Characterization of α-Crystallin-Plasma Membrane Binding

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Abstract

α-Crystallin, a large lenticular protein complex made up of two related subunits (αA- and αB-crystallin), is known to associate increasingly with fiber cell plasma membranes with age and/or the onset of cataract. To understand better the binding mechanism, we developed a sensitive membrane binding assay using lens plasma membranes and recombinant human αA- and αB-crystallins conjugated to a small fluorescent tag (Alexa350®). Both αA and αB homopolymer complexes, as well as a reconstituted 3:1 heteromeric complex, bind to lens membranes in a specific, saturable, and partially irreversible manner that is sensitive to both time and temperature. The amount of α-crystallin that binds to the membrane increases under acidic pH conditions and upon removal of exposed intrinsic membrane protein domains but is not affected at high ionic strength, suggesting that α-crystallin binds to the fiber cell plasma membranes mainly through hydrophobic interactions. The binding capacity and affinity for the reconstituted 3:1 heteromeric complex were measured to be 3.45 ± 0.11 ng/μg of membrane and 4.57 ± 0.50 × 10⁻⁴ μg⁻¹ of membrane, respectively. The present membrane binding data support the hypothesis that the physical properties of a mixed α-crystallin complex may hold particular relevance for the function of α-crystallin within the lens.

α-Crystallin is a large protein complex comprised of 30–40 copies of the A (αA-crystallin) and B (αB-crystallin) subunits in roughly a 3 αA to 1 αB molar ratio and can represent up to 50% of the total protein in vertebrate lenses (1,2). Human αA- and αB-crystallin polypeptides have molecular masses of about 20 kDa, making the 3:1 αA/αB heteromeric complex found in the lens between 600 and 800 kDa (1). Each subunit shows significant sequence homology to small heat shock proteins such as mouse HSP25 and human HSP27, but they diverge somewhat in their NH₂ and COOH termini (1,3,4). Both protein subunits are also known to exchange readily between soluble α-crystallin complexes in a time- (>6 h) and temperature- (>25 °C) dependent manner, suggesting that the heteromeric complex has a dynamic quaternary structure (1,5).

In addition to their many similarities, αA- and αB-crystallin have some notable differences. First, αA-crystallin is expressed almost exclusively in lens fiber cells, whereas αB-crystallin is expressed strongly in both the lens and non-ocular tissues such as heart, liver, and brain (6). Second, a study utilizing two-dimensional ¹H NMR spectroscopy demonstrated that αA-crystallin is more stable than αB-crystallin with respect to the denaturing effects of extreme temperature, pH, and chaotropic agents such as urea and guanidine (7). Interestingly, studies using knockout mice suggest major differences in the in vivo roles of αA- and αB-crystallins. Deletion of αB-crystallin has little effect on lens morphology; however, the removal of αA-crystallin causes early onset cataract associated with accumulation of inclusion bodies.

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containing large amounts of αB-crystallin (8,9). From these observations, it seems likely that these two subunits are not functionally equivalent and that there may be some functional significance in the combination of αA and αB subunits found in the native complex.

Despite these observations, the biological roles of α-crystallin are not yet firmly established. It is thought to give vertebrate lenses their refractive and transparency properties through short range ordering, which could result from the repulsive behavior of the native protein complex (10–13). In addition, α-crystallin has been shown to inhibit stress-induced aggregation of proteins in vitro, reflecting a potential in vivo role in maintenance of lens transparency (14–18). Finally, α-crystallin is known to bind subcellular elements such as intermediate filaments and plasma membranes (19–22), but these interactions are not well characterized, nor is it clear what role they may play in lens biology (23).

It is well known that the amount of soluble α-crystallin found in the cytoplasm of lens fiber cells falls steadily with increasing age and that this change is mirrored by an increase in the water-insoluble fraction of α-crystallin (24–28). The prevailing explanation for these observations is that the amount of soluble α-crystallin available to bind partially denatured proteins becomes depleted with age, and the resulting high molecular mass aggregates slowly become insoluble. Interestingly, the amount of crystallin protein, especially α-crystallin, bound to the membrane also increases dramatically with increasing age and/or cataract formation (29,30). It could be that the progressive insolubilization of α-crystallin is due, in part, to increased membrane binding that is also associated with aging and cataract formation. These observations suggest that increased membrane association of α-crystallin may be closely correlated with the loss of transparency in the lens, thus underscoring the need to understand this interaction in greater detail.

