X-Ray- and Neutron-Scattering Studies of α-Crystallin and Evidence That the Target Protein Sits in the Fenestrations of the α-Crystallin Shell

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Purpose. α-Crystallin, a ubiquitous molecular chaperone, is found in high concentrations in the lens. Its structure and precise mechanism of action, however, are unknown. The purpose of these experiments was to further the understanding of the chaperone function of α-crystallin.

Methods. X-ray- and neutron-solution–scattering studies were used to measure the radius of gyration of bovine lens α-crystallin when complexed with its target protein β-crystallin in both normal and heavy-water–based solutions. Spectrophotometry was used as a chaperone assay.

Results. The radius of gyration of α-crystallin on its own and when mixed with β-crystallin was 69 ± 1 Å at 35°C and increased with the temperature. In contrast to H_2O-buffered solutions, the radius of gyration did not increase significantly in D_2O-buffered solutions up to 55°C, and at 70°C was, on average, some 15 to 20 Å smaller.

Conclusions. Bovine lens α-crystallin in solution can be modeled as a fenestrated spherical shell of diameter 169 Å. At physiological temperatures, a weak interaction between α- and β-crystallin occurs, and β-crystallin is located in the fenestrations. Deuterium substitution indicates that the superaggregation process is controlled by hydrogen bonding. However, the chaperone process and superaggregation appear not to be linked. (*Invest Ophthalmol Vis Sci.* 2007;48:2695–2700) DOI: 10.1167/iovs.06-0559

The primary protein component of the mammalian lens is α-crystallin, which can approach 50% of the total dry weight of the lens.[1] Its main role is as a molecular chaperone, and it protects its target proteins, β- and γ-crystallin, from unfolding in response to environmental stresses. α-Crystallin has two isoforms, α-A and α-B, each of which has an approximate mass of 20 kDa, and is expressed independently in most of the tissues of the body, with α-B-crystallin by far the more prevalent.[2,3] It seems that it is only in the lens that the two isoforms are coexpressed, and in this tissue the two coaggregate into a heterogeneous population with an average diameter of ~15 nm and an overall mass of ~700 kDa.[4] This heterogeneity arises from the unusual solution properties of the α-crystallin subunit, so that the aggregation process is driven both by ionic and hydrophobic interactions. The resultant number of subunits in each assemblage can vary considerably. α-Crystallin also contributes to the maintenance of short-range order in the lens cytoplasm, helping to achieve the refraction of light and lens transparency in the visible spectrum.[5]

α-Crystallin has a key role as a molecular chaperone, protecting target proteins against reduction-induced precipitation,[6] heat-induced aggregation,[7] and enzyme inactivation.[8] It is involved in several pathologic situations, especially ischemic heart,[9] neurologic disorders,[10] and protein misfolding diseases. The structure of α-crystallin, however, its relationship with its target protein, and the mechanism of action all remain unknown.

Previously, we studied the low-angle x-ray diffraction patterns from gels of α-crystallin and from intact lens as a function of temperature.[11] In solutions, in gels and in the intact lens the α-crystallin aggregate underwent extensive structural changes and became much larger in response to increasing temperature, with a major transition at ~50°C. A moderate increase in the spacing and intensity of the dominant x-ray reflection was observed in the temperature range of 20°C to 45°C, followed by an accelerated increase from 45°C to 70°C. We used the term superaggregation to describe the process of the enlargement of the α-crystallin aggregates with increasing temperature. These results confirmed earlier electron microscopic, circular di, and nondenaturing gel observations of a temperature transition at ~50°C at low concentrations of α-crystallin in vitro.[12] This transition was now seen to occur also at physiological concentrations and in situ. Although the previous x-ray diffraction results were valid for both low and high concentrations of α-crystallin, investigating structural phenomena of this protein at almost physiological concentrations (as performed on α-crystallin gels) is clearly important and may resolve outstanding problems concerning the operation of the system. Our previous study made it clear that α-crystallin function in the lens is closely associated with a highly dynamic particle structure. In the present study, we used x-ray- and neutron-solution–scattering techniques to study the radius of gyration (Rg) of α-crystallin in solutions of either water or deuterium oxide through the temperature range 20°C to 70°C and to obtain data for its modeling as a fenestrated chaperone.

