Observation of Critical Phenomena in a Protein-Water Solution

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(Received 26 January 1989)

We report measurements of the intensity of the scattered light and the turbidity of aqueous solutions of the bovine lens protein, γ_{II}-crystallin, near the critical point for binary liquid phase separation, in a region of the phase diagram which is metastable with respect to protein-crystal formation. We have determined the magnitudes and critical divergences of the osmotic compressibility κ_T and the static correlation range ξ. The corresponding critical exponents are $\gamma = 1.21 \pm 0.05$ and $\nu = 0.68 \pm 0.1$.

PACS numbers: 64.70.Ja, 05.70.Jk, 82.60.Lf, 87.15.Da

The study of phase transitions involving biologically important molecules can be of fundamental physiological and medical significance. A striking example is the role of binary liquid phase separation in forms of mammalian cataract.[1-3]

This phenomenon has stimulated previous investigations[4,5] by Tanaka *et al.* of critical phenomena associated with binary phase separation in aqueous solutions of the protein lysozyme. They measured the optical turbidity τ as a function of temperature T for $T > T_c$, where T_c is the critical temperature. From their results for $T \gg T_c$, they deduced the osmotic compressibility κ_T. For T close to T_c, they also deduced the correlation range ξ assuming that κ_T could be described using the mean-field power law. Under this assumption they presented values of $\kappa_T(T)$ and $\xi(T)$ and reported mean-field exponents for γ and ν.

We have recently demonstrated the existence of binary liquid phase separation and critical phenomena in aqueous solutions of the bovine lens protein γ_{II}-crystallin.[3] We observed reversible coexistence between two macroscopic, isotropic, and optically clear liquid phases which differed in protein concentration. We also found that this liquid-liquid phase separation is metastable: For the concentrations and temperatures at which liquid-liquid phase separation occurs, the equilibrium state of the mixture consists of a solution phase coexisting with a crystalline phase.[3] We report here light scattering studies of this system in the vicinity of the critical point for the liquid-liquid phase separation. The required metastable states near the critical point were achieved by suppressing the nucleation of the solid phase, as described previously.[3] We measured both the optical turbidity τ and the Rayleigh ratio $R(\theta)$ along two isochores near the critical isochore. Our measurements span 3 orders of magnitude in reduced temperature, $3\times10^{-5} < (T-T_c)/T_c < 4\times10^{-2}$. We made use of the fact that as the scattering angle θ approaches 0, $R(\theta)$ is a direct measure of κ_T, and is to a very good approximation independent of ξ. Use of $\lim_{\theta\to 0} R(\theta) \equiv R(0)$, in combination with our measured values of τ, permitted us to deduce ξ at each temperature without prior assumptions as to the critical behavior of κ_T: The method is described below. From our analysis we obtained values for γ and ν which correspond to three-dimensional Ising-model values.

Bovine γ_{II}-crystallin is a compact globular protein with known amino-acid sequence and molecular weight 2.1×10^4 g/mol.[6,7] It was isolated and purified from fresh calf lenses by column chromatography as described.[3] The final γ_{II}-crystallin fraction was demonstrated to be $>99\%$ pure by analytical isoelectric focusing, using gels containing 6 M urea, followed by densitometric scanning, as described.[8] The purified protein was concentrated to approximately 240 (mg protein)/(ml solution) by ultrafiltration. It was then dialyzed exhaustively against a 50-mM sodium phosphate buffer (pH $=7.00$, ionic strength $=0.11$ M) which contained sodium azide, and which had been thoroughly degassed and saturated with nitrogen. Individual samples were centrifuged at $2500\times g$ for 1-2 h at 30 °C, sealed under nitrogen and stored in the dark at room temperature. These final steps were taken in order to prevent protein oxidation and to delay the onset of crystallization.[3] Protein concentrations were determined following known dilution by using optical absorption at 280 nm with a specific absorption coefficient of $A_{280}^{0.1\%,\,1\,\mathrm{cm}} = 2.4$. Reported values for $A_{280}^{0.1\%,\,1\,\mathrm{cm}}$ range from 2.0 (Ref. 9) to 2.8.[10]

To estimate the critical concentration c_c we quenched sufficiently concentrated solutions to temperatures below T_c and centrifuged them at the quench for 30 min at $1000\times g$. Two visually clear, macroscopic phases formed, separated by a sharp meniscus. We measured the protein concentrations of each phase and found that the average of these concentrations was between 240 and 260 mg/ml for each of four different quench temperatures. For the present protein-50-mM buffer solutions, the estimated c_c, 250 ± 10 mg/ml, was quite similar to the c_c previously reported for the protein-100-mM buffer solutions,[3] 244 ± 10 mg/ml. However, T_c was affected by the buffer concentration: $T_c = 282.9 \pm 0.1$ K for 50-mM buffer; $T_c = 278.5 \pm 0.2$ K for 100-mM buffer.

