Interfacial Water Structure Controls Protein Conformation

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A phenomenological theory of salt-induced Hofmeister phenomena is presented, based on a relation between protein solubility in salt solutions and protein−water interfacial tension. As a generalization of previous treatments, it implies that both kosmotropic salting out and chaotropic salting in are manifested via salt-induced changes of the hydrophobic/hydrophilic properties of protein−water interfaces. The theory is applied to describe the salt-dependent free energy profiles of proteins as a function of their water-exposed surface area. On this basis, three classes of protein conformations have been distinguished, and their existence experimentally demonstrated using the examples of bacteriorhodopsin and myoglobin. The experimental results support the ability of the new formalism to account for the diverse manifestations of salt effects on protein conformation, dynamics, and stability, and to resolve the puzzle of chaotropes stabilizing certain proteins (and other anomalies). It is also shown that the relation between interfacial tension and protein structural stability is straightforwardly linked to protein conformational fluctuations, providing a keystone for the microscopic interpretation of Hofmeister effects. Implications of the results concerning the use of Hofmeister effects in the experimental study of protein function are discussed.

1. Introduction

It is known, but not understood why, that certain salts, often called "chaotropes", destabilize many proteins when added to their solutions and conversely "kosmotropes" stabilize them.[1−3] It is experimentally established that the effects are dominated by anions (rather than cations), which can be ranked according to their ability to stabilize or destabilize protein conformation. It was also found that this series of ions is correlated with their ability to precipitate proteins. These effects, that are often called Hofmeister effects after their first systematic investigator,[1] are not restricted to proteins and have been observed more widely (e.g., in colloidal suspensions[4]). Disturbingly, however, over the years, a relatively small number of persistent exceptions have also been observed.[3] (Note that the Hofmeister series has been established with globular rather than fibrous proteins.)

A number of attempts have been made to provide a theoretical foundation for the effects (see the comprehensive reviews of Collins and Washabaugh,[2] Cacace et al.,[3] and a recent update[4]). To date, however, they have only been partially successful, and there is still no unifying formalism covering the entire spectrum of salts from salting out (i.e., precipitants, also known as structural stabilizers) to salting in (i.e., solubilizers, also known as structural destabilizers). Elaborate models postulating diverse salt effects on polyelectrolytes mediated by electrostatic or Lifshitz−van der Waals forces[5,6] have periodically surfaced in the literature, but their direct application to such complex phenomena as protein conformational changes and especially explaining the behavior of the "exceptions" still seems to be a great challenge.

Our aim in this paper is to demonstrate that a thermodynamical formalism based on protein−water interfacial tension is able to serve as a guide to understand Hofmeister effects on protein conformational changes. A crucial divagation from previous treatments aiming at the same target is that we do not make use of the artificial contrivance of placing the protein into solution from air or vacuum; therefore, we are not constrained to use air−water interfacial tension as a key quantity to explain Hofmeister phenomena in the entire range of observed behavior. In the main body of this paper, we develop the theory (in the form of a general equation) linking protein−solution interfacial tensions to the concentration of Hofmeister salts. We also present new measurements of the infrared spectra of water-containing kosmotropes and chaotropes in order to demonstrate a microscopic interpretation of our general equation in terms of water structural changes mediated by hydrogen bonding. We go on to discuss the implications of our general equation for protein conformation. What emerges from this is a useful classification of dynamical conformational type according to the profile of the total free energy as a function of interfacial area. New measurements of the conformational fluctuations of myoglobin and the thermal destabilization of bacteriorhodopsin

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in the presence of different salts are presented, in order to provide examples of the unexpected behavior of the rarer protein classes, which are however readily interpretable using our theory.

2. The "Interfacial Tension" Concept

We can rationalize Hofmeister effects by considering the contribution of interfacial energy to the total free energy change of the protein. In general, for any process (e.g., dissolution, conformational change), we have

$$\Delta G_{\text{total}} = \Delta G_{\text{protein interior}} + \Delta G_{\text{interfacial}} \quad (1)$$

Precipitation, for example, diminishes the protein−solvent interfacial area, and segregation or conformational destabilization (denaturation of a globular protein) increases it. In the following, we use this principle (the same as that used in the thermodynamical establishment of hydrophobic interactions[7]), together with verified theoretical and experimental equations for solubility, so as to establish a general relationship between protein−water interfacial tension and salt concentration.

