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Fluctuations and the Hofmeister Effect

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ABSTRACT The Hofmeister effect consists in changes of protein solubility triggered by neutral electrolyte cosolutes. Based on the assumption that salts cause stochastic fluctuations of the free energy barrier profiles, a kinetic theory of this phenomenon is proposed. An exponentially correlated noise, of amplitude proportional to the salt concentration, is added to each energy level, and the time-dependence of the mean protein concentration is calculated. It is found that the theory yields the well-known Setschenow equation if the noise correlation time is low in comparison to the aggregation time constant. Experimental data on salting-in agents are well fitted, whereas, in the case of salting-out cosolutes, two independent dichotomic fluctuations are needed to fit the data. This may result from the fact that, in both cases, the low-concentration regime is dominated by salting-in electrostatic contributions, whereas, at high salt concentrations, electron donor/acceptor interactions become important; these have opposite effects. The theory offers a novel way to metricate Hofmeister effects and also leads to thermodynamic quantities, which account for the influence of salts. The formalism may also be applied to describe kinetic phenomena in the presence of cosolutes.

INTRODUCTION

Hofmeister effects encompass a large variety of phenomena induced by salts in protein-containing systems, including changes of protein solubility, protein denaturation and, changes in enzyme kinetics. In his original work, Hofmeister (1888) reported modifications of protein solubility caused by salts present in the solution, and built up the Hofmeister series (HS) by ordering various ions according to their effectiveness in this sense. An impressive number of later works show that, with minor exceptions, the same HS emerge in studies of denaturation, depolymerization, and dissociation of proteins, and of inhibition or activation of enzymes (Collins and Washabaugh, 1985; Cacace et al., 1997).

Careful thermodynamic studies, based on a long series of preferential interaction measurements, have shown that precipitants are preferentially excluded from the vicinity of globular proteins, whereas salts that increase protein solubility exhibit weak preferential binding (Arakawa and Timasheff, 1982; Arakawa et al., 1990a,b). The principal mechanism of salting-out consists of the salt-induced increase of the surface tension of water (Melander and Horváth, 1977). Thus, a compact structure becomes energetically more favorable because it corresponds to smaller protein–solution interfacial area. Salting-in is less well understood. The selective binding model of Schellman (1987, 1990) leads to a very intuitive picture of how water may be substituted by cosolvent molecules at specific, independent sites of the protein. The main difficulty in treating electrolyte cosolutes consists of the inapplicability of the Debye–Hückel theory at high values of protein charge and ionic strength, characteristic in Hofmeister phenomena. The review paper by Timasheff (1993) offers a clear overview of experimental findings and useful thermodynamic quantities. In general, those ions that are most effective in causing protein precipitation also are most effective in preventing denaturation, whereas those that increase protein solubility favor denaturation, too (Robinson and Jencks, 1965). Exceptions are also known: some salting-out agents act as destabilizers of the native structure because of specific interactions between cosolutes and various protein sites (Arakawa et al., 1990b).

Hofmeister phenomena on membrane proteins have captured much interest in recent years. Conformational equilibrium studies performed on the artificial visual pigment 9dm-Rho revealed that well-known stabilizers fail to act so on the membrane protein because the latter is mainly stabilized by the phospholypid bilayer. All of the studied salts have shifted the equilibrium between the MI and MII conformations toward the less compact MII (Vogel et al., 2001). A similar conclusion has been drawn from kinetic studies of bacteriorhodopsin, a light-driven proton pump (Dér and Ramsden, 1998). Denaturants like NaSCN accelerated the decay of the spectral intermediate M_2, an effect attributed to loosening of the pump structure, whereas the stabilizer NaF had no kinetic effect in comparison to the Hofmeister-neutral NaCl. Fluctuation analysis of electric current through ion channels formed by the polyene antibiotic roflamycoin, has provided new insight into the microscopic mechanism of Hofmeister phenomena (Grigorjev and Bezrukov, 1994): Reversible binding of anions to the channel structure causes fast fluctuations of channel conductance between (at least) two open channel states. The dwell time in the higher conductance state parallels the HS, more chaotropic salts being bound for longer times. Studies of halide ion adsorbtion onto Sephadex G-10 gel have led to

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analogous conclusions (Collins, 1995). Using aqueous column chromatography, this author found that the more chaotropic ions adsorb more strongly.

The problem addressed in the present paper, from a theoretical point of view, is that of protein solubility. Consider an aqueous solution of some globular protein in the absence of cosolutes. The corresponding protein solubility, S_0, is the concentration above which an equilibrium mixture of dissolved and aggregated proteins exists. The aggregation is supposed to occur without denaturation of the proteins and, thus, the process is reversible (Lehninger, 1975; Arakawa et al., 1990a). The thermodynamic equilibrium may be characterized by the equation

$$\mathbf{P}_{\text{aggregate}} \underset{k_{12}}{\overset{k_{21}}{\rightleftharpoons}} \mathbf{P}_{\text{solution}}, \tag{1}$$

where **P** stands for a protein molecule, the state 1 refers to the aggregate and 2 denotes the solution. The rate constant of the $1 \rightarrow 2$ process is written as k_{21}, and the reverse process is described by k_{12}. Such a notation neatly simplifies the kinetic equations (Nagle, 1991).

If one adds pure water to the system at equilibrium, the direct reaction will be favored, and the kinetics of saturation will be described by the equation

$$\frac{\mathrm{d}c_2}{\mathrm{d}t}(t) = k_{21}c_1 - k_{12}c_2(t). \tag{2}$$

Provided that enough protein is present in the aggregate, a new saturation state will be reached by the solution: the dissolved protein concentration, $c_2(t)$, will approach again the solubility, S_0. The subscript zero stands for the absence of cosolutes. The concentration c_1 depends on the noncovalent protein–protein interactions that stabilize the aggregate. Consequently, we assume that c_1 is roughly constant, proportional to the aggregate density, whichever the composition of the surrounding aqueous solution is. A similar assumption is the cornerstone of the weak interaction model of Hofmeister ion interactions (Baldwin, 1996), which considers the chemical potential of the aggregate as a constant, independent of salt concentration.

The solution of Eq. 2, corresponding to the initial condition $c_2(0) = 0$, reads

$$c_2(t) = S_0[1 - \exp(-k_{12}t)], \tag{3}$$

where $S_0 = c_1k_{21}/k_{12}$ is the protein solubility in the absence of cosolutes.

