Phase behavior of mixtures of human lens proteins Gamma D and Beta B1

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We have experimentally determined the coexistence surface characterizing the phase behavior of γD-βB1-water ternary solutions. The coexistence surface fully describes the solution conditions, i.e., temperature, protein concentration, and protein composition, at which liquid-liquid phase separation occurs in a ternary solution. We have observed a significant demixing of γD and βB1 i.e., large difference of composition in the two coexisting phases. This demixing suggests that the energy of the γD-βB1 attractive interaction is significantly smaller than the energy of the γD-γD attractive interaction. We also observed the lowering of the phase separation temperature upon increasing of the fraction of βB1 in solution. We provide a theoretical analysis of our experimental data, which enables a quantitative description of our principal experimental findings. In this way, we have evaluated the magnitude and temperature dependence of the relevant interprotein interaction energies. Our findings provide insight into the factors essential for maintaining lens proteins in a single homogeneous phase, thereby enabling lens transparency.

cataract | crystallin | phase separation | mixture

The cytoplasm of eye lens fiber cells contains a concentrated multicomponent mixture of predominantly crystallin proteins (1, 2). Crystallins were classified into three categories: α, β, and γ crystallin (3). The β and γ crystallins have a common polypeptide chain fold and together form a super family of β-γ crystallins. To maintain lens transparency this multicomponent mixture must be free of large density variations, which produce light scattering. Conversely, inhomogeities in the protein density produced by aggregation and phase transitions can lead to cataract (4, 5).

Aggregation and phase transitions of aqueous solutions of protein mixtures are generally driven by attractive interprotein interactions (6, 7). A large body of work has been devoted to studies of interprotein interactions in single component aqueous solutions of eye lens proteins (8–13). In order to obtain further insight into maintenance of transparency in eye lens, aqueous solutions of crystallin mixture with high concentration must also be investigated. Observation of liquid-liquid phase separation (LLPS) in protein solutions provides a direct tool to probe collective interprotein interaction at concentrations up to 400 mg/mL (which is approximately the total protein concentration in a human eye lens) (14–17). Significant researches have been conducted on the LLPS of solutions of crystallin mixtures. LLPS of mixtures of different types of bovine γ crystallins has been thoroughly investigated (14, 15). Recently, phase separation of α-γ mixtures was also studied (18, 19).

Due to the abundances of β (~50% of eye lens protein) and γ (~20%) crystallins in human eye lens (20), studies of their mixtures are of particular importance. In relation to cataract disease, γD and βB1 are two especially interesting members of the β-γ crystallin family. Mutations of γD crystallin are found in many types of hereditary cataracts (9–12, 21). βB1 is subjected to extensive age-related modifications such as N terminus truncation and deamidation, and it occurs in large amounts in the age associated cataracts (20). Here, we present a study of the LLPS of mixtures of human γD and βB1 crystallin in aqueous solution.

In this work, the numbers of γD and βB1 molecules in a ternary solution are respectively denoted by N_1 and N_2. The total protein "concentration" ϕ, can be defined as the volume fraction of proteins, where $(N_1+N_2)\Omega_P/V$. Here V is the solution volume, and Ω_P is the effective volume of a single globular protein molecule, which we will assume to be the same for γD and βB1 crystallins (8, 22). We will refer to the mole fraction of βB1, $x=N_2/(N_1+N_2)$, as the protein "composition" of this solution.

A thermodynamic state of γD-βB1-water ternary solutions can be fully determined using three state variables (T,ϕ,x) where T is the solution temperature. The LLPS of a solution with a particular ϕ and x occurs at a well defined temperature $T_{\rm ph}$, which is a function of ϕ and x. Therefore, LLPS of γD-βB1-water ternary solution is described by a coexistence surface, $T_{\rm ph}(\phi x)$, in the three-dimensional (T,ϕ,x) coordinate space. In this work, we experimentally constructed this coexistence surface by measuring the cloud point curves, $T_{\rm ph}(\phi)x$, i.e., the constant x cross-sections, at several different x. We also experimentally determined the constant $T_{\rm ph}$ cross-sections of the coexistence surface, $x(\phi)_{T_{\rm ph}}$, known as binodal curves, at different $T_{\rm ph}$. Upon LLPS at given $T_{\rm ph}$, the initially homogeneous protein solution will separate into a protein-poor phase (phase I) with small $\phi^{\rm I}$ and protein-rich phase (phase II) with large $\phi^{\rm II}$. A binodal curve consists of pairs of points representing protein concentrations and compositions of protein-poor phases $(\phi^{\rm I}x^{\rm I})$ and protein-rich phases $(\phi^{\rm II}x^{\rm II})$ coexisting at given $T_{\rm ph}$. The line connecting a pair of points is called a tie-line. At the so called critical point the difference between composition and concentration of the coexisting phases becomes infinitesimally small i.e., the tie-line length becomes zero, and the direction of tie-line becomes tangential to the binodal curve. The set of critical points form a critical line on the coexistence surface.

Generally, LLPS of a ternary protein solution involves "protein condensation" and "protein demixing." Protein condensation refers to the formation of two phases with different total protein concentration, ϕ. This concentrational separation is analogous to the "usual" LLPS of a protein-water binary solution. Protein demixing refers to the formation of two phases with differing protein compositions, x. The relative importance of protein condensation and protein demixing in LLPS of any specific two proteins-water ternary system depends on the characteristics of the interprotein interactions.

