α-Crystallin Chaperone-like Activity and Membrane Binding in Age-Related Cataracts†

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ABSTRACT: α-Crystallin, the major protein component of the vertebrate lens, is thought to play a critical role in the maintenance of transparency through its ability to inhibit stress-induced protein aggregation. However, during aging and cataract formation the amount of membrane-bound α-crystallin increases significantly while high molecular weight complexes (HMWCs) comprised of α-crystallin and other lens crystallins accumulate. These and other recent data suggest a possible link between cataract formation, the formation of high molecular weight α-crystallin aggregates, and the progressive increase in membrane association of α-crystallin. To better understand these processes, we characterized the chaperone-like activity (CLA) and subunit exchange of membrane bound α-crystallin. In addition, we measured the membrane binding properties of in vitro constituted HMWCs to understand the mechanism by which increased α-crystallin is bound to the membrane of old and cataractous lens cells in vivo. Membrane-associated α-crystallin complexes have measurably reduced CLA compared to complexes in solution; however, membrane binding does not alter the time required for α-crystallin complexes to reach subunit exchange equilibrium. In addition, HMWCs prepared in vitro have a profoundly increased membrane binding capacity as compared to native α-crystallin. These results are consistent with a model in which increased membrane binding of α-crystallin is an integral step in the pathogenesis of many forms of cataracts.

The human lens is a tissue that must remain transparent throughout the lifetime of the individual in order to maintain visual acuity. Fiber cells in the nuclear region of the lens have lost all organelles and have been shown to be protein synthesis deficient, indicating an overall lack of protein turnover (*1*, *2*). Indeed, proteins synthesized during lens development are thought to remain in those cells throughout the lifetime of the individual, thus requiring a mechanism for stabilizing old and damaged proteins. However, this mechanism and its age-related failure (i.e., cataract formation) are not fully understood.

Lens fiber cells are rich in the small heat shock protein α-crystallin, which makes up nearly 50% of the total soluble protein and can reach concentrations in excess of 300 mg/mL (*3*). It is comprised of two closely related subunits, αA-crystallin (WTαA)[1] and αB-crystallin (WTαB), in roughly a 3:1 molar ratio in humans (*3*). α-Crystallin has been shown to inhibit stress-induced protein aggregation in vitro, and it has the ability to confer heat shock resistance to cell culture models (*4*−*6*). Indeed, this chaperone-like activity (CLA) is thought to play the critical role of preventing old and damaged proteins in the lens from forming inclusion bodies, which would result in light scattering and cataract (*5*).

Aside from CLA, α-crystallin is also known to selectively associate with certain types of intermediate filaments. One such lens-specific filament is known as the beaded filament (*7*). It has been proposed that α-crystallin may play a role in cell structure through aiding in the remodeling of the cytoskeleton during cell differentiation, although no direct in vivo evidence for such a model has yet been produced (*7*−*9*). α-Crystallin also associates with lens plasma membranes in vivo and in vitro, yet no function for this interaction has been demonstrated (*10*−*13*). To date, the biological significance of both these interactions is largely unknown.

The process of aging in the lens has been studied in detail from both biochemical and cellular aspects. Immunocytochemistry has shown that the nuclear region of the lens forms a syncytium, likely through membrane fusion during terminal fiber cell development (*14*). Interestingly, recent studies have demonstrated that, during aging and/or cataract formation, the border between the lens cortex and the nuclear syncytium becomes progressively less permeable to small metabolites that are thought to be required for maintaining the optimal

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[1] Abbreviations: WTαA, wild-type αA-crystallin; WTαB, wild-type αB-crystallin; PBS, phosphate-buffered saline; CLA, chaperone-like activity; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; FRET, fluorescence resonance energy transfer; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; SPH, egg sphingomyelin; Chol, cholesterol; HMWC, high molecular weight complex; *F*, fluorescence intensity.

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osmotic, nutritional, and oxidative balance (*15*−*17*). The plasma membrane itself also changes with age. The phospholipid content shifts such that hydrocarbon chain length and saturation increase, which suggests that fiber cell membranes become more rigid and less permeable (*18*, *19*). These events are all thought to play important roles in the mechanism of age-related nuclear cataract formation.

Many changes in the protein content of the lens have also been correlated with aging and the onset of cataract. Truncated, deamidated, glycated, and oxidized crystallins are common in old and cataractous lenses (*20*−*24*). High molecular weight complexes (HMWCs) comprised of α-crystallin associated with a wide variety of other lens proteins accumulate in the lens as age increases (*25*, *26*). It is thought that these complexes arise directly from α-crystallin's ability to bind damaged proteins and prevent their aggregation into inclusion bodies. In addition, α-crystallin is known to fractionate increasingly with the water-insoluble fraction of the lens with age, although the nature of that insoluble fraction has not been studied in detail (*27*−*31*). In separate reports, the amount of crystallin protein, particularly α-crystallin, found on the membranes during cataract formation is dramatically increased over the basal levels (*10*). These observations suggest that the water-insoluble pool of α-crystallin could be largely synonymous with the membrane-bound pool (*11*).