The light scattering photometer was derived from an instrument developed by Haller, Destor, and Cannell.[11] A vertically polarized argon-ion laser, $\lambda_0 = 488$ nm, was used. Scattered light was detected at twelve fixed angles θ ($11.5° \leq \theta \leq 162.6°$). The key features of the measurement protocol were the following: (i) rapid and repeated sequential sampling of the transmitted beam intensity, I_t, and the scattered intensity at each angle, $I(\theta)$; (ii) division of $I(\theta)$ by I_t; and (iii) averaging of $I(\theta)/I_t$ [denoted by $\langle I(\theta)/I_t \rangle$] with use of a dust-discrimination procedure.[11] This protocol reduced inaccuracies due to drifts in laser power and photomultiplier response, and it automatically corrected $I(\theta)$ for sample turbidity, provided that cylindrical scattering cells were used.[11] The instruments' temperature controller maintained sample temperature stability to better than ± 0.001 K.

To determine $R(\theta)$, we measured $\langle I_{\rm ref}(\theta)/I_t \rangle$ from the pure reference solvents, benzene and toluene (spectrophotometric grade, Aldrich, Milwaukee, WI), which have known Rayleigh ratios, $R_{\rm ref}$. $R(\theta)$ of $\gamma_{\rm II}$-crystallin solutions were calculated from[12]

$$R(\theta) = [\langle I(\theta)/I_t \rangle / \langle I_{\rm ref}(\theta)/I_t \rangle](n/n_{\rm ref})^2 R_{\rm ref}, \quad (1)$$

where $R_{\rm ref}$ is the Rayleigh ratio of the reference solvent, and n and $n_{\rm ref}$ are the refractive indices of the solution and the reference solvent, respectively. At 23 °C and $\lambda_0 = 488$ nm, we used $R_{\rm ref} = 39.6 \times 10^{-6}$ cm^{-1} for toluene, and $R_{\rm ref} = 35.4 \times 10^{-6}$ cm^{-1} for benzene.[13] $R_{\rm ref}$ and $n_{\rm ref}$ were compensated for temperature dependence using reported values.[14,15] Measurements of $R(\theta)$ of a $\gamma_{\rm II}$-crystallin sample, which was repeatedly removed from and replaced into the spectrometer, agreed to within 1% over a 72-h period. The instrument calibration was monitored using pure water whose $R(0)$ we determined to be $(2.51 \pm 0.02) \times 10^{-6}$ cm^{-1} at $\lambda_0 = 488$ nm, independent of temperature from 10 to 55 °C by comparison to toluene and benzene.

To verify that multiple scattering was not affecting our results, we checked the cell-path-length dependence of $R(0)$ by comparing light scattered from the same sample in three different sizes of scattering cells: 15- and 5.6-mm-diam cylindrical cells and one 1.85-mm path-length rectangular cell. $R(0)$ obtained with both the 5.6- and 1.85-mm cells agreed within experimental uncertainty at all measured temperatures. The absolute magnitudes of $R(0)$ and τ did not vary between cells by more than 2% for $T - T_c > 3$ °C. For $T - T_c < 3$ °C only the data obtained using the 5.6- and 1.85-mm cells were used.

At laser powers in excess of 0.5 mW, with a beam waist of diameter 100 μm, beam spreading was observed. These effects were eliminated by using beam powers in the range 0.1–0.4 mW. In this range no dependence of $\langle I(\theta)/I_t \rangle$ on incident beam power was observed. As an added precaution, the sample was illuminated only during data collection periods, lasting fewer than 5 min at each temperature.

We calculated the absolute magnitude of the osmotic compressibility $\kappa_T = (\partial\pi/\partial\phi)^{-1}$, where π is the osmotic pressure and ϕ is the volume fraction of protein, as follows. A linear fit to the measured values of $R(\theta)$ as a function of $q(\theta)^2$, where $q(\theta) = (4\pi n/\lambda_0)\sin(\theta/2)$, for scattering angles $11.5° \leq \theta \leq 44.8°$, was extrapolated to $q = 0$ to determine $R(0)$. κ_T was calculated from the thermodynamic formula,[16] applicable to binary mixtures,

$$[1/\kappa_T] = (k_B T/\bar{v}_p)[4\pi^2 n^2 (dn/dc)^2/\lambda_0^4] c [1/R(0)], \quad (2)$$