In a γD-βB1-water ternary solution, the interprotein interactions are described by three effective energies of pair-wise interaction: E_{11} for a γD-γD pair, E_{12} for a γD-βB1 pair, and E_{22} for a βB1-βB1 pair. To observe LLPS in a protein-water binary system

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the interprotein interactions must be attractive i.e., the corresponding E_{11} or E_{22} must be negative. In such a binary solution, the maximum LLPS temperature (T_c) is directly proportional to the magnitude of the interprotein interaction. Since T_c of the γD-water binary solution is around 277 K (10), while T_c of the βB1-water binary solution is around 249 K (8), it follows that the magnitude of E_{11} is larger than that of E_{22}. As x increases from 0, some γD-γD contacts with energy E_{11} are replaced by γD-βB1 contacts with energy E_{12}. Since x is relatively small under our experimental condition the number of βB1-βB1 contacts is negligible. The difference in contact energies E_{12} and E_{11} drives demixing. When the magnitude of E_{12} is smaller than that of E_{11}, βB1 is repelled from the γD protein-rich phase. When βB1 preferentially partitions into the protein-dilute phase, adding βB1 lowers the LLPS temperature of the γD solution. This phenomenon is analogous to the suppression of the freezing point by adding a solute into water. Indeed, in our experiments we observed and measured both reduction of phase separation temperature and strong protein demixing. We present a theoretical analysis, which provides a quantitative interpretation of our data.

Results

Dependence of T_{ph} on ϕ at Fixed x. In Fig. 1 we present the results of the measurements of the cloud point curves $T_{ph}(\phi)_x$, i.e., the constant x sections of the coexistence surface. $T_{ph}(\phi)_x$ is reported at $x = 0$, 0.08, 0.16, and 0.29. The entire cloud point curve shifts to lower temperature as x increases. For ternary mixtures with $x > 0.29$, phase separation cannot be observed because T_{ph} falls below the freezing point of the solutions. The ϕ value at which T_{ph} is maximal first decreases and then increases with increasing x.

The clouding of the solution upon lowering the temperature occurs at lower temperatures than the clarification that occurs upon temperature rise. This hysteresis is shown in Fig. 1 by vertical bars between clouding and clarification temperatures. The hysteresis is a nonequilibrium phenomenon that depends on the rate of sample cooling and heating and also on the kinetics of relaxation processes in the solution such as nucleation rate of droplets of the condensed phase, merging or sedimentation of droplets, and possible crystallization. This hysteresis is the main source of uncertainty in determination of T_{ph}. At the same time the hysteresis can provide qualitative information on the nucleation rate of droplets of the condensed phase. In the γD-water binary system the observed hysteresis was too small to be seen in Fig. 1. (When the γD volume fraction was below ~0.1, the emerging droplets sedimented too fast for the clarifying temperature to be measured. See *Materials and Methods*) Upon adding βB1, hysteresis increases as shown in Fig. 1. This observation implies that the presence of βB1 increases the nucleation energy barrier for formation of droplets in bulk solutions.

The positions of the critical points on these cloud point curves are shown in Fig. 1. In protein-water binary systems, the critical point is at the maximum of the cloud point curve (23, 24). Here, the critical point of the γD-water binary solution is at $\phi_c = 0.13 \pm 0.01$ and $T_c = 277.3 \pm 0.1$ K. At $x > 0$, the critical points are not necessarily located at the maxima of $T_{ph}(\phi)_x$ curves, and cannot be determined solely from cloud point measurements. The projection of the critical line shown in Fig. 1 is derived from the results of partitioning measurements described in the next section.

Binodal Curves of Coexisting Phases at Constant T_{ph}. In Fig. 2, we present the results of partitioning measurements. Protein concen-

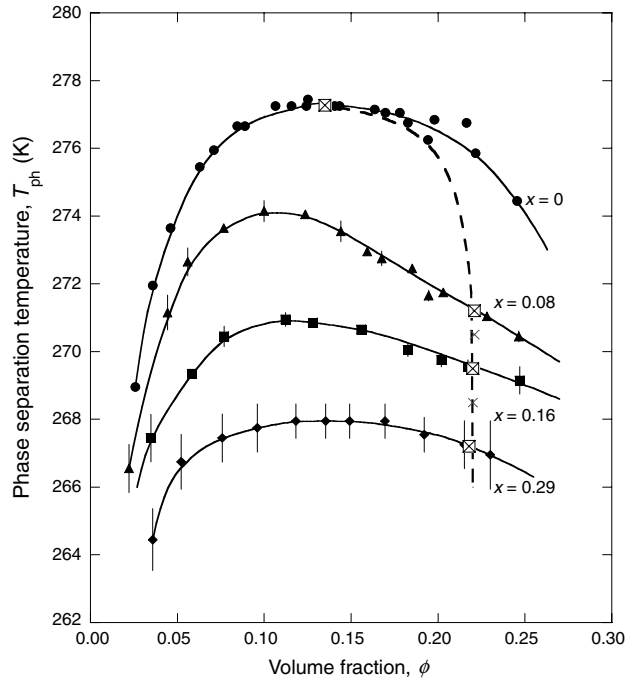

Fig. 1. $T_{ph}(\phi)$'s are presented at $x = 0$ (*circles*), 0.08 (*triangles*), 0.16 (*squares*), and 0.29 (*diamonds*). Solid curves are eye guides for the cloud point curves $T_{ph}(\phi)_x$. Vertical bars represent the hysteresis. The projection of the critical point line to the constant x plane is represented by the dashed curve. The intersections of critical line and cloud point curves are marked by the crossed squares.