This equation tells us that the kinetics of saturation is determined solely by the aggregation rate constant, k_{12}. It yields the speed at which the dissolved protein concentration approaches its saturation value (see the *curve a* in Fig. 1).

We note that Eq. 3 refers to the time-dependence of the concentration in the vicinity of an aggregate, not in the whole solution. As the solution becomes saturated in this thin layer, the concentration in distant points in the bulk solution increases because of diffusion. The speed of this process is controlled by the diffusion coefficient of the solute.

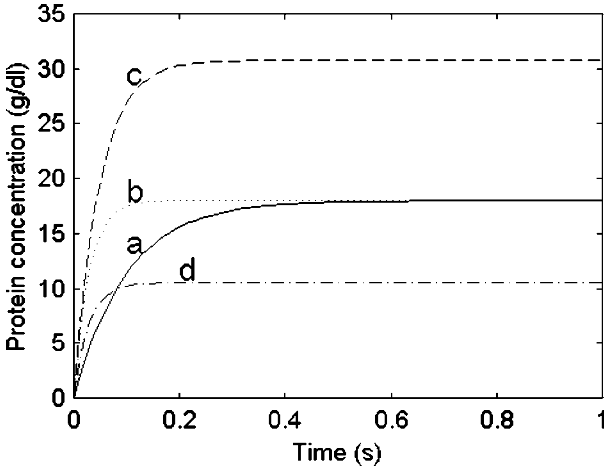

FIGURE 1 Time dependence of the noise-averaged protein concentration. Curve *a* (*solid line*) is the solution of the kinetic equation in the salt-free case (no noise), *b* corresponds to symmetric noise amplitudes $a_{21} = a_{12} = \alpha = 5.825$ kcal · l · mol^{-2}, *c* is given by $a_{21} = \alpha$ and $a_{12} = 0.7 \cdot \alpha$, and *d* results from addition of noise with $a_{21} = 0.7 \cdot \alpha$ and $a_{12} = \alpha$. The aggregation rate constant, k_{12}, has been set to 10 s^{-1}.

The thermodynamic equilibrium described by Eq. 1 is shifted by most salts. Some of them favor aggregation, reducing protein solubility, but others increase it; they are called salting-out and salting-in agents, respectively.

In spite of the large variety of forces present (such as electrostatic, Lifshitz–Van der Waals, structural) the interactions responsible to Hofmeister phenomena seem to be dominated by electron donor/acceptor (hydration) forces. Salts that stabilize water structure are called kosmotropes and, in most cases, have salting-out effect, whereas the so-called chaotropes destabilize water structure and usually show salting-in behavior (Collins, 1995; Cacace et al, 1997).

Disregarding some well-known exceptions (Arakawa et al., 1990b), kosmotropes are known to stabilize also protein structure, whereas chaotropes usually destabilize it (Collins and Washabaugh, 1985; Cacace et al., 1997). Generally speaking, kosmotropes tend to tighten inter- and intramolecular structure-making interactions in aqueous solutions of proteins; on the contrary, chaotropes tend to loosen them. Weakening the binding forces (i.e., raising potential energy minima) is supposed to lead to increased flexibility of macromolecules, giving rise to various changes in their kinetic behavior, as well. It has recently been shown, e.g., that chaotropic salts may accelerate reactions of proteins by loosening their structure (Dér and Ramsden, 1998). An altered level of flexibility, in contrast, is expected to correlate with an altered level of fluctuations of thermodynamic parameters of the system on the basis of the Fluctuation-Dissipation Theorem (Callen and Welton, 1951). In the following, we outline a theory describing the effect of salts on protein solubility using a dichotomous fluctuation formalism. The theoretical results are compared with a characteristic set of experimental data.

THEORY

The main assumption of the theory is that the overall effects of a given salt on the reaction of Eq. 1 may be summarized in stochastic free energy fluctuations, with amplitudes proportional to the salt concentration.

For a quantitative description, we use the activated complex theory as a starting point (Eyring, 1935), and include additional salt-induced fluctuation terms in the free energies of the reactants and the activated complex. Let G_1, G_2, and G_{ac} denote the Gibbs free energies of the aggregate, the monomer, and the activated complex states, respectively. Then,

$$\tilde{G}_1 = G_1 + c_s a_1 \xi_1(t), \tag{4a}$$

$$\tilde{G}_2 = G_2 + c_s a_2 \xi_2(t), \tag{4b}$$

$$\tilde{G}_{\mathrm{ac}} = G_{\mathrm{ac}} + c_s a_{\mathrm{ac}} \xi_{\mathrm{ac}}(t), \tag{4c}$$

where $\sim$ refers to the presence of a cosolute of concentration c_s.

For the sake of mathematical simplicity, the last terms of the above equations, $\xi(t)$, are chosen to be symmetric dichotomous noises, normalized to unity. By definition, such a noise has the properties,

$$\xi(t) \in \{-1, 1\}, \tag{5a}$$

$$\langle \xi(t) \rangle = 0, \tag{5b}$$

$$\langle \xi(t)\xi(t') \rangle = \exp(-\lambda |t - t'|), \tag{5c}$$

where $\langle \cdots \rangle$ denotes the mean value of the enclosed quantity. The last equation contains the noise correlation parameter, λ, which is the average frequency of jumps of the random function, $\xi(t)$, from one value to the other (λ^{-1} is the noise correlation time). The correlation function given by Eq. 5c is a measure of the probability that ξ has the same value at t and t'.

We assume that $\xi_1(t)$, $\xi_2(t)$, and $\xi_{\mathrm{ac}}(t)$ have the same noise correlation times, and, keeping in mind that all these fluctuations correspond to the same physical process, we identify these noises, $\xi_1(t) = \xi_2(t) = \xi_{\mathrm{ac}}(t) = \xi(t)$, and say that all the fluctuations described in Eq. 4 take place simultaneously but with different amplitudes. From the physical point of view, this means that cosolutes cause fluctuations of the free energy landscape that governs the reaction undergone by the protein. These may be attributed to fluctuations in the hydrogen bond network built between the protein and adjacent water molecules or reversible binding of ions to specific sites of the protein (Grigorjev and Bezrukov, 1994). Thus, using the notations of Eq. 1, the rate constants of protein solution and aggregation, in the presence of a cosolute, have the form

$$\tilde{k}_{\mathrm{ij}} = k_{\mathrm{ij}} \exp\left(-\frac{a_{\mathrm{ij}} c_s}{RT} \xi(t) \right), \tag{6}$$

where $i, j = 1, 2$ $(i \neq j)$, k_{ij} are the rate constants in the absence of salts, R is the universal gas constant, T is the absolute temperature and the noisy term $a_{\mathrm{ij}}\xi(t)$ stands for $|a_{\mathrm{ac}} - a_{\mathrm{j}}|\xi(t)$; the magnitude of free energy barrier fluctuations corresponding to the process $j \rightarrow i$ is given by $a_{\mathrm{ij}} \cdot c_s$.

