.

reasonable reparameterization of the excluded volume, because its contribution to the preferential hydration parameter is shown to be small for large crowders.⁵⁹

The change of preferential hydration parameter has often been attributed to the change of hydration,^{19–22} in contrast to molecular crowding and steric exclusion analyses. However, our analysis does not support the dominance of protein hydration in preferential hydration parameters, as was already discussed previously by one of us.²⁵

We believe that the analyses presented in this paper will be useful in the interpretation of cosolvent-induced equilibrium shifts routinely exploited for protein stabilization both in laboratories as well as in the cells surviving under (or adapted to) extreme conditions.^{1–8}

Note added in proof.

“It should be pointed out that Eq. (16) is accurate if $|\nu_{21}^0| \gg |n_1^0 v_2^0|$. This can be derived from Eqs. (5) and (6) at $n_3 \rightarrow 0$. Further application and generalization of this relationship to the cosolvent-induced equilibrium shifts will be reported elsewhere.

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