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''Sticky hard spheres'' model of proteins near crystallization: A test based on the osmotic compressibility of lysozyme solutions

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In order to establish a simple model of protein interparticle interaction near crystallization conditions, we have measured the osmotic compressibility of the metastable fluid phase of hen egg-white lysozyme within the fluid-solid coexistence region. Light scattering measurements were performed in an extended volume fraction range at pH = 4.7 as a function of temperature. A sufficient amount of NaCl has been added in order to severely screen the electrostatic interactions. Below 0.2 M NaCl, the system can still be kept in a metastable fluid phase for a sufficiently long time before nucleation of the crystal phase takes place. The experimental compressibility up to particle volume fractions $\Phi \approx 0.23$ is compared to the theoretical expression for adhesive (''sticky'') hard spheres, and the agreement is found satisfactory. We also determined the values of the Baxter τ adhesiveness parameter, which shows an apparent linear temperature dependence within the investigated temperature range. These results suggest that the main temperature dependence of the interparticle interaction potential is simply through the Boltzmann $k_B T$ factor. [S1063-651X(98)51408-0]

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All current attempts to understand and predict crystallization processes in protein solutions, which are essential in order to develop systematic strategies and reliable crystal growth protocols in structural biochemistry, would greatly benefit from a description of protein phase behavior in terms of simple models of interacting colloidal particles. Proteins can be treated as charged colloids which generally crystallize by adding a substantial amount of salt (well beyond what is needed to screen electrostatic interactions), thus exploiting what is generally called the ''salting-out'' effect [1]. Although the physical origin of salting out is still poorly understood, there is growing evidence that globular proteins, in the presence of a sufficient amount of added salt, behave as hard spheres interacting via an attractive potential with a range much shorter than the particle radius. The most relevant feature of the phase diagram for such a system is that the fluid-fluid phase separation is always metastable with respect to the fluid-solid transition [2]. This fact has been experimentally observed both for the eye protein γ-crystalline and for hen egg-white lysozyme [3–6]. Recently, it has been pointed out that this metastable fluid-fluid coexistence curve should play a fundamental role in crystallization kinetics, for the large concentration fluctuations near the metastable critical point should considerably speed up the nucleation processes [7]. Most proteins, however, fail to crystallize even if prepared in strongly supersaturated solutions, and rather show aggregation processes leading to amorphous aggregates, which may eventually, but not necessarily, restructure into low-quality crystals [1].

The simplest interaction that may account for all the above observations is the Baxter adhesive hard-sphere (AHS) potential [8], which is the limit for $\delta \to 0$, that is, for vanishing range, of

$$\frac{V(r)}{k_B T} = \begin{cases} \infty, & r < \sigma \\ \ln[12\tau\delta/(\sigma+\delta)], & \sigma \leqslant r \leqslant \sigma+\delta \\ 0, & r > \sigma+\delta \end{cases} \qquad (1)$$

where r is the interparticle distance between spheres of diameter σ, and τ is a parameter inversely proportional to the strength of the attractive interaction. Besides showing a metastable fluid-fluid coexistence, this limiting model suggests that, for sufficiently short range potentials, amorphous aggregation processes taking place near a dynamic percolation threshold could hinder the transition to the ordered phase [9]. Similar effects have indeed been observed with latex particles interacting via a very short range surfactant-induced depletion potential, and explained in terms of dynamical percolation [10].

Rosenbaum, Zamora, and Zukoski [11] have been recently able to show that the phase diagram of lysozyme is consistent with what is numerically predicted for a short range attractive Yukawa potential [2], by measuring at the same time the stability limit of the fluid region and the second osmotic virial coefficient B_2, and by assuming that B_2 can be related to the τ parameter by the AHS expression

$$\frac{B_2^{\mathrm{HS}} - B_2}{B_2^{\mathrm{HS}}} = \frac{1}{4\tau}, \qquad (2)$$

where $B_2^{\mathrm{HS}} = 2\pi\sigma^3/3$ is the second virial coefficient for hard spheres. The strength of this beautiful result is, however partly limited by the fact that a) the value of the second virial coefficient is weakly dependent on the exact form of the interparticle potential, being only a first order correction to the independent particle behaviour, and b) the exact freezing line for AHS is poorly known due to intrinsic instabilities of the solid phase which are probably connected with the presence of the percolation line [12].