In order to link solubility with interfacial tension, we consider the situation when a solid and its solution (e.g., in water) are in chemical equilibrium. It is a textbook result (see, e.g., ref 8) that

$$\mu_s = \mu_l + RT \ln x \quad (2)$$

where μ_s and μ_l are the chemical potentials of the solid and pure solute (the individual, isolated solute molecules), respectively, and x is the mole fraction of the solute in saturated solution (i.e., its solubility).[9]

Let us now consider the same solid (e.g., a protein) in another solvent (e.g., an aqueous solution of a Hofmeister salt). Analogously to eq 2, we now have

$$\tilde{\mu}_s = \mu_l + RT \ln \tilde{x} \quad (3)$$

where the tilde marks the quantities relevant to the other solvent. Note that μ_l is the same in both eqs 2 and 3 while μ_s and $\tilde{\mu}_s$ will differ only in an interfacial term, γA, since the bulk of the solid is the same in both cases. Subtracting eq 2 from eq 3, we get

$$\tilde{\mu}_s - \mu_s = (\tilde{\gamma} - \gamma)A_{pw} = RT \ln \tilde{x}/x \quad (4)$$

where γ and $\tilde{\gamma}$ are the interfacial tensions of pure water and (Hofmeister) salt solution, respectively, and the subscripts "p" and "w" refer to "protein" and "water", respectively. Although both x and $\tilde{x}$ are small ($<10^{-5}$) for most cases of practical interest, given that protein molecules are large, for real solutions, we can replace the ratio $\tilde{x}/x$ by $\tilde{a}/a$, where $a = \alpha x$ and $\tilde{a} = \tilde{\alpha}\tilde{x}$ are the corresponding activities of the dissolved protein molecules, with the α being the activity coefficients. In different salt solutions, the activity coefficients for the same protein are unlikely to differ significantly from each other; hence, we can reasonably approximate $\tilde{a}/a$ by the ratio of solubilities in molar concentration, $\tilde{S}/S$. Equation 4 now yields

$$\ln \frac{\tilde{S}}{S} = \frac{(\tilde{\gamma} - \gamma)A_{pw}}{RT} \quad (5)$$

Over 100 years ago, Setschenow established an empirical law linking the solubility of a protein with cosolute (salt) concentration:[10]

$$\ln \frac{\tilde{S}}{S} = K_s c \quad (6)$$

where K_s is the Setschenow constant (it is positive for kosmotropes and negative for chaotropes[3]) and c is the cosolute concentration. This law has meanwhile accumulated a convincing body of empirical support at high concentrations.

Comparing eqs 5 and 6, and denoting the difference of the protein−solution interfacial tensions in the presence and absence of salt in the solution by $\Delta\gamma$, we get the main theoretical result of this paper:

$$\tilde{\gamma} - \gamma = \Delta\gamma = \frac{RT K_s c}{A_{pw}} \quad (7)$$

We can interpret eq 7 by making use of the analogy between our treatment and that previously given for the hydrophobic interaction:[7] Hofmeister salts change the hydrophobic/hydrophilic properties of protein−water interfaces, with kosmotropes making them more "hydrophobic" and chaotropes making them more "hydrophilic". (Here, we use these terms in their general, phenomenological sense, namely, exclusively with respect to surface free energy changes.) As we shall see later, such a unified concept that brings kosmotropic and chaotropic effects under the same umbrella is rather useful in interpreting the diversity of Hofmeister effects on protein conformation.

Equation 7 can be considered as a generalization of previous results that gave rise to interpretations of kosmotropic salting out in a similar way.[12−14] After these pioneering papers, the surface tension of salt solutions in air has long been considered an experimental physical quantity that provides an approximate guide to Hofmeister effects.[2,3] This has been an important perception that gave a strong hint that the origin of Hofmeister effects must be connected to salt-induced water structure changes. However, it gave a straightforward (and qualitative) interpretation only for one side of the Hofmeister phenomena, namely, kosmotropic salting out, whereas the other side (chaotropic effects) remained elusive within this framework. (Other factors had to be considered to describe the solubilizing effect of chaotropes, such as a generalized electrostatic interaction between chaotropic ions and the protein.[12] Although such effects might in fact occur, but being specific to the protein−electrolyte interface, they could not be included in the air−water surface tension formalism, whereas they are naturally involved in protein−water surface tension.) More formally, as the Setschenow constant, K_s, can either be positive or negative, showing that kosmotropic and chaotropic effects are of opposite sign, the salt-induced change of protein−water interfacial tension, $\Delta\gamma$, can also be either positive or negative, according to eq 7. This is in sharp contrast to the behavior at the air−water interface, characterized by the Heydweiller equation[11]

$$\tilde{\gamma}_a - \gamma_a = K_H c$$

where the subscript "a" denotes the air−water interface; the Heydweiller constant, K_H (also called the surface tension increment in the literature), is always positive,[2] with chaotropes merely having smaller values than kosmotropes. In other words, it means that, although the surface tension increment indeed carries some information relevant to Hofmeister phenomena, it cannot reproduce the correct sign of the effects. This is why eventually it became clear that, despite its merits, air−water surface tension cannot be used as a general description of the free energy changes associated with salt-induced changes of

