Why pH Titration in Protein Solutions Follows a Hofmeister Series

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Measurements of pH in single-phase cytochrome c suspensions are reported. The pH, as determined by a glass electrode, has a fixed value. With the addition of salt, the supposedly fixed pH changes strongly. The pH depends on salt type and concentration and follows a Hofmeister series. A theoretical interpretation is given that provides insights into such Hofmeister effects. These occur generally in protein solutions. While classical electrostatic models provide partial understanding of such trends in protein solutions, they fail to explain the observed ion specificity. Such models neglect electrodynamic fluctuation (dispersion) forces acting between ions and proteins. We use a Poisson−Boltzmann cell model that takes these ionic dispersion potentials between ions and proteins into account. The observed ion specificity can then be accounted for. Proteins act as buffers that display similar salt-dependent pH trends not previously explained.

I. Introduction

Salts, with a common cation, but differing in anion, have different effectiveness in stabilizing protein suspensions. This fact, the first demonstration of specific ion effects, is due to Hofmeister effects.[1−3] The effectiveness of salts could be arranged in a sequence that later seemed to be universal. Hofmeister specific ion effects now refer to the relative effectiveness of anions or cations on a wide range of phenomena. The effects appear, for instance, with the solubility of lysozyme,[4] charge of lysozyme[5,6] and cytochrome c,[7] and pH.[8] They appear in phenomena ranging from the surface tension of electrolytes[9] to enzymatic action to bacterial growth. We here give a theoretical explanation that appears to account for such ion-specific effects that show up with measurements of pH with a glass electrode.

The only ionic parameters included in textbook descriptions of pH measurements in protein and salt solutions are the ionic charges and the ionic radii of the interacting species. The experiments on protein solutions here reported show that the measured pH increases with salt concentration. Further, the magnitudes of the effects are very different, depending on salt type. Thus the same apparent pH is obtained when the proteins are in a 0.1 M NaSCN solution or in a 0.5 M NaCl solution. Neither effect can be explained by standard electrostatic theories. Significantly, the measured pH increases with increasing anion polarizability.

A Poisson−Boltzmann mean-field cell model is used to demonstrate that this and other phenomena observed in various protein solutions can be better understood once ionic dispersion potentials acting between ions and proteins are included in the theoretical formalism. Such forces are omitted in the conventional theory.

Results of pH measurements with a glass electrode in cytochrome c solutions will be presented in section II. The theoretical framework of the Poisson−Boltzmann cell model that we shall need will be described in section III. In section IV we give numerical examples obtained from the cell model for solution pH in cytochrome c protein solutions. We end in section V with some general conclusions on previously inexplicable pH measurements in buffer and protein solutions.

II. Experimental Section: pH Titration of Cytochrome c Solutions

Materials. Cytochrome c from horse heart (product no. C7752) was purchased from Sigma Chemical Company. This product is obtained using a procedure that avoids the trichloroacetic acid (TCA), which is known to promote dimer formation. The salts used, NaCl, NaSCN, and Na_2SO_4 (grade >99.9%), were supplied by Aldrich (Milan, Italy); standardized solutions of sodium hydroxide, 0.1 N, and hydrochloric acid, 2 N, were purchased from Merck. Bi-distilled water, with a resistance greater than 18.2 MΩ cm, was obtained with a Millipore Milli-Q system (Organex).

Samples. Protein stock solutions were prepared by dissolving cytochrome c powder in water to about 0.113 g/mL. The titrant solutions (HCl and NaOH) were added with NaX ($X^- = SCN^-$ or Cl^-) to keep the salt concentration constant during the titration experiment. Each sample was obtained by mixing equal volumes of the protein stock solution and pure Milli-Q water or solutions of 0.2 or 0.5 M NaX ($X^- = SCN^-$ and Cl^-) to obtain starting samples at protein volume fraction $\phi = 0.05$ (about 4.5 mM) and 0, 0.1, 0.25, and 0.5 M NaX ($X^- = SCN^-$ and Cl^-). Each experiment was carried out by adding a fixed amount of HCl 2 N (20 μL) to the starting cytochrome c solution (volume fraction $\phi = 0.05$) and then titrating the protein back with the NaOH solution. The final protein concentration was about 2 mM.