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In order to firmly establish whether a highly screened solution of charged proteins can be mapped onto the AHS model it is necessary to directly compare data for the osmotic pressure in a wide concentration range to the full theoretical AHS osmotic equation of state. Since the equilibrium solubility of a protein in salting-out conditions is generally quite low, most of the experimental data must unfortunately be taken in the metastable region of the phase diagram, and this in turn is possible only provided that the crystal nucleation kinetics is much slower than the experiment time-scale. The main purpose of this paper is to show that, by exploiting the peculiar supersaturation properties of lysozyme solutions in an intermediate salt concentration range, and by employing careful sample preparation protocols, this result can indeed be obtained. The resulting osmotic compressibility data closely conform to what is expected for adhesive hard sphere. Moreover, by studying the osmotic compressibility curve as a function of temperature T, a direct relation between the stickiness parameter τ and T will be derived, and conclusions about the temperature dependence of the attractive part of the potential will be made.

Six times recrystallized egg-white lysozyme was obtained from Seikagaku (Japan, Lot # E96301), and used without further purification. The lyophilized compound was dissolved in 50 mM NaAcO buffer, and the pH of the solution was adjusted to 4.7 by addition of acetic acid. The ionic strength of the solution at pH$=4.7$ is estimated to be about 30 mM. This batch solution was centrifuged, and the final concentration, as determined by absorption at $\lambda=280$ nm, was found to be $c_0\approx 330$ mg/ml. Mother solutions at 0.1 and 0.2 M NaCl were prepared by adding small amounts of 3 M NaCl in NaAcO buffer. Ionic-shock induced aggregates were slowly dissolved by gently stirring the solutions while they were kept at $T\approx 40$ °C. Dilutions were prepared by volume, adding appropriate amounts of NaCl in NaAcO buffer. A straightforward calculation, using a value of about 12 for the surface charge of lysozyme at pH$=4.7$, shows that at 0.1 M NaCl the residual effect of the electrostatic repulsion is almost negligible. On the other hand, preliminary observations of the crystallization kinetics for NaCl concentration larger than 0.2 M, show that nucleation is sufficiently slow only within a limited supersaturation range, corresponding to particle volume fractions lower than about 10%. The NaCl concentration range between 0.1 and 0.2 M therefore represent a useful, although narrow window where extensive supersaturation can be reached while working on a system where short range attractive (''salting-out'') forces become dominant.

Rectangular light scattering cells having an optical path of 5 mm and a minimum working sample volume of about 400 μl, were carefully cleaned by flushing with about 50 ml of 0.2 μm filtered buffer in a closed-circuit filtering system. The diluted samples where then fed into the cells through a low protein-retention 0.2 μm filter. This careful cleaning protocol was essential not only to avoid dust problems in the scattering measurements, but also to severely reduce inhomogeneous crystal nucleation, which was found much more effective in the presence of dust. Light scattering data were taken with the experimental setup described in detail elsewhere [13]. Incident, transmitted and scattered intensities were sampled and monitored by a multiplexing setup. Measurements were made at a fixed scattering angle of 90°. The concentration dependence of the refractive index of the lysozyme solutions was measured by an Atago 3T Abbe refractometer at the sodium D-line for different temperatures and corrected for dispersion, giving an almost temperature-independent value $dn/dc\approx 0.197$ cm^3/g. Sample cells were kept in a thermostated room at $T\approx 28$ °C, and transferred just before the measurements into the scattering apparatus thermostat, which allows one to control the sample temperature to within 0.01 °C. The sample temperature was measured by a thermistor in close thermal contact with the cell and the scattered intensity monitored until they both reached a stable value, when acquisition was started and intensity values cyclically measured and averaged over about one minute. The concentration of the sample in the cell was carefully cross-checked after measurement both by UV absorption and refractive index increment. The particle volume fraction Φ was calculated from the concentration by using the lysozyme specific volume $v=0.71$ ml/g. Measurements where taken on samples of increasing concentration up to values where crystallization kinetics became noticeable on a time scale of a few hours. For the 0.2 M NaCl preparations there was clear evidence for nucleation enhancement by critical fluctuations observed in most concentrated samples at low temperatures. Indeed, liquid-liquid phase separation, followed by fast crystallization, was observed at temperatures below 10 °C for lysozyme concentration larger than about 250 mg/ml. This is qualitatively consistent with the results by Muschol and Rosenberger [5], although the boundary of the coexistence curve at pH$=4.7$ seems to be at higher temperatures than those observed at pH$=4.5$. Liquid-liquid phase separation boundaries are extremely sensitive to the charge value, which decreases about 10% by increasing the pH from 4.5 to 4.7, and there might also be dependence on the buffer concentration. Hence, we do not regard this discrepancy as particularly puzzling. In a few cases, we also noticed that partial mixing during dilution, evidenced by the appearance of Schlieren patterns in the cell, lead to an appreciable increase of turbidity even at room temperature, followed by incipient demixing. However, if the cell is immediately transferred to a thermal bath at temperatures above 35 °C and gently rocked, turbidity disappears, the sample becomes optically uniform and remains so even after being brought back at room temperature. This latter observation, which has also been made in previous studies [14], confirms however that specific sample manipulation protocols can have profound effects when dealing with metastable solutions.