In our recent report on the characterization of the congenital cataract associated αA-crystallin mutation, R116C, we concluded that increased membrane binding capacity was one possible mechanism by which this mutant could cause cataracts (*32*). This conclusion raised the question about the possible role of membrane association in age-related cataracts. Given that the amount of membrane-associated crystallin protein increases with age and cataract formation, we proposed that increased membrane association of α-crystallin could be a common link between some types of congenital cataracts and age-related cataracts (*32*).

In the present study, we sought to understand the effect of known changes in the α-crystallin population on membrane binding to better understand the increased crystallin protein membrane association in old and cataractous lenses. To this end, we measured the CLA and subunit exchange of membrane-bound α-crystallin complexes as well as the binding capacities of two types of HMWCs. We found that membrane association did not prevent α-crystallin from acting like a chaperone nor did it alter subunit exchangeability, suggesting that other nonmembrane binding crystallins could become membrane bound through the CLA of membrane-associated α-crystallin. Interestingly, we also found that reconstituted HMWCs show significantly increased membrane binding capacities over native α-crystallin. Our data support a model in which increased membrane association of α-crystallin and other lens proteins, whether caused by HMWC formation in the cytoplasm, CLA of membrane-bound α-crystallin, or genetic mutation, is a critical event during cataract pathogenesis. This model may provide an explanation for the presence of the metabolite barrier seen in older lenses.

EXPERIMENTAL PROCEDURES

Overexpression and Purification of Human Recombinant αA- and αB-Crystallin. Human WTαA and WTαB crystallins were cloned into the pET23d expression plasmid (Novagen) and then expressed and purified from *Escherichia coli* (strain BL21) cultures essentially as described previously (*13*). Primary separations were performed on a DEAE-Sepharose anion-exchange column at pH 8.0, and final fractionations were performed on a Sephacryl S400HR (Pharmacia Biotech Inc.) column in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3). All proteins were determined to be >99% homogeneous as judged by the presence of a single band on a Coomassie blue stained SDS−PAGE gel (data not shown). Proteins were stored at −80 °C until use.

Protein Conjugation to AlexaFluor350 and AlexaFluor430. Purified recombinant α-crystallins and human aldose reductase (HAR) were conjugated with either AlexaFluor350 or AlexaFluor430 fluorescent tags as described by the manufacturer (Molecular Probes, Eugene, OR). Conjugated proteins were separated from nonreacted AlexaFluor reagent using prepacked desalting columns according to the manufacturer's protocol (Econo-Pac 10DG column, Bio-Rad, Hercules, CA). The purified conjugates were analyzed using A_{280}/A_{346} (for AlexaFluor350) or A_{280}/A_{434} (for AlexaFluor430) readings in a Varian Cary 1E UV/vis spectrophotometer. Protein concentration and degree of conjugation were determined for the AlexaFluor350 conjugates with the equations:

$$[\text{protein}] = \frac{[A_{280} - (A_{346} \times 0.19)] \times \text{dilution}}{\epsilon_{\text{protein}}} \quad (1)$$

$$\text{mol of dye/mol of subunit} = \frac{A_{346} \times \text{dilution}}{19000[\text{protein}]} \quad (2)$$

where 0.19 is a correction factor for the absorbance of AlexaFluor350 at 280 nm, 19000 is the molar extinction coefficient for AlexaFluor350, A_{280} and A_{346} are the measured absorbance values at 280 and 346 nm, respectively, and $\epsilon_{\text{protein}}$ is the molar extinction coefficient for α-crystallin. The specific activities of these α-crystallin conjugates were determined by analyzing known amounts in a Hoefer DynaQuant spectrofluorometer. The average specific activities were then calculated and expressed in F/μg of protein (F = fluorescence intensity). Likewise, concentration and conjugation efficiency for the AlexaFluor430 conjugates were calculated using the equations:

$$[\text{protein}] = \frac{[A_{280} - (A_{434} \times 0.28)] \times \text{dilution}}{\epsilon_{\text{protein}}} \quad (3)$$

$$\text{mol of dye/mol of subunit} = \frac{A_{434} \times \text{dilution}}{16000[\text{protein}]} \quad (4)$$

where 0.28 is a correction factor for the absorbance of AlexaFluor430 at 280 nm, 16000 is the molar extinction coefficient for AlexaFluor430, A_{280} and A_{434} are the measured absorbance values at 280 and 434 nm, respectively, and $\epsilon_{\text{protein}}$ is the molar extinction coefficient for α-crystallin.

Lens Plasma Membrane Fractionation. Bovine lenses were isolated and decapsulated, and the cortical fiber cells were removed using a scalpel. The membrane preparation was then performed using the cortical fiber cells according to Russell et al. (*33*). The dry weight was quantified by drying aliquots

of the membrane suspension in preweighed centrifuge tubes in a spin vacuum (Heto VR1, Denmark). Weight measurements were performed on an analytical balance (Mettler-Toledo, Columbus, OH), and the concentration (μg/mL) of membrane in the suspension was determined by averaging at least four replicate measurements.

