Solution properties of γ -crystallins: Hydration of fish and mammal γ -crystallins

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Abstract: Lens γ crystallins are found at the highest protein concentration of any tissue, ranging from 300 mg/mL in some mammals to over 1000 mg/mL in fish. Such high concentrations are necessary for the refraction of light, but impose extreme requirements for protein stability and solubility. γ -crystallins, small stable monomeric proteins, are particularly associated with the lowest hydration regions of the lens. Here, we examine the solvation of selected γ -crystallins from mammals (human γ D and mouse γ S) and fish (zebrafish γ M2b and γ M7). The thermodynamic water binding coefficient B_1 could be probed by sucrose expulsion, and the hydrodynamic hydration shell of tightly bound water was probed by translational diffusion and structure-based hydrodynamic boundary element modeling. While the amount of tightly bound water of human γ D was consistent with that of average proteins, the water binding of mouse γ S was found to be relatively low. γ M2b and γ M7 crystallins were found to exhibit extremely low degrees hydration, consistent with their role in the fish lens. γ M crystallins have a very high methionine content, in some species up to 15%. Structure-based modeling of hydration in γ M7 crystallin suggests low hydration is associated with the large number of surface methionine residues, likely in adaptation to the extremely high concentration and low hydration environment in fish lenses. Overall, the degree of hydration appears to balance stability and tissue density requirements required to produce and maintain the optical properties of the lens in different vertebrate species.

Keywords: crystallin; protein hydration; protein hydrodynamics; hydrodynamic modeling; protein interactions

Introduction

Crystallins are unusual proteins in that their major role in the organism is to be present in lens tissue at high enough concentration to impart a high tissue

refractive index to serve as a refractive element to camera eyes while maintaining transparency.¹ Although at first this appears to be a simple task, and in different species numerous unrelated proteins have been coopted for this purpose,² many crystallins have adapted to this function in very sophisticated ways.³ Prototypes for this adaptation are γ -crystallins, a major protein component of the eye lens in vertebrates, which are found in a relative abundance that correlates with the highest tissue protein densities and the lowest water content.^{2–4} A

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common characteristic of this family is a tightly folded Greek key structure⁵ composed of amino acids that impart an unusually high molecular refractive index increment^{6–8} and remarkably high structural stability,⁹ and surface properties to modulate solvation and protein interactions as required for solubility at very high concentrations in excess of 500 mg/mL.^{3,5,10–12} Among the proposed mechanisms in γ -crystallins thought to enhance solubility are the modulation of the surface hydration and the creation of extended networks of charges at the protein surface,⁵ and the creation of molecular electrostatic dipole moments.¹¹ Solubility is a key property since aggregates lead to light scattering and, as a consequence, cataract formation.^{13–15} This difficulty is exacerbated due to the absence of protein turnover, requiring individual copies of γ crystallin to remain soluble for the lifetime of the whole organism.¹⁶ Thus, the study of the exceptional structural stability and origin of the probably unique solubility of γ crystallins ties in to research in cataract, and, more generally, protein misfolding diseases,^{17–20} but is also of fundamental interest in exploring the physical properties of highly concentrated protein solutions.^{21,22}

Among the γ crystallins, fish γ M crystallins may have the most extreme properties, as the aquatic environment eliminates the corneal refractive power and requires animals to have eyes with lenses of much higher density. With concentrations in excess of 1000 mg/mL^{9,10} and with >50% of the lens total mass being γ M crystallin,²³ γ M crystallins must exist close to the packing limit. A common feature of γ M crystallins is an extraordinarily high content of methionine, in some species approaching 15% of total amino acids^{24–26} (which may be compared with 2.3% average abundance in the predicted human proteome⁶). This has been hypothesized to be related to their structural stability,²⁶ and to enable intermolecular interactions.^{24,27} An alternative explanation for the high abundance of methionine is the concomitant increase in the molecular refractive index,⁶ which would permit lower total tissue protein concentrations at the same refractive power, and thereby significantly diminish both the osmotic pressure and the driving force for aggregation.⁷ Evidence for the systematic evolutionary replacement of amino acids with low refractive index increment by those with high refractive index increment has been found not only in γ crystallins, but in crystallins across different phyla among structurally unrelated proteins, including, for example, the glutathione-S-transferase derived S-crystallins of squid.⁶

Recently, Mahler et al. determined by NMR the first atomic solution structure of a γ M crystallin, zebrafish (*Danio rerio*) γ M7.²⁸ This has provided the opportunity for more detailed studies of structural stability of vertebrate γ crystallins, and allowed to

delineate an unfolding pathway.²⁸ This work did not support a correlation of the number of methionine residues with higher heat stability.²⁸ However, intriguingly, it was found that many of the methionine residues are at the surface, well-ordered and often stabilized by interactions with surface aromatic side chains, and it was hypothesized that they may play roles in intramolecular domain interactions and in intermolecular interactions.²⁸ In addition, they play a dual role in increasing the molecular refractive index increment, as they systematically replace residues of lower refractive index increment in mammalian γ crystallins.²⁸

