Enthalpy and Entropy of Hydration of Bovine Crystallins*

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The transparency of the lens of the eye is the result of a short range order in the packing of crystallin molecules within the fiber cells. Short range order depends on crystallin-crystallin as well as water-crystallin interactions. Light scattering measurements can provide information on the hydration of crystallins. Light scattering intensities were obtained as a function of scattering angle, concentration, and temperature on dilute solutions of βH, β−, and γ fractions of bovine lens crystallins. The temperature dependence of the second virial coefficient was negative for the β crystallin fractions and positive for the γ fraction as well as that for α crystallin (Wang, X., and Bettelheim, F. A. (1989) Proteins Struct. Funct. Genet. 5, 166–169). The partial molar enthalpy values of the solutions were negative for the β crystallin fractions, indicating a tendency for homo- and heterodimer and -oligomer association. The enthalpy values were positive for the α and γ fractions. The negative values of the enthalpy of solutions differentiate the β crystallins from the other crystallins. The partial molar entropy values of solutions of β− and γ fractions were identical, those of the oligomeric βH fraction were higher, whereas those of α crystallin were a magnitude larger than those of the smaller crystallin molecules.

α, β, and γ crystallins are the major structural proteins in mammalian lenses. Their function is to provide a transparent assembly with a refractive index gradient that is capable of focusing light onto the retina (1–3). α crystallins are heteropolymers with a molecular weight of about 600,000–1,000,000 (4–6). γ crystallins, on the other hand, are a family of compact monomers with a molecular weight of 20,000 having two similar globular domains (7–10). They are located mostly in the regions of the lens with the highest refractive index (11). β crystallins are of intermediate size between α and γ crystallins forming oligomers from a variety of subunits (12–14).

Thus, the lens crystallins can be subdivided into heterogeneous groups on the basis of their apparent size. The largest group is that of α crystallin, which is made of aggregates of two subunits αA and αB (each of them with a mass of about 20 kDa). These subunits are individual gene products exhibiting a very low rate of evolutionary change (15, 16). They exist as polydispers high molecular weight aggregates.

β crystallins are also aggregates of many subunits with extensive polydispersity (12, 13). Sequencing studies showed that bovine β crystallins have three acidic (βA2, βA3/1, and βA4) and three basic (βB1, βB2, and βB3) subunits with apparent mass of 23, 25, and 23 kDa and 32, 26, and 27 kDa, respectively (14). Bovine βH crystallin with apparent molecular mass of 160–200 kDa contains all the β subunits in different combinations, whereas the βH crystallin fraction with apparent molecular mass of 46–70 kDa lacks βB1 subunits (14). The subunits form homo- and heterodimers and -oligomers. The structure of the βB2 homodimer have been studied extensively (17, 18). Each domain is formed from two “Greek key” motifs, and the connecting peptide is extended. The secondary structure is made of β sheets (18). The dimer stability is provided by the intersubunit β sheet interfaces and the C-terminal extension in the βB2 dimerization (18) and the N-terminal extension in the βA3 dimerization (19).

The γ crystallin fraction contains the monomeric γ crystallins (7–10) and also monomeric γS crystallin, which in contrast to the other γ crystallins is completely denatured in 8 M urea at room temperature (20). These have a mass of approximately 20 kDa. The three-dimensional structures of γ crystallins have been elucidated from x-ray diffraction data for γB (7, 10), γC (8), and γE (9) crystallins. Each is made of a two domain structure in which each domain has two Greek key motifs.

These crystallins are the products of different genes, although β and γ crystallins may have had a common ancestral gene. The various crystallin families and their individual members are differentially expressed during development (15) leading to different mixtures of crystallins along the optical axis. The particular packing of these crystallins depends on the size as well as on the interaction of the crystallins with themselves and with the aqueous surrounding. The combination of these effects results in a protein gradient along the optic axis, and because each crystallin family has its individual refractive index contribution (21), it also results in a refractive index gradient.

In addition to the structural features of crystallins, their solvation properties and their specific interactions in homo- and heteroaggregations are of importance (22–26). The transparency of the lens depends on its hydration (2, 3). The hydration of the lens is a complex phenomenon (27–30). It involves interaction of the crystallins with water, protein-protein interactions, crystallin distribution, and gradient in the lens. The hydration of the lens can be better understood if the role of the individual factors in the hydration process are known. The present study was designed to probe the stability of the crystallins in aqueous solutions by calculating the thermodynamic parameters of solution from light scattering measurements. These parameters are important in assessing the interaction of water with single crystallin molecules, one aspect of the total hydration process.