Lipid Vesicle Formation. Dipalmitoylphosphatidylcholine (DPPC) and sphingomyelin (SPH) vesicles were formed using sonication as recommended by the manufacturer (Avanti Polar Lipids, Inc., Alabaster, AL). Briefly, lipids in chloroform were dried down to a film under argon and rehydrated for 1 h in PBS with stirring above the lipid-phase transition temperature. The rehydrated lipids were then sonicated until the solution became clear. The dry weight was quantified by drying aliquots of the vesicle suspension in preweighed centrifuge tubes in a spin vacuum (Heto VR1, Denmark). Weight measurements were performed on an analytical balance (Mettler-Toledo, Columbus, OH), and the concentration (μg/mL) of vesicle in the suspension was determined by averaging at least four replicate measurements.

Chaperone-like Activity (CLA) Measurements. Two types of measurements were used to determine the CLA of vesicle-bound α-crystallin. First, to determine the overall effect of vesicle binding on the total CLA of α-crystallin, DPPC vesicles were incubated with WTαA or WTαB for 15 h at 37 °C to allow binding to reach completion. Then the entire sample, containing both bound and unbound α-crystallin as would be found in vivo, was used in heat denaturation assays as described previously with human aldose reductase (HAR) as the substrate protein (*34*). In the second experiment, sphingomyelin vesicles were incubated for 15 h at 37 °C with WTαA or WTαB to allow binding to reach completion. The vesicles were then isolated from the soluble α-crystallin by centrifugation at 375000*g* for 2 h. These vesicles, with α-crystallin bound, were used in similar heat denaturation assays to measure the CLA of the vesicle-associated complexes in isolation.

Subunit Exchange Measurements. The rate of subunit exchange between soluble and membrane-bound α-crystallin was measured using fluorescence resonance energy transfer (FRET). AlexaFluor350-conjugated WTαA or WTαB were used as the fluorescence donors, and AlexaFluor430-conjugated WTαA or WTαB were used as the fluorescence acceptors. Lens membranes (500 μg) were saturated with the fluorescence donor conjugates for 6 h at 37 °C. Following the incubation, the membranes were pelleted (14000*g*, 30 min, 4 °C) and the supernatant was discarded. The pellets were resuspended in PBS containing either the corresponding fluorescence acceptor conjugate or nonlabeled α-crystallin (no acceptor) and then incubated for various times. At each time point, the samples were centrifuged for 30 min at 4 °C, and the pellet was analyzed for fluorescence. Fluorescence intensity of the donor in the presence (F_{da}) and absence (F_d) of the acceptor was measured to determine the efficiency of energy transfer (E) as calculated using the equation:

$$E = 1 - \frac{F_{da}}{F_d} \tag{5}$$

The time required to reach half-completion, $T_{0.5}$, was determined through curve fit analysis and used to determine the rate at which exchange reached equilibrium. The degree of removal was determined at the same time points by competition of bound fluorescence donor complexes with unlabeled α-crystallin as described previously and then compared to the time course of subunit exchange (*32*).

High Molecular Weight Complex Formation. Insulin/WTαA HMWCs were formed by incubation of WTαA−AlexaFluor350 conjugates and insulin (from bovine pancreas, Sigma, MO) under reducing conditions (10 mM dithiothreitol) at 37 °C for 30 min. Simultaneously, light scattering was monitored at 390 nm in a Cary 1E UV/vis spectrophotometer to verify suppression of insulin B chain aggregation by the WTαA conjugate. The resulting sample was centrifuged for 10 min at 10000*g* to remove any insoluble aggregates formed. The fluorescence specific activity was verified to ensure that the bound insulin did not alter the fluorescence intensity of the core α-crystallin complex. These soluble HMWCs were used in membrane binding experiments and were analyzed on a Superose 6 column as described below for the purification of HAR/WTαA HMWCs. Insulin stock solutions (10 mg/mL) were made by dissolving 10 mg of insulin (Sigma, St. Louis, MO) in 1 mL of dH_2O with 3 μL of 1 N NaOH added to dissolve the protein.

HAR/WTαA HMWCs were formed by incubation of WTαA and HAR at 52 °C for 1 h with monitoring by light scattering at 390 nm to verify suppression of HAR aggregation (data not shown). The resulting sample was filtered using a syringe filter (0.22 μm) and injected on a Superose 6 gel filtration column in PBS on a Pharmacia FPLC system (0.3 mL/min flow rate). The peak corresponding to the HAR/WTαA HMWC was pooled and then conjugated to AlexaFluor350 under standard conditions. After the conjugate was desalted, the protein was quantified using the Bradford protein assay with WTαA as the standard then used in membrane binding assays (*35*).

A standard elution curve for the Superose 6 column was constructed using IgM (~900 kDa), thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa). The molecular weights of the α-crystallin aggregates were calculated using their peak retention volumes.

Membrane Binding Assays. Membrane binding assays were performed essentially as previously described (*13*). AlexaFluor350-conjugated protein samples (see above) were incubated with bovine cortical fiber cell plasma membranes in binding buffer (PBS supplemented with 5 mM $MgCl_2$). Following the incubation, the sample was centrifuged at 14000*g* for 30 min at 4 °C and decanted. The pellets, containing plasma membrane and bound fluorescent protein, were then analyzed for fluorescence. The binding capacity was calculated from experiments in which 500 μg of membranes was used with a varied amount of conjugated protein. The horizontal asymptote of the saturation curve represents the maximum amount of protein able to bind a fixed amount of membrane.