The observation that methionine residues in γ M7 crystallin are largely at the protein surface²⁸ raises the question to what extent they impact protein hydration. Solvation of the protein surface provides a large energetic contribution for protein stability that partially has to be overcome for protein-protein contacts and aggregates to form, and is therefore an important contributor to the delicate balance of protein solubility.²⁹ In fact, some deleterious mutations of human γ crystallin leading to cataract have been shown to be due to changes in local solvation properties rather than protein structure.^{17,18,20} Furthermore, the overall amount of tightly bound water will have an impact on the maximal protein density that can be achieved, as well as the tissue refractive index. However, contrary to expectation, previous experimental studies directly measuring different γ crystallin hydration parameters have shown unusually high estimates on the order of 1 g water per gram of proteins or higher.^{30–32}

In this work, we have set out to resolve this apparent contradiction, and re-examined the hydration of members of the γ crystallin family. Specifically, we studied zebrafish γ M7 and γ M2b crystallin to complement the recent structural and unfolding studies.²⁸ As members of the group of most extreme γ crystallins, we expect they may represent the salient features of the high stability and solubility of this family, and shed additional light on the role of the high methionine content of γ M crystallin (13.3% γ M2b and 9.8% in γ M7) on hydration. For comparison, we also studied mouse γ S crystallin, a major γ -crystallin in the mammalian lens cortex, which has only average methionine content (2.3%), and human γ D, a major crystallin of the human lens nucleus. To this end, we have determined thermodynamic hydration parameters by contrast variation sedimentation equilibrium (SE) analytical ultracentrifugation experiments. Further, we have compared experimental translational frictional ratios with structure-based hydrodynamic predictions from the boundary element method BEST,³³ and applied a recently developed approach for the structure-based prediction of hydration in HyPred.³⁴ Finally, we studied the concentration-dependence of hydrodynamic interactions, which are

mediated by solvent interactions and report on weak interparticle interactions.

Results

Thermodynamic water binding coefficient

As a first measure of hydration, we carried out SE experiments to assess the thermodynamically bound water layer that cannot be penetrated by inert cosolvents. Following the strategy laid out by Ebel et al.,³⁵ and using sucrose as a probe, we conducted SE at a range of cosolvent concentrations, and determined for each condition the buoyant molar mass of the hydrated particle, from which in the invariant particular model the mass of the associated water can be deduced [Eq. (3)]. Just like the related SE method of Edelstein and Schachman,³⁶ the density contrast analysis requires high sample purity, as contaminants or products of reversible associations would provide different offsets in the signal profiles and consequently in the buoyant molecular weights at the different solvent densities, and therefore lead to bias in the mass and density estimate of the hydrated particle. We have established the purity of γ M7, γ M2b, and γ S preparations by sedimentation velocity (SV), but found the preparation of human γ D protein not to meet the very stringent requirements of this method.⁷⁴ Due to the extended time of the experiment, SE experiments were conducted at 10°C to minimize the impact of potential protein degradation. Figure 1 shows a representative set of equilibrium profiles of γ M7 at different rotor speeds

Figure 1. Representative SE data of γ M7 crystallin from a series conducted at different sucrose concentrations. Top: Absorbance equilibrium data in working buffer with 15% sucrose acquired at 250 nm acquired at rotor speeds of 12,000 rpm (purple), 18,000 rpm (blue), and 30,000 rpm (cyan). Symbols are experimental data, for clarity showing only every other data point, and the solid lines are best-fit distributions from a global fit of all data at different solvent densities in a single invariant particle model. Bottom: Residuals of the fit.

at a single sucrose concentration (of 15%), and Figure 2 shows the dependence of the best-fit buoyant molar mass as a function of sucrose concentration

Figure 2. Plot of the density increment measured in SE as a function of sucrose concentration for γ M7 (A), γ M2b (B), and γ S (C). Symbols are the experimental values determined separately at each buffer condition from the analysis of equilibrium profiles at multiple rotor speeds, and the line is the best-fit straight line of the invariant particle model [Eq. (3)]. For comparison, also shown are the theoretical relationships of the density increment for given partial-specific volume for no water binding ($B_1 = 0$, red dashed line), and for water binding at commonly expected levels ($B_1 = 0.3$, blue dotted line).