To date, few studies have examined the in vitro conditions that promote binding of α-crystallin to membranes. In a previous study, the interaction of native α-crystallin with membranes was shown to be markedly sensitive to ionic strength while reaching an optimum at 37 °C and a pH of 7.5 (22). In addition, saturable binding to protein-free phospholipid vesicles has been observed, but the measured capacity is reduced dramatically upon incorporation of cholesterol (31–33). Finally, it has been postulated that αB-crystallin is largely unable to bind membranes in the absence of αA-crystallin (20,23).

To understand better the nature of α-crystallin membrane association and the relative contribution of each α-crystallin subunit to membrane binding, we developed a binding assay using extracted bovine cortical fiber plasma membranes and recombinant human αA- and αB-crystallins. The effects of time, temperature, ionic strength, and pH on the membrane association of both homopolymeric complexes and the 3:1 heteromeric complex were compared. In addition, the reversibility, binding capacities, and binding affinities for each complex were determined. Our results show that α-crystallin homopolymeric complexes composed of αA- or αB-crystallin interact with lens fiber cell plasma membranes in a similar but distinct manner and that membrane binding of the heteromeric complex containing αA and αB subunits in a 3:1 molar ratio differs from either of the homopolymeric complexes.

**EXPERIMENTAL PROCEDURES**

**Overexpression and Purification of Recombinant Human αA- and αB-Crystallin**

Cloning of human αA-crystallin cDNA was described previously (15). A full-length human αB-crystallin cDNA was obtained as an IMAGE clone (GenBank accession number N35834) from Genome Systems, Inc. (St. Louis). Coding regions of each cDNA were cloned into pET23d(+) (Novagen) for overexpression in *Escherichia coli* strain BL21, and purification was performed essentially as described previously, with two minor changes (15). First, primary
separation was performed on a DEAE-Sepharose ion exchange column at pH 8.0. Second, all columns were operated at 4 °C. Proteins were estimated to be >99% homogeneous judging from the appearance of a single band following SDS-PAGE\(^1\) and Coomassie Blue staining. The 3:1 αA to αB crystallin heteromeric complex was made by combining the two proteins in the appropriate molar ratio and incubating them for 24 h at 37 °C in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na\(_2\)HPO\(_4\), 1.4 mM K\(_2\)HPO\(_4\), pH 7.3) (2,5,34,35).

**Overexpression and Purification of Recombinant Human γD-Crystallin**

Cloning, overexpression, and purification were performed exactly as described previously (15). The protein was stored at −80 °C in PBS until use.

**Lens Plasma Membrane Fractionation**

Bovine lenses were isolated, decapsulated, and the outer half (*i.e.* cortical fiber cells) removed using a scalpel. Membrane preparation was then performed using the cortical fiber cells as described by Russell *et al.* (36). The dry weight was quantified by drying aliquots of the membrane suspension in pre-weighed 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 determined by averaging at least four repetitions.

**α-Crystallin Conjugation to Alexa350®**

Purified recombinant αA-and αB-crystallin were conjugated to the Alexa350® fluorescent tag (molecular weight 410) as described by the manufacturer (Molecular Probes, Eugene, OR). Briefly, α-crystallin subunits were mixed with Alexa350® powder in PBS supplemented with 100 mM sodium bicarbonate. Conjugation was allowed to proceed for 1 h at room temperature. The reaction was stopped with the addition of hydroxylamine. Conjugated protein was separated from nonreacted Alexa350® using a prepacked desalting column according to the manufacturer’s protocol (Bio-Rad Econo-Pac 10DG column). The purified Alexa350®-conjugated α-crystallin subunit was analyzed using \(A_{280}/A_{346}\) readings in a Varian Cary 1E UV-visible spectrophotometer. Protein concentration and degree of conjugation (*i.e.* conjugation efficiency) were determined with the following equations,

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

\[
\text{mol of dye/mol of subunit} = \frac{A_{346} \times \text{dilution}}{19,000 \times [\text{protein}]} \quad (\text{Eq. 2})
\]

where 0.19 is a correction factor for the absorbance of Alexa350® at 280 nm, 19,000 is the molar extinction coefficient for Alexa350®, \(A_{280}\) and \(A_{346}\) are the measured absorbance values at 280 and 346 nm, respectively, and \(\varepsilon_{\text{protein}}\) is the molar extinction coefficient for α-crystallin.