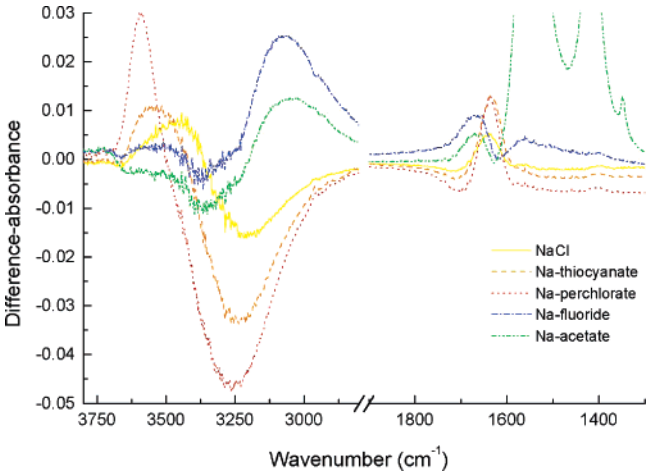

Figure 1. Difference FTIR spectra (aqueous salt solution − pure water) of monovalent sodium salts. The anions are chloride, thiocyanate, perchlorate, fluoride, and acetate.

protein solubility and conformation. Instead, on the basis of eq 7, we propose protein−water interfacial tension to be such a quantity.

In general terms, the interfacial tension is determined by the cohesion and adhesion free energies within and between phases separated by the interface, respectively. In the particular case of a protein−salt solution interface, it depends on the following three factors: (a) the nature of surface-exposed amino acid residues, (b) the strength of H-bonding between water molecules, and (c) the excess surface concentration of the cosolute (Hofmeister salt) (see, e.g., ref 8). From factor a, it trivially follows that $\Delta\gamma$ should depend on the choice of the protein. In fact, K_s in eq 7 depends also on the protein, besides the nature of the salt. Addition of a Hofmeister salt as a cosolute to the system is expected to affect factors b and c. Although, to our knowledge, protein−water interfacial tensions have not yet been measured directly, there are a number of experimental data available in the literature concerning parameters relevant to factors b and c,[3,15,16] that should be informative regarding protein−water interfacial tensions. Since cohesion forces between water molecules are expected to play a primordial role in determining surface tensions at both air−water and protein−water interfaces, we carried out new Fourier transform infrared (FTIR) experiments in different salt solutions to check whether the effect of Hofmeister salts on the strength of H-bonds in water is consistent with our theory.

2.1. Infrared Spectroscopy of Salt Solutions. Analytical grade salts were dissolved in triply distilled water at a concentration of 0.500 M. Infrared spectra were recorded at room temperature (21.0 °C) on a single beam FTIR spectrometer (Bruker) using an attenuated total reflection cell. The spectrum of the pure (triply distilled) water was subtracted in order to yield difference spectra. Figure 1 shows that the most striking spectral differences due to the presence of the different salts are around 3400 cm^{-1}, that is, the intramolecular OH stretching band. A shift to larger wavenumbers implies weakened intermolecular H-bonds, and conversely, a shift to smaller wavenumbers implies strengthening (note that very similar difference spectra can be obtained by measuring the IR spectra of water at different temperatures (data not shown), in agreement with the fact that lower temperatures strengthen the H-bonding and higher temperatures weaken it[17]).

In order of increasing weakening, the order is fluoride < acetate < chloride < thiocyanate < perchlorate. Fluoride and acetate strengthen compared to pure water, and the others weaken, with chloride giving the smallest changes—in other words, the sign of the effect crosses over at chloride. This already gives a clue that there is a link to the similar change of sign of the Setschenow constant.

