JOURNAL OF CHEMICAL PHYSICS VOLUME 121, NUMBER 18 8 NOVEMBER 2004

The Kirkwood–Buff theory and the effect of cosolvents on biochemical reactions

Seishi Shimizu[a)]
York Structural Biology Laboratory, Department of Chemistry, University of York Heslington, York, North Yorkshire YO10 5YW, United Kingdom

Chandra L. Boon
Department of Physics, University of Windsor, Windsor, Ontario N9B 3P4, Canada

(Received 25 June 2004; accepted 20 August 2004)

Cosolvents added to aqueous solutions of biomolecules profoundly affect protein stability, as well as biochemical equilibria. Some cosolvents, such as urea and guanidine hydrochloride, denature proteins, whereas others, such as osmolytes and crowders, stabilize the native structures of proteins. The way cosolvents interact with biomolecules is crucial information required to understand the cosolvent effect at a molecular level. We present a statistical mechanical framework based upon Kirkwood–Buff theory, which enables one to extract this picture from experimental data. The combination of two experimental results, namely, the cosolvent-induced equilibrium shift and the partial molar volume change upon the reaction, supplimented by the structural change, is shown to yield the number of water and cosolvent molecules bound or released during a reaction. Previously, denaturation experiments (e.g., *m*-value analysis) were analyzed by empirical and stoichiometric solvent-binding models, while the effects of osmolytes and crowders were analyzed by the approximate molecular crowding approach for low cosolvent concentration. Here we synthesize these previous approaches in a rigorous statistical mechanical treatment, which is applicable at any cosolvent concentration. The usefulness and accuracy of previous approaches was also evaluated.
© *2004 American Institute of Physics.* [DOI: 10.1063/1.1806402]

I. INTRODUCTION

Proteins are surrounded by aqueous solution. Water molecules influence biochemical reactions, such as protein folding, protein-ligand interaction, and the allosteric effect.[1–3] In addition to water, various classes of cosolvent molecules present in the aqueous solution affect these processes profoundly: (a) denaturants, such as guanidine hydrochloride (GuHCl) and urea, which are used routinely in today's biochemical laboratories (and have been known for a long time);[1,4–7] (b) osmolytes, i.e., organic metabolites such as sugars, polyols, and amino acid derivatives, which are known to stabilize the native structure of proteins and accumulate in the cell to counteract protein denaturation in a harsh environment;[8–13] (c) crowders, which do not act directly on the biomolecules, whose presence affects biochemical reactions mainly via the excluded volume effect or "crowding" effect.[10–15] Thus, an understanding of the cosolvent effect would have a direct impact on many branches of biology, biophysics, and biochemistry.

The goal of this paper is to explain the effect of cosolvents on biochemical equilibria, from the way that water and cosolvents interact with biomolecules involved in the reaction. Insight into these interactions can be extracted from experimental data. The guideline of its procedure is laid out by Kirkwood–Buff (KB) theory,[16–20] a rigorous and fundamental statistical mechanical theory, which has previously been applied to analyze the water release upon reaction[21] and the interactions between cosolvents and the native state of proteins,[22,23] in addition to a number of systems involving simpler solutes.[24–31] This paper extends these previous approaches into the role of cosolvents on biochemical reactions, including protein denaturation, protein-ligand binding, and allosteric transitions.

Why is the new approach necessary? What is the advantage over the previous approaches developed to the same end? The following brief review will clarify that the previous approaches are based upon unrealistic assumptions or oversimplified treatments of the protein-solvent interactions.

We focus first on the approaches to protein denaturation. Previous approaches aimed to elucidate the effectiveness of denaturants on proteins, based upon protein-denaturant interactions. This effectiveness was first quantified by Pace and co-workers[6,7,32] via the *m* value, which is the proportionality constant relating the denaturant concentration to the change in free energy upon protein denaturation. (In short, the larger the *m* value, the more susceptible the protein is to denaturation.[32]) The question now is how to interpret the *m* value from the interaction between the protein and the solvent molecules. Myers *et al.*[32] demonstrated that the *m* values are proportional to the change of solvent-accessible surface area (ΔSASA), when the protein denatures from its native structure to the fully extended unfolded structures. This means that the *m* value reflects the degrees of expansion of proteins upon denaturation. The ΔSASA proportionality has been derived by earlier theoretical models,[2,33] and has

[a)]Fax: +44-1904-328266. Electronic mail: shimizu@ysbl.york.ac.uk

 © 2004 American Institute of Physics

Downloaded 02 Nov 2004 to 144.32.128.75. Redistribution subject to AIP license or copyright, see http://jcp.aip.org/jcp/copyright.jsp

been a foundation of the most of theoretical models developed thus far.

However, recent experiments suggest that denatured states take compact structures, even in the presence of highly concentrated denaturants.[34] Therefore, the fully extended unfolded structures assumed in the previous models does not reflect reality. In addition, size-exclusion chromatography has cast doubts upon the correlation between the m value and the expansion in size of proteins upon denaturation.[35,36] This suggests that the ΔSASA proportionality of the m values may not be an accurate assumption.

In addition to the unrealistic assumption imposed on denatured state structures, previous models have made simplifying assumptions about protein-solvent interactions. Schellman[37] developed a thermodynamic model in which water and denaturant molecules bind weakly and competitively to the binding sites assumed to exist on protein surfaces. This model attributed the cause of protein denaturation to the weak binding of denaturants to proteins. Record and co-workers[38,39] generalized this approach into a more sophisticated model in which the weak binding of denaturants can be described in terms of the difference in denaturant concentration between the ''local'' and the ''bulk'' domains. However, Schellman's model simplified the protein-solvent interaction into stoichiometric binding. The problem with regards to this simplification is described elsewhere.[10,21,37] Record *et al.*[38,39] assumed that the solution structure change by the presence of the protein is limited to the first hydration shell, which was shown by computer simulations to be too short ranged.[24,40] These are the reasons why a rigorous statistical mechanical theory is required for the analysis of the cosolvent effects.

Recently, Schellman[41] proposed a refreshing statistical mechanical approach in which both stoichiometric solvent binding and the excluded volume effect are taken into account, and concluded that the competitive contributions of these two effects makes up the m value. This is in contrast to the previous models in which the solvent binding is the sole determinant of the m values.[37–39] What is the source of the difference in pictures? Schellman's theory is based upon the McMillan–Mayer (MM) theory of solution,[42] up to and including the second virial coefficient. The second virial approximation (SVA) is applicable only at a low cosolvent concentration limit, however, solvent denaturation takes place at high denaturant concentrations (i.e., several molars). This necessitates a theory which can be used at such concentration region, and the KB theory can fulfill this requirement. The question regarding the contribution from excluded volume can be answered within this framework.