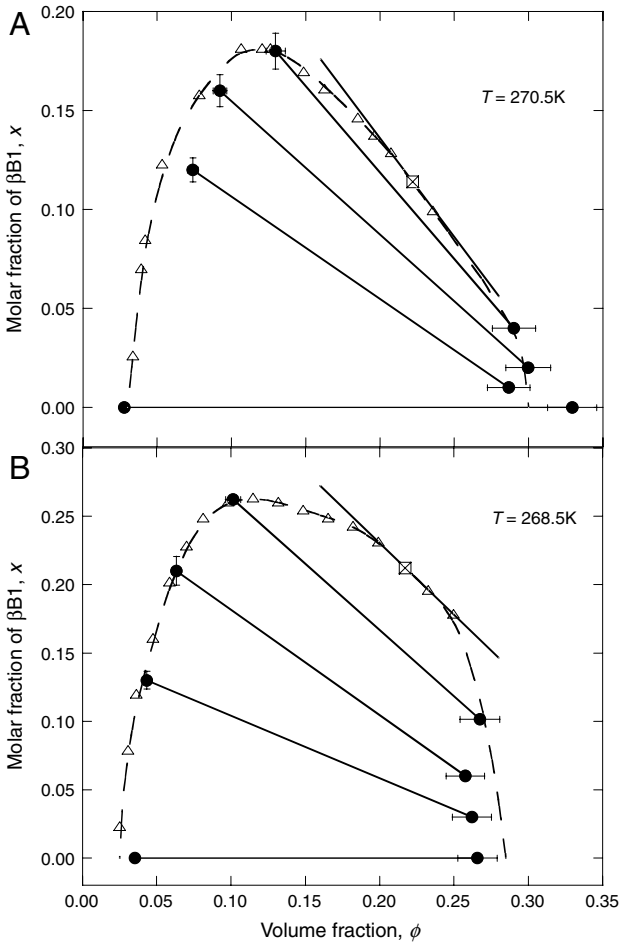

Fig. 2. Isothermal cross-sections of coexistence surface at $T_{ph} = 270.5$ K (*A*) and 268.5 K (*B*). The solid lines are tie-lines which connect pairs of points (*solid circles*) representing coexisting protein-rich and protein-poor phases in partitioning measurements. Dashed lines are eye guides for the binodal curves through data points of cloud-point measurements (*open triangles*) and partitioning measurements. The critical points are represented by crossed squares.

trations and compositions of coexisting protein-poor phase (ϕ^{I}, x^{I}) and protein-rich phase (ϕ^{II}, x^{II}) are reported at $T_{ph} = 270.5$ K and 268.5 K. Each pair of points for coexisting phases is connected by tie-lines. The slopes of tie-lines characterize the relative importance of protein condensation and demixing to LLPS. The horizontal tie-line signifies no demixing. The slopes of tie-lines are negative and significantly increase, as the proportion of βB1 increases.

In the vicinity of the critical point, the pairs of points on binodal curve that correspond to coexisting phases are difficult to determine by partitioning measurements, due to the slow dynamics of LLPS, and the small difference between coexisting phases. The binodal curve, $x(\phi)_{T_{ph}}$, here can be determined more accurately by interpolating the cloud point data, $T_{ph}(\phi)_x$. The points deduced from such interpolation are presented in Fig. 2. The two sets of data from partitioning measurements and from T_{ph} measurements are consistent within experimental error. Because the two coexisting phases coincide at the critical point, the position of the critical point (ϕ_c, x_c) at a given temperature can be estimated by extrapolating tie-lines to $\phi^{II} = \phi^{I} = \phi_c$, on the $x(\phi)_{T_{ph}}$ boundary as described in the *Discussion* section. At $T_{ph} = 270.5$ K, $x_c = 0.11 \pm 0.01$, and $\phi_c = 0.22 \pm 0.01$. At $T_{ph} = 268.5$ K, $x_c = 0.21 \pm 0.01$, $\phi_c = 0.22 \pm 0.01$.

While conducting partitioning measurements, after two weeks of incubation, we observed many crystals in the protein-rich bottom phase. These crystals have a rhombus shape (Fig. 3) identical to the geometry of crystals formed in the γD solutions (25), and we believe that these indeed are γD crystals. The formation of crystals implies that γD can readily crystallize in mixtures with βB1 and confirms that LLPS is metastable with respect to crystallization and that LLPS facilitates crystallization (17, 26).

Coexistence Surface of γD-βB1-Water Ternary Solution. In Fig. 4 we show the coexistence surface of the γD-βB1 system reconstructed from both cloud point measurements and partitioning experiments. The coexistence surface, at each point, is described by three derivatives: $(\partial T_{ph}/\partial\phi)_x$, $(\partial T_{ph}/\partial x)_{T_{ph}}$, and $(\partial x/\partial\phi)_{T_{ph}}$. These three derivatives are related mathematically by:

$$(\partial T_{ph}/\partial\phi)_x = -(\partial T_{ph}/\partial x)_\phi (\partial x/\partial\phi)_{T_{ph}} \quad [1]$$

In Fig. 4, we observe that $(\partial T_{ph}/\partial x)_\phi$ is always negative. Thus, according to Eq. **1**, $(\partial x/\partial\phi)_{T_{ph}}$ and $(\partial T_{ph}/\partial\phi)_x$ always have the same sign, and $(\partial x/\partial\phi)_{T_{ph}}$ must be equal to zero when $(\partial T_{ph}/\partial\phi)_x$ is equal to zero. Thus, the line of maxima of the cloud point curves coincides with the line where the binodal curves have maxima. This "ridge line" on the coexistence surface is shown as the red dashed line in Fig. 4. On the lower ϕ side of the ridge line, both $(\partial x/\partial\phi)_{T_{ph}}$ and $(\partial T_{ph}/\partial\phi)_x$ are positive. On the higher ϕ side, these two derivatives are both negative. Interestingly, in the low ϕ region ($\phi < \sim 0.1$), T_{ph} with good accuracy is a linear function of x, as shown in Fig. 5. The extrapolation of T_{ph} to 100% βB1 solution, i.e., $x = 1$, agrees well with $T_{ph} = 249 \pm 2$ K of pure βB1 solutions (8).

