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LETTER TO THE EDITOR

Protein crystallization: scaling of charge and salt concentration in lysozyme solutions

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Abstract. We studied the crystallization of lysozyme solutions by adding sodium chloride at pH = 4.5, 5.9 and 7.8. A universal crystallization boundary is found if data are scaled according to the salt concentration normalized by the square of the charge at the appropriate pH. Calculations show that this finding is consistent with recent attempts to rationalize protein crystallization using second virial coefficients.

1. Introduction

Understanding protein crystallization is important for biology for a number of reasons. First, crystals are needed for diffraction studies to elucidate the three-dimensional structures of proteins [1]. Secondly, crystalline proteins occur in normal and diseased tissues [1]. Finally, there is growing interest in using protein crystals in biotechnology, as a means of batch purification and as 'organic zeolites' for enzymatic reactions [2]. In the laboratory, crystallization is usually induced by adding salt, alcohol or polymer to dilute protein solutions. In current practice, the conditions under which crystallization occurs are determined by trial and error. This method is time consuming, so that crystal growth is now the major bottleneck in protein crystallography. Furthermore trial and error is wasteful, especially since new proteins are often available only in very small quantities. A better understanding of protein crystallization will therefore be welcome on a number of biological fronts.

These biological implications motivate a growing number of physical scientists to study protein crystallization. Another motivation is the peculiar nature of protein crystals. For example, they can contain a high proportion of solvent (typically 50%). Studying the formation of these crystals may therefore open up new vistas in crystal nucleation and growth. Finally, proteins can be seen as 'model colloids', as they are particles with well-defined properties. Some of these properties are hard to achieve in synthetic inorganic or polymeric colloids. For example, proteins are monodispersed (single-sized). Perhaps more interestingly, they tend to carry in the order of 10 electronic charges, while the charge of synthetic colloids is usually 10–100 times higher. The low charge, coupled with their relatively small sizes (in the 1–5 nm range), render proteins an ideal model system to study a class of phase transitions caused by

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(classical) charge correlation effects that is arousing much current interest [3, 4]. Studying these phase transitions in synthetic colloids requires difficult experiments at very low salt concentrations (in the μM range) [3]. The same effects are expected to occur for protein solutions at or above mM salt concentrations [4].

The mechamism whereby salt or polymeric additives induce crystallization is under dispute. In outline, the biological community tends to see the salt or the polymer competing for water molecules, thus lowering the amount available for solvation of the protein [1]. The basic process is the same as evaporation, except that the additives 'remove' water from the proteins without any water molecules ever leaving the solution. Physical scientists, however, tend to appeal to more generic explanations. In the case of salt, many authors have explored proteins and salt ions as objects interacting via Coulomb and van der Waals interactions (e.g. [5]), while acknowledging that this cannot be the whole story—different equi-valent salts have different efficiencies in inducing crystallization (the 'Hofmeister' effect).

We study the crystallization of lysozyme induced by sodium chloride. Lysozyme is a well-characterized small polypetide and is a standard model for studying protein crystallization [6–12]. We map out the crystallization boundary at three different pHs. A universal crystallization boundary is obtained if data are scaled according to salt concentration normalized by the square of the charge at the appropriate pH.

2. Experiments and results

Three-times crystallized, dialysed and lyophilized chicken egg-white lysozyme (Sigma Chemical Company) was used without further purification. Protein was dissolved in buffer at 150 mg cm^{-3}. For pH = 4.5, 50 mM sodium acetate titrated with HCl was used. To obtain pH = 5.9 and 7.8, we used 0.1 M mixtures of $Na_2HPO_4.7H_2O$ and NaH_2PO_4 (based on appendix in [1]). A very small amount of sodium azide was added to avoid bacterial growth. To remove dust, each stock solution was centrifuged at 13 000 rpm for 5 minutes. NaCl solution (3 M) and deionized water were added to give 200 μl samples sealed in glass vials for observation at room temperature (22.5$\pm$1 °C).

At low salt concentrations, c_s, the samples remained homogeneous solutions. When c_s was high enough, crystals formed. These were observed by eye but confirmed under the optical microscope in some cases. At the highest c_s various non-equilibrium aggregation phenomena were observed. At this temperature, liquid–liquid phase separation did not occur [13, 14]. Here we concentrate on the crystallization boundary.

The observed phases as functions of ϕ and c_s are shown in figure 1. The protein volume fraction, ϕ, is related to the mass concentration c_p by

$$\phi = \bar{v} n_p = \bar{v} \frac{N_A c_p}{M_w} \tag{1}$$

where $\bar{v}$, n_p, M_w are the molecular volume, number density and molecular weight of the protein, and N_A is Avagadro's number. Lysozyme is approximately an ellipsoid with volume $\bar{v} = (\pi/6) \times 4.5 \times 3.0 \times 3.0$ nm^3 [14]; its molecular weight from the amino acid sequence is $M_w = 14\,320$ g mol^{-1}. The data (figure 1) show that crystallization occurs at lower c_s for higher values of the pH.