As will be shown, the KB theory, being a rigorous theory, is able to evaluate whether the interpretation proposed previously is accurate. The widely-popular molecular crowding approach has attributed the effect of stabilizers (i.e., osmolytes and crowders) on the biochemical equilibria to the change upon reaction of the covolume, the volume around the biomolecules from which the cosolvents are excluded.[11,12,14,15] The major factor which determines the covolume change is the second virial coefficient; it describes the distribution of cosolvents around the biomolecules and it makes the dominant contribution to changes of biochemical equilibria. We have shown that this interpretation, which neglects the contribution from the distribution of water around the biomolecules, is an excellent approximation for crowders, but a poor approximation for osmolytes, when describing the solvation of the native protein structures.[23] Is this approach accurate and useful for the interpretation of various biochemical equilibria? How can one know whether this approach is quantitatively accurate? These questions will be answered in this paper by using the KB theory.

Based upon the necessities pointed out above, we will describe in the subsequent sections an analysis of the cosolvent-induced modulation of biochemical reactions based upon the KB theory. We will clarify the mechanism of solvent denaturation, as well as the cosolvents' role on protein-ligand binding and allosteric effects, by using experimental data available in the literature.

II. A STATISTICAL MECHANICAL FOUNDATION

Here we briefly review the KB theory used to extract information regarding protein-solvent interaction from experimental data.[21–23]

A. Biochemical reactions and preferential interaction

Consider a system that consists of water $(i=1)$, biomolecule $(i=2)$, and cosolvent $(i=3)$ molecules, at the density n_i and the chemical potential μ_i. The biomolecule at infinite dilution (i.e., $n_2 \to 0$) undergoes a reaction $\alpha \to \beta$, whose equilibrium constant is K. The equilibrium change upon the addition of cosolvents can be quantified by[10,21]

$$RT\left(\frac{\partial \ln K}{\partial \mu_3}\right)_{T,P,n_2} = \Delta \nu_{23}, \tag{1}$$

where n_i represents the molarity (density) of species i. $\Delta \nu_{23}$ is the change of preferential interaction parameter $\Delta \nu_{23}$ upon the reaction. The preferential interaction parameter for the state σ $(=\alpha$ or $\beta)$ is defined by

$$\nu_{23}^{\sigma} = -\left(\frac{\partial \mu_2^{\sigma}}{\partial \mu_3}\right)_{T,P,n_2}, \tag{2}$$

which can, in principle, be measured by dialysis equilibrium,[10] if, under the given conditions, the biomolecule only takes the state σ.

The preferential interaction parameter ν_{23}^{σ} signifies the excess of cosolvents (as compared to water) solvating the protein.[10] Similarly, it is convenient to introduce the preferential hydration parameter defined as

$$RT\left(\frac{\partial \ln K}{\partial \mu_1}\right)_{T,P,n_2} = \Delta \nu_{21}; \tag{3}$$

which signifies the excess of water (as compared to cosolvent) solvating the protein.[10] The cosolvent-induced equilibrium shift can also be interpreted by the change of preferential hydration parameter as

Downloaded 02 Nov 2004 to 144.32.128.75. Redistribution subject to AIP license or copyright, see http://jcp.aip.org/jcp/copyright.jsp

$$\left(\frac{\partial \ln K}{\partial \mu_1}\right)_{T,P,n_2} = \Delta \nu_{21}; \tag{4}$$

the two parameters are related to each other according to

$$\nu_{21}^{\sigma} = -\frac{n_1}{n_3}\nu_{23}^{\sigma}. \tag{5}$$

The m value can be related to the change of preferential interaction parameter upon denaturation as[10,33,37]

$$m = -\left(\frac{\partial \Delta \mu_2^*}{\partial n_3}\right)_{T,P,n_2\to 0} = \left(\frac{\partial \mu_3}{\partial n_3}\right)_{T,P,n_2\to 0}\Delta \nu_{23} = -\frac{n_3}{n_1}\left(\frac{\partial \mu_3}{\partial n_3}\right)_{T,P,n_2\to 0}\Delta \nu_{21}. \tag{6}$$

B. Kirkwood–Buff theory and the determination of excess solvation numbers

The KB theory connects $\Delta \nu_{23}$ to the changes in excess number of component i around the protein (ΔN_{2i}) as[17,20–23]

$$\Delta \nu_{23} = \Delta N_{23} - \frac{n_3}{n_1}\Delta N_{21}. \tag{7}$$

ΔN_{2i} is defined by[17,20]

$$\Delta N_{2i} = n_i N_A \Delta G_{2i} = n_i N_A (G_{2i}^d - G_{2i}^n), \quad G_{2i}^{\sigma} = \int d\vec{r}[g_{2i}^{\sigma}(\vec{r}) - 1], \tag{8}$$

where $g_{2i}^{\sigma}(\vec{r})$ is the correlation function at the separation $\vec{r}$ between the protein at state σ and the component i, N_A is Avogadro's number, and G_{2i}^{σ} is called the KB parameter. Although equations similar to Eq. (7) have been derived previously from thermodynamic models,[10,37] KB theory gave clear physical meaning to ΔN_{2i} as defined by Eq. (8).[20,21] A justification of Eq. (7) will be given in Sec. III A.

To determine ΔN_{21} and ΔN_{23} from experimental data, the following KB relationship between ΔN_{2i} and the partial molar volume change upon denaturation ΔV_2[21–23] is useful:

$$\Delta V_2 = -V_1 \Delta N_{21} - V_3 \Delta N_{23}, \tag{9}$$

where V_i is the partial molar volume of the species i. It must be emphasized here that the ΔV_2 can be measured experimentally via high pressure experiments and densitometry.[43–48] Recent applications of pressure-perturbation calorimetry also enabled to calculate the volume change upon denaturation in the presence of various cosolvents.[49,50] ΔN_{21} and ΔN_{23}, or ΔG_{21} and ΔG_{23}, can now be determined from experimental data by solving Eqs. (7) and (9). In solving these equations, no assumption is necessary regarding the protein-solvent binding, nor the structure of the native and the denatured states. In this way, the KB theory has synthesized the preferential interaction and volumetric approaches in order to deduce the solvation changes on biochemical reactions.

To explain the mechanism of cosolvent-induced equilibrium shift, previous investigations have focused upon cosolvents' accumulation on (or exclusion from) the protein surfaces.[32,37–39] How can we connect our rigorous statistical mechanical approach to these intuitive approaches? To do so, we note that ΔN_{21} and ΔN_{23} contain contributions from the change of the excluded volume upon reaction ΔV_E. The excluded volume of the state σ is the volume of the region into which solvent molecules cannot penetrate (i.e., $g_{2i}^{\sigma} = 0$). This volume gives a negative contribution to N_{2i}, and the rest of the N_{2i} should represent the *excess number of molecules i from the solvation shell*. How can we extract such excluded volume contribution? To do so, let us compare the density of the species i inside the inpenetrable region and at a point far from the protein surface. The density of the species i is zero in the former, and is n_i at the latter. Therefore, the contribution to the excess number from the entire region of volume V_E is $-n_i V_E$. Upon biochemical reaction, the excluded volume contribution to ΔN_{2i} becomes $-n_i \Delta V_E$. Subtracting this quantity from ΔN_{21} yields the *change of excess number of molecules i in the solvation shell upon biochemical reaction*,[21–23]

$$\Delta N'_{2i} = \Delta N_{2i} + n_i \Delta V_E, \tag{10}$$

in which ΔV_E will later be estimated from protein structure data (see Sec. IV A). It will also be demonstrated that accurate knowledge of ΔV_E may not always be necessary. We emphasize here that Eq. (10) is not a direct result of the KB theory. We also emphasize that this phenomenological scheme has already been proposed and exploited by Chalikian and co-workers,[44–48] by Schellman[41] and by us.[21–23]