where c is the concentration of protein in g/cm^3, k_B is Boltzman's constant, dn/dc is the refractive-index increment, and $\bar{v}_p$ is the partial specific volume of the protein in the solvent. We used $dn/dc = 0.20$ cm^3/g, determined for γ-crystallin solutions,[10] and $\bar{v}_p = 0.71 \pm 0.01$ cm^3/g (determined using a Mettler/Paar DMA 602/60 digital density meter, by C. R. Middaugh). The range of reported values of $A_{280}^{0.1\%,\,1\,\rm cm}$ mentioned above, implies that the literature value[10] of dn/dc, or that c, may contain a systematic error. Such an error would in no way alter our deduction of γ and ν. However, it would alter the apparent numerical magnitude of κ_T. Equation (2) neglects scattering from the solvent, which would have a magnitude only 10^{-4} times the observed intensity even at $T - T_c = 10$ °C.

The values of κ_T (in cgs units) obtained by this method are shown in Fig. 1, plotted as a function of reduced temperature $(T - T_c)/T_c$ for $c = 244$ mg/ml. This concentration corresponds to a volume fraction $\phi = 0.17$. T_c was found to be 282.92 ± 0.05 K. The solid circles correspond to data obtained using the 5.6-mm cylindrical scattering cell, the open circles to the 1.85-mm cell, and the triangles to the 15-mm cell. It should be noted that the use of the 1.85-mm cell permitted us to approach T_c

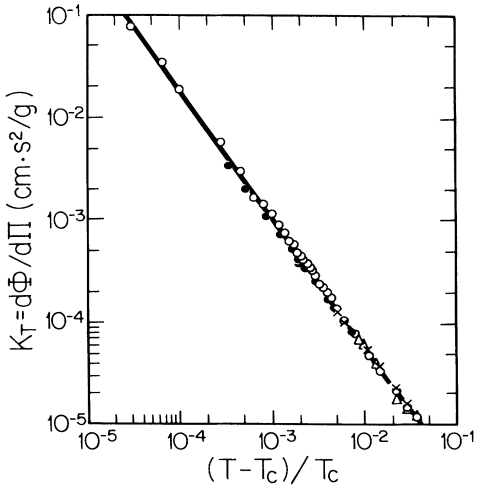

FIG. 1. Inverse osmotic compressibility as a function of reduced temperature: ○'s, 1.85-mm cell; ●'s, 5.6-mm cell; △'s, 15-mm cell; ×'s, from turbidity measurements.

to within 8 mK, while obtaining reliable values for $R(0)$ and τ. The values of κ_T shown in Fig. 1 are well characterized by $\kappa_T = \kappa_T^0[(T-T_c)/T_c]^{-\gamma}$ for $2.9\times10^{-5} \leq (T-T_c)/T_c \leq 3.6\times10^{-2}$, with $\gamma = 1.21 \pm 0.05$ and $\kappa_T^0 = (2.21 \pm 0.05)\times10^{-7}$ cm sec^2/g. The solid line in Fig. 1 corresponds to this power law. At $c = 220$ mg/ml, we found $\gamma = 1.21 \pm 0.06$ and $\kappa_T^0 = (2.17 \pm 0.4)\times10^{-7}$ cm sec^2/g. It is important to note that these measurements of γ and κ_T^0, taken at two different concentrations near c_c, agree within experimental uncertainty. Thus, although we may not have located c_c precisely by the method described above, we believe that the the consistency of these measurements indicates that we have nevertheless determined γ and κ_T^0 to within the stated uncertainty.

In principle, it is possible to determine ξ from a direct measurement of the angular dependence of $R(\theta)$. However, our values for $R(\theta)$ at small reduced temperatures led to unreliable estimates of ξ because of multiple scattering associated with sample turbidity. Previous theoretical and experimental work[17] indicates that measurements of $R(\theta)$ at intermediate θ are affected much more by incipient multiple scattering than $\lim_{\theta\to 0} R(\theta)$, or $\lim_{\theta\to\pi} R(\theta)$. We verified that multiple scattering was not affecting our results for $R(0)$ by comparing measurements from the two smaller scattering cells as described above.