EXPERIMENTAL PROCEDURES

Materials—Bovine eyes were obtained from a local slaughterhouse. The lenses were removed and processed within 6 h postmortem. Lenses were homogenized in 7 volumes of 0.05 M Tris buffer at pH 7.5, which also contained 0.1 M KCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, and...
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0.02% NaN₃. After centrifugation the supernatant was applied to a Sephadex G-200 column, and four fractions (α, β₁, β₂, and γ fractions) were collected and identified by SDS gel electrophoresis (33). The four crystallin fractions were dialyzed against distilled water using 4 kDa of exclusion dialysis membrane. The two β-fractions were lyophylized, and the γ fraction and a part of the β₁ fraction were concentrated at room temperature under a vacuum to a 5% concentration to avoid low temperature denaturation (31). The crystallins were dissolved in 0.1 M Na₂HPO₄ buffer, pH 7.4, or diluted from their concentrated solutions to have a final 0.1 M buffer concentration.

Methods—Light scattering measurements were performed in a model 3000 Universal Light Scattering Photometer (C. N. Wood Manufacturing Co.). The wavelength of the light source (mercury lamp) was 546 μm. The light scattering intensities of crystallin solutions were measured as a function of protein concentration, scattering angle, and temperature. The temperature control was achieved by thermocouple-regulated heating of the scattering cell wrapped with nickel-iron, and chromium heating wires at the top and the bottom of the cell (32). The temperature was maintained within 0.05 °C of the set temperature. The crystallin solutions were centrifuged at 15,000 rpm for 15 min before the light scattering measurements to eliminate dust particles and incompletely dissolved aggregates. The concentration of centrifuged solution was established by measuring their absorbance at 280 nm and comparing it with previously established calibration curves for each crystallin fraction. The actual readings of the instrument in volts were converted to the appropriate Rayleigh ratio by establishing the instrument constant. The light scattering of tryply distilled and filtered water at a 90 °C scattering angle was measured, and the instrument constant was calculated using the absolute scattering of water at this angle and wavelength: 1 × 10⁻⁶ cm⁻¹ (33). A secondary standard of an opaque light scattering plate was used to recalibrate the instrument after each series of angular measurements to eliminate errors due to voltage fluctuation in the light source. Light scattering measurements were taken between 20 and 60 °C scattering angles at temperatures of 16–27 °C.

RESULTS

In Fig. 1 typical light scattering data are presented in the form of a Zimm plot (33). The sample in this case was β₁ crystallin at 19.0 °C. This β₁ crystallin was concentrated at room temperature under a vacuum to a 5% concentration and sequentially diluted for a light scattering study. Another β₁ fraction was lyophylized and dissolved in buffer. It gave a Zimm plot with identical parameters, indicating the fact that β₁ fraction was lyophylized and dissolved in buffer. It gave a crystallin at 19.0°C. This

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In Fig. 2 second virial coefficients of β₁ (○), β₂ (●), and γ [14] crystallins as a function of temperature.

where c is the concentration of the protein in g/cm³ and Rᵣ is the Rayleigh ratio for unpolarized light (34) given by the equation

\[ Rᵣ \approx (Iₒ/I₁) \cdot (r^2/1 + cos^2θ) \]  

(1)

where θ is the scattering angle, Iₒ is the intensity of the scattered beam at angle θ, I₁ is the intensity of the incident beam (at a scattering angle of 0), and r is the distance between the scattering volume of the sample and the detector in cm. The x axis contains both concentration and scattering angle. K’ is an arbitrary constant (100 in Fig. 1) to spread the diagram, giving about equal weight to the concentration and to the scattering angle. The intercept of the two extrapolated lines with the y axis is inversely related to the Mᵣ, weight average molecular weight of the sample; the slope of the 0 concentration line is related to the Rₒ, radius of gyration, a size parameter. Finally the slope of the 0 scattering angle line yields the second virial coefficient from which the enthalpy and entropy of solution of the crystallins can be calculated.

The weight average molecular weights calculated from the intercept of the Zimm plots were as follows: 200,000 for β₁, 81,200 for β₂ (indicating mostly tetrameric form), and 19,800 for γ crystallin. These are in agreement with literature values (7, 14).

The second virial coefficients obtained from the Zimm plots are presented in Fig. 2 as a function of the reciprocal temperature. The straight line plot corresponds to the prediction of Flory’s theory (35). It is interesting to note that in both β fractions the second virial coefficient decreases with temperature while in α crystallin (32), and in γ crystallin it increases with temperature.

The second virial coefficient A₂ is expressed as

\[ A₂ = (1/2 - x₁)N/V₂NᵢV₁ \]  

(2)

where V₂ is the partial specific volume, V₁ is the molar volume of the solvent, Nᵢ is Avogadro’s number, and x₁ is an interaction parameter related to the partial molar free energy of the solution. The x₁ interaction parameter has enthalpy (keleton) and entropy (ψ₂) contributions (34, 35)

\[ (1/2 - x₁) = φ₁ - k₁ = φ₁(1 - \eta/T) \]  

(3)

where Τ is the absolute temperature and η is the Flory temperature; at this temperature the interaction parameter becomes V₂, and the second virial coefficient equals zero. A plot of A₂ against [1/T] such as in Fig. 2 yields the entropy parameter as an intercept and the enthalpy parameters and Flory’s temperature as the slope. From these the partial molar enthalpy (ΔH) and the partial molar entropy (ΔS) can be calculated.

\[ ΔH = RTk₁V₁^{1/2} \]  

(4)

\[ ΔS = Rφ₁V₁^{1/2} \]  

(5)

where R is the gas constant and V₁ is the volume fraction of the solute.