III. PREFERENTIAL INTERACTION AND CROWDING APPROACHES

A. Preferential interaction at low cosolvent concentrations

Here we present a justification that Eq. (7) is indeed the correct expression for the preferential interaction parameter ν_{23}. This clarification is necessary, because different expressions have so far been derived for ν_{23} in the literature.[51]

MM theory has been used to relate the preferential interaction parameter to the solution structure. This theory, when employing the SVA (which applies only to the limit $n_3 \to 0$, as denoted by the superscript 0), gives the following expression for the preferential interaction parameter changes:[12,41]

$$\Delta \nu_{23}^0 = n_3 \Delta G_{23}^0 = \Delta N_{23}^0, \tag{11}$$

whereas KB theory, at the same limit, yields[21]

$$\Delta \nu_{23}^0 = n_3(\Delta G_{23}^0 - \Delta G_{21}^0) = \Delta N_{23}^0 - \left(\frac{n_3}{n_1}\Delta N_{21}\right)^0, \tag{12}$$

which is different from Eq. (11). Moreover, Wills and Winzor have derived Eq. (12) by using the MM theory,[14,15] both results [Eqs. (11) and (12)] have been derived from the same MM theory. Which is the correct form of $\Delta \nu_{23}^0$? What is the relationship and the difference between Eqs. (11) and (12)?

These questions are important, because if Eq. (11) is accurate, then the preferential interaction is determined only by the distribution of cosolvents, but not water. In addition, as will be explained in Sec. IV B, these expressions have

Downloaded 02 Nov 2004 to 144.32.128.75. Redistribution subject to AIP license or copyright, see http://jcp.aip.org/jcp/copyright.jsp

different physical implications regarding the explicit contribution of the excluded volume change upon reaction to $\Delta\nu_{23}^0$.

To answer these questions in an intuitive manner, we adopt here a thermodynamic rederivation of KB theory based upon the Gibbs–Duhem equation.[18,19,21] Consider two parts of a biomolecular solution at infinite dilution. The first part contains a biomolecule,

$$0=S'dT+dP+d\Pi-n_2d\mu_2-n_1^*d\mu_1-n_3^*d\mu_3, \quad (13)$$

and the other part is infinitely far from the biomolecule,

$$0=SdT+dP-n_1d\mu_1-n_3d\mu_3. \quad (14)$$

The above are the Gibbs–Duhem equations under constant temperature for each part, where * represents the average densities around the biomolecules, Π denotes osmotic pressure, and S and S' represent the entropy per volume of the respective systems. The excess numbers can be calculated from the densities, according to[18,19]

$$n_i^*-n_i=n_2N_{2i}. \quad (15)$$

In addition, since the biomolecule is in infinite dilution, the first-order term of the osmotic virial expansion is sufficient to express the osmotic pressure Π as $\Pi=kTn_2$.

Now we clarify the origins of Eqs. (11) and (12).

(Case I) Under constant T, P, and n_2, combining Eqs. (13)–(15) yields

$$\nu_{23}=-\left(\frac{\partial\mu_2}{\partial\mu_3}\right)_{T,P,n_2}=N_{23}-\frac{n_3}{n_1}N_{21}, \quad (16)$$

which leads to Eq. (12).

(Case II) Under constant T, μ_1, and n_2, combining Eqs. (13)–(15) yield

$$-\left(\frac{\partial\mu_2}{\partial\mu_3}\right)_{T,\mu_1,n_2}=N_{23}. \quad (17)$$

If we assume that

$$\left(\frac{\partial\mu_2}{\partial\mu_3}\right)_{T,P,n_2}\simeq\left(\frac{\partial\mu_2}{\partial\mu_3}\right)_{T,\mu_1,n_2}, \quad (18)$$

then Eq. (11) follows. The key is to approximate $dP=0$ by $d\mu_1=0$, reminiscent of the different possible "standard states" in the solvation process.[52–54]

The above intuitive argument offers a useful insight into the approximation involved in Eq. (11), which is the foundation of some of the SVA employed in the literature.[12,41]

B. The condition for the dominance of cosolvent distribution in preferential interaction

Whether Eq. (11) holds is an important question when interpreting the cosolvent effect. If Eq. (11) is accurate, the interpretation of cosolvent action can be significantly simplified. We only have to consider the distribution of cosolvent molecules in order to understand $\Delta\nu_{23}^0$.

The molecular crowding picture belongs to this case, because it considers the large exclusion of cosolvents from proteins as a dominant contribution. Schellman's interpretation of cosolvent effects also relies upon the dominance of the cosolvent distribution. The accuracy, however, could not be assessed previously. Here we show the condition upon which Eq. (11) is accurate.

For convenience, we start from the preferential hydration form of Eq. (11),

$$\Delta\nu_{21}^0\simeq-n_1^0\Delta G_{23}^0, \quad (19)$$

where $-\Delta G_{23}^0$ is known as the second cross virial coefficient in molecular crowding literature.[11,12,14,15] This equation is compared to the result of the KB theory,

$$\Delta\nu_{21}^0=n_1(\Delta G_{21}^0-\Delta G_{23}^0), \quad (20)$$

which, as shown before, has an extra ΔG_{21}^0. By using Eq. (9) at the limit $n_3\to0$, this term can be related to the partial molar volume change as[17,20,21]

$$\Delta V_2^0=-V_1^0\Delta N_{21}^0=-\Delta G_{21}^0. \quad (21)$$

It follows from Eqs. (21) and (20) that the accuracy of the molecular crowding approximation can be examined using the following condition:

$$|\Delta\nu_{21}^0|\gg|n_1^0\Delta V_2^0|. \quad (22)$$

This means that the accuracy of the molecular crowding approximation can be examined solely from the experimental data.

IV. PROTEIN DENATURATION AND STABILIZATION BY COSOLVENTS

To clarify the molecular-based mechanism of cosolvent-induced modulation of biochemical equilibria, now we apply the KB theory presented in Sec. III to experimental data available in the literature.

A. Choice and analysis of experimental data

In order to solve Eqs. (7) and (21), data from preferential interaction and volumetric changes are necessary. In spite of a wealth of preferential interaction data[10–13,43] and volumetric data[44,55] for protein-ligand binding and allosteric effects, the combination of preferential interaction and volumetric data for the same biochemical reaction is available in strictly limited cases.[43,55] We have used the data from experiments involving allosteric transition of haemoglobin,[56] the hexokinase-glucose and cytochrome P450-camphor binding reactions[57–59] in order to calculate ΔN_{21} and ΔN_{23}.