Despite difficulties with multiple scattering at smaller reduced temperatures, it was still possible[18] to determine ξ using information from both (i) the measured optical turbidity τ and (ii) the measured $R(0)$. The method is based on calculating the optical turbidity by integrating the intensity of the scattered light over all angles. If the Ornstein-Zernike (OZ) form $R(\theta) = R(0)[1 + q(\theta)^2\xi^2]^{-1}$ applies, then[18]

$$\tau = R(0)\pi H(\alpha), \tag{3}$$

where

$$H(\alpha) = [(2\alpha^2+2\alpha+1)/\alpha^3]\ln(1+2\alpha) - 2(1+\alpha)/\alpha^2, \tag{4}$$

with $\alpha = 2(k_0\xi)^2$ and $k_0 = 2\pi n/\lambda_0$. We determined τ experimentally by measuring the transmitted beam intensities I_t for both pure water and γ_{II}-crystallin solution, with constant incident beam intensity, in the 1.85-mm path-length cell. In the extreme hydrodynamic limit ($\alpha \ll 1$) $H(\alpha)$ is independent of ξ and has the value $\frac{8}{3}$. Thus for $\alpha \ll 1$, the measured values of τ can be used to calculate $R(0)$ indirectly, independently of ξ. The values of $R(0)$ inferred from τ when $\alpha \ll 1$ were then used in Eq. (2) to determine κ_T. For $T - T_c > 2$ °C [$(T-T_c)/T_c > 7\times10^{-3}$], where $\alpha \ll 1$, the values of κ_T determined indirectly from τ (see points "×," Fig. 1) agreed with those we determined directly from $R(0)$.

For larger α, the measured values of τ were no longer sufficient by themselves to indirectly determine $R(0)$, and therefore to compute κ_T. Thus for $T - T_c < 2$ °C in the present case, there are no deduced values of κ_T corresponding to the points designated "×" in Fig. 1. Instead, the measured values of τ, along with the directly measured $R(0)$, were used in Eq. (3) to determine $H(\alpha)$. Then ξ was calculated from Eq. (4). The results are shown as open circles in Fig. 2. The dependence of ξ upon temperature is well characterized by $\xi = \xi_0[(T-T_c)/T_c]^{-\nu}$, with $\xi_0 = (6 \pm 1)\times10^{-8}$ cm and $\nu = 0.68 \pm 0.1$, as shown by the solid line. We note that the diameter of the protein,[6] $(35\text{–}55)\times10^{-8}$ cm, and the nearest-neighbor interparticle spacing, approximately 1.3×10^{-6} cm, were less than ξ over the temperature range in which ξ was determined.

Sufficiently far from T_c we were able to verify our deductions of ξ from $R(\theta)$ with use of the OZ form $R(\theta) = R(0)[1+q(\theta)^2\xi^2]^{-1}$. In the temperature range $3\times10^{-3} \leq (T-T_c)/T_c \leq 1\times10^{-2}$, the values of ξ so determined are shown as solid circles in Fig. 2 and agree quite well with the power-law dependence of ξ deduced from the measurements of κ_T and τ. Only in this temperature region could we overcome multiple-scattering effects on $R(\theta)$ and determine ξ.

We have compared the deductions of ξ made with use of Eqs. (3) and (4) to those that would result if a modified OZ form[19,20] $R(\theta) = R(0)[1+q(\theta)^2\xi^2]^{-1+\eta/2}$ were to apply, thereby modifying Eq. (4). Using $\eta = 0.1$ as a worst-case example we found that the deduced value of ν increases by the very small amount of 0.02. In all calculations we have assumed that the solution index of refraction n is independent of temperature. A weak anomaly of n near T_c, such as has been found for the methanol-cyclohexane system,[21] would not affect our results.

These data clearly demonstrate that over the temperature ranges studied $\gamma = 1.21 \pm 0.05$ and $\nu = 0.68 \pm 0.1$. It is interesting that such exponents, compatible with 3D Ising-model values, are found even in the present case of

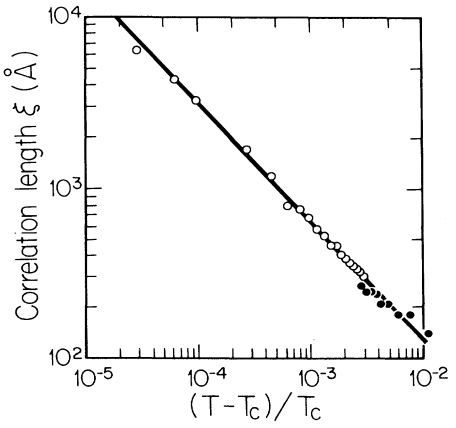

FIG. 2. Correlation length as a function of reduced temperature: o's, from turbidity and osmotic compressibility measurements; ●'s, from angular dependence of scattered intensity.

a biological macromolecular polyelectrolyte solution, in a metastable region of its aqueous phase diagram.

This work was supported by the National Eye Institute of the National Institute of Health under Grant No. 5-RO1-EY05127, by National Science Foundation Grants No. 84-08630-DMB and No. 84-18718-DMR, and by the Jessie B. Cox Charitable Trust.

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