Taking into account results from Raman spectroscopy[15] and neutron scattering,[16] it would be straightforward to infer from our results that salt effects on the surface tension of water are direct consequences of changes in the cohesive H-bonded water network, that is, controlled by electron donor−acceptor interactions between salt and water. Recent experimental evidence of Omta et al., however, has called this inference into question by showing that salt-induced changes in H-bonding are restricted to the hydration shell of the ions and do not extend into the bulk.[18] In order to resolve this apparent controversy, we recall the theoretical treatment of ref 19. There it has been pointed out that (anionic) kosmotropes, at high concentrations, are excluded from interfaces, while (anionic) chaotropes have the opposite tendency, at least whenever the cations can approach the surface (as in the case of most proteins, the majority of which are negatively charged at physiological pH). This effect, which is in agreement with experimental observations for proteins at high salt concentrations,[3] is just a consequence of the water structure-making or structure-breaking properties of kosmotropic or chaotropic anions, respectively. Depletion of cosolutes from an interface is known to cause an increase, and accumulation, a decrease, of interfacial tension (see, e.g., ref 19). This means that, irrespective of whether the effect of ions on the H-bond structure of water is global or local, kosmotropes will inevitably increase, while chaotropes inevitably decrease protein−water interfacial tension at high salt concentration (where Setschenow's law is valid), in perfect agreement with our theory.

Contrary to protein−water interfaces, the air−water interface behaves differently. Because of the strongly repulsive electrostatic image forces, ions are always depleted from the water layer adjacent to the air surface, giving rise to an increase of surface tension upon addition of any salt (i.e., K_H is always positive). The reason why chaotropes have smaller effects than kosmotropes[20] presumably arises from the opposite effect of these ions on the water cohesion forces (due to H-bonding). (Realizing the possible role of solute binding to the protein surface upon protein denaturation, Lin and Timasheff introduced a binding term in the free energy, whose contribution may compensate for that of the usual surface tension increment.[21] The main conceptual problem here, similar to that encountered in refs 12 and 13, is that K_H, being characteristic of the air−water interface, does not show up at protein surfaces, and consequently, there is nothing to compensate for in such cases.) Note that our treatment concerns the "classical" Hofmeister series of Brønsted-neutral (i.e., neither acidic nor basic) salts; the microscopic mechanism of action of other known kosmotropic and chaotropic agents (e.g., polyols, urea derivatives, etc.) may be different. Nevertheless, it is known that even nonionic kosmotropes or chaotropes are respectively excluded from, or accumulated at, protein surfaces.[3] We anticipate therefore that our arguments concerning the primordial role of interfacial tension are sufficiently general to allow eq 7 to be extended to such cases, whatever their microscopic interpretation is,[22] but this remains to be verified.

2.2. Use of the Term "Interfacial Tension" on a Molecular Scale. The interfacial tension, γ, is a well-defined macroscopic quantity for pure liquids contacting a solid, and contains all relevant information, including the usually dominant, and just discussed, electron donor−acceptor interactions typified by hydrogen bonding as well as Lifshitz−van der Waals interac-

tions. There is an old debate, however, concerning the use of surface tensions at microscopic or mesoscopic dimensions, such as in the world of macromolecules. The physical rationale for its use comes from the definition of interfacial tension itself: $\gamma_{pw} = \delta G/\delta A$, where δA denotes the change of water-exposed surface area, due, for example, to a conformational change of a protein. Since accessible surface areas can be established for proteins,[23] a free energy change associated with δA, in principle and often in practice, can also be defined.[24] Note that some authors call attention to the problem of using such a definition for a quantitative decription of protein free energies (see, e.g., ref 25). On the other hand, others argue (see, e.g. refs 26 and 27) that the interfacial tension between the solute and water and the accessible surface area of the solute are the basic factors contributing to creating a cavity in water, although the macroscopic surface tension must be corrected by a factor for the radius of curvature of the microscopic cavity. Without entering into this discussion in detail, it seems plausible to assume that a molecular-sized surface should respond in a manner qualitatively similar to that of a macroscopic surface of the same kind to changes in the thermodynamic activity and hydrogen-bonding properties of bulk water (see also ref 13). Consequently, the concept of interfacial tension should give a useful guide to what is happening at molecular interfaces.

At the same time, one should bear in mind that the concept of macroscopic surface tension to the protein−water interface is a simplification; therefore, all arguments based directly on eq 7 are only of qualitative nature, and it is clear that a precise analysis of microscopic surfaces requires further generalization of the models based on macroscopic quantities. In a recent work, it has been pointed out that, when describing the energy cost of cavity formation, fluctuation-related terms replace surface tension when working at microscopic dimensions.[28] Nevertheless, as we demonstrate below, the interfacial tension approach turns out to be rather fruitful in the sense that it helps to make order in "chaos" by enabling a model based on simple physical considerations for the interpretation of the most diverse manifestations of Hofmeister effects to be constructed. Furthermore, this model offers a natural way for extension down to atomic dimensions, implying the existence of Hofmeister ion-dependent fluctuations at the interface.