Although m values have been measured extensively for GuHCl and urea,[32] the partial molar volume change accompanying the denaturation has rarely been measured.[55] GuHCl denaturation of hen-egg lysozyme ($m=10.445$ kJ/mol/M and $\Delta V_2=-55$ ml/mol) (Refs. 60 and 61) and tendamistat ($m=4.62$ kJ/mol/M and $\Delta V_2=-41.2$ ml/mol) (Ref. 62) are the only monomeric systems with two-state folding equilibria, which report ΔV_2 over a wide denaturant concentration range. The concentration dependence of ΔV_2 in both cases were reported to be negligible.[60–62]

In the treatment of GuHCl denaturation, one encounters a difficulty in treating dissociative salt solutions via KB theory.[18,19,24,63] We therefore adopt a conventional approximate treatment in which cations and anions are assumed to

Downloaded 02 Nov 2004 to 144.32.128.75. Redistribution subject to AIP license or copyright, see http://jcp.aip.org/jcp/copyright.jsp

TABLE I. The contribution of water and stabilizers upon the dissociation of protein-ligand complexes.

Reactions	Cosolvents	$\Delta\nu_{21}^0$ (mol/mol)	$n_1^0\Delta V_2^0$ (mol/mol)	ΔG_{21}^0 (ml/mol)	ΔG_{23}^0 (ml/mol)	ΔV_E (ml/mol)
Hexokinase-glucose dissociation	PEGs	326[a]	−4.2[b]	232.4	−17812	521[a]
Cytochrome P450-camphor dissociation	Osmolytes[c]	19[c]	−1.6[c]	88.6	−963	115[d]
Haemoglobin $T\rightarrow R$	PEGs and osmolytes[e]	65[e]	0[d]	0	−1178	*n/a*[f]
Lysozyme denaturation	GuHCl	−233[g]	−3.0[g]	55	2123	396[h]
Tendamistat denaturation	GuHCl	−99.5[i]	−2.27[i]	41.2	922	*n/a*

[a]From Ref. 57.
[b]From Ref. 58.
[c]From Ref. 59.
[d]From Ref. 21.
[e]From Ref. 56.
[f]Subject to the uncertainty of the structural data (Ref. 21).
[g]From Refs. 60 and 61.
[h]Estimated by Ref. 46. See also footnote (Ref. 72).
[i]From Ref. 62.

be indistinguishable.[63] Consequently, ΔN_{23} here refers to the number of total ions and $V_3/2$ was used to signify the volume of each ion. The remaining parameters in Eqs. (5) and (6) were calculated from thermodynamic data for aqueous GuHCl solutions: V_1 and V_3 were calculated from density data,[64] and $(\partial\mu_3/\partial n_3)_{T,P,n_2\rightarrow 0}$ were obtained from vapor pressure data.[64]

ΔV_E is estimated from the biomolecular structures following the method developed by Chalikian and co-workers.[44–46] In this method, ΔV_E consists of intrinsic volume, i.e., van der Waals volume, and the thermal volume, i.e., volume of inaccessibility due to thermal motion on a thin layer around the biomolecular surface.[44–46] Although it is, in principle, difficult to define the excluded volume, we adopt the method of Chalikian and co-workers, because of its success in explaining volumetric properties of proteins.[44–48]

We have previously reported ΔV_E for protein-ligand reactions calculated from structural data.[21] In contrast, ΔV_E for protein denaturation faces a difficulty regarding the determination of the denatured state structures with sufficient accuracy. This is because the denatured state consists of an enormous number of possible configurations.[34–36] Therefore, an accurate evaluation of excluded volume for such an ensemble is extremely difficult at this stage.[41] Therefore we are forced to make a bold approximation by using an empirical model proposed by Chalikian and Filfil (CF) (Ref. 46) for this purpose. The CF model estimates ΔV_E from the molecular weight of the protein and the degree of unfolding σ ($\sigma=0$ corresponds to the native state, and $\sigma=1$ to the fully extended structure). σ for lysozyme has been estimated to be similar to acid denaturation,[47,48] i.e., $\sigma=0.7$–0.8.[60,61] Therefore, we use $\sigma=0.75\pm0.05$ for lysozyme.[65]

B. The dominance of cosolvent distribution on the preferential interaction change upon biochemical reactions

Here we focus on the cosolvent effects on biochemical equilibria at the limit $n_3\rightarrow 0$. Applying the theory presented in Sec. III B, we can now determine the mechanism of the cosolvent-induced shifts of equilibria, including both protein stabilizers and denaturants.

We first investigate the role of stabilizers. Table I summarizes the experimental data collected from the literature for systems in which $\Delta\nu_{21}^0$ and ΔV_2^0 were available. It is demonstrated clearly for all these cases that Eq. (22) is satisfied. Therefore, cosolvent distribution, described by the change of second cross virial coefficient ($-\Delta G_{23}^0$), accounts for the most of the $\Delta\nu_{21}^0$.

Specifically, the osmolyte-induced modulation of the allosteric transition of haemoglobin, as well as crowder-induced modulation of glucose-hexokinase binding, were first attributed to the change of hydration upon the reaction (i.e., equivalent to $\Delta N_{21}'$).[56,57] This interpretation was controversial,[66–68] and it was shown previously that the hydration change is grossly overestimated by osmotic stress analysis.[21] Table I shows clearly that the stabilizer-induced modulation can be attributed almost entirely to ΔG_{23}^0. This quantity was previously shown to be irrelevant to $\Delta N_{21}'$.[23] Since stabilizers are excluded from protein surfaces,[22,23] $-\Delta G_{23}^0$ reflects the change in the exclusion of osmolytes from protein surfaces, which is the dominant cause of the stabilizer-induced modulation of the allosteric effect. This conclusion supports the previous interpretation based upon the covolume changes.[11,14,15,69]

The above analysis is relevant to another important and unresolved question. How do osmotic and hydrostatic pressures modulate the biochemical equilibria?[43,70,71] It was reported that the effect of osmotic pressure is much larger than the effect of hydrostatic pressure for protein-DNA interactions, and that the equilibrium shift caused by the two pressures may even take different signs.[43,70,71] Therefore, the two pressures were considered to work differently to modulate the biochemical equilibria.[43,70,71] The reason, however, as well as its mechanism and implication, were unclear.[43,70,71] Although it was recognized that these pressures affect different aspects of the equilibrium, the precise difference between

Downloaded 02 Nov 2004 to 144.32.128.75. Redistribution subject to AIP license or copyright, see http://jcp.aip.org/jcp/copyright.jsp

the two was not understood.[43] It is noteworthy here that the "osmotic pressure" in this context refers simply to the change of water activity due to the addition of cosolvents, and does not involve any dialysis equilibrium from which the osmotic stress actually emerges.[66,67] This is the very reason why Timasheff showed that the use of osmotic pressure in this context is a misnomer.[66,67] Indeed, the osmotic pressure effect is represented by[21]

$$RT\left(\frac{\partial \ln K}{\partial \mu_1}\right)_{T,P,n_2} \equiv -\frac{RT}{V_1}\left(\frac{\partial \ln K}{\partial \Pi}\right)_{T,P,n_2} = \Delta \nu_{21}^0, \tag{23}$$

i.e., the modulation of the equilibrium via water activity μ_1. Therefore, the question of "osmotic" versus hydrostatic pressures is now reduced to the comparison in magnitude between $\Delta \nu_{21}^0$ and ΔV_2^0, the theory presented in Sec. III B: the water activity changes modulate the equilibrium via the preferential hydration change $n_3(\Delta G_{23}^0 - \Delta G_{21}^0)$, whereas the hydrostatic pressure change estimates $-\Delta G_{21}^0$. Therefore, much larger equilibrium modulation observed by osmotic pressure than by hydrostatic pressure suggests that the second virial coefficient change is the major contribution to the osmolyte-induced equilibrium shift for protein-DNA interactions.