Methods. pH measurements were performed at room temperature using a Crison Basic2 pH meter. A combined glass electrode was used and calibrated using standard solutions at

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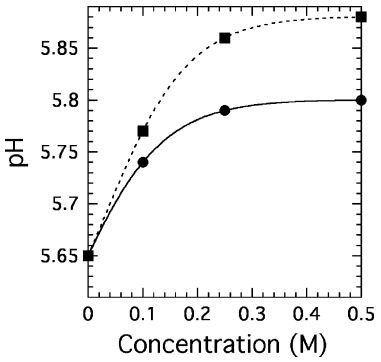

Figure 1. Experimental pH of cytochrome c solutions as functions of salt concentration as measured after dissolving the protein and the corresponding salt. Squares and circles correspond to NaSCN and NaCl, respectively, as different choices of background salt.

pH 4.0, 7.0, and 10.0 purchased from Merck. Each curve represents an average of between three and five titration experiments. The curves obtained were corrected by subtraction of the titrant concentration necessary to adjust an identical volume of solution not containing the protein (i.e., water, 0.1 M NaCl, 0.5 M NaCl, or 0.1 M NaSCN) to each corresponding pH value.

Experimental Results. The experimental results of Figure 1 show that the pH in a solution of cytochrome c as measured with a glass electrode depends strongly on the choice of background salt and on the salt concentration. The measured pH in the acid regime increase with added salt concentration and with the increased anionic polarizability, as can be seen in Figure 2a. By contrast, in the basic regime (pH > 9), the measured pH decreases with increasing salt concentration; cf. Figure 2b. Note that one obtains the same measured pH if one replaces a smaller concentration of NaSCN with a much larger concentration of NaCl. This cannot be explained within classical theories that are based on the Nernst equation.

III. Theory

We use a Poisson−Boltzmann cell model[10] to investigate solution pH in a suspension with a cytochrome c protein concentration c_p. The globular proteins are modeled as charged spheres with uniformly distributed ionizable charge groups.[6,7] In the cell model the charge-regulated sphere occupies a spherical volume equal to the inverse protein concentration. The condition of electroneutrality requires that

$$Z_p + n_{H^+} + n_+ z_+ + n_- z_- - n_{OH^-} = 0 \qquad (1)$$

Here Z_p is the effective number of charges on each protein (which, depending on pH, can be positive or negative), and n_{H^+} and n_{OH^-} are the total number of free hydronium and hydroxide ions in the cell. $n_\pm$ and $z_\pm$ are the total number of and charge number of ions in the cell. Since we focus here on systems with a pH less than 6, we can neglect the low concentration of hydroxide ions. This cell model enables us to determine how many of the hydronium ions are present in solution and how many are bound to proteins. The protein is modeled as a dielectric, homogeneously charged, hard sphere of radius r_p. Such a model should hopefully capture the main features of rigid proteins with a high degree of structural stability.

Bulk pH is equal to minus the logarithm of the hydronium ion chemical potential, taken to be constant in the cell[11,12]