The partial molar enthalpies of solution for α, β₁, β₂, and γ crystallins are presented in Table I as a function of concentra-
tion of crystallins. The $\Delta H$ of $\alpha$ crystallin is taken from a previous publication (32) and is presented for comparison. Both $\alpha$ and $\gamma$ crystallins possess positive enthalpy of solution (endothermic), whereas the $\beta$ crystallin fractions have small negative enthalpy of solutions (exothermic).

The partial molar entropy values of solutions of $\alpha$, $\beta_A$, $\beta_L$, and $\gamma$ crystallins are also given in Table I as a function of concentration. It is interesting to note that $\beta_L$ and $\gamma$ fractions have identical partial molar entropies of solutions.

**DISCUSSION**

The present study was intended to illuminate the interaction of crystallins with the aqueous environment. We calculate the partial molar enthalpy and entropy of solutions from the temperature dependence of the second virial coefficients of light scattering measurements. These are extrapolated values, and as such the numerical values of $A_2$ had a standard deviation of $\pm 2.5\%$, and the temperature dependence of $A_2$ is statistically significant at the 90% confidence level. Thus, it is better to focus on the relative trends shown by the different crystallins than on the absolute values of partial molar enthalpies and entropies.

The strong concentration dependence of the partial molar enthalpy of $\alpha$ crystallin indicates polydispersity. The partial molar enthalpy represents the energy expended when 1 mol of crystallin is dissolved in a solution of specified concentration having infinitely large volume. A positive $\Delta H$ value means that in order to solvate the crystallin, to surround it with a bound water layer, energy input is necessary. This is what happens in $\alpha$ and $\gamma$ crystallins. The $\beta$ crystallin fractions have small but negative partial molar enthalpy values. This implies that the water-$\beta$ crystallin interaction is less energetic than the average of water-water and crystallin-crystallin interaction. This indicates a tendency for dimeric and oligomeric aggregation.

The partial molar entropy values are positive for all crystallin fractions. Thus the randomness on the dispersion of a crystallin in water and the gain in segment mobility of the polypeptide chain upon solvation are greater than the contribution of water immobilization in the bound solvation layer. The entropy of solution values follow the molecular size of the crystallins. $\alpha$ crystallin has entropy values 1 order of magnitude greater than the other groups. The monomeric $\gamma$ fraction and the mainly tetrameric $\beta_L$ fraction have identical partial molar entropy of solutions indicating the compact nature of these proteins. The $\beta_A$ fraction that is largely made of oligomeric aggregates of subunits has larger albeit the same order of magnitude of partial molar entropy values as those of the smaller molecular weight compounds.

The combination of enthalpy and entropy of solution may explain the behavior of the different crystallins in immobilizing water in their solvation layer. The $\beta_L$ crystallin has greater bound (nonfreezable) water content than $\alpha$ crystallin (24). Even though the mass of $\beta_L$ is three to five times smaller than that of $\alpha$ crystallin and thus does not gain that much in segment mobility in solubilization, it still binds more water than $\alpha$ crystallin. The negative enthalpy of solution implies strong solute-solute interactions. A strong attraction, possibly among the Greek motifs, would trap more water in the bound form around the extended connecting peptides. The hydration of $\alpha$ crystallin is largely entropy driven. The $\beta_L$ and $\gamma$ crystallins immobilize water to about the same degree in their solvation layer (26) in the form of nonfreezable water. However, when the same tendency is probed by water vapor sorption, the $\gamma$ fraction has much less bound water in the solvation layer (24). This may be the result of the negative enthalpy of solution of the $\beta_L$ crystallin, which enables strong interactions in the dimerization trapping more water around the dimer than around the monomeric $\gamma$ crystallins.

**CONCLUSIONS**

Bovine lens uses three different families of crystallins to build a protein concentration gradient along the optic axis. For transparency and hence for optimal packing all three crystallins are necessary. The main finding of this study was that the slope of the temperature-dependent second virial coefficients and hence the enthalpy of hydration of $\beta$ crystallins are negative, whereas that of $\alpha$ and $\gamma$ crystallins are positive. Thus, one could propose that although the $\gamma$ crystallins by their size may provide an optimal packing, the $\beta$ crystallins by their tendency of strong intermolecular association and water immobilization also enhance close packing.

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