Now we turn to the GuHCl denaturation of proteins. Table I shows that $\Delta \nu_{21}^0$ is again much larger in magnitude than ΔV_2^0. This suggests that the GuHCl-induced shift of denaturation equilibria can also be attributed to ΔG_{23}^0, the same quantity which characterizes the stabilizer-induced shift of binding and allosteric transition.

What can the ΔG_{23}^0 for denaturation tell us about its mechanism? Table I shows that the ΔG_{23} increases greatly upon denaturation. Protein denaturation is accompanied by an expansion of protein conformation, and therefore by an increase of excluded volume (volume in which $g_{23}=0$). Since the excluded volume contributes *negatively* to KB parameter [Eq. (8)], the contribution of excluded volume change to ΔG_{23} should be negative. The observed *positive* ΔG_{23} suggests that, upon denaturation, the contribution from the region which satisfies $g_{23}>1$ increases. This means, in agreement with Schellman,[41] that the accumulation of GuHCl on protein surface drives ΔG_{23}, and that the accumulation of GuHCl is larger around the denatured state than the native state.

As in protein denaturation, ΔV_E increases when ligands dissociate from proteins. As shown in Table I, this process is accompanied by a *negative* ΔG_{23}, i.e., the opposite sign of the protein denaturation. This suggests that an increase in the stabilizer-exclusion accompanies the protein-ligand dissociation. This also implies that the SVA [Eq. (11)] may not only be accurate for osmolytes and crowders, but also for denaturants.

The above discussion indicates that an intuitive comparison between ΔG_{23} and ΔV_E may be used to judge how equilibria are modulated by cosolvents. This extends the usefulness of molecular crowding approaches which have been applied mostly to the study of stabilizers.

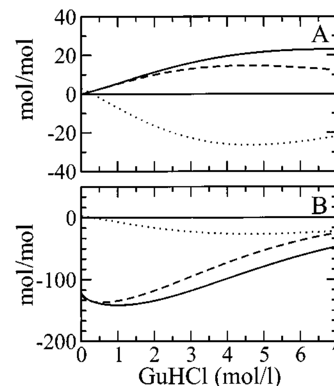

FIG. 1. The role of water and GuHCl upon the GuHCl denaturation of hen-egg lysozyme at 25 °C. (a) $\Delta \nu_{23}$ (solid line), ΔN_{21} (dotted line), and ΔN_{23} (dashed line) against GuHCl concentration. (b) $\Delta \nu_{21}$ (solid line), ΔN_{21} (dotted line), and $-n_1/n_3 \Delta N_{23}$ (dashed line) against GuHCl concentration.

C. Kirkwood-Buff interpretation of denaturation

The SVA approach in the preceding section enabled one to analyze the limit $n_3 \to 0$. This is useful for the osmolyte modulation of biochemical equilibria, where osmolyte concentration is usually not high. However, solvent denaturation occurs at several molars of denaturants. Therefore, the elucidation of solvent denaturation requires the analysis at higher denaturant concentrations. In contrast to the previous MM approaches, our KB approach can determine ΔG_{21} and ΔG_{23} *at any cosolvent concentration.*

1. GuHCl-induced denaturation

Figures 1 and 2 show the analysis of GuHCl-denaturation of lysozyme. At $n_3=0$, ΔN_{21} is positive, suggesting the positive contribution due to the increase of hydration overrides the negative contribution from ΔV_E upon denaturation. At lower GuHCl concentration, $\Delta \nu_{23}$ is determined dominantly by ΔN_{23}, as seen in the preceding section. This is shown more clearly in Fig. 1(b) where the contribution from ΔN_{23} [i.e., $-n_1/n_3 \Delta N_{23}$ (Refs. 21–23)] determines dominantly the preferential hydration parameter $\Delta \nu_{21}$. As the GuHCl concentration increases, ΔN_{21} decreases, and the contribution from ΔG_{21} and ΔN_{21} becomes more prominent in $\Delta \nu_{23}$, wherein ΔG_{21} and ΔG_{23} have opposite signs. The negative ΔG_{21} suggests that at higher GuHCl concentration, the $g_{21}>1$ region does not increase significantly upon denaturation; the increase cannot compensate for the negative contribution to ΔG_{21} from the excluded

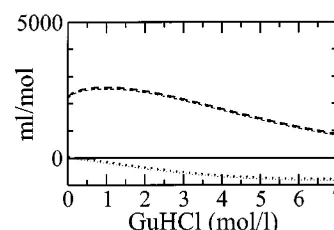

FIG. 2. The change of KB parameters upon the GuHCl denaturation of hen-egg lysozyme. ΔG_{21} (thick dotted line) and ΔG_{23} (thick dashed line) are plotted against GuHCl concentration. Corresponding thin lines were calculated assuming $\Delta V_2=0$.

Downloaded 02 Nov 2004 to 144.32.128.75. Redistribution subject to AIP license or copyright, see http://jcp.aip.org/jcp/copyright.jsp

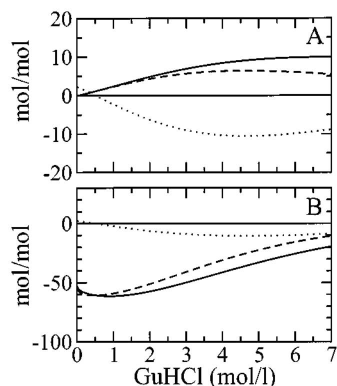

FIG. 3. The role of water and GuHCl upon the GuHCl denaturation of tendamistat at 35 °C. (a) $\Delta\nu_{23}$ (solid line), ΔN_{21} (dotted line), and ΔN_{23} (dashed line) against GuHCl concentration. (b) $\Delta\nu_{21}$ (solid line), ΔN_{21} (dotted line), and $-n_1/n_3\Delta N_{23}$ (dashed line) against GuHCl concentration.

volume change ΔV_E. This suggests that the GuHCl accumulates more significantly than water upon denaturation, and therefore the hydration change upon protein denaturation decreases. This is qualitatively consistent with the previous solvent-exchange viewpoint.[33,37–39] In Figs. 1 and 2, this contribution from hydration becomes more significant as the GuHCl concentration increases. Especially after about $4.5M$, the decrease of ΔG_{23} is more prominent than the increase of ΔG_{21}, suggesting the significant dehydration due to the accumulation of GuHCl in the hydration shell. The same scenario applies for GuHCl-denaturation of tendamistat, whose results are shown in Figs. 3 and 4.

