Subunit Exchange of αA-Crystallin*

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α-Crystallin, the major protein in the mammalian lens, is a molecular chaperone that can bind denaturing proteins and prevent their aggregation. Like other structurally related small heat shock proteins, each α-crystallin molecule is composed of an average of 40 subunits that can undergo extensive reorganization. In this study we used fluorescence resonance energy transfer to monitor the rapid exchange of recombinant α-crystallin subunits. We labeled αA-crystallin with stilbene iodoacetamide (4-acetamido-4'-(iodoacetyl)amino)-stilbene-2,2'-disulfonic acid), which serves as an energy donor and with lucifer yellow iodoacetamide, which serves as an energy acceptor. Upon mixing the two populations of labeled αA-crystallin, we observed a reversible, time-dependent decrease in stilbene iodoacetamide emission intensity and a concomitant increase in lucifer yellow iodoacetamide fluorescence. This result is indicative of an exchange reaction that brings the fluorescent αA-crystallin subunits close to each other. We further showed that the exchange reaction is strongly dependent on temperature, with a rate constant of 0.075 min⁻¹ at 37 °C and an activation energy of 60 kcal/mol. The subunit exchange is independent of pH and calcium concentration but decreases at low and high ionic strength, suggesting the involvement of both ionic and hydrophobic interactions. It is also markedly reduced by the binding of large denatured proteins. The degree of inhibition is directly proportional to the molecular mass and the amount of bound polypeptide, suggesting an interaction of several αA-crystallin subunits with multiple binding sites of the denaturing protein. Our findings reveal a dynamic organization of αA-crystallin subunits, which may be a key factor in preventing protein aggregation during denaturation.

α-Crystallin, the major lens protein of the mammalian eye, is a member of the small heat shock protein family (1, 2). Like other small heat shock proteins, α-crystallin is a high molecular mass complex consisting of a large number of subunits. The two polypeptides of α-crystallin found in the lens of the mammalian eye, αA and αB, are encoded by evolutionarily related genes and share more than 50% identity in amino acid sequence (3, 4). For many years, α-crystallin was thought to be lens-specific. However, recent advances in detection methods have revealed much wider non-lenticular tissue distributions in heart, thymus, skin, lung, retina, and brain (5–8).

In the past, α-crystallin was considered to play only a structural role in maintaining the transparency of the lens. Recent studies have demonstrated that α-crystallin can prevent the thermally induced aggregation of a diversity of denaturing proteins (9–14). These findings suggest that α-crystallin possesses chaperone-like property that prevents aggregation of denatured lens proteins, thus preserving the transparency of the lens and reducing the probability of developing cataract (15). Its protective function is further supported by the detection of an elevated amount of αB-crystallin under heat and hypertonic stress (16–18) and in a number of neurological degenerative diseases including Creutzfeldt-Jacob disease (19, 20), diffuse Lewy body disease (21), Alzheimer’s disease (22), and Alexander’s disease (23). However, the exact physiological role of α-crystallin in these diseases is still unclear.

α-Crystallin is normally isolated as an oligomeric complex with an average of 40 subunits and a molecular mass of 8 × 10⁵ Da (3). However, its size distribution can vary from 3 × 10⁵ to 5 × 10⁶ Da, depending on the age of the tissue from which it is isolated (24–29), and the temperature, calcium concentration, pH, and ionic strength of the assay conditions (30–33). Moreover, calf lens α-crystallin that was separated into five subpopulations with distinct molecular masses has been shown to rapidly return to its original distribution upon mixing (34). Exchange of subunits between native and phosphorylated forms of α-crystallin has also been detected by isoelectric focusing (35). These findings suggest that the subunits of α-crystallin are capable of freely associating and dissociating to form large multimeric protein complexes.

In this study we developed a fluorescence resonance energy transfer method to monitor the exchange of recombinant αA-crystallin subunits. Using this technique, we have determined the effect of pH, Ca²⁺, and ionic strength on the rate of subunit exchange. We further found a strong dependence of subunit exchange on temperature, with an activation energy of 60 kcal/mol. Binding of large denatured proteins to αA-crystallin markedly reduced the exchange rate, indicating an association of the polypeptides with several αA-crystallin subunits. The multiple interactions may explain why the binding of denatured proteins to αA-crystallin is irreversible.