The stochastic nature of $\tilde{k}_{\mathrm{ij}}$ may be viewed as an expression of the fact that the presence of ions in the vicinity of the aggregate depends on diffusion, which is a stochastic process. Ions cause perturbations of the hydrogen bond network, or may temporarily bind, which may favor or hinder aggregation. Eq. 6 states that these kinetic switches occur at random time points, with an average frequency λ, equal to the inverse of the noise correlation time. Given the complexity of the problem, here we do not attempt to find a microscopic interpretation of Eq. 6, but simply investigate the consequences of dichotomous fluctuations of free energies, and ask whether they can account for experimental results.

Along with the two-state model of the roflamycoin channel discussed in the Introduction (Grigorjev and Bezrukov, 1994), we mention two more theories that present formal similarities with ours. Barrier structure fluctuations, attributed to conformational transitions in ionic channels, have been found to account for nonlinear dependence of channel conductance on ion concentration (Läuger et al., 1980, Läuger, 1985). The theory is based on shifts between two different energy profiles of an ion channel with two main barriers and one main ion-binding site. The second example is a recently proposed model of an ion pump, the stochastic energization–relaxation channel model (Muneyuki et al., 1996; Muneyuki and Fukami, 2000). It treats the pump as a multi-ion channel with two main conformational states, corresponding to distinct potential profiles. It is assumed that switches between these hinge on external energy supply, and occur stochastically. Comparison with experimental data on bacteriorhodopsin shows that the model successfully reproduces basic features of active transport.

We next turn to study the kinetic consequences of the assumption that Hofmeister cosolutes cause fluctuations of the rate constants of protein reactions (Eq. 6). Using the properties given in Eq. 5, we observe that, for any real number, α, one may expand, $\exp(-\alpha\xi(t)) = \cosh \alpha - \xi(t)\sinh \alpha$, because $\xi^2(t) = 1$. Using this relation, the kinetic equation (Eq. 2) in the presence of fluctuations becomes

$$\begin{aligned} \frac{\mathrm{d}c_2}{\mathrm{d}t}(t) &= k_{21}c_1(C_{21} - \xi(t)S_{21}) \\ &\quad - k_{12}(C_{12} - \xi(t)S_{12})c_2(t), \end{aligned} \tag{7}$$

where c_1 is again constant (see the comment following Eq. 2), and we used the short-hands

$$C_{\mathrm{ij}} = \cosh\left(\frac{a_{\mathrm{ij}}c_{\mathrm{s}}}{RT}\right) \quad \text{and} \quad S_{\mathrm{ij}} = \sinh\left(\frac{a_{\mathrm{ij}}c_{\mathrm{s}}}{RT}\right). \tag{8}$$

We are interested in the time evolution of the fluctuation-averaged protein concentration, $\langle c_2\rangle(t)$, and its equilibrium value, $\lim_{t\to\infty}\langle c_2\rangle(t) \equiv S$, which is the protein solubility in the presence of noise (attributed to salts).

Taking the mean of both sides of Eq. 7, we obtain

$$\frac{\mathrm{d}\langle c_2\rangle}{\mathrm{d}t} = -k_{12}(C_{12}\langle c_2\rangle - S_{12}\langle \xi c_2\rangle) + k_{21}c_1C_{21}, \tag{9}$$

where a second unknown function appears, $\langle \xi c_2\rangle(t)$. The differential equation needed to complete the system may be obtained by applying the Shapiro–Loginov theorem (Shapiro and Loginov, 1978; Loginov, 1996; Fulinski, 1998), which states that, for any differentiable function, $f(t)$,

$$\frac{\mathrm{d}}{\mathrm{d}t}\langle \xi f\rangle = -\lambda\langle \xi f\rangle + \left\langle \xi\frac{\mathrm{d}f}{\mathrm{d}t}\right\rangle. \tag{10}$$

Thus:

$$\frac{\mathrm{d}\langle \xi c_2\rangle}{\mathrm{d}t} = -k_{12}\left[-S_{12}\langle c_2\rangle + \left(C_{12} + \frac{\lambda}{k_{12}}\right)\langle \xi c_2\rangle\right] - k_{21}c_1S_{21}. \tag{11}$$

The system composed of Eqs. 9 and 11 is exactly solvable. The equilibrium value of the mean protein concentration is found by solving the algebraic equations, which result as the derivatives vanish. The result is the protein solubility,

$$S = S_0\,\frac{\cosh\left[\dfrac{(a_{21} - a_{12})c_{\mathrm{s}}}{RT}\right] + r\cdot\cosh\left(\dfrac{a_{21}c_{\mathrm{s}}}{RT}\right)}{1 + r\cdot\cosh\left(\dfrac{a_{12}c_{\mathrm{s}}}{RT}\right)}, \tag{12}$$

where $r = \lambda/k_{12}$, and S_0 is the protein solubility in the salt-free case.