and solution density. From the slope and intercept values for the partial-specific volume and the hydration parameter, B_1 can be extracted. The partial-specific volume estimate determined in this way for γ M7 was 0.717 g/mL at the experimental temperature of 10°C. When transformed to standard conditions at 20°C to account for average thermal expansion of proteins,³⁷ a partial-specific volume of $\bar{v}^{(SE,20)} = 0.721$ g/mL was determined, very close to the value of 0.723 (0.713 – 0.735) g/mL determined previously by H₂O/H₂¹⁸O density contrast SV.⁷⁴ This lends credence to the hydration parameter B_1 , which from the same sucrose density contrast analysis [Fig. 2(A)] is calculated to be 0.115 g/g (68% confidence interval 0.109 – 0.122 g/g). Alternatively, from a global fit of all data at all densities with a single invariant particle model, a value of 0.16 (0.07 – 0.23) g/g was found for γ M7. For γ M2b, the analogous SE analysis resulted in slightly higher best-fit estimates for B_1 of 0.151 (0.148 – 0.155) g/g [Fig. 2(B)], and for mouse γ S a value of 0.220 (0.218 – 0.230) g/g was obtained [Fig. 2(C)].

Tightly bound water by hydrodynamics

Next, we aimed at characterizing the most tightly bound water layers thought to be relevant for hydrodynamic friction.³⁸ We have already experimentally determined the translational frictional ratios of diverse γ -crystallins in a parallel study focused on structural stability and density.⁷⁴ For example, for γ M7 we have measured a sedimentation coefficient of 2.403 S, corresponding to a frictional ratio of 1.17. The biggest contribution to the uncertainty of experimental s -values arises from systematic errors,³⁹ with a conservative estimate being 1%, or ± 0.024 S. This leads to a value for the Stokes radius of $R_S = 2.13$ (2.02 – 2.23) nm, with confidence intervals here reflecting joint uncertainties in the experimental estimates of s -value and errors in the partial-specific volume of 0.723 (0.713 – 0.735) g/mL from H₂O/H₂¹⁸O density contrast SV. With the recently reported solution structure of γ M7 from NMR²⁸ it is now possible to unravel the contributions of the detailed protein shape to the translational friction and assess hydrodynamic hydration. Figure 3 shows an example of a tessellated approximation of the shape of γ M7, drawn from an ensemble of 15 conformations, for each of which the boundary element method in BEST was used to predict precise hydrodynamic translational and rotational friction properties.³³ This calculation assumes by default a uniform hydration layer of 1.1 Å, which was found by Aragon and Hahn to describe well the translational and rotational diffusion properties of average proteins.³³ The ensemble average diffusion coefficient so predicted was 9.54×10^{-7} cm²/s, corresponding to a Stokes radius of $R_S^{(BEST)} = 2.25$ nm. With the known mass and density, this corresponds to a theoretical

Figure 3. Hydrodynamic model of γ M7 crystallin; example taken from an ensemble of 15 solution structures that were tessellated with 8230 triangles for precise hydrodynamic boundary element calculation in BEST. In conjunction with a series of tessellations, extrapolated to infinite number of triangles, this resulted in an isotropic translational diffusion coefficient of $(9.539 \pm 0.052) \times 10^{-7}$ cm²/s, corresponding to a Stokes radius of 2.25 nm.

frictional coefficient of $f/f_0^{(BEST)} = 1.24$, from which a theoretical sedimentation coefficient of $s_{20,w}^{(BEST)} = 2.263$ (2.165 – 2.344) S can be expected (with the uncertainty arising mainly from the propagated error of the partial-specific volume⁴⁰). This s -value is significantly lower than the experimental value of 2.403 ± 0.024 S, suggesting that the standard assumptions on hydration may not apply for γ M7. This is consistent with the lower than average thermodynamic water binding coefficient B_1 determined above for this protein. Phrased in terms of Stokes radii, the difference between the structure-based prediction and the experimental value would be consistent with an average hydration layer that is at least 0.2 Å thinner than the typical 1.1 Å.

Similar boundary element calculations in BEST were performed for the other crystallins, with the exception of γ M2b for which no structure is available. For mouse γ S the solution structure 1ZWM⁴¹ led to a theoretical frictional ratio, based on standard hydration assumptions, of 1.30. This value is distinctly higher than the experimental value of 1.21. For human γ D crystallin unfortunately, we found no solution structure with a precise match for the molecules used in SV experiments, as the NMR structure 2KLJ⁴² reports an additional N-terminal Q. Nevertheless, the boundary element hydrodynamic predictions in BEST with standard assumptions led to very similar frictional ratios as for the crystal structure 1HK0, with values of 1.22 and 1.21 for 1HK0 and 2KLJ, respectively. These values are fully in agreement with the experimentally determined value of 1.21, suggesting that for human γ D

crystallin the layer of tightly bound water has similar average thickness as for average proteins.