3. Protein Free Energy Landscape

What are the implications of eq 7 for protein stability and structural fluctuations? It is known empirically that kosmotropes usually stabilize native protein conformations (e.g., their melting temperatures increase) and chaotropes destabilize (their melting temperatures decrease). It is also widely accepted that fluctuations—closely related to conformational stability via the fluctuation−dissipation theorem[29]—are essential for understanding structure−function relationships in proteins,[30] and it is therefore important to understand the influence of salt solutions in which the proteins are dissolved on these fluctuations. Indeed, fluctuations are intimately related to function. In some cases, "loosening" a protein (i.e., enabling fluctuations of a greater magnitude) has been demonstrated to improve its functional efficiency.[31]

In order to analyze this question formally, we need to consider that similarly to precipitation−dissolution processes that involve diminution or increase of the protein−solvent surface area, many working proteins (functional proteins such as enzymes) oscillate between "open" and "closed" conformations, which also implies water-exposed surface area changes. As already discussed (see section 2), the interfacial component of the accompanying free energy changes can be written as

$$\Delta G_{\text{interfacial}} \propto \gamma_{pw}\Delta A_{pw} \tag{8}$$

In the presence of salts, we have $\tilde{\gamma}_{pw} = \gamma_{pw} + \Delta\gamma_{pw}$ (eq 7) and hence the contribution of the salt-dependent part to the free energy change is

$$\Delta\Delta\tilde{G}_{\text{interfacial}} = \Delta\gamma_{pw}\Delta A_{pw} = K_s cRT \tag{9}$$

In terms of the free energy change (dependence), the addition of salt adds a linear term to the free energy, increasing or decreasing as explained above according to the position of the salt in the Hofmeister series.

Proteins exist within a range of A_{pw}, with the lower bound given by the compressibility limit and the upper bound by the persistence limit (i.e., the protein cannot be indefinitely expanded without destroying it). Within these bounds, the protein can fluctuate, changing its interfacial area, L (Figure 2).

Several consequences follow: chaotropes stabilize proteins (i.e., reduce the amplitude of their fluctuations, and hence raise the melting temperature) whose native conformation is open (family 2) and destabilize those whose conformation is closed (family 1). Kosmotropes have precisely the opposite effect, which is why it is far from generally true that chaotropes invariably destabilize. That idea probably arose because natively open conformations are relatively rare.

Hence, we conclude that salts (in particular those at the extremities of the Hofmeister series) will almost certainly change the fluctuation amplitude of a protein. Note that this rationalizes the results of our previous paper,[32] where a fluctuation formalism was used to derive the Setschenow equation in the context of protein aggregation. In that work, it was shown that if we assume salt-induced fluctuations of protein free energy levels, the Setschenow equation can be derived, but no strict rationale for this assumption was given.

In most proteins hitherto investigated, the closed conformation has revealed itself as being the most common one (family 1). In this case, chaotropes will destabilize, while kosmotropes stabilize conformation, as has been shown in a vast accumulation of experimental examples.[2,3] In the remainder of this paper, we illustrate the behavior of the two rarer and less-investigated families (0 and 2, in Figure 2) via experimental examples, as case studies. Our choice fell on two proteins considered to be paradigmatical for their class: the water-soluble myoglobin (Mb, the most abundant protein in animals) and the membrane protein bacteriorhodopsin, a typical representative of the 7-alpha helix family.

3.1. Myoglobin. For the first example, we have carried out spectral measurements. The Soret band of Mb is due to the heme group embedded in the protein, and the temperature dependencies of its width and peak position are sensitive to the conformational fluctuations of the protein: a steeper temperature dependence indicates an increased level of fluctuations in the heme pocket.[33] Therefore, we measured the temperature dependence of the Soret band in the absorption spectrum of Mb, in the presence of different salts. Lyophilized myoglobin (Sigma) was dissolved in water and intensively treated with carbon monoxide in order to obtain the MbCO form. The protein concentration was adjusted to 1.0×10^{-5} M, and the pH was kept at 7.0 by 20 mM HEPES−NaOH buffer. In order to avoid metMb formation, a slight excess of sodium dithionite (3×10^{-4} M) was added to the solution immediately before the measurements. Analytical grade salts were added to give a final concentration of 1.00 M.