The time dependence of the mean concentration is given by

$$\langle c_2\rangle(t) = S\left\{1 + \frac{W-1}{2}\exp\left[-k_{12}\left(\frac{r}{2} + C_{12} + \sqrt{\left(\frac{r}{2}\right)^2 + S_{12}^2 t}\right]\right. - \frac{W+1}{2} \times \exp\left[-k_{12}\left(\frac{r}{2} + C_{12} - \sqrt{\left(\frac{r}{2}\right)^2 + S_{12}^2}\right)t\right]\right\}, \tag{13}$$

where C_{12}, S_{12} are the notations introduced in Eq. 8, and

$$W = \frac{1}{\sqrt{\left(\dfrac{r}{2}\right)^2 + \sinh^2\left(\dfrac{a_{12}c_{\mathrm{s}}}{RT}\right)}} \times\left[\frac{r}{2} + \frac{\sinh\left(\dfrac{a_{12}c_{\mathrm{s}}}{RT}\right)\sinh\left(\dfrac{(a_{12} - a_{21})c_{\mathrm{s}}}{RT}\right)}{\cosh\left(\dfrac{(a_{12} - a_{21})c_{\mathrm{s}}}{RT}\right) + r\cdot\cosh\left(\dfrac{a_{21}c_{\mathrm{s}}}{RT}\right)}\right]. \tag{13a}$$

Eq. 13 describes the kinetics of saturation of the solution near the aggregate if salts are present. It is the counterpart of Eq. 3 from the salt-free case. In the limit of vanishing fluctuations ($a_{\mathrm{ij}} \to 0$), the dimensionless expression W approaches unity, the second term on the right-hand side of Eq. 13 drops out, so does the ratio r, and the expression reduces to Eq. 3. These two solutions are compared in Fig. 1. Curve *a*, drawn as a solid line, represents the salt-free evolution of the protein concentration. The corresponding solubility value, $S_0 = 18$ g/dl is typical for deoxy-Hb S (Poillon and Bertles, 1979). The aggregation rate constant, k_{12}, has been set to 10 s^{-1} just for the sake of illustration; its actual value has no importance in the present analysis because the experimentally available quantity is the equilibrium solubility. The remaining curves illustrate the influence of various dichotomic fluctuations: *b* corresponds to a symmetric noise ($a_{12} = a_{21} = \alpha$), *c* is generated by adding fluctuations of higher amplitude to the energy barrier of the direct process ($a_{21} = \alpha$; $a_{12} = 0.7\alpha$), and *d* is a result of the reverse case ($a_{12} = \alpha$; $a_{21} = 0.7\alpha$). Here $\alpha = RT_1/c_{\mathrm{s}_1} = 5.825$ kcal·l/mol^2, is a typical noise amplitude (R is the universal gas constant, and $T_1 = 293.15$ K and $c_{\mathrm{s}_1} = 0.1$ mol/l are chosen as reference values of the temperature and salt concentration, respectively.) The ratio $r = \lambda/k_{12}$ has been chosen to be 10^6 for reasons that will be argued later in this section.

The experimentally accessible quantity is the asymptotic value of the noise-averaged protein concentration, S. It is given by Eq. 12, which tells us that a symmetric noise does not shift the chemical equilibrium. This is observed also on Fig. 1, by comparing curves *a* and *b*. It is interesting that the kinetics is influenced (the saturation accelerated) also by symmetric fluctuations.

Hofmeister effects, in the context of protein solutions, consist of the salt-induced modifications of the protein solubility (Hofmeister, 1888; Cacace et al, 1997). These can be modeled by asymmetric free energy fluctuations of the reactants if one admits that the dimensionless parameter, $r = \lambda/k_{12}$ is of order 10^6 or higher. This means that the noise correlation time, λ^{-1}, is negligibly small as compared to the aggregation time constant, k_{12}^{-1}. One is led to this conclusion by comparing Eq. 12 with experimental results. It is a

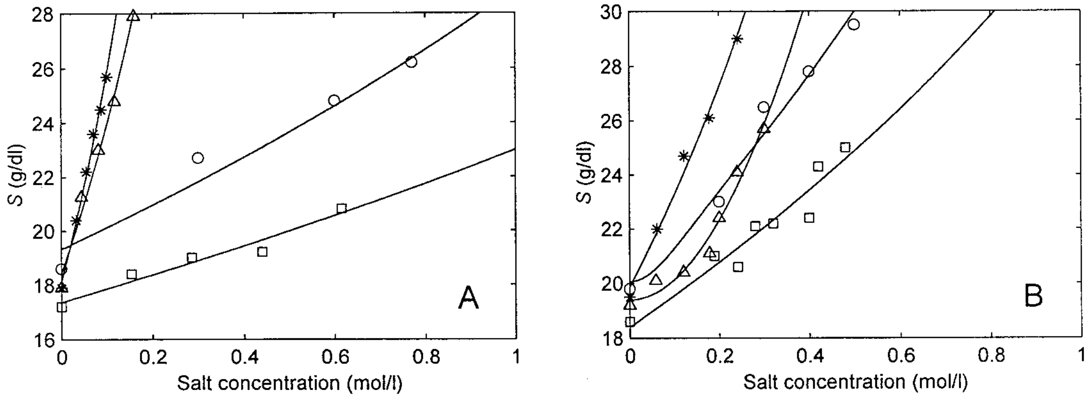

FIGURE 2 Plots of deoxy-Hb S solubility in the presence of salting-in cosolutes, based on experimental data reported by Poillon and Bertles (1979). The solid lines result from free energy fluctuation theory, Eq. 15, with parameters given in Table 1. Here *S* denotes protein solubility in the presence of (*A*) chloride salts: $CaCl_2$ (*), $MgCl_2$ (△), LiCl (○), RbCl (□) and (*B*) sodium salts: NaSCN (*), NaI (△), $NaClO_4$ (○) and NaBr (□).

well-known fact that, above a certain value of the salt concentration, the protein solubility depends exponentially on salt concentration, according to the Setschenow equation (Setschenow, 1889; Green, 1932; Cohn and Edsall, 1943; Robinson and Jencks, 1965; Arakawa et al., 1990a; Cacace et al., 1997),

$$\log \frac{S_0}{S} = K_s c_s, \tag{14}$$

where K_s is a phenomenological solubility constant, which is a measure of the lyotropic effect exerted by a given salt. Salting-in salts have negative Setschenow constants, whereas salting-out agents correspond to positive K_s. This is the reason why K_s is also known as salting-out constant.