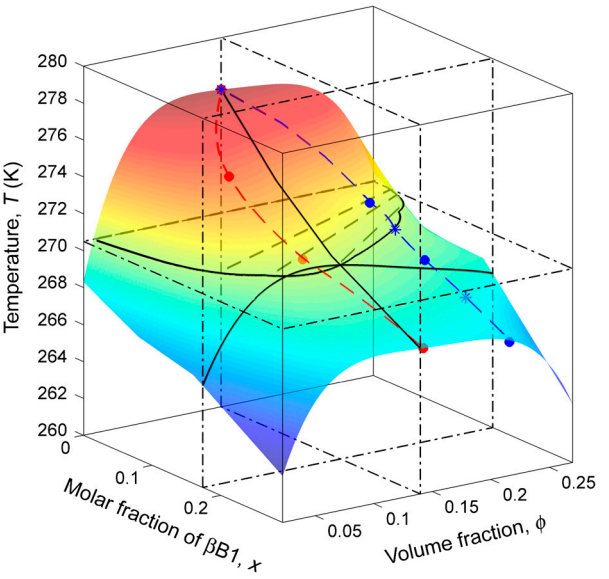

Fig. 4. Illustration of LLPS coexisting surface of γD-βB1-water ternary system. The colors from red to blue indicate the T_{ph} gradient on the coexistence surface from high to low temperature. The black solid lines are representative cross-sections of the coexistence surface at constant ϕ, x, and T_{ph}. The black dashed lines represent tie-lines of coexisting phases in a constant T_{ph} plane. The red dashed line is the eye guide for the ridge line drawn through the experimental maximum points (*red circles*) in cloud-point measurements. The blue dashed line is the eye guide for the critical point line drawn through the experimental critical points (*blue circles*).

We know experimentally critical points (ϕ^c, x^c) at three different temperatures: at $T_{ph} = 277.3$ K, from the position of the maximum on the coexistence curve in the pure γD solution (Fig. 1), and at $T_{ph} = 270.5$ K, and $T_{ph} = 268.5$ K, from the critical points on the binodal lines (Fig. 2). Using these three points we can draw the curve of T_{ph} (ϕ^c, x^c), and determine the location of the critical points on each of the cloud point curves (Fig. 4). We observe that the critical line is significantly shifted from the ridge line toward large ϕ. This observation is the result of strong demixing effects. The derivative $(\partial x/\partial\phi)_{T_{ph}}$ is negative, signifying preferential partitioning of βB1 into the protein-poor phase, and its magnitude increases with x.

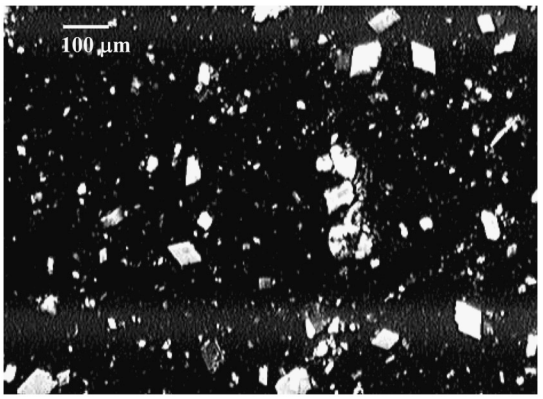

Fig. 3. Polarization microscopy image of γD crystals. Crystals were taken from the protein-rich phase ($\phi = 0.27$ and $x = 0.11$) in a partitioning measurement after two weeks incubation at 268.5 K. Image was taken at room temperature.

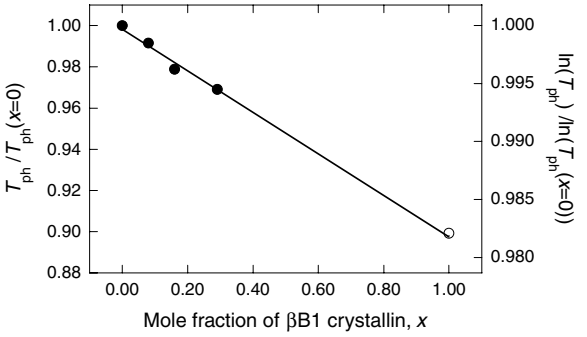

Fig. 5. T_{ph} of a γD-βB1 mixture solution at $\phi = 0.071$ and different x. T_{ph} (*left* vertical axis) and natural logarithm of T_{ph} (*right* vertical axis) are plotted as a function of βB1 crystallin fractions, x, as the solid circles. The open circle represents T_{ph} and logarithm of T_{ph} of pure βB1 solution deduced from βB1-PEG-water ternary system.