EXPERIMENTAL PROCEDURES

Materials—Lucifer yellow iodoacetamide (LYI) and 4-acetamido-4'-(iodoacetyl)amino)-stilbene-2,2'-disulfonic acid (AIAS) were purchased from Molecular Probes, Eugene, OR. Ovotransferrin, α-lactalbumin, insulin, and melittin were obtained from Sigma. They were used in the experiments without further purification. Restriction enzymes and Tag polymerase were purchased from New England Biolabs and Promega, respectively. Escherichia coli strain BL21 DE3, pET7 Blue T-cloning vector, and the pET 20b⁺ expression vector were obtained from Novagen. Rat lens epithelial Agt-11 cDNA library was a generous gift of Dr. S. Bhat, Jules Stein Eye Institute, UCLA.

Cloning of αA-Crystallin from Rat Lens Epithelium—DNA of αA-
crystallin was obtained from the rat lens epithelial Agt-11 DNA library by polyclone antibody reaction amplification using sense primer 5'-TCACATTGACACGGTTCCGAGG-3' and antisense primer 5'-TGGAGACGAGGGTGCCGAGG-3'. The polyclone antibody reaction was carried out in a 25-μl volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 50 μg/ml DNTP, 0.1 μCi/ml [3H]lysine, 1 μCi/ml [3H]methionine and 2.5 units of Taq polymerase. Amplification was performed for 35 cycles with conditions for denaturation at 94°C for 1 min, annealing at 54°C for 2 min, and extension at 72°C for 3 min. The 525-base-long polyclone antibody reaction product was gel-purified, ligated into pT7 blue vector, and subsequently subcloned into the pET 20b expression vector. The amino terminus of the recombinant subunit was fused to a conservative substitution Phe near the amino terminus. Comparison of the DNA sequence of our construct with that of the published rat sequence (36) also indicated a single base change converting Ser-129 to Cys. Both errors were subsequently corrected by mutagenesis.

**Expression and Purification of Recombinant αA-Crystallin—** BL21DE3 cells containing the pET 20bαA-crystallin plasmids were grown in 500 ml of LB broth to a cell density between 0.6 and 1.0 at 600 nm and induced with 0.5 mM isopropl-β-D-thiogalactopyranoside for 3 h. Cells were harvested by centrifugation at 4,000 × g for 10 min, resuspended in 20 ml of ice-cold buffer containing 50 mM Tris, pH 7.9, 0.1 mM NaCl, 2 mM EDTA and lysed by sonication. The cell particulates in relative fluorescence intensity at 415 nm in 15 min.

The effect of pH on subunit exchange was determined using the same assay with the following changes: melittin, 0.1 mM NaCl, 50 mM sodium phosphate, pH 7.5, at room temperature for 30 min (37); insulin, 20 mM DTT, 50 mM sodium phosphate, pH 7.0, at room temperature for 60 min (36); α-lactalbumin, 2 mM EDTA, 50 mM DTT, 0.1 mM NaCl, 50 mM sodium phosphate, pH 7.0, at 37°C for 90 min; ovotransferrin, 20 mM DTT, 0.1 mM NaCl, 50 mM sodium phosphate, pH 7.9, at 42°C for 90 min. After binding, equal amounts of AIAS-labeled and LYI-labeled αA-crystallin containing the bound polypeptide were incubated at 37°C for 15 min, and the relative fluorescence intensity of the preparation was determined as described in the previous section.

**Analytical Methods—** Protein concentrations were determined by Coomassie Blue binding (38) using γ-globulin as a standard. SDS-polyacrylamide gel electrophoresis of proteins was performed by the method of Laemml (39). The concentrations of LYI and AIAS were determined from their absorption spectra using molar extinction coefficients of 15,000 cm−1 M−1 at 435 nm and 35,000 cm−1 M−1 at 355 nm, respectively.

**RESULTS**

**Expression of Recombinant αA-Crystallin—a**αA-Crystallin cDNA was constructed in vector pET 20b** carrying a strong bacteriophage T7 promoter. The resulting expression construct was used to transform E. coli BL21DE3 cells containing a chromosomal copy of the inducible T7 RNA polymerase gene. The level of expression of αA-crystallin in this system was greater than 50% that of the total proteins. When the cell lysate was separated into soluble and particulate fractions by centrifugation, more than 90% of the recombinant αA-crystallin was found in the soluble fraction (data not shown). The high expression level of αA-crystallin in the soluble fraction allowed purification of the recombinant protein to greater than 95% purity by successive Mono-Q ion exchange chromatography and gel filtration chromatography.

Purified recombinant αA-crystallin and native lens αA-crystallin were equally effective in preventing the thermally-induced aggregation of proteins. Their conformations, as determined by circular dichroism, were identical (data not shown). They also interacted to form a multimeric subunit complex of 800 kDa, which is similar in size to native α-crystallin isolated from lens.