In the above-mentioned limit of low noise correlation time ($r \gg 1$), Eq. 12 yields

$$S = S_0 \frac{\cosh(a_{21}c_s/RT)}{\cosh(a_{12}c_s/RT)}, \tag{15}$$

which turns into the Setschenow equation if c_s is high enough,

$$S \cong S_0 \exp\left[-\frac{(a_{12}-a_{21})c_s}{RT}\right] \quad \text{(for high } c_s\text{)}. \tag{16}$$

Comparison with Eq. 14 yields the expression of the Setschenow constant as a function of noise amplitudes,

$$K_s = \frac{a_{12}-a_{21}}{2.303 \cdot RT}. \tag{17}$$

It shows that Hofmeister effects stem from the asymmetry of fluctuations. The above formula strongly resembles that obtained by Timasheff and co-workers in the framework of a thermodynamic formalism that accounts for salt exclusion from (or enrichment in) the vicinity of the protein surface (Timasheff, 1993). In the next section, we discuss possible connections between noise amplitudes and the thermodynamic quantities introduced by these authors.

Experimental results on deoxy-Hb S in the presence of salting-in cosolutes (Poillon and Bertles, 1979), are well fitted by the function of Eq. 15. The results of the nonlinear least-squares fit are shown on Fig. 2. In the next section, further details of the fit procedure are also given. The experimental points are represented by markers, for chloride salts (Fig. 2 *A*) and sodium salts (Fig. 2 *B*), together with the plots of the fit function (*solid lines*). The corresponding parameters are specified in the first three columns of Table 1.

The fit program has also been used for testing the assumption of high *r* values. To this end also, *r* was allowed to vary, and, starting from the value $r_0 = 10$, the fit program implemented for Eq. 12 yielded good results for *r* of order 10^4–10^7, depending on the particular salt. Together with the analytical arguments concerning the Setschenow law, this led us to adopt Eq. 15, as theoretical basis for fitting the experimental data in the case of the salts presented in Table 1.

In the above form, our fluctuation theory remains unsatisfactory in the case of salting-out agents, because it is not able to account for the salting-in behavior observed at low concentrations. This effect is believed to appear due to electrostatic interactions described by the Debye–Hückel theory (Melander and Horváth, 1977; Arakawa et al., 1990a; Cacace et al., 1997). Nonetheless, the high concentration limit is known to be dominated by electron donor/acceptor forces (Cacace et al., 1997), and, in the case of strongly salting-in electrolytes, one of these is overheliming, at variance with the context of salting-out cosolutes, where the two factors are comparable and compete each other. To account for such a complex behavior, observed also in the case of salts that are weakly salting-in (such as NaCl) or weakly salting-out (like KCl), it is necessary to extend the above theory.

TABLE 1 Parameters obtained by least-squares fit of the deoxy-Hb S data of Poillon and Bertles (1979), in the case of salting-in salts

Salt	x_{21}	x_{12}	S_0	K_s	c_{sa} (mol/l)	χ^2_m	ν	Q
$CaCl_2$	47.6312	47.2653	18.1149	−1.5364	<0.1	0.2473	3	0.9696
$MgCl_2$	39.0354	38.7654	18.4187	−1.1335	<0.1	0.6944	2	0.7067
NaSCN	26.8714	26.7082	19.8770	−0.6854	<0.1	0.5834	2	0.7470
NaI	0.3399	0.1840	19.4040	−0.6576	1.5	0.9353	4	0.9194
$NaClO_4$	1.0127	0.9296	20.0687	−0.3489	0.1	1.4953	2	0.4735
NaBr	8.5586	8.4964	18.4207	−0.2609	<0.1	2.3468	5	0.7994
LiCl	7.8532	7.8117	19.3442	−0.1740	<0.1	1.4427	2	0.4861
RbCl	11.7756	11.7466	17.3699	−0.1215	<0.1	0.3597	2	0.8354

The theory based on a single dichotomic noise in the free energy landscape of the protein (Eq. 15) provides a good fit in the case of monotonous dependence of protein solubility on salt concentration. The above salts belong to this category as can be seen on Fig. 2. The noise amplitudes are given by $a_{ij} = \alpha \cdot x_{ij}$, $(i, j = 1, 2; i \neq j)$, where x_{ij} denotes dimensionless amplitudes and $\alpha = 5.825$ kcal · l · mol^{-2}. Here S_0 is the fitted value of the protein solubility in the absence of salts, an adjustable parameter in this model. The Setschenow constant, K_s, describes the asymptotic behaviour of protein solubility according to the Setschenow law: $\log(S_0/S) = K_s \cdot c_s$ The salts included in the table obey this law starting from salt concentrations c_{sa}. Inequalities are used when these values are small, of order 10^{-2}, such that, practically in the whole experimentally investigated concentration range, the Setchenow law is satisfied. For each salt, the minimal value of the chi-square merit function (Press et al., 1993) is denoted by χ^2_m; the number of degrees of freedom, ν, is the difference between the number of experimental points and that of model parameters. The last column is a measure of the goodness-of-fit: Q is the probability that the merit function exceeds χ^2_m by chance.

To each of the two competing classes of interactions we associate a dichotomic noise. Let us denote them by $\xi(t)$ and $\xi'(t)$, and implement the independence of their effects by the properties,

$$\xi(t) \in \{-1, 1\} \quad \xi'(t) \in \{-1, 1\} \tag{18a}$$

$$\langle \xi(t) \rangle = 0 \quad \langle \xi'(t) \rangle = 0 \tag{18b}$$

$$\begin{aligned} \langle \xi(t)\xi(t') \rangle &= \exp(-\lambda|t - t'|) \\ \langle \xi'(t)\xi'(t') \rangle &= \exp(-\lambda'|t - t'|) \end{aligned} \tag{18c}$$

$$\langle \xi(t)\xi'(t') \rangle = 0 \tag{18d}$$

These relations define two independent, symmetric dichotomic fluctuations of unit norm. Consequently, just like in the previous case, the rate constants are written as

$$\tilde{k}_{ij} = k_{ij} \exp\left(-\frac{1}{RT} (a_{ij}\xi(t) + a'_{ij}\xi'(t))c_s \right), \tag{19}$$

where a_{ij} and a'_{ij} are the noise amplitudes and the other notations are like in Eq. 6.

