Studies on the α-crystallin target protein binding sites: sequential binding with two target proteins

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**Purpose:** α-Crystallin belongs to a class of small heat shock proteins and is shown to prevent aggregation of several proteins. We have shown that the temperature-induced structural perturbation leads to several fold enhanced activity. The purpose of this study was to investigate the availability and specificity of the hydrophobic sites that might become available at elevated temperatures. Specifically, we address the following question: Is there an increased exposure of fixed number of hydrophobic sites as a function of temperature or does a new set of sites become available at elevated temperatures?

**Methods:** α-Crystallin target protein complexes were made at two different temperatures and this complex was investigated for its chaperone-like activity towards the same target protein and also other target proteins. DTT-induced aggregation of insulin, α-lactalbumin, thermal aggregation of βL- and γ-crystallin, and photo-aggregation of γ-crystallin were used as model systems. Increased light scattering was used to monitor the progress of aggregation.

**Results:** α-Crystallin target protein complex prepared at 37 °C temperature was effective against thermal aggregation of βL-crystallin as well as non-thermal aggregation at elevated temperatures. However, the complex prepared at high temperature was ineffective at lower temperatures as well as with other target proteins at both temperatures.

**Conclusions:** More target protein binding sites become available at elevated temperatures. The sites available at low temperature are a subset of the total sites available at elevated temperatures.

α-Crystallin, a major structural protein of the eye lens, plays a prominent role in the maintenance of the eye lens transparency and its refractive properties [1]. It is a heteroaggregate of two highly homologous 20 kDa gene products, αA-, and αB-crystallin, in a molar ratio of 3:1 (αA/αB) in most mammalian lenses [2]. Studies over the past few years have shown that the subunits of α-crystallin are expressed in several non-lenticular tissues such as heart, kidney and brain [3,4] and the expression of αB-crystallin is enhanced several fold during stress and disease conditions [5,6]. Furthermore, the two subunits αA-, and αB-crystallin share significant sequence homology with the ubiquitous family of small heat shock proteins [7-9]. The high degree of sequence homology of αA-, and αB-crystallins with small heat shock proteins, the demonstration of their non-lenticular expression, and association with numerous pathological conditions suggested non-structural role(s) for the proteins. Horwitz [10] showed that α-crystallin displays chaperone-like activity in preventing the thermal aggregation of βL- and γ-crystallins as well as that of enzymes such as alcohol dehydrogenase, citrate synthase and carbonic anhydrase. Using non-thermal models of aggregation such as DTT-induced aggregation of insulin and the photo aggregation of γ-crystallin, we showed that α-crystallin possesses less chaperone-like activity below 30 °C [11,12], the activity is about 8-fold higher at 40 °C as compared to that at 27 °C [11]. This led us to hypothesize that α-crystallin prevents the aggregation of non-native proteins by providing appropriately placed hydrophobic surfaces and a structural transition above 30 °C enhances the protective ability by increasing or reorganizing these hydrophobic surfaces. Similar temperature-dependent activation of several other chaperones is also known [13,14]. The structural alteration may constitute a general mechanism for activation of chaperones under heat shock conditions.

Earlier structural studies by others [15-17] as well as from our laboratory [12] showed that α-crystallin undergoes two structural transitions as a function of temperature: one around 30 °C and the other around 55 °C, resulting in the concomitant exposure of hydrophobic surfaces. The first transition involves subtle tertiary structural changes and quaternary structural reorganization in the heteroaggregate, while the second transition involves large secondary structural alterations. Dudich et al [18] showed that mammalian HSP25/27 also undergoes similar structural transitions around 36 °C and 60 °C. Studies on the temperature-dependence of the chaperone-like activity of α-crystallin against the refolding-induced aggregation of GuHCl-unfolded βL-crystallin also showed two transitions corresponding to the structural transitions seen in α-crystallin [19]. The observed enhancement of chaperone-like activity of α-crystallin at the physiologically relevant temperature of 37 °C appears to correspond with its first structural transition.