Predicted hydration density distribution

Because of the unique structural feature of surface methionine residues in γ M7 and its unusually low hydration both by hydrodynamic and by thermodynamic water binding coefficient, we took advantage of the solution structure of this γ M crystallin²⁸ to apply the recently developed HyPred approach to predict the detailed distribution of water molecules in the vicinity of the γ M7 crystallin surface.³⁴ HyPred is based on atom- and residue-dependent water distribution functions predetermined from all-atom explicit solvent molecular dynamic simulations of model proteins.³⁴ These local water distribution functions were shown to be protein-independent, and therefore can be used to assemble estimates of the detailed hydration shell surrounding any protein.^{34,43,44}

Figure 4 shows the surface of γ M7 crystallin (grey) and different depictions of the three-dimensional hydration density distribution: Panel A shows likely water locations (transparent blue) calculated as regions where the density exceeds a threshold,³⁴ and Panel B shows a cross-section of the distribution depicted as a color temperature plot, with dark blue areas having low hydration density and cyan to yellow areas having higher hydration density. In both Panels of Figure 4, methionine residues are highlighted in yellow. As might be expected from their nonpolar nature, it may be discerned from Figure 4 that methionine residues are generally surrounded by regions of lower hydration densities. HyPred reports the hydration density on a 0.5 Å rectangular grid and retains the information, which atom and residue type a particular volume element of the hydration shell is associated with, as well as its distance from that dominating atom. This permits the more detailed analysis of hydration proximal to certain residues. Integrating the hydration density distribution γ M7 crystallin for all volume elements associated with methionine residues at a distance between 15 Å and 50 Å, which approximately comprises the first 2 – 3 hydration layers, yields an average solvent density value of 0.924 g/mL, as opposed to the average value of 1.013 g/mL obtained over the same range in proximity to all other residues. Figure 5 shows the hydration density distribution collapsed into a profile of distance from the protein surface, and averaged over all volume elements with a given distance from the dominating atom. It can be discerned that the hydration density profile of γ M7 crystallin shows significantly lower hydration in the first two peaks of the distribution proximal to methionine residues (red line) as compared to all other residues (black line), in particular

Figure 4. Solution structure of γ M7 crystallin²⁸ and its hydration shell as predicted in HyPred.³⁴ A: The protein surface is displayed in gray, with methionine residues highlighted in yellow. Transparent cyan depict regions of the hydration shell surrounding the protein where the water density exceeds a threshold of 1.5 e/Å. B: γ M7 crystallin displayed as in (A), with a planar cross-section of the hydration density distribution. The hydration density is shown discretized on a rectangular grid with 0.5 Å mesh size and with the local density indicated by the color temperature (low values dark blue, higher values cyan to yellow).

in the range of the first hydration peak at distances below ~ 2.2 Å.

We also carried out HyPred analyses on mouse γ S crystallin and human γ D crystallin. The order of predicted total average water density between 15 and 50 Å is consistent with the experimental thermodynamic measures of bound water, with a value of 1.0028 g/mL for γ M7, 1.0155 g/mL for γ S, and 1.0344 g/mL for γ D. The crystallins studied here have linker peptides of different sequences and lengths. As a control, to address whether these could be the main origin of the differences in hydration, we have calculated the average predicted hydration density for the volume elements of the hydration shell between 15 and 50 Å for which the atoms of the linker are closest protein atoms. For γ M7 with the linker LHHGSF (residues 84–89) this resulted in a value of 0.9937 g/mL (in a region with a total volume of 1695 Å³); for γ S with the linker

Figure 5. Average radial hydration density distribution of γ M7 crystallin predicted by HyPred for pixels closest to methionine residues (red) or to all other residues (black).

LSSGGQA (residues 87–93) this resulted in a value of 1.0162 g/mL (in a total volume of 1811 Å³); and for γ D with the linker HSGSH (residues 84–88) this resulted in a value of 1.0334 g/mL (in a region with a total volume of 720 Å³).

Weak protein-protein and hydrodynamic interactions

Finally, we were interested in assessing the magnitude of hydrodynamic interactions and the presence of weak protein-protein interactions which can be mediated by protein-solvent interactions. Weak protein interactions were previously measured for mammalian crystallins¹²; we focused the present experiments on fish γ M crystallins. Weak interactions can be observed from the concentration dependence of sedimentation coefficients, because even transient interactions amount to changes in the macromolecular distance distribution and thereby modulate their long-range hydrodynamic interactions: if particles exhibit repulsive interactions, they will on average be less able to sediment in their mutual flow-field and experience a stronger concentration-dependent retardation of sedimentation (with the solvent back-flow in the closed sample volume constituting the leading term⁴⁵), and vice versa, attractive interactions will lead to a diminished concentration-dependent retardation or even an increase of SV with concentration. That the concentration-dependence of sedimentation can be an excellent method to detect weak interactions with K_d up to the low mM range was illustrated recently by Patel et al.⁴⁶

To this end we conducted SV experiments at a range of concentrations. The resulting relative decrease plot of the sedimentation coefficient as a function of concentration is shown as symbols in

Figure 6. Concentration dependence of the sedimentation coefficient as a measure of hydrodynamic and intermolecular interactions. Symbols show the experimental sedimentation coefficients relative to the extrapolated sedimentation coefficient at infinite dilution in working buffer without (black triangles) and with 150 mM NaCl (cyan circles), for γ M7 (A) and γ M2b (B) crystallin. For both, the theoretical prediction for noninteracting spheres is illustrated as straight dotted lines, corresponding to nonideality coefficients k_s of 0.007 mL/mg (γ M7) and 0.0078 mL/mg (γ M2), respectively.