RESULTS

Chaperone-like Activity of Membrane-Bound α-Crystallin. Chaperone-like activity is the best studied aspect of α-crystallin's biological function; however, little is known about the effect of membrane association on that activity. To measure the CLA of membrane-bound complexes, phospholipid vesicles were employed as the primary binding template

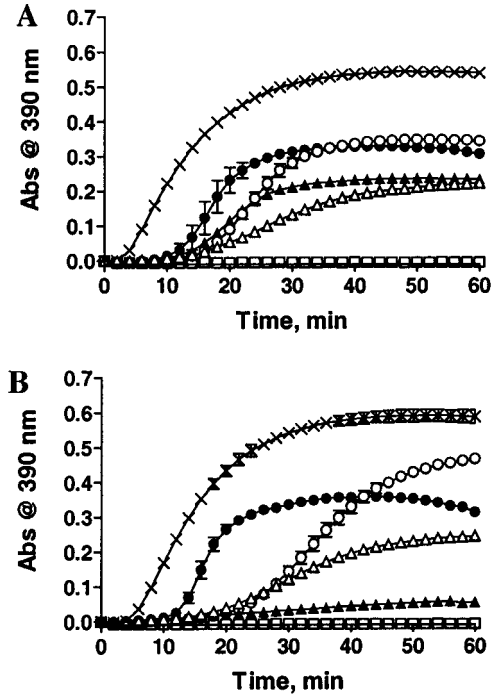

FIGURE 1: CLA of membrane-bound α-crystallin. (A) WTαA (1 nmol) and WTαB (2 nmol) were incubated with DPPC vesicles, and the mix of bound and soluble α-crystallin was assayed for CLA using the heat denaturation assay with HAR (2 nmol) as the substrate. A small difference ($p = 0.0010$) was seen between WTαA in the presence (△) and absence (▲) of DPPC vesicles. Curves with WTαB in the presence (○) and absence (●) of DPPC vesicles were not significantly different ($p = 0.1713$). For an aggregation positive control, HAR was incubated in the presence of DPPC vesicles but in the absence of α-crystallin (×). For an aggregation negative control, α-crystallin was incubated in the presence of DPPC vesicles but in the absence of HAR (□). (B) WTαA and WTαB were bound to SPH vesicles and then fractionated away from the soluble α-crystallin. The purified vesicles with α-crystallin bound (2 nmol of α-crystallin/assay) were then used in assays as before (with 2 nmol of HAR) with similar aggregation positive (×) and negative (□) controls. A measurable difference ($p = 0.0010$) was seen between SPH-bound WTαA (△) and unbound WTαA (▲). Likewise, a measurable reduction ($p = 0.0058$) in activity was seen between SPH-bound WTαB (○) and unbound WTαB (●).

because DPPC and SPH vesicle suspensions are essentially clear while fractionated lens membranes cause significant light scattering. The CLA was measured on two different mixtures for each homocomplex. First, DPPC vesicles were incubated with and without α-crystallin homocomplexes for 15 h at 37 °C to allow binding to reach completion. As controls, α-crystallin homocomplexes were incubated without vesicles under identical conditions. Then the entire sample volume, containing a mixture of approximately 65% bound and 35% unbound α-crystallin (data not shown), was used in heat denaturation chaperone assays with human aldose reductase (HAR) as the substrate (Figure 1A). For WTαA samples, a molar ratio of 1:0.5 (HAR to WTαA) was used, whereas the ratio was 1:1 for WTαB assays. We found a small difference in the CLA curves ($p = 0.0010$) resulting from WTαA mixtures as compared to the vesicle-free α-crystallin controls, although the total amount of HAR aggregation remained similar. For WTαB, the mixture containing both bound and unbound protein had indistinguishable CLA compared to the soluble protein alone ($p = 0.1713$). Crystallin-free reactions confirmed that the presence of vesicles did not alter the amount of HAR aggregation, while α-crystallin-only controls showed no aggregation in the absence of HAR.

The second experiment was similar to the DPPC experiments above, except that the vesicles, made of SPH and containing bound α-crystallin, were first isolated from the soluble pool before being used in the CLA assays with HAR in order to directly measure the CLA of bound α-crystallin in isolation (Figure 1B). All assays were done with a 1:1 molar ratio of HAR to α-crystallin. We found that the activity of bound complexes was reduced ($p = 0.0010$ for WTαA samples and 0.0058 for the WTαB samples) compared to the activity of an equal amount of soluble α-crystallin; however, significant CLA of bound complexes was observed.