The Soret band of the MbCO absorption spectrum was measured from 500 to 350 nm with 0.5 nm resolution, over the

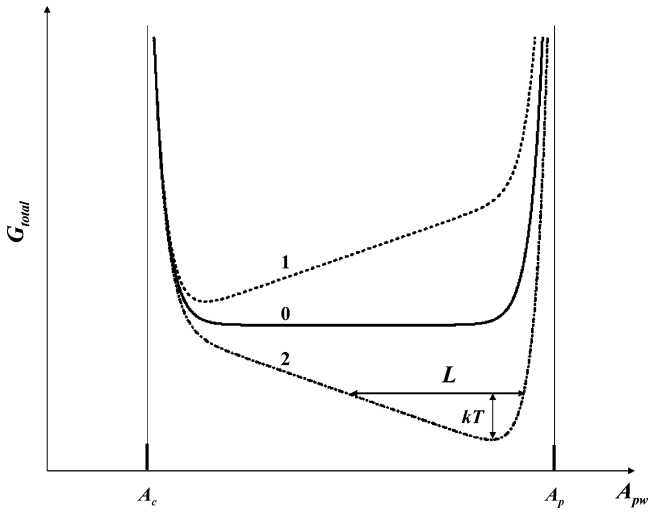

Figure 2. Sketch of the protein energy landscapes (total free energy as a function of interfacial area) corresponding to the three archetypical protein families, defined according to the theory presented in section 2. Family 0 (solid line), flat-bottomed conformation (rare). Family 1 (short-dashed line), closed conformation (the most typical); this corresponds to $\gamma_{pw} > 0$, a corollary of which is that such a protein will spend most of its time close to the compression limit, A_c. Family 2 (dashed−dotted line), open conformation (fairly rare), corresponding to $\gamma_{pw} < 0$, a corollary of which is that such a protein will spend most of its time close to the persistence limit, A_p. The quantity L, which we call the lability parameter, is defined as the allowed range of A_{pw} at a height of kT above the energy minimum (drawn for a natively open protein) is a measure of lability, or fluctuation amplitude.

TABLE 1: Data Fitting Parameters Pertaining to the Myoglobin Soret Band (Γ Is the Homogeneous Lorentzian Width, while the *S* Parameters Denote the Linear Coupling Constants of the High-Frequency Modes at the Wavelengths Indicated in the Indices[34])

salt	Γ/cm^{-1}	S_{350}	S_{676}	S_{1100}	S_{1374}
NaCl	220	0.06	0.09	0.01	0.10
NaCOOH	240	0.07	0.08	0.01	0.10
NaSCN	234	0.08	0.09	0.01	0.10

temperature range 0−40 °C. A model peak was fitted to the Soret band, and peak position and width were extracted using the parameters given in Table 1.

Figure 3 shows the Soret band peak frequencies and Gaussian widths for the entire temperature range investigated with the values of the slopes given in Table 2. A systematic effect of the salts on the slopes of both width and peak frequency is apparent, much more pronounced for the latter than for the former, in which the differences are barely statistically significant, from which we can deduce the particular features of the shape of the potential well sketched out in Figure 2. A steeper temperature dependence of peak frequency indicates increased local dynamics of the heme pocket, providing evidence for increased protein flexibility.[34]

The highest fluctuations appear for the Hofmeister-neutral salt, NaCl: *both kosmotropes and chaotropes act in the same direction, diminishing the fluctuations*. We therefore infer that myoglobin (MbCO) belongs to the "flat-bottomed'' (Figure 2) family 0 of proteins. Clearly, if the shape of the protein potential were a simple parabola (in contrast to the shapes shown in Figure 2), the sign of the salt-dependent term in eq 9 would not affect the magnitude of the fluctuations. However, a protein is a complex, multiatom construction with multiple stable states,[35] whose shape is better approximated by a rectangle than by a parabola (Figure 2). In the flat-bottomed class, the addition of a linear term, whether of positive or negative slope, will