Figures 2 and 4 also show that ΔG_{21} and ΔG_{23} by assuming $\Delta V_2=0$. The accuracy of this approximation suggests the possibility that ΔG_{21} and ΔG_{23} may be calculated even without the volumetric data ΔV_2, when the m value is sufficiently large. As it is clear from Figs. 2 and 4, this approximation yields accurate ΔN_{21} and ΔN_{23} at high GuHCl concentrations. In this region, Eq. (9) yields

$$\frac{\Delta N_{23}}{\Delta N_{21}}=-\frac{V_1}{V_3}, \tag{24}$$

which shows that the excess number ratio is determined by partial molar volume ratio. This is reminescent of the cross section ratio used to estimate the stoichiometry of water-denaturant exchange in the thermodynamic models.[38,39]

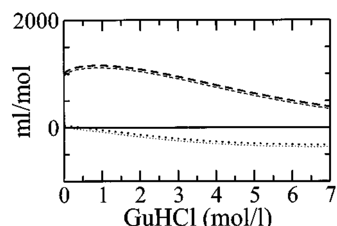

FIG. 4. The change of KB parameters upon the GuHCl denaturation of tendamistat. ΔG_{21} (thick dotted line) and ΔG_{23} (thick dashed line) are plotted against GuHCl concentration. Corresponding thin lines were calculated assuming $\Delta V_2=0$.

More experimental data are necessary to assess whether this useful approximation can be applied to a wide range of proteins and cosolvents.

The deviation of $\Delta\nu_{23}$ from linearity is can be attributed to $(\partial\mu_3/\partial n_3)_{T,P,n_2\to 0}$, when m is constant. Using KB expression for this term,[16–20] Eq. (6) is rewritten as

$$\Delta\nu_{23}=\frac{m}{2kT}n_3[1+n_3(G_{33}-G_{31})]. \tag{25}$$

G_{33}, calculated from experimental value by Chitra and Smith,[24] shows the self-association of GuHCl at low GuHCl concentration, and the decrease thereof at higher concentration. G_{31}, on the other hand, does not change significantly over the change of n_3. Therefore, $G_{33}-G_{31}$ becomes more negative as n_3 increases, and is responsible for the deviation from linearity. This shows that the self-association of denaturant contributes to the preferential interaction parameter.

2. The contribution of the excluded volume effect

Here we investigate how much the excluded volume effect contributes to the m value and to N'_{21} and N'_{23}.

Solvent exchange models,[33,37] as well as local-bulk partitioning model[38,39] have shown that the binding (or accumulation) of denaturant on protein surfaces determines the m value. The binding of denaturants is accompanied by the release of hydrating water via solvent exchange.[33,37–39] SVA,[41] on the other hand, suggested that the m value is determined not only by the solvent exchange, but also by the excluded volume effect: these two large contributions compensate for each other to makeup the m value.

Which of the above explanation is correct? This question can be answered by the theoretical framework of this paper. Since the excess number contribution from the hydration shell was calculated as in Eq. (10), Eq. (7) can be transformed into

$$\Delta\nu_{23}=\Delta N'_{23}-\frac{n_3}{n_1}\Delta N'_{21}, \tag{26}$$

which means that the contribution from the excluded volume changes ΔV_E in ΔN_{21} and ΔN_{23} cancel out. Therefore, KB theory supports the solvent exchange and local-bulk partitioning models that ΔV_E does not contribute to $\Delta\nu_{23}$. The contribution of ΔV_E to $\Delta\nu_{23}$ concluded from the SVA is due to an artifact of the approximation employed [Eq. (18)]. Although the contribution of N_{21} terms is negligibly small for GuHCl denaturation at lower concentrations, neglecting this term leads to a contribution of excluded volume to the m values, inconsistent with KB theory and the thermodynamic models.

The basis of the above argument is Eq. (10). Although this equation is not strictly the result of the KB theory and should be considered phenomenological, this dissection has a clear physical meaning as pointed out in Sec. II. Moreover, in spite of the difference regarding how to estimate ΔV_E (see below), SVA by Schellman employs the same theoretical framework of dissecting the second virial coefficient $-G^0_{23}$ into the contributions from ΔV_E and from the accumulation (exchange) of solvent molecules around the protein. There-

Downloaded 02 Nov 2004 to 144.32.128.75. Redistribution subject to AIP license or copyright, see http://jcp.aip.org/jcp/copyright.jsp

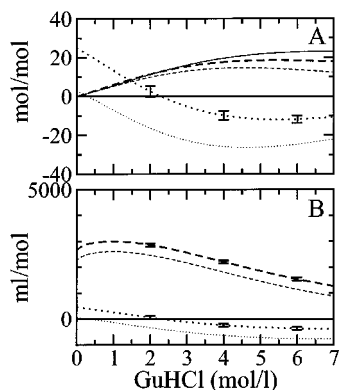

FIG. 5. The contributions of water and GuHCl from the hydration shell upon the GuHCl-denaturation of hen-egg lysozyme at 25 °C. (a) $\Delta N'_{21}$ (thick dotted line) and $\Delta N'_{23}$ (thick dashed line) against GuHCl concentration are compared with ν_{23} (thin solid line), ΔN_{21} (thin dotted line) and ΔN_{23} (thin dashed line). (b) $\Delta G_{21}+\Delta V_E$ (thick dotted line) and $\Delta G_{23}+\Delta V_E$ (thick dashed line) are plotted against GuHCl concentration and compared to ΔG_{21} (thin dotted line) and ΔG_{23} (thin dashed line). The error bars are calculated by the assumed variation in the degrees of unfolding σ (see text), which is not shown for $\Delta N'_{23}$ where the error bar is very small.

fore, the analysis presented here suggests, at least within the phenomenological dissection scheme employed by the volumetric analysis[44–48] and by Schellman,[41] that ΔV_E does not contribute explicitly to $\Delta \nu_{23}$.

Next we investigate the contribution of ΔV_E in $\Delta N'_{23}$. Figure 5 suggests that the contribution of ΔV_E (i.e., the difference between $\Delta N'_{23}$ and ΔN_{23}) is small in ΔN_{23}. This indicates that the accumulated GuHCl in the vicinity of proteins makes up the majority of $\Delta N'_{23}$, and consequently of the m value. This is consistent with the common view that the accumulation of GuHCl is the cause of denaturation.[5–7,10,33,37] This also shows that the contribution from the hydration change $(-n_3\Delta N'_{21}/n_1)$ is small. However, the above contention is subject to the accuracy of the estimation of ΔV_E employed here. It is emphasized that, due to the experimental and computational difficulty, the accurate estimation of ΔV_E is extremely difficult at this stage.[72–74] Nevertheless, it is at least clear, from a discussion which does not involve numerical values of ΔV_E presented in Sec. IV, that the accumulation of GuHCl is the driving force of denaturation and that ΔV_E is an opposing contribution, which is qualitatively in agreement with Schellman's.[41] We believe that the CF model gives a better estimation of ΔV_E than the steric polypeptide model based approach adopted by Schellman.[72] However, a better estimation of ΔV_E is necessary to examine this issue. To this end, a precise characterization of denatured state ensembles is indispensable.

V. CONCLUSION

In this paper, we demonstrated that the combination of preferential interaction and volumetric experiments can give valuable information on how cosolvent molecules modulate the biochemical equilibria. The KB theory is used to formulate the theoretical foundation of this approach.