The kinetic equation in the presence of fluctuations becomes

$$\begin{aligned} \frac{dc_2}{dt} &= k_{21}c_1(C_{21} - \xi S_{21})(C'_{21} - \xi' S'_{21}) \\ &\quad - k_{12}(C_{12} - \xi S_{12})(C'_{12} - \xi' S'_{12})c_2. \end{aligned} \tag{20}$$

It will be averaged over noise, and supplemented with the Shapiro–Loginov theorem (Shapiro and Loginov, 1978; Loginov, 1996), which yields two equations like Eq. 10 (one for ξ and another for ξ'), along with

$$\frac{d}{dt} \langle \xi\xi' f \rangle = -\lambda \langle \xi\xi' f \rangle - \lambda' \langle \xi\xi' f \rangle + \left\langle \xi\xi' \frac{df}{dt} \right\rangle. \tag{21}$$

The resulting system is

$$\dot{y}(t) = -k_{12} \cdot M \cdot y(t) + k_{21}c_1 b, \tag{22}$$

where

$$y(t) = \begin{bmatrix} \langle c_2 \rangle \\ \langle \xi c_2 \rangle \\ \langle \xi' c_2 \rangle \\ \langle \xi\xi' c_2 \rangle \end{bmatrix} \quad b = \begin{bmatrix} C_{21}C'_{21} \\ -S_{21}C'_{21} \\ -S'_{21}C_{21} \\ S_{21}S'_{21} \end{bmatrix}, \tag{22a}$$

and

$$M = \begin{bmatrix} C_{12}C'_{12} & -S_{12}C'_{12} & -S'_{12}C_{12} & S_{12}S'_{12} \\ -S_{12}C'_{12} & C_{12}C'_{12} + r & S_{12}S'_{12} & -S'_{12}C_{12} \\ -S'_{12}C_{12} & S_{12}S'_{12} & C_{12}C'_{12} + r' & -S_{12}C'_{12} \\ S_{12}S'_{12} & -S'_{12}C_{12} & -S_{12}C'_{12} & C_{12}C'_{12} + r + r' \end{bmatrix}. \tag{22b}$$

Again, C_{ij} and S_{ij} denote hyperbolic cosines and sines (Eq. 8), and similar (primed) quantities are related to the second noise. The protein solubility is the equilibrium value of $\langle c_2 \rangle$. It is given by the first component of the column vector

$$y_{ech} = S_0 \cdot M^{-1} \cdot b. \tag{23}$$

Numerical fitting and the analytic requirement related to the exponential dependence of S on salt concentration in the asymptotic domain again suggest that the physically relevant limit is that of low noise correlation times as compared to k_{12}^{-1}. In this limit, the expression of protein solubility becomes

$$S = S_0 \frac{\cosh[a_{21}c_s/RT] \cdot \cosh[a'_{21}c_s/RT]}{\cosh[a_{12}c_s/RT] \cdot \cosh[a'_{12}c_s/RT]}, \tag{24}$$

and the comparison with Eq. 14, made in the high c_s limit, yields the Setschenow constant

$$K_s = \frac{1}{2.303 \cdot RT} [(a_{12} - a_{21}) + (a'_{12} - a'_{21})]. \tag{25}$$

TABLE 2 Parameters obtained by least-squares fit of the deoxy-Hb S data of Poillon and Bertles (1979), in the case of salting-out and weakly salting-in agents

Salt	x_{21}	x_{12}	x'_{21}	x'_{12}	K_s	c_{sa} (mol/l)	χ^2_m	ν	Q
NH_4Cl	0.1345	0.3442	0.5884	0.3442	−0.1451	2	0.2775	3	0.9642
NaCl	15.2084	15.1527	0.0752	0.1287	−0.0089	3	0.9551	6	0.9873
CsCl	0.4959	0.3888	0.0510	0.1638	0.0242	4	$7.1045 \cdot 10^{-9}$	0	0
KCl	0.4127	0.3539	0.0657	0.1392	0.0614	2.5	0.2121	7	1.0000
$MgSO_4$	30.4022	30.1211	0.0346	0.3767	0.2556	3	1.6714	12	0.9998
KF	12.4838	12.4122	0.1186	0.2759	0.3599	1	0.5996	3	0.8965
$(NH_4)_2SO_4$	0.1215	0.4836	0.9420	0.7178	0.5791	1.2	0.1166	3	0.9898
K_2HPO_4	0.6273	0.4185	0.0000	0.4185	0.8807	2	4.5701	15	0.9952
Na_2SO_4	1.0864	0.6868	0.0000	0.6868	1.2058	1.5	3.3851	2	0.1840
Cs_2SO_4	19.2298	19.0742	0.0000	0.4746	1.3394	1.5	1.5453	1	0.2138

The underlying theory contains two independent dichotomic fluctuations, yielding Eq. 24, which accounts for the complex behavior of solutions that show a maximum or a plateau in the plot of protein solubility versus salt concentration. The noise amplitudes are obtained by multiplying the dimensionless ones (x_{ij} and x'_{ij}) by α. Its value and the remaining notations are given in the comments that follow Table 1.

ANALYSIS OF EXPERIMENTAL DATA

As an example, in the following, we use the fluctuation theory to analyze the experimental results of Poillon and Bertles (1979) regarding the polymerization of deoxy-Hb S. These authors have presented a comprehensive data set for protein solubility in the presence of various lyotropic salts at 30°C and pH = 6.8. Given the experimental errors of the measurements of about ±4% and no individual error bars, we considered an error of 1 g/dl for each point, because most equilibrium protein solubilities are situated in the range of 15–30 g/dl. This estimation needs to be done carefully because it has impact on the absolute value of the merit functions and, consequently, also on the goodness-of-fit evaluation based on the chi-square probability function (Press et al, 1993). More specifically, $Q = 1 - \mathrm{P}(0.5\nu, 0.5\chi^2_m)$ is the probability that the chi-square will exceed the value χ^2_m by chance, even for a correct model. Here $\mathrm{P}(a, x)$ stands for the incomplete gamma function (Abramowitz and Stegun, 1984), and χ^2_m is the minimum of the merit function. It is determined by a nonlinear least-squares program, based on the *fmins* function of Matlab 5.2 (The MathWorks, Inc., Natick, MA). The number of degrees of freedom of the chi-square distribution, ν, equals the number of experimental points minus the number of model parameters. A model is considered acceptable if the values of Q are higher than about 0.1 (see Tables 1 and 2).

Bearing these in mind, we may conclude that the effects of salts with pronounced chaotropic character are well summarized in a single dichotomic fluctuation. The experimental results (Poillon and Bertles, 1979) are plotted in Fig. 2 using markers, and the continuous line is given by Eq. 15 with three adjustable parameters: the protein solubility, S_0, in the absence of salts and the noise amplitudes, a_{21} and a_{12}. Treating S_0 as an adjustable parameter has slightly improved the fit, and the difference between the measured value and that resulted from fitting the data turned out to be less than the experimental error of 4%.