Methods

All proteins used in the study were produced at the Nuffield Laboratory of Ophthalmology, Oxford University, as described by Derham and Harding,[13] and are wild-type proteins with all isoforms present. Low-angle x-ray and neutron-scattering experiments were conducted at Station 2.1 at the Daresbury SRS (Synchrotron Radiation Source) and beam-line D11 at the ILL (Institut Laue-Langevin) research reactor (Grenoble, France), respectively. We used a 2-mg mL^{-1} protein concentration in solutions containing 100 mM NaCl and 0.02% (wt/vol) NaN_3 buffered with 50 mM imidazole at pH 7.5. These solutions were

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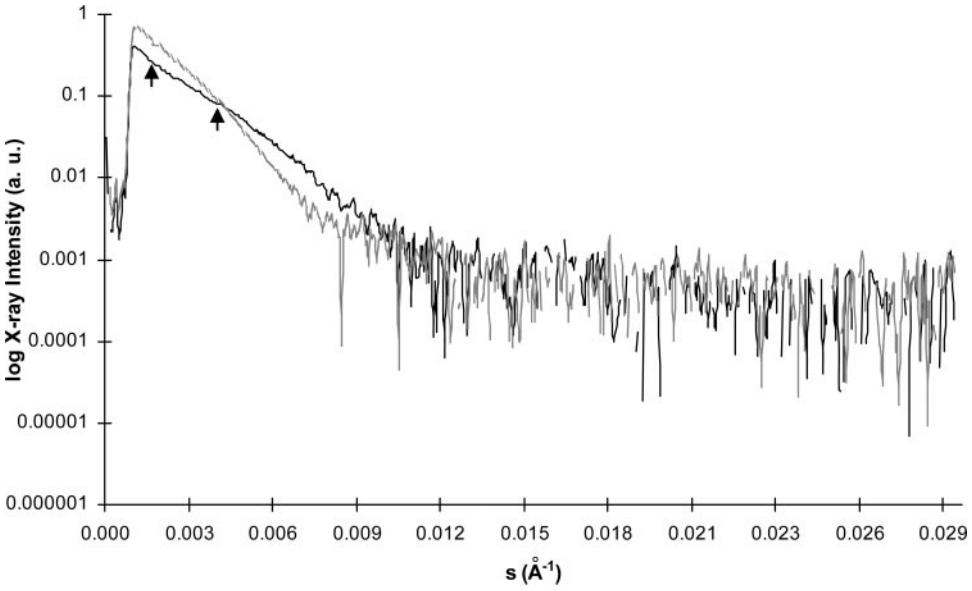

FIGURE 1. X-ray-scattering intensity profiles of α-crystallin at 35°C (*black line*) and 65°C (*grey line*). *Arrows*: limits of the Guinier region for the 35°C intensity profile.

also made with heavy water (deuterium oxide; D_2O) rather than with normal water. For deuterium solutions, pD = pH +0.4, in all experiments, Guinier analysis was used to determine the average Rg of the protein aggregates as a function of temperature, in accordance with our earlier work.[11] Rg is derived from the Guinier region of the solution-scattering x-ray and neutron intensity profiles. Typical small-angle x-ray-solution–scattering profiles of α-crystallin at two different temperatures, 35°C and 65°C, are shown in Figure 1. The increase in intensity with increased temperature is due to the superaggregation process, discussed later. The two arrows indicate the limits of the Guinier region at 35°C in inverted space (S): 2×10^{-3} to 4×10^{-3} Å^{-1} in this case. Such regions were then used to fit the Guinier approximation equation[14]

$$I = I_0 \exp(-4\pi^2 S^2 Rg^2/3),$$

where I is the scattered intensity and I_0 the forward scattering intensity. Rg is the root mean square distance of the electrons of the molecules in solution from the centers of their electronic masses and, therefore, is a measure of the overall size of the molecules. Exposure times were 1 and 5 minutes for the x-ray and neutron experiments, respectively.

For the optical density experiments, similar solutions of 0.1 mg mL^{-1} α-crystallin, 0.25 mg mL^{-1} β-crystallin, or both combined, dissolved in buffered normal or heavy water were placed in 1-mL cuvettes preheated to 55°C in a spectrophotometer (model 930; Kontron America, Poway, CA) recording at 360 nm.[15] The solutions were left for 3 minutes to equilibrate at 55°C, determined with a thermocouple thermometer (Comark, Beaverton, OR).