The data are replotted in figure 2 where the vertical axis is now c_s/Q^2. The charge, Q (in units of the electronic charge, e), is taken from titration experiments and calculations [15]. At pH = 4.5, 5.9 and 7.8, the average charge on lysozyme was Q =11.4, 9.4 and 8 respectively. Furthermore, at pH $\geqslant$ 4.5, Q was independent of salt concentration in the range 0.1 M $< c_s <$ 1 M. In this scaled representation, data from the three different pHs collapse to give a universal crystallization boundary.

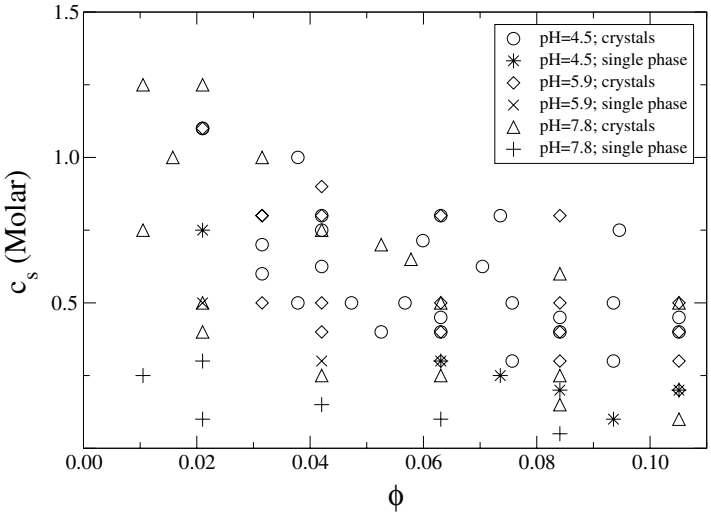

Figure 1. Phase diagram of lysozyme solutions with added sodium chloride at three different pHs. The axes give the dimensionless protein volume fraction ϕ (see text) and the salt concentration c_s in molar. Crystals coexisting with solution: pH = 4.5 ◯, pH = 5.9 ⋄, pH = 7.8 △; single-phased solution: pH = 4.5 ∗, pH = 5.9 ×, pH = 7.8 +.

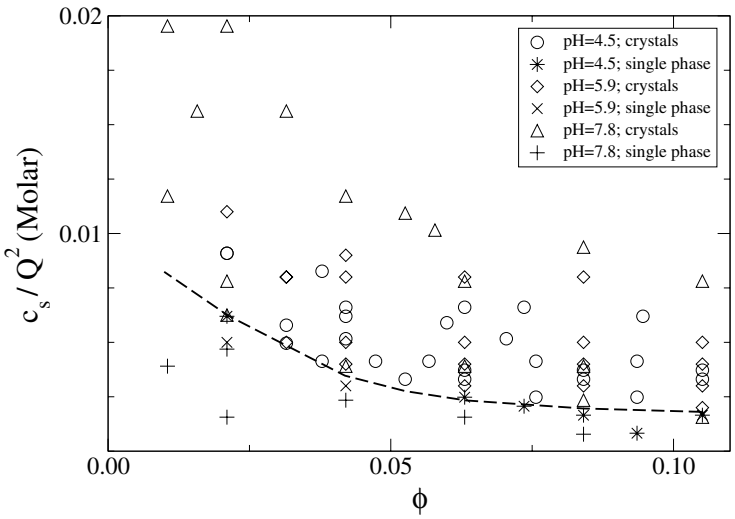

Figure 2. Scaled phase diagram. The vertical axis gives c_s/Q^2, where $Q = 11.4$, 9.4 and 8 for pH = 4.5, 5.9 and 7.8 respectively. Symbols have the same meaning as in figure 1. The broken curve indicates the approximate position of the universal crystallization boundary in this representation.

3. Discussion

One way to rationalize the c_s/Q^2 scaling shown in figure 2 is via recent work correlating protein crystallization with the second virial coefficient, B_2. For particles interacting with pair potential $U(r)$ when their centres are separated by r [16]

$$B_2 = 2\pi \int_0^\infty \left(1 - \mathrm{e}^{-U(r)/k_BT}\right) r^2 \mathrm{d}r. \tag{2}$$

George and Wilson [17] and Zukoski and co-workers [7, 18] found that many different globular proteins show a quasi-universal crystallization boundary in the (ϕ, B_2) plane. At constant protein volume fraction, crystallization starts at the same B_2 for all proteins and precipitants studied, including lysozyme/NaCl.