The precise mechanistic details of the temperature-dependent enhancement of chaperone-like activity of α-crystallin are not known due to limited structural information about the molecule. The observed temperature-dependent transition in
the structure, exposure of hydrophobic surfaces and the resulting enhancement of the chaperone-like activity raise interesting questions. For instance, is there an increased exposure of a fixed number of hydrophobic sites as a function of temperature or are new hydrophobic surfaces exposed/generated at higher temperatures in the oligomeric assembly of α-crystallin? We made an attempt to answer this question by using two classes of target proteins, those that undergo non-thermal aggregation and others that undergo thermal mode of aggregation. We have generated the complexes of α-crystallin with non-thermally and thermally aggregated target proteins at temperatures corresponding to either the first (37 °C) or the second structural transition (60 °C). We have compared the ability of these purified complexes to exhibit chaperone-like activity against target proteins undergoing either thermal or non-thermal modes of aggregation. Our results provide further insight into the mechanistic aspects of chaperone-like activity of α-crystallin.

**METHODS**

**Preparation of α-crystallin:** The outer cortex of calf-lenses was homogenized in 50 mM Tris-HCl buffer, pH 7.2, (containing 100 mM NaCl, 1 mM EDTA, and 0.02% sodium azide) and centrifuged at 12000 rpm at 4 °C for 20 min. The use of α-crystallin isolated from the outer cortex of calf lenses minimises the effects resulting from post-translational modifications. The soluble proteins in the supernatant were fractionated by gel filtration chromatography on a column of Bio-Gel A-1.5 M (Bio-Rad Laboratories, Hercules, CA) at 4 °C. Fractions corresponding to α-crystallin were pooled and concentrated at 4 °C using an Amicon ultra filtration unit fitted with a 30 kDa molecular weight cut-off filter. α-Crystallin was further purified by gel filtration on Bio-Gel A-5M column (Bio-Rad Laboratories), and the purified fractions were pooled and concentrated. The concentrated fractions were stored at -20 °C until required.

**Preparation of α-crystallin target protein complexes:** The complex of α-crystallin with insulin was prepared by incubating α-crystallin (0.3 mg/ml) with insulin (0.2 mg/ml) in 10 mM phosphate buffer (pH 7.2) containing 100 mM NaCl and 13.3 mM DTT at 37 °C for 20 min. The complex of α-crystallin with β-crystallin was prepared by incubating α-crystallin (0.02 mg/ml) with β-crystallin (0.2 mg/ml) in 10 mM phosphate buffer (pH 7.2) containing 100 mM NaCl at 60 °C for 10 min. The complexes were separated from any free target protein by gel filtration chromatography on a Superose 6 HR 10/30 pre packed column (10 mm x 300 mm, AP Biotech, Uppsala, Sweden). The fractions corresponding to the complex were pooled and concentrated by ultrafiltration using PM 30 membrane.

**DTT-induced aggregation of insulin and α-lactalbumin:** All chaperone assays were carried out in 10 mM phosphate buffer, pH 7.4, containing 100 mM NaCl. Insulin (0.2 mg/ml), in the presence or the absence of the required amount of α-crystallin, was equilibrated at 37 °C for 5 min with constant stirring in the sample holder using a Julabo thermo stated water bath (Julabo Labortechnik, GMBH, Seelback, Germany). The actual temperature of the sample was monitored by a Physitemp micro thermocouple thermometer system (Physitemp Instruments, Inc., Clifton, NJ). Reduction of insulin was initiated by adding 20 µl of 1 M DTT to 1.5 ml of the sample. The extent of aggregation of the insulin-B chain was measured as a function of time by monitoring 90 degree-scattering at 365 nm (excitation and emission band passes set at 1.5 nm) using a Hitachi F-4000 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan). A similar procedure was used for the aggregation of α-lactalbumin (0.2 mg/ml) in the presence of varying concentrations of α-crystallin.