RESULTS

Neutron- and x-ray–scattering experiments on α-crystallin in solution at 2 mg mL^{-1} indicate that Rg is 69 ± 1 Å at 35°C (Figs. 2A, 2B). In normal water, this Rg is essentially unchanged from 20°C to 50°C, until superaggregation occurs above 50°C.[11] In deuterium-based solutions, however, Rg shows no sign of increase between 40°C and 55°C (Table 1). At 70°C, Rg in heavy water is, on average, some 15 to 20 Å smaller than Rg in hydrogen-based solutions, indicating an absence of superaggregation.

To investigate the chaperone activity of α-crystallin in solution, we used β-crystallin, a protein that it protects in the lens, as the target protein. As demonstrated by Horwitz,[7] optical density measurements of β-crystallin in an aqueous solution at 2 mg mL^{-1} indicate that it denatures and precipitates at 55°C. Above this temperature, too, x-ray-scattering intensity was reduced, because most of the β-crystallin came out of solution (data not shown). This occurred even though one of its isoforms, βB2-crystallin, is known to unfold at high temperatures, but remains in solution, giving very high Rg (above 55°C; Fig. 3). Optical density measurements of α+β solutions revealed that partial unfolding and precipitation of β-crystallin is prevented by the chaperone activity of α-crystallin in both normal and heavy water solutions (Figs. 4A, 4B).

Measurements of Rg in the temperature range 20°C to 70°C, for β-crystallin alone (2 mg mL^{-1}), for α-crystallin alone (2 mg mL^{-1}), and for a mixture of 2 mg mL^{-1} α-crystallin plus 2 mg mL^{-1} β-crystallin (Table 2), revealed that Rg for β-crystallin is smaller than Rg for α-crystallin by approximately 20 Å (Fig. 4). Rg for proteins in solution is an average value for all molecules in the solution; thus, if α-and β-crystallins acted independently, a reduced Rg would be expected from α+β-crystallin in solution compared with α-crystallin alone, as the β-crystallin aggregate is smaller (40–200 kDa) than the α-crystallin aggregate (700 kDa). That this was manifestly not the case between 20°C and 35°C, indicates that α- and β-crystallin interact in this temperature range. Above 35°C, the Rg of α-crystallin alone and of α+β-crystallin diverged dynamically, but was almost identical (correlation coefficient, $R = 0.995$) if the absolute temperature responses of the α+β aggregates were increased by 1.9%.

DISCUSSION

α-Crystallin is a member of the small heat-shock protein (HSP) family.[13] It has not been crystallized, but crystallographic structures of two HSPs have been identified, and there are similarities.[16,17] The basic unit in both HSP-16.5 and -16.9 is a dimer of the C-terminal (hydrophilic) domains, common to all members of the small HSP family. Each dimer then interacts with two further dimers to form a tertiary structure building block—planar and with three-fold symmetry. Quaternary structures of higher symmetry arise from these building blocks, governed by the packing of the dissimilar N-terminal regions and C-terminal extensions. In HSP-16.5 this structure is roughly spherical, but has eight triangular and six square windows that give access to a central cavity.[15] In wheat HSP-16.9 two similar planar six-molecule building blocks are related by a rotated mirror plane, giving a quaternary structure that is a pair of apposed discs,