We have shown that solvent accumulation and exchange upon protein denaturation can be estimated directly by combining m value and volumetric data. The analysis presented here supports the previously proposed thermodynamic models in which the accumulation of GuHCl (and the consequent dehydration) determines the m values is the driving force of denaturation. The change of excluded volume is shown to make a small, opposing contribution. However, we realize that the estimation of the excluded volume used here leaves a room for improvement, and therefore an extensive computer simulation on the structures of denatured state ensemble is necessary to refine the estimation of its contribution. In addition, KB integrals (founded in statistical mechanics) have been used to express excess solvation numbers, and have served to generalize previous assumptions about solvent binding at protein surfaces introduced phenomenologically to explain the roles of cosolvents.

Furthermore, KB theory was applied to analyze the mechanism of osmolyte- and crowder-induced modulation of protein-ligand binding and allosteric effects. A simple comparison of preferential interaction and volumetric data was proposed to assess whether the distribution of cosolvent is the dominant factor in the cosolvent-induced equilibrium shift. The distribution of stabilizers was shown to account for the stabilizer-induced shifts of equilibria. This justifies the molecular crowding approach, which assumes that the exclusion of stabilizers from biomolecular surfaces enhances protein stabilization and protein-ligand binding.[10–15] Moreover, the KB theory, when applied to solvent denaturation, supports the classical view that the preferential accumulation of denaturant molecules on protein surfaces drive the protein denaturation.[32,37–39] We have shown that the difference of action between these two classes of cosolvents can be clarified from the comparison of the second osmotic virial coefficient change with the excluded volume change.

The above analyses have demonstrated the value of KB theory in analyzing the cosolvent effects on proteins, by generalizing the previous thermodynamic models for the cosolvent effects, as well as the molecular crowding analysis. In addition, the KB theory is free from the assumption [Eq. (18)] when applying the MM theory. Furthermore, the KB theory is applicable to any cosolvent concentration, whereas the MM theory can only be applicable to infinite dilution of cosolvents.

A remaining issue, however, is to examine an alternative explanation of protein denaturation and stabilization based upon the enhancement and breaking of "water structure."[75–83] In addition, the understanding of the cosolvent effects from the solvent exposure of each residues, as well as the contact between them, still remains to be addressed.[84–91]

The present paper established a guideline for experiments; the combination of preferential interaction, high pressure measurements, as well as the structural information is indispensable to elucidate the mechanism of chemical denaturantion of proteins. We hope further experiments will be undertaken, especially the high pressure experiments in the presence of denaturants, such as GuHCl and urea.

Downloaded 02 Nov 2004 to 144.32.128.75. Redistribution subject to AIP license or copyright, see http://jcp.aip.org/jcp/copyright.jsp

ACKNOWLEDGMENTS

This paper is dedicated to Eleanor and Guy Dodson for their long association with the University of York. The authors are grateful to T. Chalikian for helpful correspondence, and to T. Gloster for computational assistance. S.S. gratefully acknowledges an award from the Nuffield Foundation (Grant No. NAL/00920/G).