**Photo aggregation of γ-crystallin:** γ-Crystallin (0.2 mg/ml) was irradiated in the absence or the presence of α-crystallin with 295 nm light using a Hitachi F-4000 fluorescence spectrophotometer (Hitachi, Ltd.) with an excitation band pass of 20 nm for a fixed amount of time. Light scattering was measured at regular intervals using the same procedure as described above in the case of insulin.

**Thermal aggregation of βL- and γ-crystallins:** A fixed concentration of either βL- or γ-crystallin (0.2 mg/ml) was used and the concentration of α-crystallin varied. Other experimental conditions are the same as mentioned above. The thermal aggregation of βL-crystallin was initiated by incubating the sample at 60 °C, whereas that of γ-crystallin was initiated by incubating the sample at 63 °C. Percentage protection (% protection) is calculated as, (I t - I t+)/I t, where I t is the intensity of scattered light for target proteins and I t+ is the intensity of scattered light in the presence of α-crystallin.

**RESULTS & DISCUSSION**

The chaperone-like activity of α-crystallin is temperature-dependent [11,12,16]; the activity is low at temperatures below 37 °C. We have shown that α-crystallin hinders aggregation of light chain of insulin [11] as well as of α-lactalbumin [12]. α-Crystallin protects βL-crystallin from aggregation at both 37 °C and 63 °C [16]. Our results provide an explanation for the activity of α-crystallin as a chaperone and enhance the understanding of the mechanism of action of α-crystallin as a chaperone (Figure 1). The chaperone-like activity of α-crystallin in the prevention of protein aggregation. The DTT-induced aggregation of α-lactalbumin (solid squares) and insulin (solid circles) at 37 °C, UV-induced aggregation of γ-crystallin (open squares) at 37 °C, thermal aggregation of βL-crystallin (solid triangles) and γ-crystallin (open triangles) at 60 °C and 63 °C respectively. For calculation of molar ratios, molecular weights taken are: α-crystallin-20,000, βL-crystallin-27,000, γ-crystallin-21,000, insulin-6,000 and α-lactalbumin-14,200.
30 °C, and is enhanced several fold above 30 °C. Structural perturbation of α-crystallin and resultant exposure of hydrophobic surfaces is believed to mediate this temperature-dependent enhancement of activity. Earlier studies showed that α-crystallin undergoes two structural transitions at around 30 °C and 55 °C [12,15,16]. The ability of α-crystallin to solubilize the hydrophobic compound, pyrene was measured as a function of temperature. It was shown that there was a 2-fold enhancement in the amount of solubilized pyrene beyond 30 °C (as measured by absorbance of pyrene at 338 nm) and a further increase was observed beyond 55 °C (see Figure 5A in Raman et al. [12]). The Ham ratio (the ratio of the third to the first vibronic bands of the emission spectrum) of pyrene reflects on hydrophobic environment immediately surrounding it. The Ham ratio of the pyrene solubilized in α-crystallin changes from 0.78 to 0.82 beyond 30 °C and increases further to about 0.86 beyond 55 °C (see Figure 5B in Raman et al. [12]). Similarly the two transitions in the protective ability of α-crystallin in the refolding of GuHCl-denatured βL-crystallin (see Figure 1B in Raman et al. [19]) corresponded to the two transitions observed in the binding of pyrene to α-crystallin as a function of temperature [12].