Covalent attachment of Alexa350® to the α-crystallin subunits was verified by denaturing the α-crystallin conjugates with 6 M urea followed by dialysis for 16 h into 2,000 volumes of PBS.

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\(^1\)The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; F, fluorescent unit(s); CLA, chaperone-like activity; bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; BSA, bovine serum albumin; MES, 4-morpholineethanesulfonic acid; \(K\), binding constant; \(B\), binding capacity.

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using a 14000 MWCO membrane (Spectra/Por®). The number of mol of Alexa350® detected per mol of α-crystallin protein subunit before and after urea treatment and dialysis was not significantly different (data not shown).

The specific activity of the α-crystallin conjugates was determined by analyzing known amounts in a Hoefer Dyna-Quant Spectrofluorometer. The average specific activity was then calculated and expressed in $F/\mu g$ of protein ($F = $ fluorescence units). Also, emission spectra were collected using an LS50B luminescence spectrometer (Perkin-Elmer) with an excitation wavelength of 346 nm.

**Chaperone-like Activity (CLA) Measurements**

CLA measurements were determined on the Alexa350®-conjugated and nonconjugated αA- and αB-crystallin using the previously described insulin reduction chaperone assay (17,37–40). The insulin stock solution (1 mg/ml) was made by dissolving 10 mg of insulin (Sigma) in 10 ml of dH$_2$O with 10 μl of 1 N NaOH added to dissolve the protein. The activity was calculated by the following equation, then scaled such that nonconjugated α-crystallin had 100% activity.

$$\text{Activity} = \left(\frac{\Delta A_{390}(\text{insulin only}) - (\text{insulin} + \alpha - \text{crystallin})}{\Delta A_{390}(\text{insulin only})}\right) \times 100$$  
(Eq. 3)

**Molecular Mass Determination**

Conjugated and nonconjugated homopolymers were run on a Superose 6 size exclusion column (1.6 × 60 cm) controlled by a Pharmacia FPLC system at a flow rate of 0.3 ml/min. The buffer contained 20 mM Tris-HCl, pH 7.6, 200 mM NaCl, and 0.5 mM EDTA. A standard curve was constructed using IgM (~900 kDa), thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa). The molecular masses of the α-crystallin samples were calculated using their peak retention volumes.

**Trypsin Treatment of Lens Plasma Membranes**

Lens plasma membranes were pelleted by centrifugation at 14,000 × $g$ for 30 min at 4 °C and decanted. The membranes were then resuspended in buffer T (10 mM bis-Tris, pH 6.5, and 0.1 mM MgCl$_2$). Trypsin (Sigma) was added to a final concentration of 10 μg/ml, and the mix was allowed to incubate at 37 °C for 3 h. The reaction was stopped by the addition of antipain serine protease inhibitor (Sigma) to a final concentration of 10 μM. The membranes were centrifuged at 14,000 × $g$ for 30 min and decanted. The pellet was washed twice with PBS. The final pellet was resuspended in PBS containing 10 μM antipain inhibitor.

**Membrane Binding Measurements**

Alexa350®-conjugated αA- or αB-crystallin was incubated with bovine cortical fiber cell plasma membranes in binding buffer (PBS supplemented with 5 mM MgCl$_2$, except in the pH dependence measurements; see below). In control assays, bovine serum albumin (BSA) or recombinant human γD-crystallin was added to the reactions in a 1:1 ratio with α-crystallin. After the incubation, the sample was centrifuged at 14,000 × $g$ for 30 min at 4 °C and decanted. The pellet, containing plasma membrane and bound Alexa350®-conjugated α-crystallin, and the supernatant were then analyzed for fluorescence. The degree of binding ($\bar{X}$) was calculated by the following formula.
The binding capacity was calculated from experiments in which 500 μg of membranes were used with a varied amount of conjugated α-crystallin protein. The horizontal asymptote of the saturation curve represents the maximum amount of α-crystallin able to bind a fixed amount of membrane.

Other binding experiments were performed using this general approach, only with varied time (0–16 h), temperature (4–45 °C), or NaCl concentration (0–1 M). The effect of pH was determined using a previously described Tri-buffer system (Tris, MES, and acetate) that was designed to give high buffering capacity at a wide pH range (5 to 9 pH) while keeping the ionic strength constant (41).