Figure 6 for γ M7 (Panel A) and γ M2b crystallin (Panel B). We examined the concentration dependence of s -values both in the presence and absence of 150 mM NaCl (black and cyan symbols, respectively) to elucidate electrostatic contributions to the interparticle interactions. Also shown in Figure 6 is the theoretical reference of obligate hydrodynamic interactions of non-interacting spheres with stick boundary condition (thin straight line). The experimental data show a smaller concentration-dependence than predicted for noninteracting spheres, therefore indicating the presence of attractive interactions. If one would hypothesize the presence of a weak monomer-dimer association, then the sedimentation coefficient would be virtually concentration independent over a large concentration range if the equilibrium dissociation constant was $K_D = 100$ mg/mL, or in the low mM range. Thus, we can draw the following conclusions from Figure 6: With γ M7 crystallin in the absence of NaCl showing a slightly shallower slope, it is behaving closer to non-interacting particles, but with slightly attractive potential. In the presence of 150 mM NaCl the interactions become slightly stronger attractive. Similar is true for γ M2b, with apparently slightly stronger attractive interactions.

Discussion

In this work, we have examined various aspects of hydration of different members of the γ -crystallin family. We found the two zebrafish γ M crystallins to be very similar in that they exhibit unusually very low degrees of hydration by thermodynamic and hydrodynamic measures. Generally it is thought that average proteins typically bind about 0.3–0.4 g of water per gram of protein.^{47–49} However, probing the thermodynamic water binding coefficient of the γ crystallins examined in this work, by sucrose density contrast, for the γ M crystallins we arrive at values of only ~ 0.1 – 0.15 g/g. By contrast, the hydration of mouse γ S crystallin is also low, but with 0.22 g/g still in the range of ordinary soluble proteins, and equal to the hydration reported for aldolase using the same technique and same probe size.³⁵ Different from the thermodynamic bound water, hydrodynamic friction reports on tightly bound water. Usually it is thought of as the first water layer that is exposed to drag through molecular rugosity of the protein surface, manifesting stick condition through attractive polar and ionic interactions, with effective viscosity gradients towards the bulk water.³⁸ After accounting for the detailed macromolecular shape, here, too, lower values than usual are suggested by the experimental frictional coefficients in comparison with theoretical boundary element-based predictions in BEST. This was observed for both γ M and γ S crystallins with available solution structures determined by NMR. The difference was slightly larger for γ S than γ M7 crystallin, but it seems possible for γ S that slightly less extended conformations of the N-terminal arm than determined in the structure under different experimental conditions could potentially also contribute to a lower frictional ratio in the hydrodynamic experiments. Interestingly, although the thermodynamic experiments were not possible with human γ D crystallin, its hydrodynamic properties were fully consistent with hydration comparable to that of average proteins. A third view of hydration was adopted in the structure-based computational prediction of the water distribution in HyPred, which resulted overall in the same order of average density of hydration γ M < γ S < γ D, both for the entire molecules as well as for the connecting peptide between the two Greek key domains.

Our experimental results seem to be at odds with older literature reports of unusually high values of 0.9 g/g for bovine γ crystallin by SANS³² and higher values from measuring “nonfreezable” water.^{31,32} We can only speculate about the origin of the apparent discrepancy; among possible explanations could be protein conformational changes after freeze drying, isotope effects of deuterium on the hydration shell,^{50,51} or possible protein aggregation responsible for the concentration-dependence of values in Refs. 31 and 32. An only moderate hydration

of 0.27 g/g was estimated for bovine γ crystallin from a previous hydrodynamic study by Chiou et al.⁵²; however, this value was based on ellipsoidal shape models, which, as discussed by Aragon and Hahn,³³ can provide large over-estimates of the degree of hydration in hydrodynamics.