As seen in Table 1, the noise amplitude entering the barrier of the direct process, a_{21}, has to be different from that of the inverse one, a_{12}. Their difference (and hence K_s from Eq. 17) is very well assessed by the fit program: successive runs from random starting points of the parameter space yield the same result up to the seventh digit. Their sum, however, is not so well defined; its variations of about 5% leave the fit quality practically unchanged. This tells us that the model parameters obtained are not unique, they correspond to points from a credibility domain. The size of this domain depends on the experimental data set. Expressions like Eqs. 15 and 24 are sensitive to their arguments in the low concentration range. Experimental data of high accuracy, obtained also at low salt concentrations, are needed to define more precisely the noise amplitudes (i.e., to reduce the size of the credibility domain). Fig. 3 shows that the influence of Hofmeister-neutral and salting-out agents on protein solubility can be ascribed to two independent dichotomic noises. The solid lines from Fig. 3 result from Eq. 24 with four adjustable parameters (the noise amplitudes). These are given in Table 2. In the case of CsCl, the number of experimental points equals the number of model parameters, making the fit-quality assessment meaningless.

Although most chaotropic salts cause exponential increase of protein solubilities, in the case of salts from Table 2, the Setschenow law becomes valid only at high salt concentrations. The estimated lower limit of this asymptotic domain is denoted by c_{sa} in both tables.

The onset of the exponential behavior is illustrated on Fig. 4. The dotted line plots the Setschenow law (Eq. 14) and the solid lines are given by the fit function (Eq. 24). The free energy change that accompanies depolymerization is modified in the presence of salts by the amount

$$\Delta G^0_{\mathrm{D,salt}} = -RT\ln(S/S_0), \qquad (26)$$

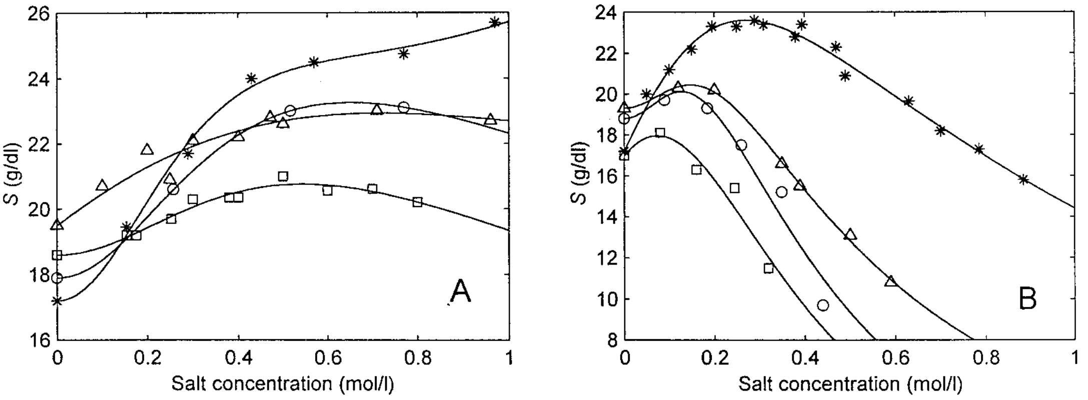

FIGURE 3 Deoxy-Hb S solubility plotted against salt concentration. The experimental data have been obtained by Poillon and Bertles (1979). The curves result from fluctuation theory. Two independent dichotomic noises in the energy barrier lead to Eq. 24, which was fitted, in the least-squares sense, to data regarding deoxy-Hb S solubility in the presence of cosolutes that are not strongly salting-in. (*A*) chloride salts situated at the limit between salting-in and salting-out behavior: NH_4Cl (∗), NaCl (△), CsCl (○), KCl (□) and (*B*) sulphate salts with salting-out character at high concentrations: $MgSO_4$ (∗), $(NH_4)_2SO_4$ (△), Na_2SO_4 (○) and Cs_2SO_4 (□).

where the argument of the logarithm is given either by Eq. 15 or by Eq. 24, depending on the particular salt. This quantity is a measure of the extent to which the salt shifts the chemical equilibrium of Eq. 1. For example, in the particular case of $MgSO_4$, it is plotted in Fig. 5. Note that, for most values of the salt concentration, the temperature-dependence of $\Delta G^0_{\mathrm{D,salt}}$ is practically linear in the interval under consideration, allowing for a straightforward split into enthalpic and entropic contributions.

A connection with the thermodynamic approach of Timasheff and co-workers, (Timasheff, 1993) may be inferred by taking the high concentration limit of Eq. 26 in the simpler case when the solubility ratio is given by Eq. 15.

The result is given by $\Delta G^0_{\mathrm{D,salt}} = (a_{12} - a_{21})c_s$, and may be compared by the corresponding thermodynamic expression, $\Delta G^0_{\mathrm{D,salt}} = \Delta\mu_2^{\mathrm{l}} - \Delta\mu_2^{\mathrm{s}}$, where $\Delta\mu_2^{\mathrm{l}}$ is the transfer free energy of a mole of dissolved proteins from water into the salt-containing solution (Arakawa et al., 1990a; Timasheff, 1993), whereas $\Delta\mu_2^{\mathrm{s}}$ is the transfer free energy of proteins in polymerized state from water into the solution of concentration c_s. Up to an arbitrary additive term, we may identify the noise amplitudes. This procedure suggests that the amplitude of the fluctuations suffered by the activation free energy of the reverse process of Eq. 1 (polymerization) is $a_{12} = \Delta\mu_2^{\mathrm{l}}/c_s$, which is a property of the three-component liquid phase. Similarly, the noise amplitude associated to the solution process, $a_{21} = \Delta\mu_2^{\mathrm{s}}/c_s$, depends only on the polymerized state. Thus, it seems reasonable to view the stochastic switches between barrier profiles as baseline fluctuations, i.e., in Eq. 4, the amplitudes are $a_{12} = a_2$, $a_{21} = a_1$, and $a_{\mathrm{ac}} = 0$. Note that, in the present approach, the noise amplitudes are considered independent of the salt concentrations and constitute the high concentration limit of the above expressions. (By high salt concentration, we mean the asymptotic domain, $c_s \geq c_{\mathrm{sa}}$, specified in Tables 1 and 2.) At low concentrations, the solubility law turns out to be nonexponential, and, in the framework of the present theory, it is given by Eq. 15 or 24.