[1] W. Kauzmann, Adv. Protein Chem. **14**, 1 (1959).
[2] K. A. Dill, Biochemistry **29**, 7133 (1990).
[3] H. S. Chan, S. Shimizu, and H. Kaya, Methods Enzymol. **380**, 350 (2004).
[4] W. Ramsden, J. Physiol. (Proc. Physiological Soc.) **28**, xxiii (1902).
[5] C. Tanford, Adv. Protein Chem. **24**, 1 (1970).
[6] C. N. Pace, CRC Crit. Rev. Biochem. **3**, 1 (1975).
[7] C. N. Pace, Methods Enzymol. **131**, 266 (1986).
[8] P. W. Hochachka and G. N. Somero, *Biochemical Adaptation: Mechanism and Process in Physiological Evolution* (Oxford University Press, Oxford, 2002).
[9] P. H. Yancey, M. E. Clark, S. C. Hand, R. D. Bowlus, and G. N. Somero, Science **217**, 1214 (1982).
[10] S. N. Timasheff, Adv. Protein Chem. **51**, 355 (1998).
[11] P. W. Davis-Searles, A. J. Saunders, D. A. Erie, D. J. Winzor, and G. J. Pielak, Annu. Rev. Biophys. Biomol. Struct. **30**, 271 (2001).
[12] A. P. Minton, Methods Enzymol. **295**, 127 (1998).
[13] D. W. Bolen and I. V. Baskakov, J. Mol. Biol. **310**, 955 (2001).
[14] D. J. Winzor and P. R. Wills, Biophys. Chem. **25**, 243 (1986).
[15] P. R. Wills and D. J. Winzor, Biopolymers **33**, 1627 (1993).
[16] J. G. Kirkwood and F. P. Buff, J. Chem. Phys. **19**, 774 (1951).
[17] A. Ben-Naim, *Statistical Thermodynamics for Chemists and Biochemists* (Plenum, New York, 1992), pp. 372–382 and 448–457.
[18] D. G. Hall, Trans. Faraday Soc. **67**, 2516 (1971).
[19] K. E. Newman, Chem. Soc. Rev. **23**, 31 (1994).
[20] R. Chitra and P. E. Smith, J. Phys. Chem. B **105**, 11513 (2001).
[21] S. Shimizu, Proc. Natl. Acad. Sci. U.S.A. **101**, 1195 (2004).
[22] S. Shimizu, J. Chem. Phys. **120**, 4989 (2004).
[23] S. Shimizu and D. J. Smith, J. Chem. Phys. **121**, 1148 (2004).
[24] R. Chitra and P. E. Smith, J. Phys. Chem. B **106**, 1491 (2002).
[25] T. Imai, M. Kinoshita, and F. Hirata, J. Chem. Phys. **112**, 9469 (2000).
[26] N. F. A. van der Vegt and W. F. van Gunsteren, J. Phys. Chem. B **108**, 1056 (2004).
[27] R. D. Mountain and D. Thirumalai, J. Phys. Chem. B **108**, 6826 (2004).
[28] J. Zielkiewicz and J. Mazerski, J. Phys. Chem. B **106**, 861 (2002).
[29] I. Shulgin and E. Ruckenstein, J. Phys. Chem. B **103**, 2496 (1999).
[30] Y. Marcus, Phys. Chem. Chem. Phys. **4**, 4462 (2002).
[31] E. Matteoli, J. Phys. Chem. B **101**, 9800 (1997).
[32] J. K. Myers, C. N. Pace, and J. M. Scholtz, Protein Sci. **4**, 2138 (1995).
[33] J. A. Schellman, Biopolymers **17**, 1305 (1978).
[34] J. Klein-Seetharaman, M. Oikawa, S. B. Grimshaw *et al.*, Science **295**, 1719 (2002).
[35] I. V. Baskakov and D. W. Bolen, Biochemistry **37**, 18010 (1998).
[36] J. Wrabl and D. Shortle, Nat. Struct. Biol. **6**, 876 (1999).
[37] J. A. Schellman, Biopolymers **26**, 549 (1987).
[38] E. S. Courtenay, M. W. Capp, R. M. Saecker, and M. T. Record, Proteins **4**, 72 (2000).
[39] E. S. Courtenay, M. W. Capp, and M. T. Record, Protein Sci. **10**, 2485 (2001).
[40] B. M. Baynes and B. L. Trout, J. Phys. Chem. B **107**, 14058 (2003).
[41] J. A. Schellman, Biophys. J. **85**, 108 (2003).
[42] W. G. McMillan and J. E. Mayer, J. Chem. Phys. **13**, 276 (1945).
[43] J. A. Kornblatt and M. J. Kornblatt, Biochim. Biophys. Acta **1595**, 30 (2002).
[44] T. V. Chalikian, Annu. Rev. Biophys. Biomol. Struct. **32**, 207 (2003).
[45] T. V. Chalikian, J. Phys. Chem. B **105**, 12566 (2001).
[46] T. V. Chalikian and R. Filfil, Biophys. Chem. **104**, 489 (2003).
[47] T. V. Chalikian, V. S. Gindikin, and K. J. Breslauer, J. Mol. Biol. **250**, 291 (1995).
[48] T. V. Chalikian, V. S. Gindikin, and K. J. Breslauer, FASEB J. **10**, 164 (1996).
[49] R. Ravindra and R. Winter, Chem. Phys. Chem. **5**, 566 (2004).
[50] R. Ravindra, C. Royer, and R. Winter, Phys. Chem. Chem. Phys. **6**, 1952 (2004).
[51] The superscript σ has been omitted throughtout this section, with an understanding that ν_{21} and ν_{23} refer to the value for a given conformational state.
[52] H. L. Friedman, J. Chem. Phys. **32**, 1351 (1960).
[53] H. Cabezas and J. P. O'Conell, Ind. Eng. Chem. Res. **32**, 2892 (1993).
[54] L. L. Lee, J. Mol. Liq. **87**, 129 (2000).
[55] S. Perrett and J. M. Zhou, Biochim. Biophys. Acta **1595**, 210 (2002).
[56] M. F. Colombo, D. C. Rau, and V. A. Parsegian, Science **256**, 2083 (1992).
[57] C. Reid and R. P. Rand, Biophys. J. **72**, 1022 (1997).
[58] R. Filfil and T. V. Chalikian, FEBS Lett. **554**, 351 (2003).
[59] C. Di Primo, E. Deprez, G. Hui Bon Hoa, and P. Douzou, Biophys. J. **68**, 2056 (1995).
[60] K. Sasahara, M. Sakurai, and K. Nitta, Proteins **44**, 180 (2001).
[61] K. Sasahara, M. Sakurai, and K. Nitta, J. Mol. Biol. **291**, 693 (1999).
[62] G. Pappenberger, C. Sauden, M. Becker, A. E. Merbach, and T. Kiefhaber, Proc. Natl. Acad. Sci. U.S.A. **97**, 17 (2000).
[63] H. L. Friedman and P. S. Ramanathan, J. Phys. Chem. **74**, 3756 (1970).
[64] G. I. Makhatadze, J. Fernandez, E. Freire, T. H. Lilley, and P. I. Privalov, J. Chem. Eng. Data **38**, 83 (1993).
[65] There is no study reported on the GuHCl denatured state structures on tendamistat, nor the CF model (Ref. 46) applicable to tendamistat (7.9 KDa; smaller than the range of applcability of the CF model 12–30 kDa). Therefore, no calculation of ΔV_E was performed for this case.
[66] S. N. Timasheff, Proc. Natl. Acad. Sci. U.S.A. **95**, 7363 (1998).
[67] S. N. Timasheff, Proc. Natl. Acad. Sci. U.S.A. **99**, 9721 (2002).
[68] V. A. Parsegian, R. P. Rand, and D. C. Rau, Proc. Natl. Acad. Sci. U.S.A. **97**, 3987 (2000).
[69] D. Winzor and P. R. Wills, Biophys. Chem. **57**, 103 (1995).
[70] C. R. Robinson and S. G. Sligar, Methods Enzymol. **259**, 395 (1995).
[71] C. R. Robinson and S. G. Sligar, Biochemistry **33**, 3787 (1994).
[72] Indeed, the estimated ΔV_E is much smaller than Schellman (Ref. 41). It is noteworthy, however, that the denatured state structures used by Schellman was estimated from short peptides, whose conformation was calculated by incorporating the steric repulsion only (Ref. 72). It was shown that such calculation gives structures far more expanded than GuHCl-denatured structures (Ref. 74). However, we emphasize that the CF model estimation adopted here employs (a) a phenomenological parameter σ expressing the degree of unfolding (Ref. 46) which has been estimated from experiments and (b) the empirical relationship between molecular weight and protein size (Ref. 46). The CF model estimation is shown to reproduce semiquantitatively the volume and compressibility changes upon protein denaturation (Ref. 46). However, the CF model should not be considered to be accurate due to its underlying assumptions. We were forced to adopt this approach, due to the lack of experimental data necessary for the characterization of the ensemble of GuHCl-denatured structures.
[73] T. P. Creamer, R. Srinivasan, and G. D. Rose, Biochemistry **36**, 2832 (1997).
[74] D. P. Goldenberg, J. Mol. Biol. **326**, 1615 (2003).
[75] H. S. Frank and F. Franks, J. Chem. Phys. **48**, 4746 (1968).
[76] F. Franks, Biophys. Chem. **96**, 117 (2002).
[77] N. Muller, J. Phys. Chem. **94**, 3856 (1990).
[78] J. L. Finney and A. K. Soper, Chem. Soc. Rev. **23**, 1 (1994).
[79] J. Tsai, M. Gerstein, and M. Levitt, J. Chem. Phys. **104**, 9417 (1996).
[80] A. Idrissi, F. Sokolic, and A. Perera, J. Chem. Phys. **112**, 9479 (2000).
[81] A. K. Soper, E. W. Castner, and A. Luzar, Biophys. Chem. **105**, 649 (2003).
[82] S. Moelbert and P. De Los Rios, J. Chem. Phys. **119**, 7988 (2003).
[83] P. E. Mason, G. W. Neilson, C. E. Dempsey, A. C. Barnes, and J. M. Cruickshank, Proc. Natl. Acad. Sci. U.S.A. **100**, 4557 (2003).
[84] P. K. Nandi and D. R. Robinson, Biochemistry **23**, 6661 (1984).
[85] Q. Zou, S. M. Habermann-Rottinghaus, and K. P. Murphy, Proteins **31**, 107 (1998).
[86] A. Wallqvist, D. G. Covell, and D. Thirumalai, J. Am. Chem. Soc. **120**, 427 (1998).
[87] S. Shimizu and K. Shimizu, J. Am. Chem. Soc. **121**, 2387 (1999).
[88] M. Ikeguchi, S. Nakamura, and K. Shimizu, J. Am. Chem. Soc. **123**, 677 (2001).
[89] G. Graziano, J. Phys. Chem. B **115**, 2632 (2001).
[90] G. Graziano, Can. J. Chem. **80**, 388 (2002).
[91] S. Shimizu and H. S. Chan, Proteins **49**, 560 (2002).

Downloaded 02 Nov 2004 to 144.32.128.75. Redistribution subject to AIP license or copyright, see http://jcp.aip.org/jcp/copyright.jsp