Quasielastic Light-Scattering (QLS) Studies of γD-βB1 Mixtures. To characterize the size and interprotein interaction of proteins in γD-βB1 solutions we have conducted QLS experiments. We first measured the diffusion coefficients, D's, of proteins in both pure γD solution and pure βB1 solution. The corresponding hydrodynamic radii were calculated using Stokes-Einstein relation: $R_h = kT/6\pi\eta D$, where k is Boltzmann's constant and η is the viscosity of buffer solution. The hydrodynamic radius, R_{h1}, of pure γD is 2.3 ± 0.1 nm, and R_{h2} of pure βB1 monomer is 3.0 ± 0.1 nm (8). The difference in hydrodynamic size is mostly due to the extended terminal arms of βB1, as the globular body of βB1 is known to be about the same as that of γD (8). We have also obtained the average R_h's in solutions with different proportion of βB1: R_h is equal to 2.3 ± 0.1 nm at $x = 0.04$ and 2.4 ± 0.1 nm at $x = 0.08$. These values are equal to the values of $R_h(x)$ calculated from the z-average of diffusion coefficients of γD and βB1 monomers, i.e., $D(x) = ((1-x)M_1{}^2D_1 + xM_2{}^2D_2)/((1-x)M_1{}^2 + xM_2{}^2)$, where M_1 and M_2 are respectively the molecular weights of γD and βB1. This equality indicates that aggregation, including homodimerization of βB1, is negligible in our experiments.

In order to characterize the interprotein interactions in γD-βB1 solution, the apparent diffusion coefficients in solutions with different proportions of βB1 ($x = 0$, 0.04, and 0.08) were measured as a function of ϕ. Within the concentration range of measurement ($0.002 \leq \phi \leq 0.021$), D decreases linearly with ϕ (Fig. 6). The large negative slope, $(dD/d\phi)_x < 0$, implies a net effective attractive interaction between the protein molecules in these solutions. The decrease in magnitude of this slope as x increases suggests that the net effective attractive interprotein interaction in solution is reduced by adding βB1. This observation is in accordance with the reduction T_{ph} by adding βB1.

Discussion

The coexistence surface of the γD-βB1-water ternary solution can be theoretically derived if the expression for the free energy is known. γD and βB1 crystallins are globular proteins of approximately the same geometrical size (8). The QLS measurements showed that βB1 crystallin in solutions with relatively low ϕ and x is in monomer form. We assume here that at our relatively low concentrations of βB1 the role of oligomerization of βB1 is negligible and our γD-βB1-water solutions can be treated as a colloidal mixture of two spherical components with the same size. In thermodynamic analysis, an incompressible solution of particles is equivalent to a compressible fluid constituted of the solute particles at pressure equal to the osmotic pressure, Π, in the solution (27). We therefore introduce a reduced Gibbs free energy $g(\Pi,T,x) \equiv G(\Pi,T,N_1,N_2)/N$. Here, $N = N_1 + N_2$, where N_1 and N_2 are respectively the numbers of γD and βB1 molecules. For a colloid mixture of two distinct spherical components with the same size, the reduced Gibbs free energy can be written as: $g = kTs_{HS} + kTs_{mix} + U$. Here s_{HS} is the entropy per particle in a solution of hard-spheres, which is well approximated by the Carnahan Starling expression (28): $s_{HS} = \ln\phi + (1 + 5\phi - 6\phi^2 + 2\phi^3)/(1-\phi)^3$. s_{mix} is the entropy per particle of mixing two distinguishable proteins: $s_{mix} = x\ln x + (1-x)\ln(1-x)$. U is the free energy associated with interprotein interactions. U is a function of the state variables: Π, T, and x.

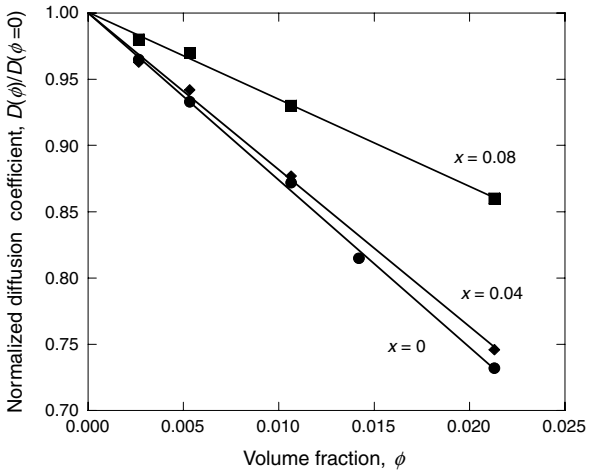

Fig. 6. Normalized diffusion coefficients $D(\phi)/D(\phi = 0)$ are plotted as a function of volume fraction ϕ, for γD solution (*circles*) and γD-βB1 mixture solutions with $x = 0.04$ (*diamonds*) and 0.08 (*squares*).

Let us first consider a γD-water binary solution, $x = 0$. In this case, g is the chemical potential of γD, and U is the energy needed to remove one γD molecule from the solution. This energy, U, can be viewed as a product of the average number of contacts, n_c, times the effective energy, E_{11}, per contact between γD molecules, i.e., $U = n_cE_{11}$. Of course, the notion of a "contact" is unambiguous only in the framework of the square-well potential model with a specific range of interaction. Nevertheless, the number of contacts has proven to be a useful concept for understanding the phase behavior of colloid systems (24, 29). Generally, U is a function of the temperature. However, a solution of globular proteins can be regarded as a colloid system with relatively weak interactions. In such systems the spatial distribution function and thus n_c is mainly governed by entropic excluded volume interaction, and therefore n_c would be independent of temperature. In effect, we describe the thermodynamic features of our system in a high temperature approximation (30). Hereafter, n_c is dependent only on the volume fraction, ϕ, which here has to be treated as a function of osmotic pressure, Π. Note that the effective free energy of a contact between protein molecules, E_{11}, may have an entropic component and thus can be temperature dependent.