The excess intensity with respect to the solvent scattered by particles much smaller than the wavelength can be written as

$$I=A\left(2n\,\frac{dn}{dc}\right)^2 k_B T c\left(\frac{\partial\Pi}{\partial c}\right)^{-1}, \tag{3}$$

where n is the index of refraction of the medium, dn/dc the refractive index increment, T the absolute temperature, $\partial\Pi/\partial c$ the isothermal osmotic compressibility, and A an instrumental constant that can be determined by calibration with respect to a sample of known scattering power. Figure 1 shows the volume fraction dependence of the reduced osmotic compressibility $(M/RT)(\partial\Pi/\partial c)$, for lysozyme solu-

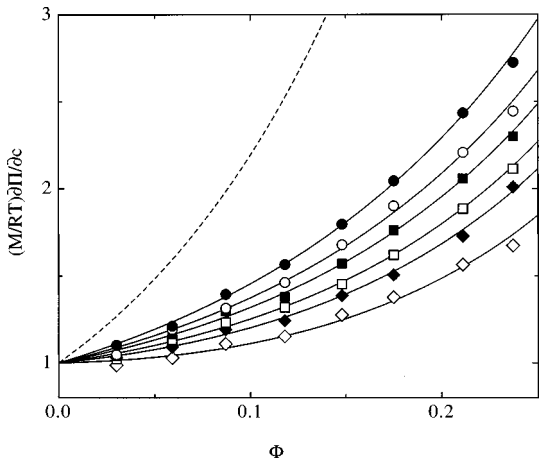

FIG. 1. Reduced osmotic compressibility of hen egg-white lysozyme in NaAcO buffer+0.1 M NaCl at pH=4.7. The experimental points refer to measurements taken at 36.8 °C (closed circles), 29.7 °C (open circles), 22.7 °C (closed squares), 17.4 °C (open squares), 12.7 °C (closed diamonds), and 8.2 °C (open diamonds). The full lines are fits to the theoretical osmotic compressibility for AHS. The dashed line presents the Percus-Yevick compressibility for hard spheres.

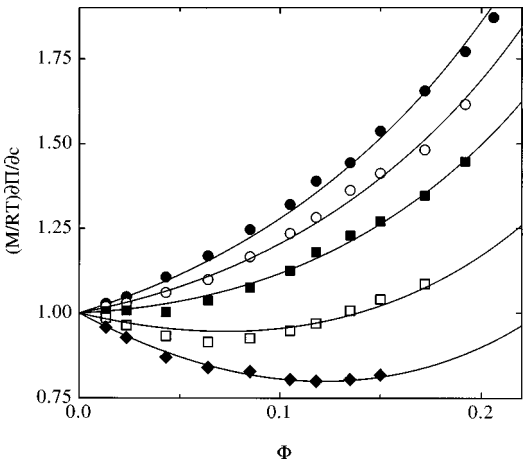

FIG. 2. Same as Fig. 1 for samples containing 0.2 M NaCl. The experimental points refer to measurements taken at 35.0 °C (closed circles), 30.0 °C (open circles), 24.5 °C (closed squares), 17.2 °C (open squares), and 12.2 °C (closed diamonds).

tions at a NaCl concentration of 0.1 M at six temperatures between 8.2 °C and 36.7 °C, which were derived from the experimental scattering data using Eq. (3). The osmotic compressibility for AHS can be analytically evaluated in the Percus-Yevick approximation as [15]

$$\frac{\partial \Pi}{\partial c} = \frac{RT}{M} \frac{(1+2\Phi-\lambda\Phi)^2}{(1-\Phi)^4}, \tag{4}$$

where

$$\lambda = 6(1-\tau+\tau/\Phi)\left(1-\sqrt{1-\frac{1+2/\Phi}{6(1-\tau+\tau/\Phi)^2}}\right).$$