α-Crystallin exhibits chaperone-like activity against several proteins, some of which aggregate around the first transition temperature, and others around the second transition temperature. The chaperone-like activity of α-crystallin against different target proteins aggregating at temperatures corresponding to either the first or the second transition is shown in Figure 1. The amount of α-crystallin required for preventing the thermally-induced aggregation of βL-, or γ-crystallin is markedly lower than that required for the DTT-induced aggregation of insulin and α-lactalbumin or the photo aggregation of γ-crystallin. For instance, a molar ratio of 0.27 μM in the case of α-crystallin to βL-crystallin and 0.21 in the case of α-crystallin to γ-crystallin, which corresponds to a W/W ratio of 0.04 mg/ml α-crystallin: 0.2 mg/ml of either βL-, or γ-crystallins, almost completely prevents the thermally-induced aggregation of βL-, or γ-crystallins. On the other hand, much higher ratios are required for comparable levels of prevention of DTT-induced aggregation of insulin (molar ratio of 0.9 or W/W ratio of 0.6 mg/ml of α-crystallin: 0.2 mg/ml insulin) and α-lactalbumin (molar ratio of 3.7 or 1 mg/ml of α-crystallin: 0.2 mg/ml α-lactalbumin) or photo-aggregation of γ-crystallin (molar ratio of 5 or W/W ratio of 1 mg/ml of α-crystallin: 0.2 mg/ml γ-crystallin) at 37 °C. The observed differences in the molar ratios required in the two modes of aggregation may be due to differences in molecular mass of the target proteins. However, as seen in Figure 1, α-crystallin prevents the thermal aggregation of γ-crystallin at 63 °C far more efficiently than its photoaggregation at 37 °C. Thus, these differences in protective ability of α-crystallin can be attributed to the fact that it exhibits more hydrophobicity around 50-60 °C compared to that at 37 °C. It has already been demonstrated that α-crystallin undergoes a structural change with temperature resulting in the enhanced exposure of hydrophobic surfaces at 55 °C as compared to 37 °C [12,16]. This increase in hydrophobicity at the elevated temperature could be due to enhanced accessibility of pre-existing hydrophobic sites at 37 °C or due to the exposure of new sites in the oligomeric assembly. Earlier studies have identified hydrophobic stretches that get exposed at elevated temperatures [20]. Post-translational modifications such as glycation and glycosylation of α-crystallin, despite causing partial unfolding of the protein, are known to decrease its surface hydrophobicity as well as chaperone-like activity [21,22]. This, perhaps, is due to overlap of the sequences involved in the modification and target protein binding [22]. Short sequences are identified as target protein binding sites in the constituent subunits of α-crystallin [23]. These sequences may be part of hydrophobic patches in the oligomeric α-crystallin. Subtle rearrangement of subunits in the oligomeric assembly as a function of temperature could alter the nature and accessibility of these hydrophobic patches.

Figure 2. Chaperone-like activity of α-crystallin target protein complexes. A: Thermal aggregation of βL-crystallin (0.2 mg/ml) at 60 °C, (solid triangles), or in the presence of 13.3 mM DTT, (open triangle); α-crystallin (0.02 mg/ml), (solid circles); α-crystallin-insulin complex (0.02 mg/ml), (open squares); 13.3 mM DTT + α-crystallin-insulin complex (0.02 mg/ml), (inverted solid triangles). Scatter profile of α-crystallin (0.02 mg/ml) + 13.3 mM DTT at 60 °C, (open circles). B: DTT-induced aggregation of insulin (0.2 mg/ml) at 37 °C, (solid circles), or in the presence of α-crystallin (0.1 mg/ml), (solid triangles); α-crystallin-βL-crystallin complex (0.1 mg/ml), (solid squares). Scatter profile of α-crystallin-βL-crystallin complex (0.1 mg/ml) + 13.3 mM DTT at 37 °C, (inverted open triangles).
and the sequences/target protein binding sites therein.

