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

Justyn W. Regini,1 J. Günter Grossmann,2 Peter Timmins,3 John J. Harding,4 Andrew J. Quantock,1 Stuart A. Hodson,4 and Gerald F. Elliott1,4

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 H2O-buffered solutions, the radius of gyration did not increase significantly in D2O-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.

METHODS. We used a 2-mg mL−1 solution of 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 nonnaturating 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 beamline 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) NaN3 buffered with 50 mM imidazole at pH 7.5. These solutions were
also made with heavy water (deuterium oxide; D₂O) 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. 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

\[ I = I₀ \exp(-4π²S²Rg²/3), \]

where I is the scattered intensity and \( I₀ \) 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. 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. 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, 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. It has not been crystallized, but crystallographic structures of two HSPs have been identified, and there are similarities. 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. 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,
with one three-fold and three two-fold axes.\textsuperscript{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 $\beta$-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\textsuperscript{-1}.\textsuperscript{18} Taking an average value of 0.72 mL g\textsuperscript{-1} for the $\beta$-crystallin aggregate, together with its molecular mass of approximately 700 kDa gives an estimated volume of $0.83 \times 10^6$ Å\textsuperscript{3}, with an uncertainty of approximately 20%. A solid sphere with this volume would have a radius of approximately 58 Å.

The relationship between $R_g$ and $r$, the geometric radius, of any body is given by $r = k' \times R_g$, where $k'$ is a constant with a numerical value that depends on the distribution of mass within the body. If $\alpha$-crystallin were a solid sphere of radius 58 Å, $k'$ would equal 1.581. Therefore, the $R_g$ 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 $\alpha$-crystallin in solution, though, $k'$ has a value of 1.225. From our observed $R_g$, the $R_g$ of such a shell would then be $\approx 84.5$ Å (diameter, 169 Å). The surface area of such a spherical shell would be approximately $9 \times 10^4$ Å$^2$ giving room to accommodate a protein volume of $0.83 \times 10^6$ Å$^3$ in a layer approximately 10-Å thick.

Support for the shell model for $\alpha$-crystallin comes from cryo-electron microscopy and three-dimensional image reconstruction studies of recombinant human $\alpha$B-crystallin that suggest some sort of hollow sphere of diameter (for a 39-subunit aggregate) of 175 ± 20 Å.\textsuperscript{19} This compares well with a fenestrated $\alpha$-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.\textsuperscript{20} have used similar arguments to show that $\alpha$-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 $S \approx 9 \times 10^{-3}$ 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...
et al.\(^1\) has used data from \(\beta\)-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\(^1\) and furthers our understanding of the possible underlying structure of \(\alpha\)-crystallin.

We have shown that the \(R_g\) of \((\alpha+\beta)\)-crystallin below 35°C is 69 ± 1 Å, the same as that of \(\alpha\)-crystallin alone (Fig. 3). This result implies that, under nonstress conditions, \(\beta\)-crystallin is not independent of \(\alpha\)-crystallin in solution, but weakly interacts with \(\alpha\)-crystallin, a contention that is backed up by nuclear magnetic resonance (NMR) studies.\(^2\) From simple mechanics, if \(\beta\)-crystallin were located on the outside of the \(\alpha\)-crystallin shell and if it were, in total, approximately the same molecular weight and density as the \(\alpha\)-crystallin, we would expect to see an approximate increase in \(R_g\) of 4 Å. Alternatively, if it were on the inside of the shell, we would expect to see an approximate 4 Å decrease in the \(R_g\) of the \(\alpha+\beta\) aggregate. Thus, our data point to a model with the targeted \(\beta\)-crystallin coplanar in

**TABLE 1.** \(R_g\) of \(\alpha\)-Crystallin in Buffered H\(_2\)O and D\(_2\)O Solutions

<table>
<thead>
<tr>
<th>Method/Temp (°C)</th>
<th>H(_2)O ± SD (Å)</th>
<th>D(_2)O ± SD (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutron scattering</td>
<td></td>
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</tr>
<tr>
<td>20</td>
<td>69.0 ± 1.12</td>
<td>68.0 ± 1.2</td>
</tr>
<tr>
<td>35</td>
<td>64.9 ± 1.09</td>
<td>65.5 ± 0.87</td>
</tr>
<tr>
<td>40</td>
<td>64.9 ± 1.14</td>
<td>63.7 ± 1.02</td>
</tr>
<tr>
<td>45</td>
<td>67.3 ± 0.98</td>
<td>60.0 ± 0.99</td>
</tr>
<tr>
<td>50</td>
<td>68.2 ± 1.02</td>
<td>59.7 ± 1.12</td>
</tr>
<tr>
<td>55</td>
<td>72.5 ± 1.15</td>
<td>59.9 ± 0.95</td>
</tr>
<tr>
<td>60</td>
<td>87.5 ± 0.95</td>
<td>62.7 ± 0.93</td>
</tr>
<tr>
<td>65</td>
<td>98.5 ± 0.92</td>
<td>67.0 ± 1.0</td>
</tr>
<tr>
<td>70</td>
<td>100.0 ± 0.84</td>
<td>71.0 ± 0.87</td>
</tr>
<tr>
<td>X-ray scattering</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>68.7 ± 1.01</td>
<td>69.4 ± 0.99</td>
</tr>
<tr>
<td>35</td>
<td>68.5 ± 1.02</td>
<td>70.6 ± 1.13</td>
</tr>
<tr>
<td>40</td>
<td>68.1 ± 1.10</td>
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<td>79.1 ± 0.96</td>
<td>70.0 ± 0.94</td>
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<tr>
<td>65</td>
<td>89.8 ± 0.90</td>
<td>73.5 ± 1.10</td>
</tr>
<tr>
<td>70</td>
<td>97.8 ± 0.86</td>
<td>77.6 ± 0.89</td>
</tr>
</tbody>
</table>

**FIGURE 3.** The light-scattering (optical density) of \(\beta\)-crystallin (\(○\)), and \(\alpha+\beta\)-crystallin (\(●\)) (subunit molar ratio of \(\alpha\) to \(\beta\), 0.5:1) at 56°C in (A) buffered H\(_2\)O solution and (B) buffered D\(_2\)O solution.
the windows in the surfaces of the α-cry stallin 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-cry stallin, are known to increase in size with temperature in normal water, intersheet hydrogen bonds are cleaved more easily and the aggregates increase in size with temperature in normal water, intersheet hydrogen bonds are cleaved more easily and the aggregated process is linked. 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,¹ we used α-cry stallin gels (300 mg mL⁻¹) and did not observe this effect.

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

Acknowledgments

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References