**Labeling of αA-Crystallin with Fluorescent Probes—** Recombinant αA-crystallin was labeled with fluorescein-5-maleimide. Subunit exchange was employed to determine the rate of subunit exchange. The reaction was initiated by mixing an equal volume of 0.4 mg/ml AIAS-labeled αA-crystallin and 0.4 mg/ml LYI-labeled αA-crystallin in 0.1 M NaCl, 2 mM EDTA at pH 7.5. The reaction was allowed to proceed for 3 h at room temperature (22°C) in the dark. Unreacted AIAS was separated from the fluorescently labeled αA-crystallin by gel filtration through a 0.2 μm filter. Polyethyleneimine was then added to the filtrate with rapid stirring to form a 0.12% solution. After incubation on ice for 2 min, the mixture was centrifuged at 15,000 × g for 10 min to remove the precipitated DNA. The supernatant was adjusted with DTT to a final concentration of 10 mM and applied onto a Mono-Q column (Pharmacia Biotech Inc.) pre-equilibrated in 100 mM NaCl, 20 mM Tris-HCl, pH 7.9. Proteins were eluted using a linear gradient of 0–1 M NaCl in the same buffer. Fractions containing recombinant αA-crystallin were concentrated and applied to a Superose 6 gel filtration column (Pharmacia) equilibrated in 100 mM NaCl, 20 mM Tris, pH 7.9. Fractions containing purified αA-crystallin were pooled, concentrated to 20 mg/ml, and stored frozen at −20°C.

**Determination of the Effect of Bound Polypeptides—** The modification of Cys-131 did not appear to perturb the conformation or the interaction of the αA-crystallin subunits. Fig. 2 shows a comparison of the gel filtration profiles between unlabeled αA-crystallin and AIAS-labeled αA-crystallin. Their average molecular masses were both 500 kDa, and their size distribution ranged from 300 to 1,000 kDa. Similar size distribution was obtained with LYI-labeled αA-crystallin.

The emission spectra of AIAS-labeled αA-crystallin excited at 335 nm (upper panel), and LYI-labeled αA-crystallin excited at 435 nm (lower panel) are shown in Fig. 1. The emission maxima of AIAS-labeled αA-crystallin and LYI-labeled αA-crystallin were at 415 and 525 nm, respectively. The significant overlap of the emission spectrum of the AIAS fluorophore with the absorption band of the LYI fluorophore indicates that they are an excellent donor-acceptor pair for fluorescence resonance energy transfer.
Determination of Subunit Exchange by Fluorescence Resonance Energy Transfer—
Both AIAS-labeled and LYI-labeled aA-crystallin were very stable at 37 °C, with no significant change in fluorescence intensity over a period of 12 h. If the oligomeric complex of aA-crystallin is static and subunit exchange does not occur after mixing the two populations of labeled aA-crystallin, the fluorescence intensity will remain the same, since the donor and the acceptor are far apart. However, the fluorescence intensity was markedly altered upon mixing of the two populations of labeled aA-crystallin (Fig. 3). The time-dependent decrease in AIAS emission intensity at 415 nm and a concomitant increase in LYI fluorescence at 525 nm were indicative of energy transfer due to the close proximity of the two fluorophores. The quenching of the fluorescence was completed in 4 h at 37 °C (Fig. 4), resulting in approximately 40% decrease of the original AIAS fluorescence intensity.

The rate of subunit exchange can be obtained by measuring either the decrease in donor fluorescence or the increase in acceptor fluorescence. The upper panel of Fig. 4 shows a plot of the emission intensity of AIAS at 415 nm as a function of time after the mixing of the two populations of labeled aA-crystallin. An exchange rate constant of 0.075 min⁻¹ was obtained by fitting the data to the exponential function $F(t) = C_1 + C_2 e^{-kt}$. The same exchange rate was determined by measuring the increase in LYI fluorescence intensity at 545 nm (lower panel). Since both measurements gave essentially the same rate constant, all subsequent measurements were obtained by monitoring only the quenching of the AIAS fluorescence.

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Reversibility of Subunit Exchange—Fig. 5 shows that the AIAS fluorescence at 415 nm rapidly recovered upon the addition of unlabeled aA-crystallin to a pre-mixed population of AIAS-labeled and LYI-labeled aA-crystallin at 37 °C. This result demonstrates that the subunit exchange reaction is reversible.