If this 'B_2 scaling' is universal, we expect our 'salt-charge scaling' to map onto it. To check whether this is the case, we need a model for $U(r)$, the inter-protein potential. The simplest way to model the interaction is to consider each protein as a macroion with radius R and charge Qe immersed in a sea of ions dissolved in a structureless solvent with dielectric constant ϵ, taken to be 80 for water. At the salt concentrations used in this study, a simple model for the interparticle potential is that due to Derjaguin–Landau–Verwey–Overbeek (DLVO) [19]:

$$U(r) = U_{\mathrm{HS}}(r) + U_{\mathrm{C}}(r) + U_{\mathrm{A}}(r). \tag{3}$$

An excluded volume component prevents the macroions from overlapping. This is modelled by a hard-sphere potential, $U_{\mathrm{HS}}(r)$. A linearized Poisson–Boltzmann form is used for the screened-Coulomb part, $U_{\mathrm{C}}(r)$; for particles with constant charge this is:

$$U_{\mathrm{C}}(r) = \frac{(Qe)^2}{4\pi\epsilon_0\epsilon r}\frac{\exp[-\kappa(r-2R)]}{(1+\kappa R)^2} \tag{4}$$

with κ the Debye screening constant. For mono-valent counterions in water at room temperature, κ is given in terms of the salt concentration by

$$\kappa = \left(0.327 \times 10^{10}\ \mathrm{m}^{-1}\right) \times \sqrt{c_s/\mathrm{M}}. \tag{5}$$

Finally, a van der Waals component, $U_{\mathrm{A}}(r)$, describes the dispersion interaction:

$$U_{\mathrm{A}}(r) = -\frac{A}{12}\left(\frac{4R^2}{r^2-4R^2} + \frac{4R^2}{r^2} + 2\ln\left[1-\frac{4R^2}{r^2}\right]\right). \tag{6}$$

Scattering measurements give the 'Hamaker' constant $A \sim 8.3k_BT$ for lysozyme [10].

The DLVO potential was integrated numerically to give the second virial coefficient as a function of Q and c_s. The hard-sphere part of the integral can be calculated analytically and factored out, so that we can write

$$b_2(c_s, Q) \equiv \frac{B_2}{B_2^{\mathrm{HS}}} = 1 + \frac{24}{\mathcal{R}^3}\int_{2R+\delta}^{\infty}\left(1 - \mathrm{e}^{-[U_{\mathrm{A}}(r)+U_{\mathrm{C}}(r)]/k_BT}\right)r^2\mathrm{d}r. \tag{7}$$

We use $R = 1.7$ nm, because a sphere of this radius has volume $\bar{v}$. The continuum approximation upon which $U_{\mathrm{A}}(r)$ is based breaks down at atomic dimensions. This is reflected in the divergence of the integral if the lower limit is $r = 2R$. We therefore integrate from $r = 2R + \delta$. Consistency then demands that we take $\mathcal{R} = R + \delta/2$ for the hard-sphere interaction radius used to calculate B_2^{HS}. To pin down a particular value for δ we look for agreement between our data and previous measurements. At $Q = 11.4$ (i.e. pH = 4.5) and $\phi \approx 0.05$ we take the crystallization boundary to be at $c_s = 0.358$ M, consistent with the data in figures 1 and 2. Using $\delta = 0.1437$ nm the calculated b_2 for these values of Q and c_s matches the value measured under similar conditions [18]. Our δ is bracketed by published values in the range of ∼0.1–0.3 nm [9–12].

The values of Q and c_s needed to give $b_2 = -0.85$ and $b_2 = -3.2$ are plotted in figure 3, where the broken lines show that, for $Q \leqslant 12$,

$$b_2(c_s, Q) \approx b_2\left(c_s/Q^2\right). \tag{8}$$

Experimental b_2 values [7–9, 20] satisfy the same scaling [21]. Equation (8) and the b_2 scaling of the crystallization boundary [17, 18] together imply the 'salt-charge scaling' evidenced in figures 2. The range $-3.2 < b_2 < -0.85$ was identified as the favourable 'crystallization