In order to investigate the nature of the increased hydrophobicity, we have formed complexes of α-crystallin with insulin at 37 °C or with βL-crystallin at 60 °C at stoichiometric ratios where approximately 85% to 95% protection is offered, separated them from any remaining free target protein and concentrated as described under Materials and Methods. These purified complexes were assayed for their chaperone-like activity at 37 °C or 60 °C. The chaperone-like activity of α-crystallin-insulin complex towards the thermal aggregation of βL-crystallin and that of α-crystallin-βL-crystallin complex towards the DTT-induced aggregation of insulin is compared in Figure 2. At 60 °C, βL-crystallin aggregates and precipitates out of solution (Figure 2A). α-Crystallin at a ratio of 1:10 w/w (0.02 mg/ml α-crystallin: 0.2 mg/ml βL-crystallin) prevents this aggregation. Under the conditions of the assay, the scatter value shown by α-crystallin alone is minimal and does not change over the time period of the assay (Figure 2A). The α-crystallin-insulin complex at a ratio of 1:10 (α-crystallin-insulin complex:βL-crystallin, w/w) also prevents the aggregation of βL-crystallin to a comparable extent. It is possible that the α-crystallin-insulin complex offers protection to βL-crystallin by the formation of disulfide bonds between the free sulfhydryl groups of insulin bound to α-crystallin and the free sulfhydryl groups in βL-crystallin. In order to test this possibility, we performed an experiment in which we assayed the protective ability of α-crystallin-insulin complex against βL-crystallin in the presence of 13.3 mM DTT. Even in presence of DTT (which prevents the disulfide bond formation between free sulfhydryl groups of insulin and βL-crystallin), the α-crystallin-insulin complex offered protection against βL-crystallin which was comparable to that in the absence of DTT (Figure 2A). Further, the lack of aggregation of βL-crystallin in the above experiment was not due to the presence of DTT, as DTT had no effect on the aggregation profile of βL-crystallin alone (Figure 2A). Thus, our results clearly show that even upon saturation of the target protein binding sites available at 37 °C, α-crystallin generates new functional target protein binding sites at an elevated temperature of 60 °C, which can prevent the aggregation of the new target protein. In contrast, α-crystallin-βL-crystallin complex formed at 60 °C fails to prevent the DTT-induced aggregation of insulin at a ratio of 0.5:1 (α-crystallin-βL-crystallin complex:insulin, w/w) at 37 °C. In fact, the scatter value observed when the chaperone-like activity of α-crystallin-βL-crystallin complex is measured against the DTT-induced aggregation of insulin is much higher than that observed for the aggregation of insulin alone. The observed scattering is not due to the release of βL-crystallin from the α-crystallin-βL-crystallin complex upon addition of DTT (Figure 2B). The over-saturation of α-crystallin with target proteins is known to result in the formation of large insoluble complexes which display high light scattering [24]. This process may involve interaction of the aggregating species with alpha-crystallin-bound target protein rather than with alpha crystallin itself resulting in insolubilization of the whole assembly like in the model proposed by Smulders, et al. [25]. The higher scatter value observed with the α-crystallin-βL-crystallin complex (Figure 2B) is probably due to this phenomenon. At the same ratio, α-crystallin offers considerable protection (Figure 2B). In our experiments, we did not observe any change in the scatter value of α-crystallin in the presence of DTT (data not shown). α-Crystallin has no disulfide bonds and it has been shown earlier that DTT has no effect on the structure of α-crystallin [26]. Our results show that saturation of all the exposed hydrophobic target protein binding sites of α-crystallin at 60 °C, abolishes its ability to prevent protein aggregation at a lower temperature of 37 °C. Taken together, these results also indicate that the smaller number of target protein interaction sites readily accessible at 37 °C is a subset of the total number of sites available at 60 °C in the oligomeric assembly of α-crystallin. More than one subunit may participate in generating these target protein-interaction sites. The elegant study by Bova, et al. [27] shows that subunit exchange in αA-crystallin is inhibited upon complexation with large target proteins, indicating that they interact simultaneously with multiple subunits of αA-crystallin.