Effects of pH—The effect of pH on the subunit exchange of aA-crystallin at 37 °C is shown in Fig. 6. For this experiment, the rate of subunit exchange was determined by the change in relative fluorescence intensity in 15 min after mixing an equal amount of AIAS-labeled aA-crystallin and LYI-labeled aA-crystallin. Except for a small decrease in the relative fluorescence intensity at pH 6.5, the subunit exchange rate at pH 7.5, 8.0, and 9.2 were the same.
Effects of Sodium and Calcium Ions—Calcium ions have been shown previously to change the subunit organization of α-crystallin (33). Fig. 7 shows that calcium chloride concentration in the range of 0–50 mM had no effect on subunit exchange. The effect of NaCl on the subunit exchange of α-crystallin is more complex (Fig. 8). Increasing the NaCl concentration from 0 to 0.1 M resulted in an increase in subunit exchange, as indicated by a decrease in relative fluorescence intensity at 15 min. However, at 1.0 M NaCl, a small decrease of the exchange rate was observed. These results suggest that the multimeric subunit organization of α-crystallin may be stabilized by both ionic interaction and hydrophobic interactions.

Effect of Temperature—The rate of subunit exchange was highly temperature-dependent. The AIAS-labeled and LYI-labeled α-crystallin was performed as described under “Experimental Procedures.” Changes in relative fluorescence intensity at 415 nm in 15 min were plotted as a function of pH.

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Subunit Exchange of \(\alpha\)-A-Crystallin

Subunit exchange was determined, and the changes in exchange rate were then plotted as a function of the protein concentration during denaturation. Fig. 10 shows a comparison of the exchange rate of \(\alpha\)-A-crystallin containing either bound melittin, insulin B chain, \(\alpha\)-lactalbumin, or ovotransferrin. Binding of short polypeptides such as melittin (2.6 kDa) and insulin B chain (3.0 kDa) to \(\alpha\)-A-crystallin either did not alter the exchange rate or had only a small effect at high concentrations. In contrast, binding of a large polypeptide such as ovotransferrin (40 kDa) markedly reduced the rate of subunit exchange. The degree of inhibition is proportional to the amount of bound ovotransferrin, with a 35% decrease in exchange rate at a 2:1 molar ratio of \(\alpha\)-A-crystallin subunit to ovotransferrin. There is also a close correlation between the size of the bound polypeptide and the inhibition of subunit exchange. Bound \(\alpha\)-lactalbumin (14 kDa) was found to be more effective than insulin but less effective than ovotransferrin in inhibiting subunit exchange. These results suggest the association of larger polypeptides with several \(\alpha\)-A-crystallin subunits, effectively cross-linking them together and preventing them from exchanging.

**DISCUSSION**

In this study we have used fluorescence resonance energy transfer (40, 41) to monitor the rapid exchange of \(\alpha\)-A-crystallin. We demonstrated that the exchange can be fit to a simple exponential function with a rate constant of 0.075 min\(^{-1}\) at 37 °C and reached a complete equilibrium within 4 h (Fig. 4). Our results are similar but not identical to those of van den Oetelaar et al. (35), who measured the mixing of isolated bovine \(\alpha\)-A- and \(\alpha\)-B-crystallins by isoelectric focusing (35). In this earlier study, a prolonged exchange reaction between \(\alpha\)-A- and \(\alpha\)-B-crystallins that did not completely terminate until after 24 h was observed. This difference can be accounted for by a number of contributing factors. First, the exchange between \(\alpha\)A- and \(\alpha\)B-crystallins may be intrinsically slower, a hypothesis that we are currently investigating. Second, the \(\alpha\)-crystallin preparations may be crucial to the subunit exchange measurement. The native bovine \(\alpha\)-crystallin used in the earlier study of van den Oetelaar et al. (35) was prepared in urea, which may change the conformation of \(\alpha\)-A-crystallin. In addition, post-translational modifications or binding of pre-existing proteins may significantly slow down subunit exchange. Similarly, the exchange rate could be accelerated in
our AIAS-labeled and LYT1-labeled αA-crystallin by the modification of the cysteine residue. Finally, the assay conditions may also have an effect on the kinetics of the exchange reaction. For example, we have found that the rate of subunit exchange is noticeably slower (Fig. 8) under the lower ionic strength conditions used in earlier experiments (35).