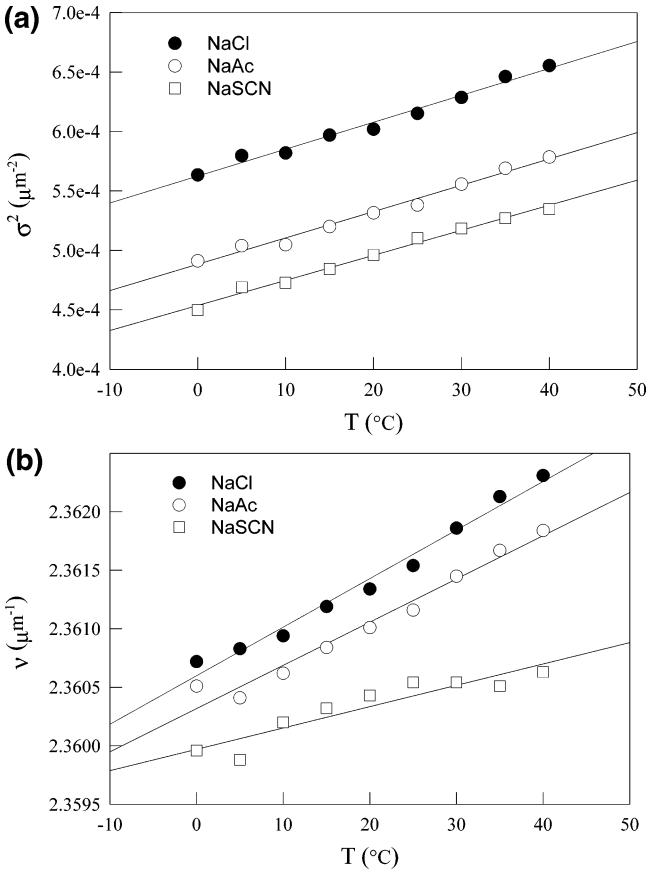

Figure 3. Temperature dependence of the spectral parameters of the Soret band for myoglobin dissolved in the presence of various salts—NaCl (Hofmeister neutral, i.e., negligible effect on native conformation), sodium acetate (Hofmeister precipitant [kosmotrope]), or sodium thiocyanate (Hofmeister solubilizer [chaotrope]): (a) Gaussian width (typical errors are $\pm 3.5 \times 10^{-6}$); (b) Soret band peak frequency (typical errors are $\pm 5 \times 10^{-5}$; points for NaSCN are displaced by -7 cm^{-1} for clarity).

TABLE 2: Temperature Dependence of Soret Band Parameters (Slopes of the Lines in Figure 3)

salt	$(d\sigma^2/dT)/\mu m^{-2}\ K^{-1}$	$(d\nu^2/dT)/\mu m^{-1}\ K^{-1}$
NaCl	$(2.26 \pm 0.10) \times 10^{-6}$	$(4.15 \pm 0.21) \times 10^{-5}$
NaCOOH	$(2.22 \pm 0.10) \times 10^{-6}$	$(3.69 \pm 0.25) \times 10^{-5}$
NaSCN	$(2.11 \pm 0.08) \times 10^{-6}$	$(1.82 \pm 0.27) \times 10^{-5}$

inevitably narrow the potential distribution (Figure 2), and hence inevitably diminish the magnitude of fluctuations, in agreement with our experimental observations.

3.2. Bacteriorhodopsin. So far, there have been only very few examples reported of proteins having an open conformation as the naturally stablest form[3] (family 2). The unusual membrane protein of *Halobacterium salinarum*, bacteriorhodopsin (bR),[36] also appears to be of such a type in terms of heat destabilization. FTIR spectroscopy evidences an α_{II} helix conformation in bR below ca. 50 °C.[37] Between 50 and 70 °C, the protein core undergoes an $\alpha_{II}-\alpha_I$ conformational change.[38] In α_I, however, the helix is known to be more tightly packed than in α_{II}.[37,39] For such a case, our theory predicts an unusual phenomenon to occur: chaotropes should have a stabilizing effect; consequently, they are expected to shift the $\alpha_{II}-\alpha_I$ transition to higher temperatures, while kosmotropes are expected to destabilize the open α_{II} protein conformation, therefore shifting the transition toward lower temperatures.

Bacteriorhodopsin-containing purple membranes were isolated according to the standard procedure.[40] Differential scanning