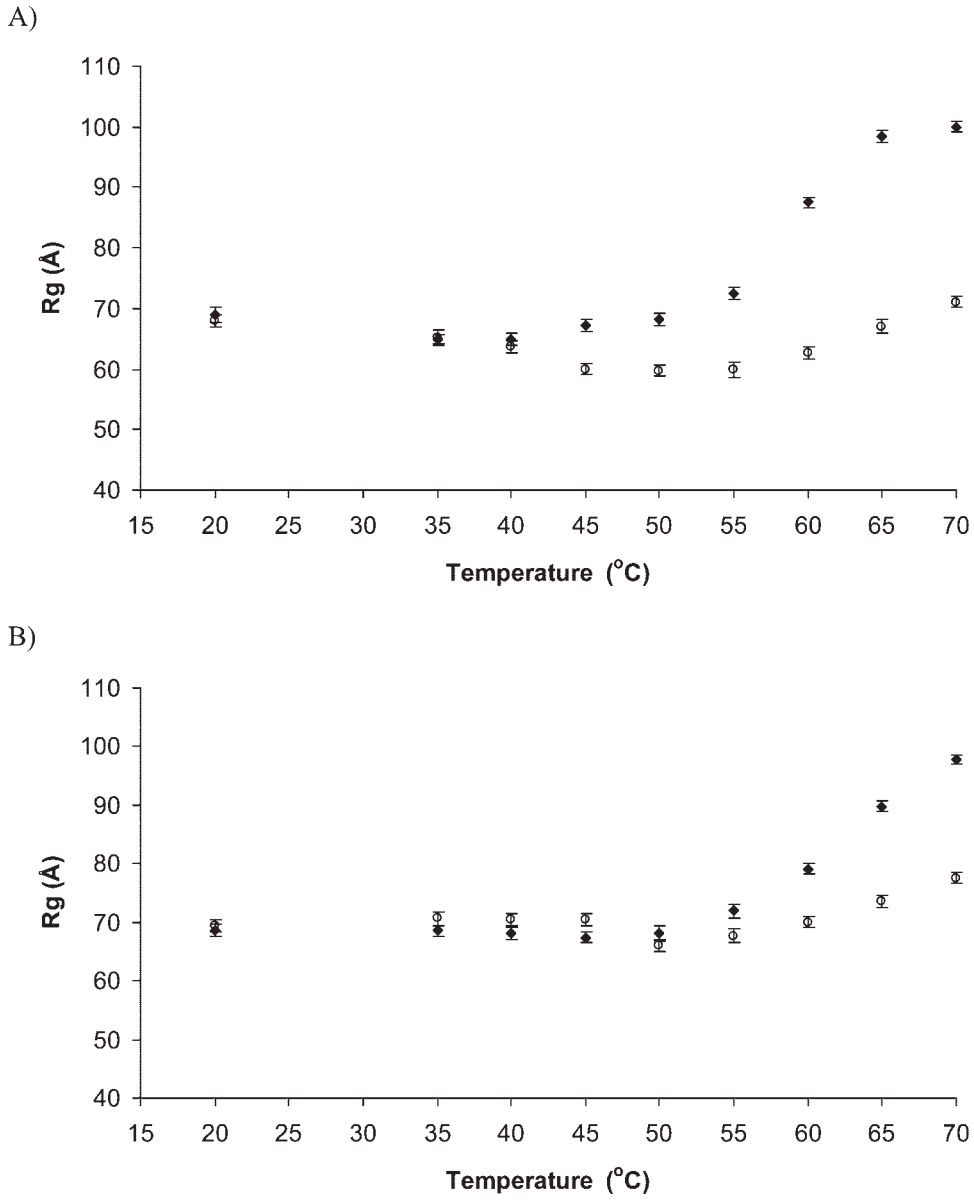

FIGURE 2. (**A**) α-Crystallin aggregate Rg in buffered H_2O (♦) and D_2O (○) solutions as a function of temperature, observed by neutron scattering. (**B**) α-Crystallin aggregate Rg in buffered H_2O (♦) and D_2O (○) solutions as a function of temperature observed by x-ray scattering. Error bars: the SD from the line of best fit in the Guinier region.

with one three-fold and three two-fold axes.[17] Again, there is a central cavity, accessible from windows on either side of the structure along the three-fold axis.

A similar structure for α-crystallin at physiological temperatures may be inferred from our data by the following reasoning. The specific volume occupancies of globular proteins are remarkably similar and lie in the range of 0.69 to 0.74 mL g^{-1}.[18] Taking an average value of 0.72 mL g^{-1} for the α-crystallin aggregate, together with its molecular mass of approximately 700 kDa gives an estimated volume of 0.83 × 106 Å,^{3} with an uncertainty of approximately 20%. A solid sphere with this volume would have a radius of approximately 58 Å.

The relationship between Rg and r, the geometric radius, of any body is given by $r = k' \times \text{Rg}$, where k' is a constant with a numerical value that depends on the distribution of mass within the body. If α-crystallin were a solid sphere of radius 58 Å, k' would equal 1.581. Therefore, the Rg of the sphere would be approximately 37 Å. This is clearly incompatible with our experimental value of 69 ± 1; thus, the packed spherical volume hypothesis is excluded by the data.

If we consider a thin solid spherical shell hypothesis for α-crystallin in solution, though, k' has a value of 1.225. From our observed Rg, the Rg of such a shell would then be ~84.5 Å (diameter, 169 Å). The surface area of such a spherical shell would be approximately 9×10^4 Å^2 giving room to accommodate a protein volume of 0.83×10^6 Å^3 in a layer approximately 10-Å thick.