The full lines in Fig. 1 are the best fit of the Eq. (4) to the experimental results, using τ as the only free fit parameter. This good fit clearly shows that the theoretical expression for the osmotic compressibility of AHS provides a very good description of the data for all temperature conditions investigated. For comparison, the Percus-Yevick compressibility for hard-spheres, which correspond to the $\tau \to \infty$ limit, is also drawn in Fig. 1 to show that in the investigated temperature range the particles are far less repulsive than hard spheres. Notice that the measured volume fractions span a range about ten times larger than those usually investigated in second virial coefficient studies, and that the results suggest an agreement between the experimental compressibility and the theoretical curve (4) to better than $O(\Phi^2)$. Figure 2 shows similar results obtained for 0.2 M NaCl solutions. Here, at low temperatures, the measured sample volume fraction had to be limited to values where metastability could be kept within the limits where experiments can be performed. Notice that in this case, for temperatures lower than about 20 °C, the second virial coefficient becomes negative. For consistency, we also checked the data obtained at low volume fractions by directly measuring the osmotic pressure Π by means of a Knauer membrane osmometer. The obtained second virial coefficient for Π were found in good agreement with those obtained by light scattering. The values obtained for the τ parameter in the two temperature runs are shown in Fig. 3. Apparently, a linear proportionality between τ and T applies to both salt concentrations in the (limited) investigated temperature ranges. The parameter τ in the Baxter potential plays the role of a generalized temperature. In order to find a correspondence with real temperatures, a more detailed model is needed. The simplest short range potential that becomes the AHS potential in the limit of vanishing range is made of a hard sphere part plus a temperature independent square well (SW),

$$V(r) = \begin{cases} \infty & 0 < r < \sigma \\ -V_0 & \sigma < r < \sigma+\delta \\ 0 & r > \sigma+\delta. \end{cases} \tag{5}$$

Identification of τ in terms of the SW parameters can be made by equating the second virial coefficient, which can be analitically evaluated for the SW potential, obtaining [15]

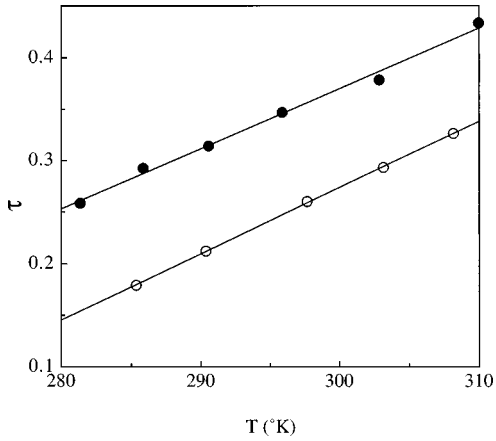

FIG. 3. Temperature dependence of the Baxter τ parameter from the fits to the two sets of measurement shown in Figs. 1 and 2. Results for 0.1 M NaCl solutions are shown by closed circles, while open circles give the results for the 0.2 M run.

$$\frac{T_0}{T}=\ln\left(1+\frac{\tau_0}{\tau}\right), \tag{6}$$

where $T_0=V_0/k_B$ and $\tau_0^{-1}=4[(1+\delta/\sigma)^3-1]\approx 12\delta/\sigma$. Equation (6) implies that, for weak adhesiveness ($\tau_0 \ll \tau$), τ and T are expected to be proportional ($\tau \approx \tau_0 T/T_0$), as is experimentally found. This would suggest that the main temperature dependence of the interparticle interaction is simply through the Boltzmann factor, while other temperature effects, such as changes in the hydration of the lysozyme particles, if present, play a lesser role. We notice that a similar temperature dependence of the τ parameter, derived from a fit of the second virial coefficient to the AHS expression, has been found by Rosenbaum and Zukoski [16], and that similar conclusions on the weak temperature dependence of the potential has been suggested by Malfois *et al.* [17].

As a conclusion, by analyzing the results obtained from this measurement of the whole osmotic equation of state of lysozyme up to high volume fractions in the metastable fluid region, we can state that an extremely simple interaction model, such as the Baxter ''sticky'' hard-sphere potential, may account fairly well for the thermodynamic properties of the system. This does not imply at all that more complicated features, related for instance to the structural properties of protein crystals, can be inferred from such a simple model. However, we believe that it represents a valuable starting point in order to predict the phase behavior and, by a more thorough investigation of the strength and range of the interactions as a function of the crystallization control parameters (salt nature and concentration, pH, and so on), to shed light on the physical meaning of the still obscure ''salting-out'' effect.

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