Subunit Exchange and Reversibility of α-Crystallin Membrane Binding. We have reported previously that membrane association of α-crystallin is partially reversible (*13*). The conclusion from those experiments was that the partial reversibility of binding is likely due to subunit exchange between membrane-bound and soluble α-crystallin rather than removal of intact complexes. To better understand the mechanism of reversibility and the in vivo equilibrium that would result, we measured α-crystallin subunit exchange between membrane-bound and soluble α-crystallin using fluorescence resonance energy transfer (FRET). Lens membranes were first saturated with AlexaFluor350-conjugated WTαA or WTαB to serve as the fluorescence donors. Upon completion of binding, the membranes containing bound α-crystallin were isolated with centrifugation, and the resulting pellet was resuspended in PBS containing either nonconjugated α-crystallin (no fluorescence acceptor) or AlexaFluor430-conjugated α-crystallin (the fluorescence acceptor) for varying times. The times to reach half-completion of subunit exchange ($T_{0.5}$) for both WTαA and WTαB (4.0 ± 0.7 and 1.5 ± 0.2 h, respectively) were not statistically different ($p = 0.7843$ and 0.7166, respectively) to the time to release 50% of removable AlexaFluor350 conjugates through competition with nonconjugated α-crystallin (Figure 2, Table 1).

Formation of α-Crystallin High Molecular Weight Complexes. High molecular weight α-crystallin complexes (HMWCs) composed of α-crystallin and a variety of other lens proteins can be formed in vivo (*25*, *36*−*39*). These HMWCs, presumably resulting from the CLA of lens α-crystallin toward damaged lens proteins, accumulate with age and with the onset of cataracts (*25*, *36*−*39*). It has also been shown that lens proteins, particularly α-crystallin, increasingly fractionate with the water-insoluble fraction of the lens with age and the onset of cataracts (*10*, *11*). To determine the effect of forming HMWCs on the membrane binding properties of α-crystallin, we made large aggregates in vitro for binding studies.

We formed two HMWCs to control for the possible binding differences between aggregates of varying composition. First, α-crystallin/insulin complexes were made by incubating equal weights of AlexaFluor-350-conjugated WTαA and insulin under reducing conditions at 37 °C for 30 min, as described in Experimental Procedures. Aggregation of insulin's B chain with and without α-crystallin was monitored at 390 nm to ensure that the aggregation was blocked by the presence of WTαA. Under these conditions,

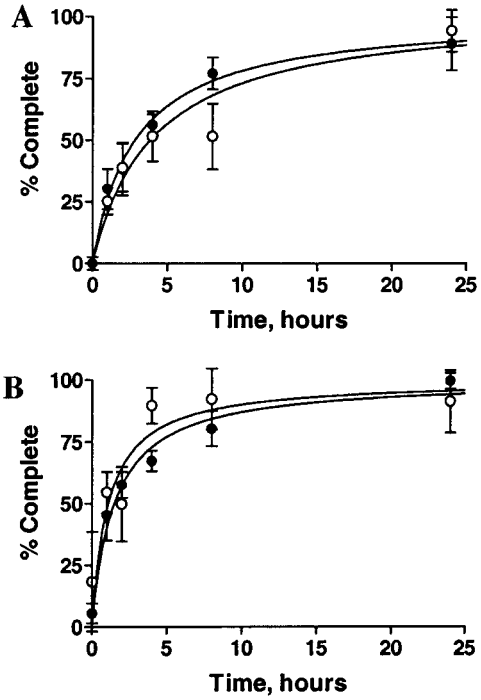

FIGURE 2: Subunit exchange of membrane-bound α-crystallin. Lens membranes were saturated with AlexaFluor350 α-crystallin conjugates which were subsequently competed off with nonconjugated (no fluorescence acceptor) or AlexaFluor430-conjugated α-crystallin. The time course curves are shown for simple competitive removal in percent complete (●) and fluorescence energy transfer efficiency in percent complete (○). 100% complete indicates the incubation had reached equilibrium; it should not be assumed that 100% of all protein was removed or exchanged. (A) The time courses for reversibility and exchange between bound and soluble WTαA were indistinguishable. (B) The time courses for reversibility and exchange between bound and soluble WTαB were also indistinguishable.

Table 1: Summary of Data[a]

	WTαA	WTαB
reversibility ($T_{0.5}$) (h)	2.9 ± 0.5	1.1 ± 0.3
exchange ($T_{0.5}$) (h)	4.0 ± 0.7	1.5 ± 0.2
p value	0.7843	0.7166

[a] The time to reach half-completion ($T_{0.5}$) for removal and subunit exchange of membrane-bound α-crystallin complexes with soluble α-crystallin complexes. An unpaired *t*-test indicated that the differences in $T_{0.5}$ values were not significantly different for either protein.

WTαA was the limiting reagent and was thus functionally saturated with insulin, as shown by the lack of complete inhibition of aggregation (Figure 3). The mixture was centrifuged briefly to clear the small amount of aggregated insulin; then the total supernatant was used in the membrane binding studies described below. Due to the use of conjugated WTαA in these HMWCs, no post-HMWC formation labeling was required, and purification of these complexes was not performed. However, they were analyzed by gel filtration on a Superose 6 column using an FPLC system. Despite the apparent saturation of α-crystallin CLA (Figure 3), we found that nearly half of the α-crystallin added to this assay remained approximately 550 kDa in size, which corresponds to the elution of pure recombinant α-crystallin (Figure 4). The remaining portion of α-crystallin was found in the first peak, which eluted in the void volume of the column, indicating a molecular mass greater than 2000 kDa. Other

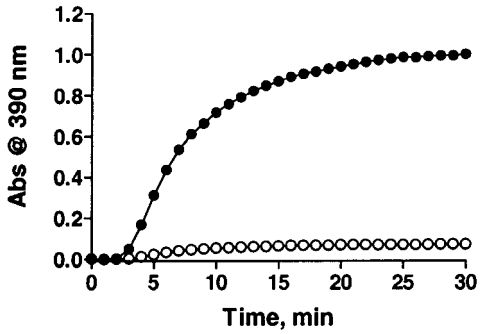

FIGURE 3: Formation of insulin/WTαA HMWCs. Insulin was incubated for 30 min at 37 °C with (○) and without (●) WTαA under reducing conditions to allow α-crystallin to bind denaturing insulin B chains. Light scattering was measured at 390 nm to observe HMWC formation (i.e., inhibition of insulin aggregation).