Our results of unusually low hydration of the γ M and γ S crystallins are in line with the biological expectation. Low hydration of γ crystallin was hypothesized on the basis of the inverse correlation of the lens tissue hydration with the γ crystallin content,²³ the solvent exposure of some hydrophobic side chains,⁵ and chiefly an unusually high number of ion pairs at the surface of mammalian γ crystallin,^{5,23,53} arranged in an extended network of alternately charged groups, which would diminish the propensity for strongly bound water.^{5,54} Especially, fish γ M crystallins have adapted to an extreme environment, mandated by the high refractive power of the lens needed for visual acuity under water. The reported protein concentrations in fish lenses of 700 mg/mL and even in excess of 1000 mg/mL^{4,9,10} are likely only approximations, usually estimated indirectly from the tissue water content or the lens refractive index (knowing that the major dry weight component is crystallin). Even though we do not yet have a clear understanding of the physical environment under these conditions, it is worthwhile to visualize in a back-of-the-envelope calculation how crowded such solutions of fish γ M crystallins inevitably must be. The hexagonal packing limit of spheres covers a volume fraction of 0.74, which, assuming our measured partial-specific volume of γ M crystallin, would accommodate 1030 mg/mL of protein. A lower concentration of 750 mg/mL corresponding to $\sim 54\%$ volume occupancy would be achieved with spheres in hexagonal packing with interparticle distances $\sim 10\%$ larger than in the close-packed hexagonal arrangement. Even though crystallin molecules are known not to exist in a state with long-range order,^{13,15} we can still derive from this simplified picture an estimate of average inter-protein distances: with Stokes radii of 2.17 nm of γ M7 crystallin, on average they would be only ~ 4 Å apart, barely accommodating two water layers.

From this perspective, it appears that tightly bound water, which when considered for a protein in dilute solution should increase the local density and protein refractive index increment, may be detrimental to the formation of highly concentrated solutions with high solution refractive index. Even though we believe exchange of less polarizable for stronger polarizable amino acids within the polypeptide chain is universally favored to increase the lenticular refractive index,^{6,7} with regard to the hydration the cost of additional volume occupancy of bound water and its impact on structural and colloidal stability may change the balance in a more complex manner.

A rather mysterious feature of γ M crystallins is their extremely high methionine content.^{24–26} We have previously proposed that their abundance serves to increase the molecular refractive index increment, permitting a lower total protein concentration and ultimately resulting in a significant reduction of protein aggregation propensity.^{6,7} Intriguingly the NMR structure of γ M7 crystallins shows 14 of the 16 methionine residues to be located at the protein surface.²⁸ While this could simply reflect fewer constraints at the surface as compared with the interior of the tightly packed protein to accommodate these residues without impairing structural stability, they might also serve additional functions at the surface to modulate hydration and interparticle interactions, which will be very sensitive to surface topography as well as electrostatics of surface residues.⁵⁵ In particular, from the HyPred predictions of the hydration shell structure it seems that the surface methionine residues show strongly reduced average water density almost across the entire range of what we estimate to be the average interprotein distance (Fig. 5). It is conceivable that this not only would reduce the overall hydration, but also energetically facilitate achieving these high concentrations. Further modulation of the hydration may arise from the fact that the majority of the surface methionine residues are in close proximity to at least one aromatic residue.²⁸ However, the effect of nonpolar residues in opposing surfaces with small separations may be significantly different from hydration typically considered at a single interface between the protein and a bulk,⁵⁶ and potentially, small molecule co-solvents partitioning in the opposing interfaces may play additional roles.

Interestingly, the depletion of hydration in the present study in the order of γ M with least hydration, γ S with low, and γ D with apparently normal hydration (as far as we could measure) mirrors the order of thermal stability of these molecules, with γ M crystallins exhibiting the lowest stability, γ S intermediate, and γ D by far the highest stability in both CD²⁸ and turbidity assays.⁷⁴ This is not unexpected considering that hydration provides a significant thermodynamic contribution to the stability of protein folding.²⁹ However, if the lower hydration of γ M crystallins can afford a higher protein concentration, then additional contributions to protein stability from increased crowding⁵⁷ at the very extreme conditions of fish lenses may provide compensatory stabilizing contributions. Vice versa, the observation that the hydrodynamic behavior of γ D is consistent with normal hydration may indicate that it faces higher intrinsic stability requirements, due to the long lifespan of the organism and its distinctly less crowded (though still highly concentrated) environment. A more moderate hydration of

γ D despite a low tissue water content would not necessarily be a contradiction, as other factors such as active water and ion transport⁵⁸ will contribute to the tissue properties. We also cannot rule out the possibility that human γ D has undergone specific subtle modifications that actually increase hydration as part of an evolutionary program to “soften” the nucleus of the primate lens. This program probably includes the elimination of some γ -crystallins and the recruitment of an enzyme, BHMT, as a crystallin expressed in the fetal primate lens.⁵⁹