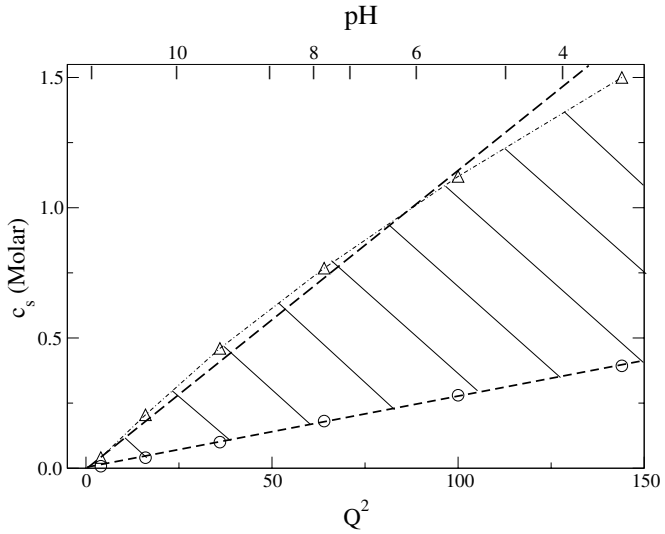

Figure 3. Contours of constant b_2 ($b_2 = -0.85$ ○; $b_2 = -3.2$ △, the chain curve links these points). Crystallization at any pH is expected within the hatched area. The broken lines show that b_2 contours are approximately linear.

window' [17, 18]; thus the conditions for crystallising lysozyme using NaCl at any pH are bounded within the hatched area in figure 3.

Note that c_s does not include buffer and the small (but not precisely known) amount of impurities in the protein. We have estimated their effects; even in the worst case our conclusions are not materially affected.

4. Summary and conclusions

A common crystallization boundary of lysozyme at different pHs is found when the salt concentration is normalized by the square of the charge at the appropriate pH. Within a DLVO framework, this 'salt-charge scaling' is consistent with a previously established scaling according to the second virial coefficient. The applicability of a simple DLVO potential for proteins has been questioned before, e.g. [5]. We have shown that this potential *is* sufficient for rationalizing the observed scaling. The physical origin of this scaling is, however, unclear; indeed it may simply be a mathematical coincidence in the range of experimentally relevant parameters.

Our argument predicts a common 'c_s/Q^2 crystallization boundary' for any particular protein irrespective of the type of salt. This contradicts the observed 'Hofmeister' effect, that different equi-valent salts do not have the same 'salting out efficiency'. Nevertheless, even if a separate c_s/Q^2 scaling relation holds for each salt, the amount of pH scanning needed in crystallization trials can be reduced (provided that there are estimates of charges from the amino acid sequence).

This investigation may be compared with previous work which shows that the second-virial crystallization boundary in a non-biological colloid–polymer mixture corresponds closely to that for many globular proteins [22]. In both cases, very simple generic colloid models are used to account for the phase behaviour of protein solutions in complex solvents.

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References

[1] McPherson A 1999 *Crystallization of Biological Macromolecules* (New York: Cold Spring Harbor Laboratory Press)
[2] Govardhan C P 1999 *Current Opinion Biotech.* **10** 331
[3] Arora A K and Tata B V R 1998 *Adv. Colloid Interface Sci.* **78** 49
[4] Warren P B 2000 *J. Chem. Phys.* **112** 2683
[5] Neal B L, Asthagiri D, Velev O D, Lenhoff A M and Kaler E W 1999 *J. Cryst. Growth* **196** 377
[6] Piazza R 1999 *J. Cryst. Growth* **196** 415
[7] Rosenbaum D F and Zukoski C F 1996 *J. Cryst. Growth* **183** 452
[8] Guo B, Kao S, McDonald H, Asanov A, Combs L L and Wilson W W 1999 *J. Cryst. Growth* **196** 424
[9] Velev O D, Kaler E W and Lenhoff A M 1998 *Biophys. J.* **75** 2682
[10] Muschol M and Rosenberger F 1995 *J. Chem. Phys.* **103** 10424
[11] Kuehner D E, Heyer C, Ramsch C, Fornefeld U M, Blanch H W and Prausnitz J M 1997 *Biophys. J.* **73** 3211
[12] Tardieu A, Le Verge A, Malfois M, Bonneté F, Finet S, Riès-Kautt M and Belloni L 1999 *J. Cryst. Growth* **196** 193
[13] Taratuta V G, Holschbach A, Thurston G M, Blankschtein D and Benedek G B 1990 *J. Phys. Chem.* **94** 2140
[14] Brodie M L, Tominc T M and Saxowsky M D 1996 *Phys. Rev.* E **53** 6325
[15] Tanford C and Roxby R 1972 *Biochem.* **11** 2192
[16] Waldram J R 1985 *The theory of thermodynamics* (Cambridge: Cambridge University Press)
[17] George A and Wilson W 1994 *Acta Cryst.* D **50** 361
[18] Rosenbaum D, Zamora P C and Zukoski C F 1996 *Phys. Rev. Lett.* **76** 150
[19] Russel W B, Saville D A and Schowalter W R 1991 *Colloidal Dispersions* (Cambridge: Cambridge University Press)
[20] Piazza R and Pierno M 2000 *J. Phys.: Condens. Matter* **12** A443
[21] Egelhaaf S U and Poon W C K, in preparation
[22] Poon W C K 1997 *Phys. Rev.* E **55** 3762