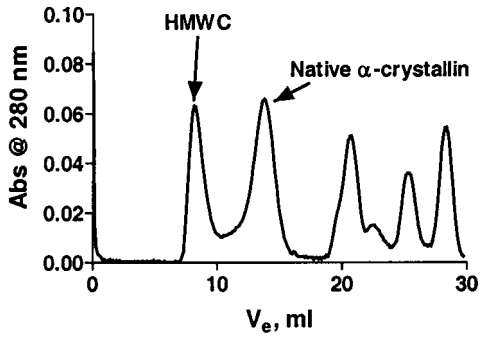

FIGURE 4: FPLC analysis of insulin/WTαA HMWCs. Insulin/WTαA HMWCs were loaded onto a Superose 6 gel filtration column on a FPLC system to determine the size distribution of α-crystallin in the sample. The α-crystallin is found in both HMWCs and native-sized complexes (see arrows) in roughly a 50:50 ratio. The column was run in PBS at room temperature at 0.3 mL/min while monitoring the eluate with A_{280} readings.

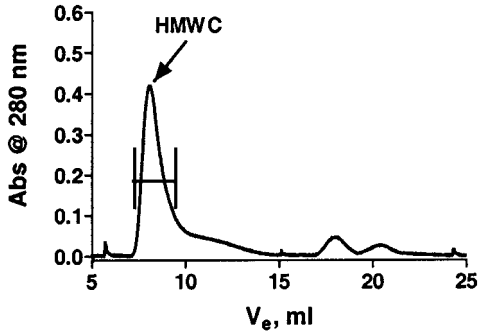

FIGURE 5: FPLC analysis of HAR/WTαA HMWCs. HAR/WTαA HMWCs were loaded onto a Superose 6 gel filtration column on a FPLC system to purify the HMW aggregate (arrow). The column was run in PBS at room temperature at 0.3 mL/min and monitored with A_{280} readings. The protein was pooled (brackets) and then conjugated with Alexa-Fluor350 for membrane binding studies.

peaks in the elution profile likely represent variously sized soluble insulin aggregates.

The second form of HMWC contained WTαA and HAR. Nonconjugated WTαA was incubated with HAR for 60 min at 52 °C to allow α-crystallin to block HAR aggregation (light scattering data not shown). The resulting solution was run on a Superose 6 gel filtration column to purify the HMWCs from all other soluble proteins (Figure 5). The HMWC peak eluting at the column void volume was pooled (see bracket in Figure 5) and conjugated with AlexaFluor350. The presence of both WTαA and HAR in the HMWC pool

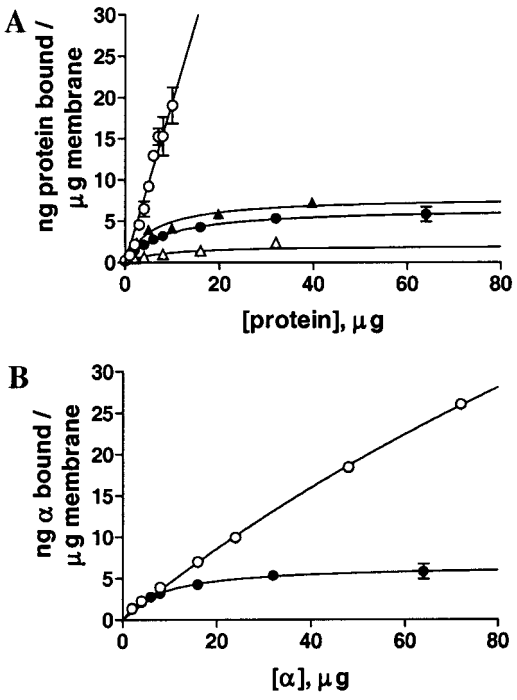

FIGURE 6: Membrane association of α-crystallin HMWCs. Lens membranes were incubated with a varied amount of α-crystallin HMWC for 6 h at 37 °C to allow binding to reach completion. (A) HAR/WTαA HMWCs (○) binding to membranes compared to the controls, heat-treated WTαA (▲), non-heat-treated WTαA (●), and HAR alone (△). (B) Insulin/WTαA HMWCs (○) binding to membranes compared to the WTαA control (●). In both cases, the binding capacities of HMWCs are dramatically increased over that of native WTαA.

was confirmed by SDS−PAGE (data not shown). Once conjugated, these complexes were used in the binding assays described below. As a control, WTαA was heated for 1 h at 52 °C alone and then used in the binding assays described below.