Finally, we considered weak protein interactions as they are mediated by solvation and the obligate hydrodynamic interactions of proteins, which have been shown to dominate macromolecular motion in the crowded intracellular milieu.⁶⁰ Consistent with previous results from small angle x-ray scattering and osmometry by Tardieu and colleagues on bovine γ -crystallin,¹² and the analysis of liquid-liquid phase separation of bovine γ S-crystallin by Benedek and coworkers,⁶¹ we observed weakly attractive interactions of both γ M-crystallins superimposed on the hydrodynamic interactions. Weakly attractive interactions between different protein species contribute significantly to liquid-liquid phase transitions and to lens transparency.^{22,61–63} We found the weakly attractive interaction to be slightly enhanced at higher ionic strength, consistent with a role of electrostatic contributions to the interparticle potential. Such an ionic strength dependence was not observed previously for bovine γ -crystallin,¹² which may reflect a different role of protein surface charges in the interparticle potentials of the two γ -crystallin families. The high-resolution crystal structure of human γ D-crystallin in comparison with that of the cataract-forming R58H¹² offers an example for the balance between ion-pair mediated protein contacts, tightly bound water, and solubility,¹² if one considers these local contacts as a snapshot of possible mutual interactions that may occur transiently in disordered solution.

In the cytosol of fiber cells, at average protein distances corresponding to as little as two hydration layers, one could expect a profound and tight interrelation between protein-solvent interactions, the structure of the solvent itself and its mobility and fluctuations,⁶⁴ protein stability, and protein-protein interactions. Although likely additional interactions will be at play in solutions with > 50% protein volume occupancy than those examined in this work, we expect that the salient effects of γ -crystallin hydration identified in theory and experiment for dilute solution here will still be at least important factors under more crowded conditions. Although challenging, principles of hydration in crowded multicomponent solutions may likely be crucial for understanding the biophysics of cytosolic environment in general.

Materials and Methods

Protein expression and purification

All crystallin molecules were expressed in *Escherichia coli* BL21(DE3)pLysS as described in detail elsewhere.^{28,65,74} Purification was achieved through anion exchange chromatography followed by size exclusion chromatography, both using the AKTA Explorer 100 (Amersham Pharmacia Biotech). The final working buffer was 50 mM Tris, pH 8.5, 1 mM dithiothreitol, and 1 mM EDTA. Purity and solubility were assessed as described in the companion paper.⁷⁴

Sedimentation equilibrium

SE experiments were also conducted in a ProteomeLab XLA/I analytical ultracentrifuge (Beckman Coulter, Indianapolis) following standard protocols.⁶⁶ For the determination of the protein partial-specific volume and hydration, crystallin was dissolved to a final concentration of 0.4 mg/mL (γ M7) or 0.1 – 0.3 mg/mL (γ S) in working buffer containing sucrose at concentrations of 0, 7.5, 15, 22.5, and 35%; 160 μ L samples were brought to SE at 10°C sequentially at rotor speeds of 12,000, 18,000, and 30,000 rpm. Absorbance data were acquired at wavelengths of 280 and 250 nm in time intervals of 6 h. Attainment of equilibrium was tested by verifying the asymptotic decrease of differences of scans using SEDFIT. SE data were analyzed using the software SEDPHAT, using the single-species model,

$$a(r) = a_0(r_0, \omega) \exp \left(\frac{\omega^2}{2RT} M \left(\frac{\partial \rho}{\partial c} \right)_\mu (r^2 - r_0^2) \right) \quad (1)$$

where $a(r)$ are the experimental radial signal profiles, with R denoting the gas constant and T the absolute temperature, M the protein molar mass, $(d\rho/dc)_\mu$ the protein density increment at constant chemical potential, and r_0 a reference radius. This model was fit globally to many sedimentation profiles acquired at multiple rotor speeds and at different detection wavelengths. The pre-exponential factor $a_0(r_0, \omega)$ was constrained for each cell by implicit mass conservation of dissolved protein in the entire solution column, treating the bottom of the solution column as an adjustable parameter in the nonlinear regression.⁶⁷ All plots of analytical ultracentrifugation concentration profiles were made with the software GUSSI (<http://biophysics.swmed.edu/MBR/software.html>).

Analysis of water binding coefficient B_1

Given the known sequence molar mass of the protein, the buoyant molar mass $M_b = M (d\rho/dc)_\mu$ and the density increment of the protein component (2) can be determined from SE profiles as described above. Our analysis of water binding follows the strategy of

Ebel et al.³⁵ who have illustrated how the density increment is related with the preferential binding parameters B_1 and B_3 of gram water or cosolvent, respectively, bound per gram of protein via,

$$(d\rho/dc_2)_\mu = (1 - \rho^0 \bar{v}_2^0) + B_1(1 - \rho^0 \bar{v}_1) + B_3(1 - \rho^0 \bar{v}_3) \quad (2)$$

where ρ^0 denotes the buffer density, and $\bar{v}_2^0$, $\bar{v}_1$, and $\bar{v}_3$ denote the protein, solvent, and cosolvent partial-specific volumes,³⁵ or

$$(d\rho/dc_2)_\mu = (1 + B_1 + B_3) - \rho^0(\bar{v}_2^0 + B_1 \bar{v}_1 + B_3 \bar{v}_3). \quad (3)$$