In a γD-βB1-water ternary solution, $x > 0$, some of the γD molecules are replaced by βB1 molecules of the same size, but with different contact energy, E_{12}. In the same high temperature approximation as described above, n_c is not affected by interparticle interactions. Thus the change of total contact energy associated with a replacement of γD with βB1 will be $n_c(E_{12} - E_{11})$. Therefore, when x is small and the contacts of βB1 molecules with each other can be ignored, we can model U as $U = n_c(E_{11} + x(E_{12} - E_{11}))$.

Having defined all the terms of the Gibbs free energy, we can now calculate the chemical potentials of the two proteins in the solution: $\mu_1 \equiv (\partial G/\partial N_1)_{N_2,\Pi,T}$ and $\mu_2 \equiv (\partial G/\partial N_2)_{N_1,\Pi,T}$ to the lowest order in x:

$$\mu_1 = kTs_{HS} + n_cE_{11} - kTx \quad \textbf{[2a]}$$

$$\mu_2 - \mu_1 = kT\ln x + n_c(E_{12} - E_{11}). \quad \textbf{[2b]}$$

The coexistence surface is defined by a set of two equations which express the equality of the chemical potentials of γD and βB1 molecules in two phases:

$$\mu_1{}^{I}(\Pi,T,x^{I}) = \mu_1{}^{II}(\Pi,T,x^{II}) \quad \textbf{[3a]}$$

$$\mu_2{}^{I}(\Pi,T,x^{I}) - \mu_1{}^{I}(\Pi,T,x^{I}) = \mu_2{}^{II}(\Pi,T,x^{II}) - \mu_1{}^{II}(\Pi,T,x^{II}). \quad \textbf{[3b]}$$

Below, we discuss two specific features of the coexistence surface, namely the slope of tie-lines and the T_{ph} dependence on x, both of which can be quantitatively analyzed even without explicit knowledge of $n_c(\phi)$.

Slope of Tie-Lines. An important experimentally observed feature of γD-βB1 solutions is that the slopes of tie-lines in the isotherm cross-section (Fig. 2) have negative values with magnitudes increasing as the average x increases. The negative slope of the

tie-lines signifies that βB1 is expelled from the dense γD phase. Because the x's are small and thus βB1-βB1 interaction is negligible, this observation implies that the effective γD-βB1 interaction is less attractive than the γD-γD interaction, i.e., $|E_{12}| < |E_{11}|$. To quantify the slope of tie-lines we substitute Eq. **2b** into Eq. **3b** and find:

$$kT_{\rm ph}(\ln x^{\rm II} - \ln x^{\rm I}) = -(n^{\rm II} - n^{\rm I})(E_{12} - E_{11}), \qquad [4]$$

where $n^{\rm I} = n_c(\phi^{\rm I})$ and $n^{\rm II} = n_c(\phi^{\rm II})$. Note that when phase separation occurs, the two coexisting phases, while having the same osmotic pressure, have two different values of ϕ and thus two different values of $n_c(\phi)$. Substituting Eq. **2a** into Eq. **3a** we find to the lowest order of x:

$$kT_{\rm ph}({s_{\rm HS}}^{\rm II} - {s_{\rm HS}}^{\rm I}) = -(n^{\rm II} - n^{\rm I})E_{11}, \qquad [5]$$

where ${s_{\rm HS}}^{\rm I} = s_{\rm HS}(\phi^{\rm I})$ and ${s_{\rm HS}}^{\rm II} = s_{\rm HS}(\phi^{\rm II})$. Combining Eq. **4** and Eq. **5**, we can obtain the relation between the steepness of a tie-line and the energies of interprotein interaction:

$$(E_{12} - E_{11})/E_{11} = (\ln x^{\rm II} - \ln x^{\rm I})/({s_{\rm HS}}^{\rm II} - {s_{\rm HS}}^{\rm I}). \qquad [6]$$

Using the Carnahan Starling expression for $s_{\rm HS}(\phi)$, we substitute the experimental data for $(\phi^{\rm I}, x^{\rm I})$ and $(\phi^{\rm II}, x^{\rm II})$ shown in Fig. 2 into Eq. **6**, and calculate values of E_{12}/E_{11} corresponding to each tie-line. These values are shown in Table 1. E_{12}/E_{11} is equal to 0.59 ± 0.02 at 270.5 K and is equal to 0.73 ± 0.03 at 268.5 K. We comment that a difference between the values of E_{12}/E_{11} at different temperatures can be expected due to the anisotropy of interprotein interactions and other entropic contributions which lead to a temperature dependence of the effective interaction energies (24, 29). The ratio E_{12}/E_{11} is a robust physical quantity which determines the steepness of tie-line. In contrast, the absolute magnitudes of the contact energies are model dependent and only the product, $n_c\, E_{11}$, is physically significant. Nevertheless, it can be said that E_{11} has a negative value, of the order of kT_c.

With E_{12}/E_{11} known, we used Eq. **6** together with the experimentally determined binodal curve, $x(\phi)_{T_{\rm ph}}$, to determine for any $(\phi^{\rm I}, x^{\rm I})$ the corresponding $(\phi^{\rm II}, x^{\rm II})$. This approach enables the construction of a family of tie-lines which can be extrapolated to the vicinity of the critical point ($x^{\rm I} \to x^{\rm II}$ and $\phi^{\rm I} \to \phi^{\rm II}$) on the binodal curve. We used this procedure to graphically locate the critical points shown in Fig. 2.