HMWC Membrane Binding. Both the insulin/WTαA and HAR/WTαA aggregates were used in lens membrane binding experiments as previously described for native α-crystallin complexes. After a 6 h incubation at 37 °C, the membranes were pelleted in a centrifuge for 30 min and then analyzed for fluorescence. For the purified HAR/WTαA aggregates, the binding capacity was linear over the measurable range and dramatically increased compared to either HAR or WTαA alone (Figure 6A). The assays were performed to the solubility limit of the HMWC, and no significant difference ($p = 0.5370$) was seen between the 52 °C treated and non-heat-treated WTαA controls (Figure 6A). We also examined the insulin/WTαA aggregate, which contained any remaining soluble insulin as well as AlexaFluor350-tagged subunits associated equally with both HMWCs and native-sized complexes. This mixture of α-crystallin complexes bound approximately half as much as the HAR/WTαA aggregates but still much more than WTαA alone (compare panels A and B of Figure 6). The insulin present in these binding assays was not fluorescently conjugated and thus did not increase the background of these readings.

DISCUSSION

Previous studies have demonstrated that, with increased age and/or the onset of cataracts, an increase in the amount of membrane-bound crystallin proteins, particularly α-crystallin, is seen (*10*, *11*). In our report on the characterization of the autosomal dominant αA R116C protein, we found that this mutation results in dramatically increased membrane binding capacity (*32*). These observations led us and others to hypothesize that increased membrane association was involved with the formation of at least some types of cataracts. In the present study, we investigated the relationship between α-crystallin CLA and membrane association with age-related changes in the lens protein population. We found a direct correlation between the formation of high molecular weight aggregates and increased membrane binding, supporting a model in which increased membrane association is a key event in the pathway leading to cataracts.

The model proposes that increased membrane association is deleterious with respect to lens transparency. It is important, therefore, to understand the relationship between the CLA of the protein complex and membrane binding. To this end, we measured the CLA of vesicle-bound α-crystallin in two slightly different assays. First, a mixture of DPPC vesicles with bound and unbound WTαA or WTαB in equilibrium was tested for activity in a heat denaturation assay with HAR as the substrate (Figure 1A). We found a small but measurable difference in the inhibition of HAR aggregation when vesicle-bound α-crystallin was in equilibrium with soluble α-crystallin. In a separate experiment, sphingomyelin vesicles with bound WTαA or WTαB were isolated from the soluble α-crystallin and used in CLA measurements. Again, we found a decrease, but not elimination, of activity (Figure 1B). The observed decrease in activity seen may be due to 25−45% of the complex being buried within the lipid bilayer, as previously postulated (*13*). Considering the extremely high concentration of soluble α-crystallin compared to the amount normally associated with the membranes in vivo, we conclude that the overall effect of membrane association on α-crystallin CLA in the lens must be very small, provided that significant amounts of α-crystallin remain soluble. In addition, neither β- nor γ-crystallin bind to membranes alone. Therefore, CLA of membrane-bound α-crystallin could at least partially account for the presence of β- and γ-crystallins on the membranes of old lenses.

It has been proposed that the active unit for α-crystallin CLA is actually a dimer or tetramer of subunits (*40*). This model implies that subunit movement into and away from intact native complexes (i.e., subunit exchange) is a requirement for activity. In addition, we previously measured partial reversibility in the membrane-bound complexes which was interpreted to be a result of subunit exchange with soluble complexes (*13*). To provide support for these proposals and to gain a better understanding of the equilibrium that exists between bound and unbound α-crystallin complexes, we measured exchange between membrane-associated and soluble α-crystallin using FRET (Figure 2). We found that the time course for subunit removal by simple competition with nonfluorescent α-crystallin subunits corresponds directly with the time course of subunit exchange between fluorescent α-crystallin conjugates as measured by FRET. These results indicate that the reversibility previously reported (*13*) is indeed subunit exchange between membrane-bound but solvent-exposed α-crystallin subunits and soluble α-crystallin complexes. The ability to function like a chaperone and