Support for the shell model for α-crystallin comes from cryo-electron microscopy and three-dimensional image reconstruction studies of recombinant human αB-crystallin that suggest some sort of hollow sphere of diameter (for a 39-subunit aggregate) of 175 ± 20 Å.[19] This compares well with a fenestrated α-crystallin sphere of 36 subunits (based on solved HSP structures), with a shell thickness of 9.8 Å and a diameter of 169 ± 2.6 Å. Carver et al.[20] have used similar arguments to show that α-crystallin aggregates must contain "space," though the model that they propose is barrel-shaped rather than spherical.

Figure 1 shows that in the experimental region beyond $S = 9 \times 10^{-3}$ Å^{-1}, the x-ray data become noisy and indistinct. This is the region where information may be sought from the particle Fourier transform, in monodisperse systems, and a shape reconstruction performed. One obvious cause of the lack of information in the data is that Fourier transforms from different-sized particles are overlaid and smear out the data. However, a recent x-ray-solution–scattering study by Spinozzi

TABLE 1. Rg of α-Crystallin in Buffered H_2O and D_2O Solutions

Method/Temp (°C)	H_2O ± SD (Å)	D_2O ± SD (Å)
Neutron scattering		
20	69.0 ± 1.12	68.0 ± 1.2
35	64.9 ± 1.09	65.3 ± 0.87
40	64.9 ± 1.14	63.7 ± 1.02
45	67.3 ± 0.98	60.0 ± 0.99
50	68.2 ± 1.02	59.7 ± 1.12
55	72.5 ± 1.13	59.9 ± 0.95
60	87.5 ± 0.95	62.7 ± 0.93
65	98.5 ± 0.92	67.0 ± 1.0
70	100.0 ± 0.84	71.0 ± 0.87
X-ray scattering		
20	68.7 ± 1.01	69.4 ± 0.99
35	68.5 ± 1.02	70.6 ± 1.13
40	68.1 ± 1.10	70.5 ± 1.09
45	67.4 ± 0.93	70.4 ± 0.96
50	68.2 ± 1.09	65.9 ± 0.91
55	71.9 ± 1.17	67.7 ± 1.20
60	79.1 ± 0.96	70.0 ± 0.94
65	89.8 ± 0.90	73.5 ± 1.10
70	97.8 ± 0.86	77.6 ± 0.89

et al.[21] has used data from αB-crystallin, which is also polydisperse, to generate theoretical scattering curves in the noisy and indistinct region at different temperatures, based on the known structure of HSP16.9. The authors then created a general aggregation shape reconstruction model for all their experimental conditions. The resultant particle shape reconstruction resembles a distorted hollow sphere with a central cavity. Although the shape reconstruction is described by the authors as a snapshot of a polydisperse sphere, it complements the cryoelectron microscopy studies just mentioned[19] and furthers our understanding of the possible underlying structure of α-crystallin.

We have shown that the Rg of (α+β)-crystallin below 35°C is 69 ± 1 Å, the same as that of α-crystallin alone (Fig. 3). This result implies that, under nonstress conditions, β-crystallin is not independent of α-crystallin in solution, but weakly interacts with α-crystallin, a contention that is backed up by nuclear magnetic resonance (NMR) studies.[22] From simple mechanics, if β-crystallin were located on the outside of the α-crystallin shell and if it were, in total, approximately the same molecular weight and density as the α-crystallin, we would expect to see an approximate increase in Rg of ~4 Å. Alternatively, if it were on the inside of the shell, we would expect to see an approximate 4Å decrease in the Rg of the α+β aggregate. Thus, our data point to a model with the targeted β-crystallin coplanar in