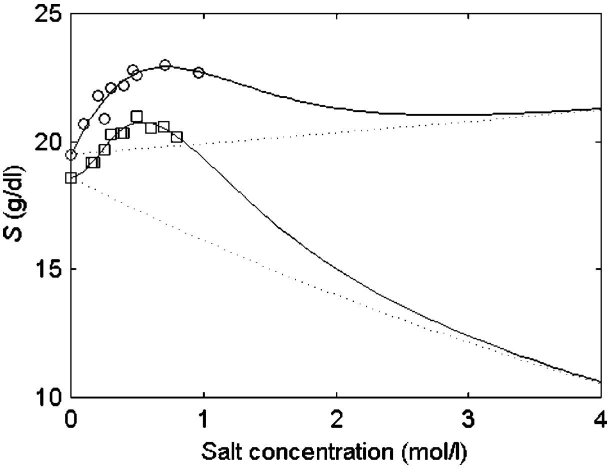

FIGURE 4 The asymptotic behavior of protein solubility versus salt concentration, described by the Setschenow law, Eq. 14, is represented by dotted lines, along with experimental points (Poillon and Bertles, 1979) and the fit function, Eq. 24, (*solid line*). The data regarding NaCl are plotted using circles and those of KCl are marked by squares. NaCl is rather ineffective in what concerns Hofmeister effects, whereas KCl has a weak salting-out action.

CONCLUSIONS

A theoretical model is proposed in which salt-induced changes in protein solubility are attributed to fluctuations of the barrier profile that governs the aggregation–solution process. The average frequency of stochastic switches be-

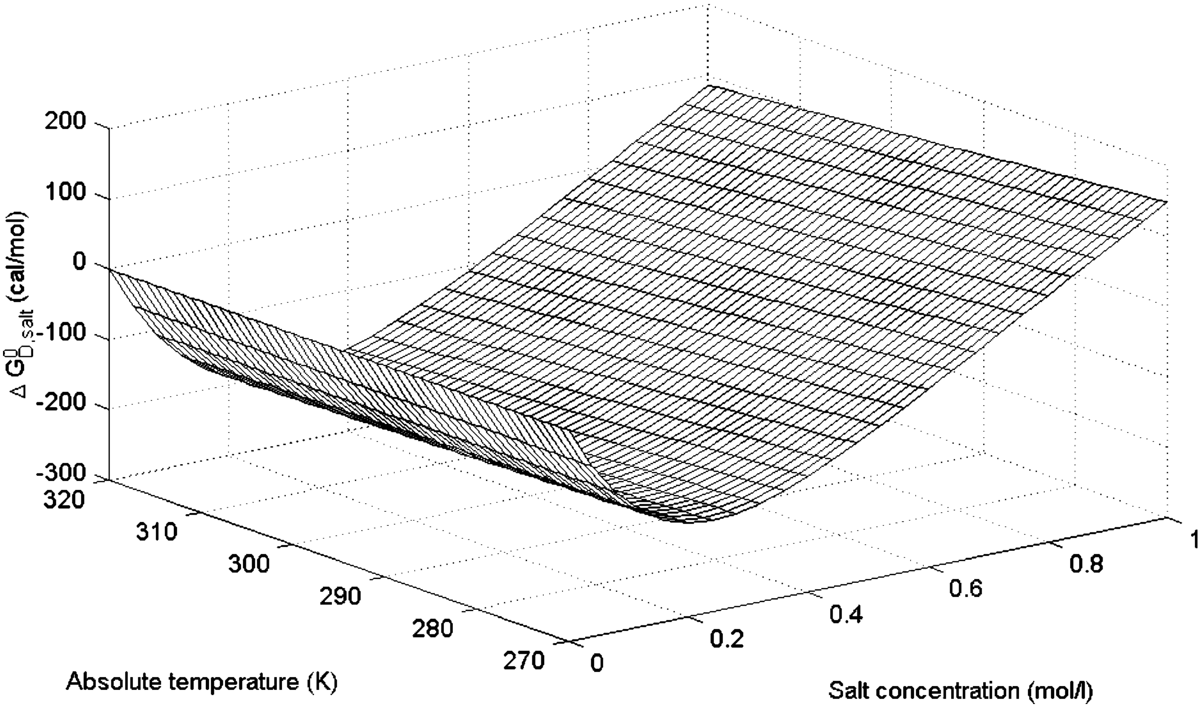

FIGURE 5 The salt-induced Gibbs free energy change, $\Delta G^0_{D,salt}$, that accompanies the depolymerization of deoxy-Hb S is plotted against temperature and $MgSO_4$ concentration, as predicted by fluctuation theory (Eqs. 26 and 24).

tween the various profiles is λ, the inverse of the noise correlation time, which turned out to be much higher than the aggregation rate constant. This conclusion is based both on numerical fit to solubility data of deoxy-HbS (Poillon and Bertles, 1979), and analytic arguments related to the empirical solubility law of Setschenow, valid for most soluble proteins at high enough cosolute concentrations (Arakawa et al., 1990a; Cacace et al., 1997). As a consequence, the protein aggregation/solution evolves according to the mean rate constants, and the noise correlation parameter drops out of the results. This may not be the case when dealing with quick reaction steps of proteins in the presence of cosolutes.

The theory presented in this paper, based only on a general assumption of salt-induced free energy fluctuations in macromolecules, is, according to our knowledge, the first formalism suitable for a satisfactory description of protein solubility data concerning Hofmeister effects along the whole range of cosolute concentrations. One of its virtues is that it identifies the concentration range in which Setschenow's law is expected to be valid, even if experimental data are available only at lower concentrations. This is at variance with earlier approaches, based on exponential fits of protein solubility data in some salt concentration intervals (Robinson and Jencks, 1965; Poillon and Bertles, 1979). Considering the good match of experimental and theoretical data, it is possible to extrapolate to solubility values lying out of the experimentally investigated range.

Given the general nature of the assumptions used in this theory, it is readily applicable to other reactions of macromolecules influenced by the presence of cosolutes. For example, it offers a method to analyze kinetic phenomena associated with Hofmeister effects.

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