Of all the biochemical parameters we have examined, temperature has the most pronounced effect on subunit exchange of αA-crystallin. The exchange rate increased markedly upon increasing the temperature from 37 to 42 °C (Fig. 9). This result is important in light of the potential roles of αA-crystallin in transcriptional regulation. Although αA-crystallin does not have a definitive nuclear localization sequence, it has been shown to translocate from the cytoplasm to the nucleus of NIH 3T3 cells under heat shock conditions (16). α-Crystallin has also been found to bind specifically downstream of the transcription initiation site on the γ-crystallin promoter (42). How does α-crystallin, which has an average molecular mass of 800 kDa, cross the nuclear membrane with an exclusion limit of approximately 70 kDa (43, 44)? We propose that the dissociation of α-crystallin into smaller subunits at high temperatures may explain its entry into the nucleus. Under normal physiological condition, subunit dissociation is largely prevented by the high activation energy barrier of 60 kcal/mol for the subunit exchange reaction, which would explain why nuclear localization is predominantly observed only at higher temperatures (16).

The interaction of αA-crystallin subunits most likely involves both ionic and hydrophobic interactions, since we have observed a decrease in the exchange rate under either very low salt or very high salt conditions. The effect of ionic strength on subunit exchange was not detected previously by isoelectric focussing (35). The negative result can be readily explained by the fact that these earlier experiments were all performed at low salt conditions to produce sharply focused protein bands. As a result, only a relatively narrow range of salt concentrations has been tested, which falls outside the region where the ionic interaction becomes significant.

Surprisingly, although changes in pH have been implicated to have a considerable effect on the subunit organization of α-crystallin (30, 31), we have found that the exchange rate is constant at pH values ranging from 7.5 to 9.2 (Fig. 7). Our result is in agreement with van den Oetelaar et al. (35), who also showed that subunit exchange is independent of pH.

Ca2+ is another biochemical parameter that we thought would have an influence on subunit exchange, since previous studies have reported the detection of large light scattering aggregates of α-crystallin when bovine lens is incubated in solution containing 4–8 mM Ca2+ (33, 45). Instead, we found that the exchange rate is independent of Ca2+ at concentrations as high as 50 mM (Fig. 7). It is not clear why α-crystallin in the lens is more susceptible to Ca2+-induced aggregation. One possible explanation is a change in subunit-subunit interaction due to post-translational modifications as the lens ages (46, 47), an effect that is absent with recombinant αA-crystallin.

Which region of the αA-crystallin molecule is involved in subunit exchange? Although several mutational analyses of α-crystallin have been reported, very little is known about the contact sites between its subunits (48, 49). Based on the high activation energy of 60 kcal/mol relative to other exchange reactions (50), we speculate that the subunit-subunit interactions may involve multiple binding sites. Recently, a stretch of 35 amino acid residues of Hsp42 has been implicated in subunit-subunit interaction (51). Comparison of the amino acid sequence of αA-crystallin to that of Hsp42 suggests residues 112–147 may be involved in the same function. Site-directed mutagenesis of αA-crystallin is currently under way to answer this question.

α-Crystallin has been shown to possess chaperone-like property that prevents protein aggregation during denaturation (9–14). Our study indicates that subunit exchange is not significantly affected by the binding of small polypeptides such as melittin or insulin B chain. In contrast, when α-lactalbumin or ovotransferrin bind to αA-crystallin, the subunit exchange rate is markedly reduced. This result implicates an association of larger polypeptides with several αA-crystallin subunits, effectively cross-linking them together and preventing them from dissociating. The multiple interactions may explain why the binding of denatured proteins to αA-crystallin is so strong and irreversible (9, 52).

Although the exact mechanism of chaperone-like activity is not still clear, it is tempting to speculate that α-crystallin most likely recognizes certain structures that are transiently exposed during unfolding of the protein. The domains for binding denatured protein and for subunit-subunit interaction are distinct, since α-crystallin containing bound proteins retains its multimeric subunit organization (37, 52). Moreover, α-crystallin is known to bind insulin B chain at room temperature (37), under which subunit exchange is largely inhibited (Fig. 9). If protein binding and subunit-subunit contact sites are located in a different part of the α-crystallin molecule, is there a relationship between subunit exchange and chaperone function? It is tempting to speculate that the rearrangement of α-crystallin subunits is essential for covering the unfolded polypeptides, thus shielding them from aggregation. This hypothesis would explain why the subunit exchange reaction (Fig. 9) and chaperone-like activity are markedly enhanced at high temperature (53).

Small heat shock proteins have been shown to protect cells from stress (54–57). Their ubiquitous tissue distribution, over-expression in a number of pathological states, and stress-induced cellular redistribution argue for their importance in safeguarding many important cellular processes. A hallmark of small heat shock proteins like α-crystallin is the large multimeric subunit organization (58, 59), which we have shown here to undergo continuous rearrangement through the exchange of subunits. The challenge in the coming years will be to explain why the oligomeric structure is important for the function of small heat shock proteins.

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