Thus, in the absence of sucrose binding to the protein ($B_3 = 0$), the slope and intercept of the experimental dependence of the density increment, or similarly buoyant molar mass, on the solvent density, can be used to determine the protein partial-specific volume and the hydration parameter B_1 .³⁵ Alternatively, the raw SE data profiles can be globally fitted with a single invariant particle model with both total molar mass $M(1+B_1)$ and total partial-specific volume $(\bar{v}_2^0 + B_1 \bar{v}_1)/(1+B_1)$ of the complete sedimenting particle inclusive hydration shell as parameters refined in the global fit.

Sedimentation velocity

SV analytical ultracentrifugation (SV) was carried out in a ProteomeLab XLA/I analytical ultracentrifuge (Beckman Coulter, Indianapolis) following standard protocols^{68,69}; 400 μ L samples of crystallin in working buffer were filled in 12 mm charcoal-filled Epon double-sector centerpieces, or 100 μ L samples in 3 mm centerpieces, respectively, and matched with an equal volume of buffer in the reference sector. Cell assemblies were inserted in an 8-hole rotor and temperature equilibrated at rest in the rotor chamber at 20°C for a period of 2 h prior to acceleration to a rotor speed of 50,000 rpm. Sedimentation data were collected at the highest possible rate with both the absorbance optical system set to a wavelength of 280 nm and the interference optical system, and scan time corrections were calculated on the basis of the computer operating system time stamps.⁷⁰ Solution density and viscosity were experimentally determined with a DMA 5000M density meter and an AMVn automated microviscometer, respectively (both from Anton Paar, Graz, Austria). For the determination of sedimentation coefficients, data were analyzed with the software SEDFIT using the $c(s)$ method.⁷¹

Analysis of hydrodynamic interactions

Repulsive hydrodynamic interactions lead to a decrease of sedimentation coefficients with increasing concentration. As shown by Batchelor using

methods of statistical hydrodynamics, for dilute suspensions of noninteracting equal sized spheres, the relative reduction of sedimentation coefficients amounts to -6.55Φ , where Φ is the occupied volume fraction⁴⁵:

$$s = s^0(1 - k_S w) = s^0(1 - 6.55\Phi) \quad (4)$$

with s^0 denoting the sedimentation coefficient in infinite dilution, w the weight concentration of the protein. For the equivalent spheres of a protein with a given frictional ratio and partial-specific volume, we take the theoretical concentration-dependence as

$$k_S = 6.55 \times \bar{v} \left\{ 0.2 + 0.8(f/f_0)^3 \right\}, \quad (5)$$

accounting for the fact that $\sim 80\%$ of the dominant backflow contributions to the concentration dependence arises from the effective volume of the fluid drag of the sedimenting particle.

Theoretical prediction of the translational friction coefficient

Hydrodynamic calculations were based on an ensemble of solution structures determined by NMR.²⁸ For each, a theoretical diffusion coefficient was calculated with the boundary element method in the software BEST.^{33,72} Briefly, this involves triangulation of the molecular surface with the program MSROLL⁷³ (kindly provided by M.L. Connolly), with all atoms being uniformly inflated by an effective radius of hydration of $1.1 \text{ \AA}$,³³ as previously determined by Aragon and Hahn from the analysis of model proteins.³³ This is followed by subtriangulation at different number N of triangles describing the surface using COALESCE (kindly provided by S. Aragon), with N chosen to be ~ 3000 , 5000 , 7000 , and 8200 . For each, the accurate solution of the Stokes equations for these tessellated structures was determined with BEST (kindly provided by S. Aragon), and calculated translational diffusion coefficients extrapolated to infinite N .

Prediction of protein hydration

Structure-based theoretical estimates of the hydration shell density distribution were obtained using the HyPred method developed by Virtanen *et al.*,³⁴ as implemented in the webserver at <http://godzilla.uchicago.edu/pages/jouko/hypred.html>. Briefly, it is based on proximal radial distribution functions (pRDFs) describing the hydration water density for each atom in the context of different amino acids. The pRDFs were calculated initially on the basis of explicit solvent molecular dynamics calculations of model proteins.³⁴ Such pRDFs were shown to be universal,^{43,44} and thus enable one to estimate the

entire water distribution around any given protein on the basis of atomic coordinates by recombination of elemental pRDFs.^{34,44} The water density distribution from HyPred was interpolated in MATLAB (Mathworks, Natick, MA) to show a specific planar cross-section and plotted in PyMOL (version 0.99rc6). A custom-written MATLAB script was used to identify hydration volume elements proximal to certain residues.

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