Reduction of Phase Separation Temperature upon Adding βB1 Crystallin. To calculate the reduction of $T_{\rm ph}$ upon increasing βB1 fraction, i.e., $\Delta T_{\rm ph}(x)$, we compare Eq. **3a** written at some fixed osmotic pressure for a γD-water binary solution with that for a γD-βB1-water ternary solution. Omitting the fixed variable Π we can write ${\mu_1}^{\rm I}(T_{\rm ph}, 0) = {\mu_1}^{\rm II}(T_{\rm ph}, 0)$ for pure γD and ${\mu_1}^{\rm I}(T_{\rm ph} + \Delta T_{\rm ph}, x^{\rm I}) = {\mu_1}^{\rm II}(T_{\rm ph} + \Delta T_{\rm ph}, x^{\rm II})$ for the γD-βB1 mixture. Expanding the second condition to the first order in $\Delta T_{\rm ph}$ and in x around $T = T_{\rm ph}$ and $x = 0$, we obtain a general formula for the change of phase transition temperature upon adding a small amount of second component (31):

$$(\Delta T_{\rm ph})_{\Pi} = \frac{kT_{\rm ph}(x^{\rm I} - x^{\rm II})}{\partial\mu_1^{\rm I}/\partial T - \partial\mu_1^{\rm II}/\partial T} = \frac{kT_{\rm ph}^2(x^{\rm I} - x^{\rm II})}{q}. \qquad [7]$$

Table 1. E_{12}/E_{11} calculated from the tie-lines shown in Fig. 2

$T_{\rm ph}$	$\phi^{\rm I}$	$\phi^{\rm II}$	$x^{\rm I}$	$x^{\rm II}$	E_{12}/E_{11}
270.5	0.07	0.29	0.12	0.01	0.58
270.5	0.09	0.30	0.16	0.02	0.61
270.5	0.13	0.29	0.18	0.03	0.59
268.5	0.04	0.26	0.13	0.03	0.72
268.5	0.06	0.26	0.21	0.06	0.72
268.5	0.10	0.27	0.26	0.10	0.76

We used here $\partial\mu_1/\partial x = -kT_{\rm ph}$, according to Eq. **2a**. Because $\partial\mu_1/\partial T$ is the entropy per molecule, q is the molecular latent heat of LLPS of the γD-water binary solution. According to Eq. **2a**, $q = kT_{\rm ph}({s_{\rm HS}}^{\rm I} - {s_{\rm HS}}^{\rm II}) + (n^{\rm I} - n^{\rm II})T_{\rm ph}(\mathrm{d}E_{11}/\mathrm{d}T)$. This latent heat, q, consists of two components: the first one, $kT_{\rm ph}({s_{\rm HS}}^{\rm I} - {s_{\rm HS}}^{\rm II})$, is associated with the entropy change due to excluded volume; the second one, $(n^{\rm I} - n^{\rm II})T_{\rm ph}(\mathrm{d}E_{11}/\mathrm{d}T)$, is the difference in entropic components of the effective interprotein interaction.

Eq. **7** gives an expression for the temperature shift at fixed osmotic pressure while the experimentally determined quantity is the temperature shift at constant concentration. In the dilute phase $\phi^{\rm I} \ll 1$, where $\Pi \approx kT\phi^{\rm I}$, the relative difference between $(\Delta T_{\rm ph})_{\phi}$ and $(\Delta T_{\rm ph})_{\Pi}$ is: $((\Delta T_{\rm ph})_{\phi} - (\Delta T_{\rm ph})_{\Pi})/(\Delta T_{\rm ph})_{\Pi} = (\partial T_{\rm ph}/\partial\phi)(\phi/T_{\rm ph})$. This difference is numerically less than 2% and can be neglected. Furthermore, in the γD-βB1 mixture, the βB1 predominantly partitions into protein-poor phase I, i.e., $x^{\rm I} \gg x^{\rm II}$. Thereby, using Eq. **5** for the expression of $(n^{\rm I} - n^{\rm II})$, we find:

$$(\Delta T_{\rm ph})_{\phi}/T_{\rm ph} \approx \frac{kT_{\rm ph}x^{\rm I}}{q} = \frac{x^{\rm I}}{(s_{\rm HS}^{\rm I} - s_{\rm HS}^{\rm II})[1 - (\mathrm{d}E_{11}/\mathrm{d}T)(T_{\rm ph}/E_{11})]}. \qquad [8]$$

Eq. **8** allows us to determine the latent heat of LLPS, q, and the entropic component of interprotein interaction in a pure protein-water solution, $(\mathrm{d}E_{11}/\mathrm{d}T)T/E_{11}$. By simultaneously fitting the experimental $(\Delta T_{\rm ph}(x))_{\phi}$ in Fig. 1 at several different $\phi^{\rm I}$'s (Fig. S1), we find $(\mathrm{d}E_{11}/\mathrm{d}T)T/E_{11} = -1.0 \pm 0.2$. This value signifies that the entropic component of the γD-γD interaction is comparable to the excluded volume entropy. The values of q's at concentrations used in the fitting procedure are listed in Table 2. The negative value of q is consistent with the idea that βB1 suppresses the phase separation temperature and thereby plays the role of phase separation inhibitor. The $T_{\rm ph}$ reduction (~7 °C with $x \approx 0.3$) due to demixing that occurs during γD-βB1 phase separation is comparable to the effect of previously discovered small molecule cataract inhibitors (32, 33) which, at high mole fractions, x, up to 0.66, chemically modify eye lens proteins. Our analysis suggests that the solution components which undergo strong demixing from the major eye lens proteins serve as important agents which may forestall a cataractogenic phase transition. Such strongly demixing agents could be viable guides in the design of cataract inhibiting drugs.