$$\mathrm{pH} = -\log[c_H \gamma_H] \qquad (2)$$

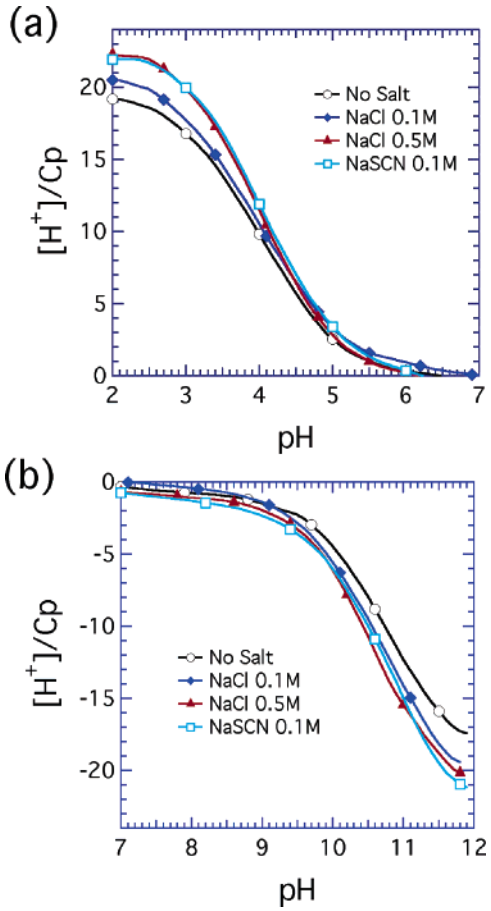

Figure 2. Experimental titration curves of cytochrome c as functions of salt concentration (no salt, 0.1 M NaCl, 0.5 M NaCl, and 0.1 M NaSCN) shown (a) in the acid region and (b) in the basic region. c_p = protein concentration.

where c_H is the hydronium ion concentration. The activity coefficient is here approximated with the purely electrostatic (very) low-density approximation[13]

$$\gamma_H \approx \exp\left[-\left(\frac{e^2}{8\pi\epsilon_0\epsilon_w kT}\right)\frac{\kappa_{eff}}{1+2r_{ion}\kappa_{eff}} + \frac{8\pi}{3}c(2r_{ion})^3\right] \qquad (3)$$

We will in a subsequent work improve the theory by using a better approximation for the activity coefficient. Here ϵ_w is the dielectric constant of water, k is Boltzmann's constant, T is temperature, and κ_{eff} is the effective inverse Debye length of the solution. We remark that the Debye length to be used is that for the whole solution not just the electrolyte. The presence of even an extremely low concentration of highly charged species such as proteins dramatically changes the Debye screening length. While the chemical potential is constant the electrochemical potential changes near interfaces.[14] The "surface pH" differs from pH by the addition of the factor $e\phi$(surface)/kT ln(10). Mörnestam et al.[10] have demonstrated that the nonspecific concentration dependence of pH titration could be understood if one plots protein charge against pH + $e\phi$(surface)/kT ln(10) rather than against pH.

The net protein charge, surface pH, solution pH, and ion distributions can be determined self-consistently via the nonlinear Poisson−Boltzmann equation

$$\frac{\epsilon_w\epsilon_0}{r^2}\frac{d}{dr}\left(r^2\frac{d\phi}{dr}\right) = -e[c_+(r) - c_-(r) + c_{H^+}(r)] \qquad (3)$$

with the ion concentrations given by

$$c_{\pm}(r) = c_{\pm}^{0} \exp(-[\pm e\phi + U_{\pm}(r)]) \quad (4)$$

Here ϕ is the self-consistent electrostatic potential, and $U_{\pm}$ is the interaction potential experienced by the ions. This interaction potential receives contributions from different sources (e.g., hard-core interaction and ion−ion interactions), but here we include only the ionic dispersion potential between the ion and the protein. For consistency this electrodynamic fluctuation contribution has to be included at the same nonlinear level in the Poisson−Boltzmann equation as the electrostatic contribution. The boundary conditions follow from global charge neutrality, and we have no charges at the cell boundary. The electric field at the protein surface is related to the solution charge as follows

$$(r_{\mathrm{p}} + r_{\mathrm{ion}})^2 \frac{\mathrm{d}\phi}{\mathrm{d}r}|_{r=r_{\mathrm{p}}+r_{\mathrm{ion}}} = -\sum_i q_{\pm}^{i}/4\pi\epsilon_0\epsilon_{\mathrm{w}} \quad (5)$$

The above equations are solved in a standard way[18] with the additional complication that we now prescribe the total amount of hydronium ions in the system as input data. Following an initial guess for the hydronium ion concentration at the cell boundary one obtains a certain amount of bound hydronium ions. Since the total amount in the system is specified we can then iterate in a straightforward way until the sum of free and bound hydronium ions is equal to what we specified initially.