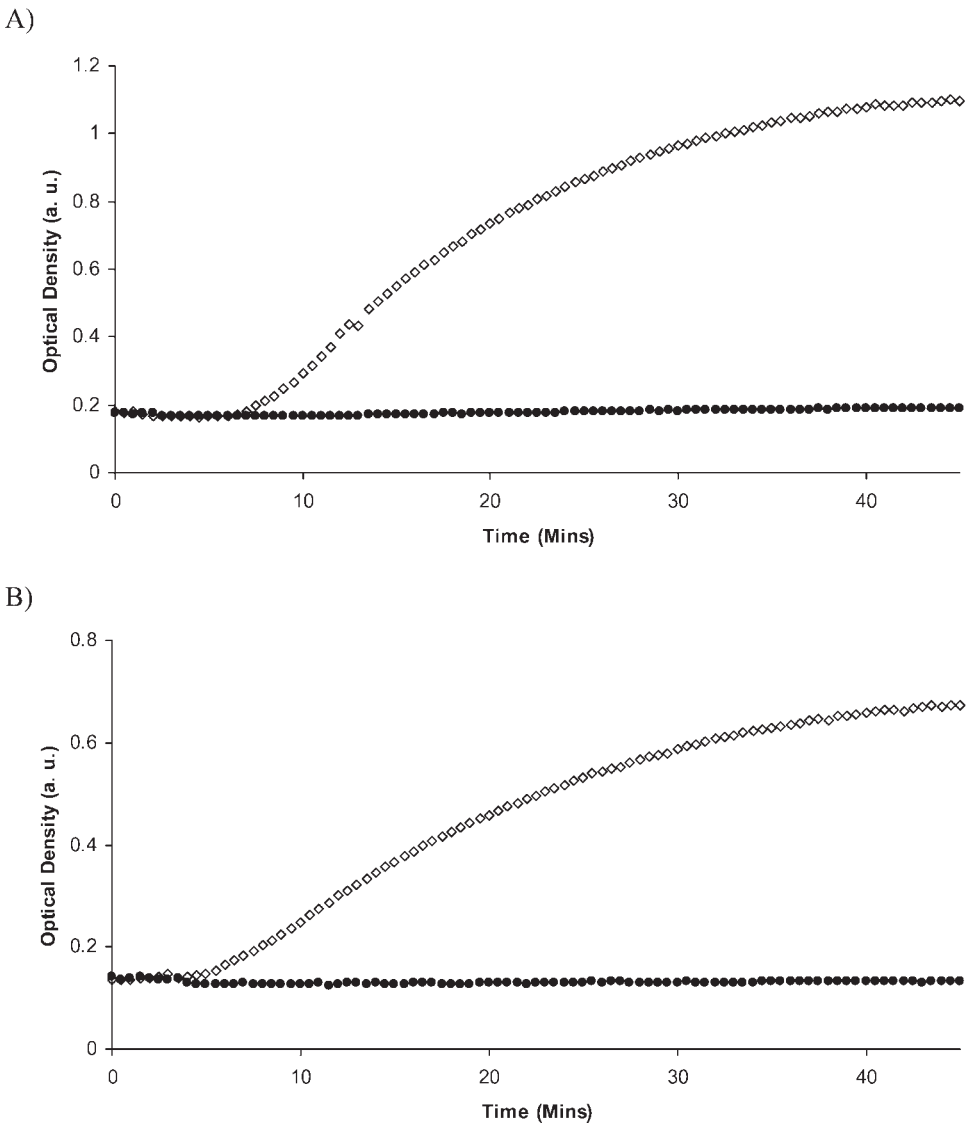

FIGURE 3. The light-scattering (optical density) of β-crystallin (◇), and α+β-crystallin (◆) (subunit molar ratio of α to β, 0.5:1) at 56°C in (**A**) buffered H_2O solution and (**B**) buffered D_2O solution.