Lee, et al. [28] reported that α-crystallin complexed with target protein at 60 °C exhibits enhanced chaperone-like activity at lower temperatures. Our results, however, are not in agreement with this report. In their studies, insulin or γ-crystallin was pre-incubated with α-crystallin at 60 °C before subjecting to aggregation by exposure to DTT or UV-light, respectively, to measure the chaperone-like activity. These results were compared with the chaperone-like activity obtained upon pre incubating the target proteins with α-crystallin at room temperature. Both γ-crystallin and insulin aggregate at 60 °C. α-Crystallin exhibits several fold higher chaperone-like activity at this temperature (Figure 1). Both the target proteins would bind α-crystallin during pre-incubation at 60 °C leaving no free protein to be aggregated at the lower assay temperature. This perhaps explains the apparent enhancement in the chaperone-like activity upon complexation at higher temperatu
temperature and measurement of activity at a lower temperature. On the other hand, Takemoto and Boyle [29] demonstrated that binding of partially denatured proteins to α-crystallin at elevated temperatures resulted in a complex with decreased ability to protect against aggregation at lower temperature. This result is in agreement with our observation that the α-crystallin-βL-crystallin complex formed at 60 °C fails to protect the DTT-induced insulin aggregation at 37 °C.

Since the structure of α-crystallin is unknown and its quaternary structure is a matter of debate [30], the precise mechanism of its chaperone-like activity is not fully understood. Several models have been proposed for its oligomeric structure including a GroEL-like toroidal structure for α-crystallin [31]. More recent image constrained analysis of cryoelectron microscopy data on αB-crystallin shows it to have an oligomeric structure with voids in it [32]. Using gold antibodies and electron microscopy, target proteins have been localized at the center of α-crystallin [33]. It is speculated that the target proteins localize in the putative hollow central cavity similar to that observed with GroEL. However a large number of insulin molecules bind to α-crystallin oligomer (Figure 1). It is difficult to envisage how so many target molecules can be accommodated in the central hollow cavity. It is more likely that the target proteins bind on the outer surface of the oligomeric assembly. α-Crystallin is a heteroaggregate of αA- and αB-crystallin, both of which can individually form homoaggregates and display chaperone-like activity, albeit to different extents. This suggests the possibility that a variety of target binding sites could be generated depending on the packing and subunit arrangement in the heteroaggregate. Studies by Sharma, et al. [23,34] demonstrate that the alcohol dehydrogenase-binding sites encompass residues 57-69 and 93-107, while melittin-binding site encompasses residues 75-82 in αB-crystallin. In view of these observations, it is of interest to investigate whether different target proteins bind preferentially to different sites on α-crystallin or compete for common binding sites. To investigate this possibility, we have purified α-crystallin-insulin complex (0.3 mg/ml α-crystallin to 0.2 mg/ml α-lactalbumin) (Figure 3). The complex fails to offer any protection against DTT-induced aggregation of α-lactalbumin at 37 °C. In fact, addition of the complex to α-lactalbumin led to the formation of larger aggregates, resulting in a higher scattering value compared to that shown by α-lactalbumin alone. Native α-crystallin (0.8 mg/ml), as shown in Figure 3, offers significant protection (about 40%). This clearly demonstrates that prior saturation of α-crystallin at a given temperature with insulin depletes its protective ability against other target proteins such as α-lactalbumin at the same temperature, indicating that both the proteins bind to the same or overlapping sites. However, such a complex offers protection at higher temperatures against the thermal-induced aggregation of proteins like β- and γ-crystallins due to the generation of new target protein binding sites at higher temperatures. This suggests a sequential exposure of hydrophobic target binding sites as a function of temperature, with low temperatures binding sites being a subset of the total number of sites available at elevated temperatures. This process of enhanced exposure of hydrophobic surfaces at elevated temperatures involves conformational changes in α-crystallin. The non-specificity of α-crystallin, due to common target protein binding surfaces, may be advantageous for its role in binding diverse aggregation-prone molecules in the lens and keep them in solution.

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REFERENCES