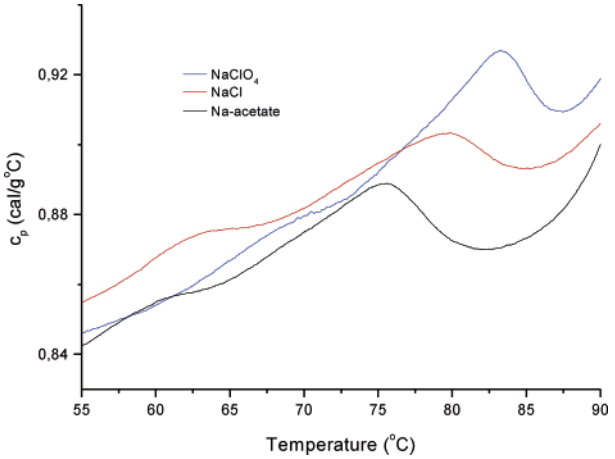

Figure 4. Heat capacity, C_p, of bacteriorhodopsin in the presence of 500 mM sodium chloride, sodium acetate, and sodium perchlorate solutions in the temperature régime corresponding to the $\alpha_{II}-\alpha_I$ helix phase transition.

calorimetry (DSC) measurements were performed in an MC-2 high-sensitivity differential scanning calorimeter (Microcal Inc., Northampton, MA) using membrane suspensions (3 mg/mL protein concentration) in 10 mM HEPES buffer, pH 7, containing 500 mM chaotropic, kosmotropic, or Hofmeister-neutral salts ($NaClO_4$, sodium acetate, and NaCl, respectively). Runs were made in the temperature range 20−105 °C, at a heating rate of 1 °C/min. Having been cooled back to room temperature, the samples containing the denatured protein were subjected to the same measuring protocol. These reheating scans then surved as baselines for the original recordings.

In Figure 4, we summarize the results of our differential scanning calorimetry (DSC) measurements to illustrate the shift of the transition to lower temperatures.

According to ref 38, the two C_p peaks in the temperature range 50 °C < T < 70 °C are attributable to two phases of the $\alpha_{II}-\alpha_I$ conformational change, often referred to as a reversible "pretransition" in the thermal denaturation of bR.[41] In the presence of chaotropic ClO_4^- ions, the pretransition is shifted to higher temperatures (83.0 °C for the main peak), as compared to the Hofmeister-neutral Cl^- (main peak at 79.5 °C). On the contrary, kosmotropic acetate ions shift the transition to lower temperatures (main peak at 76.0 °C), indicating a destabilizing effect on α_{II}, exactly as expected for protein conformations belonging to family 2 (Figure 2).

4. Concluding Remarks

The above examples demonstrate that the phenomenological formalism presented in this paper is able to account for the most diverse manifestations of Hofmeister effects on protein conformations. The existence of a few contrary examples (e.g., a chaotrope destabilizing many proteins, but stabilizing exceptions) has until now been an unsolved puzzle. Providing a useful classification of dynamic protein conformational types, our model is expected to serve as a tool to understand some important phenomena concerning protein conformation in general, and especially those related to water structure changes.

A criticism of previous attempts to interpret protein stability in terms of surface tension is that the latter, being a macroscopic quantity, is not commensurate with the former, a microscopic quantity. Nevertheless, our treatment provides a bridge connecting the two perspectives, the keystone being provided by fluctuations, which can be interpreted at the molecular scale. A protein is a complex multiatom entity that can exist in two or more stable states; this is the essence of the notion of a protein as a construction,[42] one consequence of which is that the protein's energy is concentrated into a very small number of degrees of freedom. The action of a protein depends on transitions between these stable states. In general, the water-exposed surface areas of these states will be different; hence, regardless of other details, the free energy must include an interfacial term, $A_{pw}\gamma_{pw}$. This reveals the key role of protein−water interfacial tension on protein structure and dynamics, the latter processes being coupled to (interfacial) solvent fluctuations, giving a natural explanation of the phenomenon denoted "slaving"[35] (i.e., solvent control of protein behavior); for most proteins, the presence of water is important for maintaining sufficient structural flexibility to ensure that necessary protein motions can take place. Since the sign and magnitude of interfacial tension is salt-dependent, in consequence of the above view, we also assert that a "fluctuation Hofmeister series" should parallel the precipitation series.

A methodological implication of the results concerns the use of Hofmeister effects to assist in unraveling the complicated effects of temperature change on protein function. By judiciously choosing a salt from an appropriate position in the Hofmeister series, we can separately mimic temperature effects on interfacial water structure, without directly affecting protein fluctuations. Otherwise, if temperature itself is varied, changes in both protein and interfacial water structure are inextricably mingled.

Another important field of application is protein conformational dynamics. Hofmeister effects are expected to manifest themselves, by changing transition rates and equilibrium constants, in reactions accompanying major conformational changes involving water-exposed surface area changes of macromolecules and supramolecular assemblies.[31,43] This gives us a tool to identify crucial steps of biological processes such as enzyme catalysis or substrate binding reactions.

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