The dispersion potential between a point particle (ion) and a sphere (protein) is approximated as[6]

$$U_{\pm}(r) \approx \frac{B_{\pm}}{(r - r_{\mathrm{p}})^3[1 + (r - r_{\mathrm{p}})^3/(r_{\mathrm{p}})^3]} \quad (6)$$

where the dispersion coefficient ($B_{\pm}$) will be different for different combinations of ion and spherical protein. By the term dispersion or non-electrostatic (NES) forces we mean the totality of many-body electrodynamic fluctuation forces embraced by extensions of Lifshitz theory. That is, they include many-body dipole−dipole, induced dipole−dipole, and dispersion forces proper. Using ionic polarizabilities and dielectric properties described elsewhere[6] we estimate that the dispersion coefficients are around -0.454×10^{-50}, -3.574×10^{-50}, and -10×10^{-50} J m^3 for sodium, chloride, and thiocyanate-like ions.

IV. Numerical Results: pH in Protein Solutions

It is known that the protein net charge in both potassium chloride[5] and sodium chloride[10] increases with concentration and that both charge and pH (see references in ref 6) in protein solutions depend on the choice of background salt (the cytochrome c or lysozyme charge is, for instance, larger in 0.1 M KSCN than in 0.1 M KCl). While results obtained using electrostatic estimates sometimes can be used to explain the nonspecific concentration dependence we emphasize again that it cannot accommodate any such ion specificity.

We now demonstrate how the Poisson−Boltzmann cell model, with ionic dispersion potentials included, can capture the essential features of the experimental ion specificity.

We consider the properties of a 2 mM cytochrome c protein solution with constant total (bound plus free) hydronium concentration and a varying salt concentration. In this case, the task of theoretical modeling is to find out how much of the added hydronium ion is in solution (changing pH directly) and how much is bound to the protein.

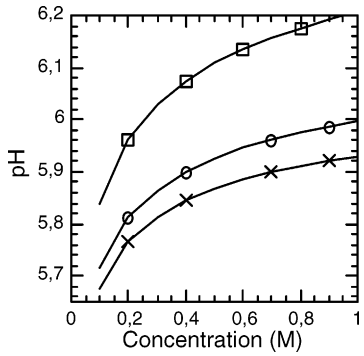

Figure 3. Calculated pH of cytochrome c solutions as functions of salt concentration. Squares and circles correspond to model calculations for "NaSCN" and "NaCl" salt, respectively. The purely electrostatic result (without ionic dispersion potentials) is marked with crosses.

As can be seen in Figure 3 the predicted solution pH increases with added salt and also with increasing ion polarizability. This means that one obtains the same pH in a system with a larger concentration of NaCl as one does in a system with a smaller concentration of NaSCN. This trend is in agreement with the experimental results for cytochrome c (cf. Figures 1 and 2). We remark that the local electrochemical potential near the protein is very well buffered. More polarizable anions are more strongly attracted toward the protein (or buffer) surface. This in turn has the consequence that more hydronium ions are attracted to the surface (or strictly speaking a region of higher surface electrochemical potential) and more bound hydronium ions (higher charge). There are then fewer hydronium ions present in solution and a higher bulk pH.

V. Conclusions

Measurement of pH via a glass electrode is an accepted, standard way to determine the pH of a solution. However, it has not been understood why the measured pH depends on the choice of salt and salt concentration and notably on the choice of protein. The same problem occurs with buffers. We have here demonstrated the important role of ionic dispersion forces that have previously been neglected from theoretical consideration. These polarizability-dependent forces seem to lie behind the ion specificity observed in pH measurements on globular protein solutions. While it is clear that ionic dispersion forces, strongly dependent on ionic polarizabilities, are a major factor underpinning the experimentally observed Hofmeister series, there may also be other things that influence ion specificity: e.g., ionic size, the interaction between polarizable ions and water molecules,[2] and for an air−water interface also changes in the ionic solvation energies as ions move into the interface region with its profile of water molecules and dissolved gases.[9] The essential point to note is that when ionic dispersion and electrostatic potentials are treated together in a nonlinear theory they give consistency between theory and experiment in a large number of systems. It was recently shown that the pH in buffer solutions follows similar ion-specific trends[15] as the pH in protein solutions.

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