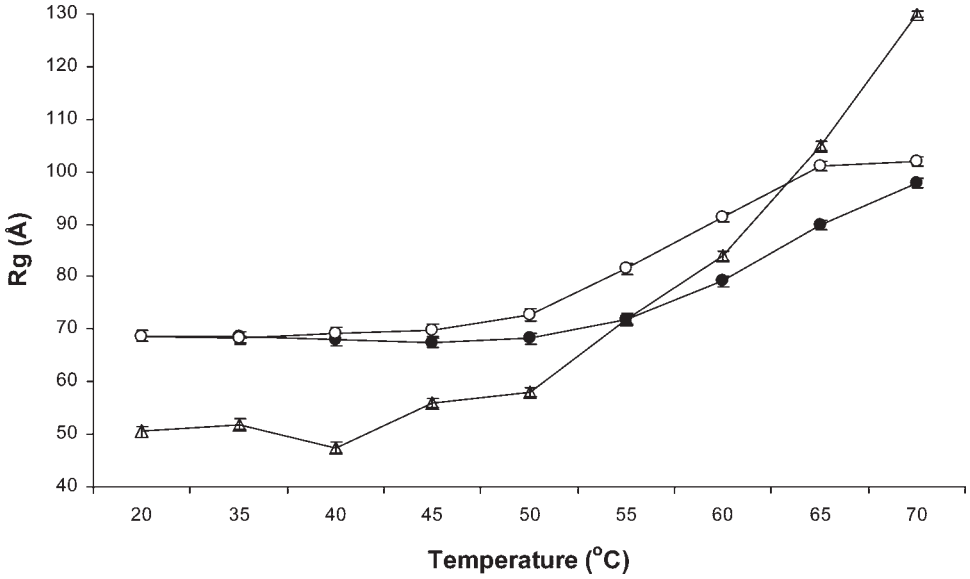

FIGURE 4. The Rg of α-crystallin (○), β-crystallin (△), and α+β-crystallin (●) (subunit molar ratio of α to β, 1.25:1), as a function of temperature observed by x-ray scattering. Error bars: SD from the line of best fit in the Guinier region.

the windows in the surfaces of the α-crystallin shell precisely because experimentally we see no change in Rg. At higher temperatures, the situation is more complicated; the subunit exchange of both isoforms of α-crystallin, are known to increase with higher association and disassociation rates with increasing temperatures.[23,24] The possibility exists that a higher number of β-monomers chaperoned by the α-crystallin, or the higher mobility of each monomer, causes an increase in the size (Rg) of the α-crystallin superaggregates. This increase may be caused by swelling induced by the chaperone function, or by aggregation on the outside of the α-aggregates, or by both of these mechanisms.

The deuterium bond is more stable than the hydrogen bond by 0.1 to 0.2 kcal $mole^{-1}$ and thus needs greater activation energy for cleavage.[25] The different aggregation process of α-crystallin in hydrogen- and deuterium-based solutions indicates the importance of hydrogen bonding. It is likely that as the aggregates increase in size with temperature in normal water, intersheet hydrogen bonds are cleaved more easily and may allow for subunit exchange and recruitment that leads to a swelling and/or mass increase of spherical shells. Particle size measurements using photocorrelation techniques show that in the temperature range from 4°C to 50°C, α-crystallin actually decreases in molecular mass and size.[26,27] From 4°C to 20°C the hydrodynamic diameter (D_H) of α-crystallin is stable but steadily decreases from 20°C and reaches a minimum at 40°C, before increasing dramatically at 50°C. As mentioned, the deuterium bond is stronger than the hydrogen bond, and it is probable that higher temperatures than 70°C are necessary before α-crystallin undergoes superaggregation in D_2O. Similarly, the decrease in the Rg of α-crystallin in D_2O that we observe from our neutron and x-ray studies for 40°C to 60°C is likely to be due to the same dynamic behavior of the particles observed by photocorrelation techniques at lower temperatures in H_2O-based solutions, but shifted to a higher temperature range. It should be noted that the decrease in particle size at ~40°C is dependent on the protein concentration. In our neutron- and x-ray-scattering studies and in the photocorrelation techniques, protein concentrations were below 10 mg mL^{-1}. In our previous study,[11] we used α-crystallin gels (300 mg mL^{-1}) and did not observe this effect.

The lack of superaggregation in deuterium-based solutions and the fact that α-crystallin is still a functional chaperone in such solutions implies that the two processes are not linked.

TABLE 2. Rg α-Crystallin, α+β-Crystallin, and β-Crystallin Observed by X-ray Scattering

Temperature (°C)	α ± SD (Å)	α+β ± SD (Å)	β ± SD (Å)
20	68.7 ± 1.01	68.7 ± 1.08	50.5 ± 1.15
35	68.5 ± 1.06	68.4 ± 1.16	51.9 ± 0.98
40	68.1 ± 1.20	69.2 ± 1.23	47.5 ± 1.14
45	67.4 ± 0.95	69.7 ± 1.14	55.9 ± 0.97
50	78.2 ± 1.09	72.7 ± 1.25	58.0 ± 0.96
55	71.9 ± 1.15	81.5 ± 1.04	71.9 ± 0.81
60	79.1 ± 0.95	91.4 ± 0.92	83.9 ± 0.91
65	89.8 ± 0.91	101 ± 0.84	105 ± 0.86
70	97.8 ± 0.86	102 ± 0.84	130 ± 0.61

The subunit molar ratio of α to β was 1.25:1. The standard deviation is taken from the line of best fit in the Guinier region.

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