In conclusion, we have experimentally determined the LLPS coexistence surface of the γD-βB1-water ternary system. Two important features were observed: (*i*) strong demixing of γD and βB1, with βB1 preferentially segregated into the dilute phase; (*ii*) a significant decrease of phase separation temperature upon the addition of βB1 to the solution. We determined the ratio of the effective energy of the γD-βB1 interaction, to that of the γD-γD interaction: $E_{12}/E_{11} = 0.7$. We also determined the relative entropic contribution $((\mathrm{d}E_{11}/\mathrm{d}T)T/E_{11} = -1.0 \pm 0.2)$ to the effective energy of the γD-γD interaction. With these two parameters our thermodynamic analysis describes quantitatively the principle features of the phase behavior of this ternary protein solution. The reduction of phase separation temperature in the presence of βB1 suggests the importance of this protein in maintaining lens transparency.

Table 2. Molecular latent heat, q, of LLPS of γD-water binary solution calculated at several $\phi^{\rm I}$

$\phi^{\rm I}$	0.030	0.040	0.050	0.060	0.071
$-q/kT_c$	11.6	10.4	9.4	8.5	7.7
$N_A q$ (kJ/mol)	−26.8	−24.0	−21.6	−19.6	−17.7

Materials and Methods

Expression, Purification, and Characterization of Human γD and βB1. Recombinant human γD and βB1 crystallins were prepared by amplification of the coding sequence from a human fetal lens cDNA library and were expressed in *Escherichia coli*, as described in our previous studies (8, 25). Overexpression and purification of γD and βB1 were carried out as described previously (8, 25). The final products were analyzed by electrospray ionization mass spectrometry (ESI-MS model LCQ, ThermoQuest), which confirmed the molecular masses of 20,607 g/mol for γD and 27,892 g/mol for βB1.

Solution Preparation. The purified γD and βB1 proteins were dialyzed exhaustively into sodium phosphate buffer (0.1 M, pH 7.1) that contained sodium azide (0.02%) and DTT (20 mM). Solutions containing dilute γD and βB1 in phosphate buffer were concentrated by ultrafiltration (Amicon, 10 kDa) and centrifugation (Amicon Ultra, 10 kDa). The concentrations of γD and βB1 in the solutions were determined using a HPLC system (System Gold I26, Beckman Coulter) with a UV detector and a reverse phase column (Jupiter 5μ C18 300A, phenomenex). Reverse phase HPLC was precalibrated with standard γD and βB1 solutions without DTT. The concentrations of standard solutions were measured by an UV spectrometer at 280 nm using the extinction coefficient value of 2.09 $mg^{-1} \cdot mL \cdot cm^{-1}$ for γD and 2.06 $mg^{-1} \cdot mL \cdot cm^{-1}$ for βB1 (www.expasy.org). Protein volume fractions in solution were calculated by multiplying the mass concentration by the specific volume of protein, 0.71 mL/mg (15).

Measurement of T_{ph}. A test tube containing the sample was placed in a thermostated light-scattering stage, whose temperature was initially set above T_{ph} so that the solution was transparent. The transmitted intensity of a 4-mW He-Ne laser was recorded by a photodiode. The temperature of the sample was then step wise lowered by 0.1 K every 5 min. At a well defined temperature, T_{cloud}, the sample became visibly cloudy. The temperature was then step wise raised by 0.1 K every 5 min. The minimum temperature at which the solution became clear again was denoted by $T_{clarify}$. The phase separation temperature T_{ph} is estimated as the average of $T_{clarify}$ and T_{cloud}. The difference between T_{cloud} and $T_{clarify}$ is hysteresis which reflects the nucleation rate (14, 15). Since hysteresis depends on kinetic processes, all the cooling and heating steps were set with a standard time interval (5 min). In pure γD solutions with ϕ lower than 0.1, sedimentation of the protein-rich phase occurs in a time small compared to 5 min. In this domain we chose T_{ph} to be equal to T_{cloud}.

Measurement of γD-βB1 Partitioning. The solutions having known ϕ and x were quenched to a temperature below T_{ph} in a thermostated water bath. After an incubation time of one week, a sharp interface formed between two liquid phases. The formation of the sharp interface was taken as an indication that equilibrium was reached. The γD and βB1 crystallins in both phases were separated and their concentrations were measured using reverse phase HPLC. Using these concentrations we calculated ϕ and x in each of the two coexisting phases.

Quasielastic Light Scattering. All protein samples were filtered through a 0.02 μm Millipore filter and placed in a test tube. QLS experiments were performed on a light-scattering apparatus using a PD2000DLS^{PLUS} correlator (Precision Detectors) and a Coherent He-Ne laser (35 mW, 632.8 nm; Coherent Radiation). The measurements were performed at a scattering angle of 90°. The measured correlation functions were analyzed by the Precision Deconvolve 5.5 software (Precision Detectors). The correlation functions were used to calculate the apparent diffusion coefficients, D, of proteins in solutions with given ϕ and x. $D(\phi = 0)_x$ were obtained by extrapolating $D(\phi)_x$ to $\phi = 0$. The hydrodynamic radii, R_h's, of proteins in solutions with fixed x were calculated from $D(\phi = 0)_x$ using Stokes-Einstein relation.

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