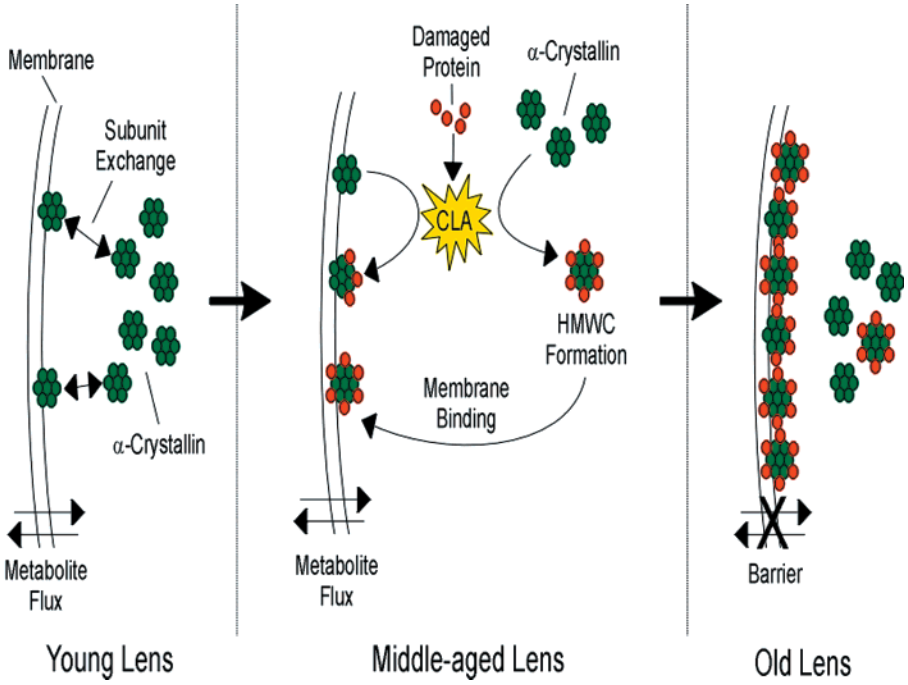

FIGURE 7: Schematic of the HMWC binding model and its proposed role in barrier formation. In young lenses, an equilibrium is established between membrane-bound and soluble α-crystallin via subunit exchange. These lenses are also characterized by free metabolite diffusion between the nuclear syncytium and the cortex. As age increases, soluble lens proteins accumulate damage and begin to denature. Both membrane-bound and soluble α-crystallin inhibit stress-induced protein aggregation. However, CLA results in the formation of HMWC both in solution and on the membrane, although the soluble HMWCs quickly become membrane bound. These lenses are still able to maintain metabolite diffusion. With time, so many HMWCs accumulate on the lens membranes that the flow of small molecules between the nucleus and cortex is blocked, forming the barrier previously described in old and cataractous lenses (*15*−*17*).

undergo subunit exchange while bound to the membrane also supports the proposal in which subunit exchange must be maintained for activity (*40*). Finally, these observations suggest that membrane-bound and cytoplasmic α-crystallins are normally in exchange-mediated equilibrium in vivo.

It is well-known that HMWCs, comprised of α-crystallin and a variety of other proteins, form in the lens with age (*25*, *36*−*39*). It is believed that these complexes arise as a direct result of α-crystallin binding to damaged proteins in the lens, thus preventing the formation of light scattering protein inclusion bodies (*41*, *42*). However, the formation of such complexes is accompanied by the accumulation of lens proteins on the plasma membrane of fiber cells (*10*, *11*). To determine if these events are related, we formed high molecular weight α-crystallin complexes in vitro using HAR and insulin as substrates and measured the membrane binding of the resulting HMWCs (Figure 6). These complexes have a significantly higher membrane binding capacity than does native α-crystallin. We also show that, with purified HAR/WTαA HMWCs, the binding is roughly double that of insulin/WTαA complexes (compare panels A and B of Figure 6). This may be explained by the fact that only half of the WTαA in the insulin HMWC mixture is in large aggregates and the other half is present in native-sized complexes (Figure 4). Interestingly, these data suggest that HMWCs formed in vivo could be sequestered to the membrane, away from the soluble native α-crystallin. In addition, HMWC binding could account for the membrane-associated β- and γ-crystallins in old and cataractous lenses. On the basis of these observations, we conclude that the increased binding in these samples, as compared to native WTαA, is directly proportional to the concentration of HMWCs. This demonstrates another strong link between the CLA of α-crystallin and increased membrane association of the crystallins.

In the present study, we have shown that known age-associated changes in α-crystallin, namely, HMWC formation, have a dramatic impact on membrane association. Given that these changes occur concurrently with increased age and with cataract formation, it is reasonable to hypothesize that the build up of membrane-bound crystallins is a key component within the mechanism of age-related cataract formation. This model is further supported by the recent characterization of the αA R116C mutant in which we proposed that its self-forming, polydisperse HMWC and increased membrane association are involved with formation of that type of congenital cataract.

To date, it is unclear what effect α-crystallin membrane association has on the membrane that could lead to cataract formation. Although little is known about the maintenance of membrane transparency in the lens, it is possible that extensive α-crystallin binding could create light scattering centers on membranes that accumulate to a point where a cataract is formed. However, an additional possibility relates to the metabolite barrier reported to form in old and cataractous lenses (*15*−*17*). The loss of metabolite flux in the lens is believed to lead to a drop in reduced glutathione in the nucleus and, consequently, to high levels of lipid and protein oxidation (*15*). This oxidation would presumably result in further protein damage leading directly to increased formation of HMWCs with α-crystallin. Therefore, we propose that the barrier between the nucleus and cortex of the lens is formed at least partially as a result of excess HMWC membrane binding (Figure 7). α-Crystallin binding could potentially disrupt the lipid bilayer to the point of

causing membrane instability, which could have deleterious effects on the function of membrane channels. Another possibility is that these large complexes could directly or indirectly block the pores of gap junctions and aquaporins through high-capacity interactions, such as those seen in the present binding assays.

Our data, in conjunction with the numerous cited reports from other investigators regarding the distribution, CLA, and membrane association of α-crystallin as well as morphological and biochemical observations of the aged and cataractous lens, support a model in which excess membrane association of α-crystallin is a critical event in some forms of cataracts. Indeed, enhanced membrane binding by α-crystallin could provide a possible mechanism by which the metabolite barrier is formed in old and cataractous lenses.

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