Reversibility was assessed by first binding Alexa350®, conjugated aA- or aB-crystallin to a membrane sample and then decanting the unbound α-crystallin protein following the standard centrifugation. Next, the membrane-α-crystallin complex was resuspended in either binding buffer only or binding buffer containing a molar excess of nonconjugated α-crystallin and incubated at 37 °C for 24 h. As controls, the second incubations were also performed in the presence of α-crystallin and either human γD-crystallin or BSA. The degree of removal was determined through fluorescence analysis of the pellet and supernatant immediately after the second incubation.

The binding constants were determined by performing a series of binding assays with varied membrane (i.e., substrate) concentration. The apparent binding constant ($K_{\text{app}}$) was calculated for a wide range of membrane concentrations, according to the equation below, and then graphed in a $\log_{10} K_{\text{app}}$ versus $\log_{10}$ [membranes] plot.

$$K_{\text{app}} = \frac{X}{(1 - X) \times [\text{membranes}]}$$

(Eq. 5)

The overall binding constant ($K$) for noncooperative binding events was then obtained by the inverse logarithm of the y axis of the linear fits. For the cooperative binding event, the binding constants ($K_{\text{high}}$ and $K_{\text{low}}$) were determined by the inverse logarithms of the y axes of the horizontal asymptotes from nonlinear regression fitting.

RESULTS

α-Crystallin Overexpression and Purification

αA- and αB-crystallin were both overexpressed in E. coli as described previously. Purification led to homogeneous proteins of greater than 99% purity, as judged by SDS-PAGE and Coomassie Blue staining (Fig. 1, lanes 2 and 3).

Membrane Fractionation

Plasma membranes were extracted from 20 bovine lenses as described under “Experimental Procedures.” The final product was analyzed with SDS-PAGE, which showed no detectable α-crystallin upon zinc negative staining and Coomassie Blue staining. The only visible band was at a position corresponding to the major intrinsic membrane protein MP26 (Fig. 1, lane 1) (36).
The membrane sample was also analyzed by transmission electron microscopy. As expected, the trilayered gap-junction-associated membrane structures were clearly visible at a magnification of × 90,000. The membranes were smooth on both sides, indicating that α-crystallin complexes normally associated with lens membranes were removed by the extraction procedure (data not shown).

**Analysis of α-Crystallin Alexa350® Conjugates**

Conjugation of Alexa350® to either αA- or αB-crystallin was performed numerous times throughout the course of the present study. The conjugation reaction proved to be very consistent for both α-crystallin subunits (Table I). On average, 0.73 ± 0.12 mol of Alexa350® was attached per αA-crystallin subunit, whereas 1.0 ± 0.12 mol was conjugated to each αB-crystallin subunit. In addition, the absorbance and emission spectra, characterized by an absorbance peak at about 346 nm and an emission peak at about 440 nm, matched those published previously (42) (data not shown).

To determine the specific activity, known amounts of each Alexa350® conjugate were analyzed for fluorescence. The specific activity was determined by taking an average of measurements at a wide range of protein concentrations (Table I). These results demonstrated the high sensitivity and reproducibility of detection. Linearity of the fluorescence extended to at least 100 μg of protein conjugate, with low error (data not shown).

The average molecular masses for Alexa350®-conjugated αA- and αB-crystallin homopolymeric complexes were measured on a Pharmacia FPLC Superose 6 column and then compared with nonconjugated αA- and αB-crystallins (summarized in Table I). αA-Crystallin without the Alexa350® moiety had an average molecular mass of 540 ± 5 kDa, whereas the Alexa350® conjugate was 526 ± 4 kDa. Similar results were obtained for αB-crystallin. No significant changes in molecular mass of the complexes were seen upon conjugation with Alexa350®. The elution profile of conjugated protein complexes was indistinguishable from that obtained with nonconjugated controls.

CLA of the Alexa350® conjugates was tested using the insulin reduction assay. The activity of αA-Alexa350® was calculated as the percentage of activity seen with an equimolar quantity of nonconjugated αA-crystallin. αB-crystallin was treated in the same manner. The summarized data indicate no measurable change in CLA for either α-crystallin subunit after modification with Alexa350® (Table I).

**Verification That Alexa350® Does Not Alter α-Crystallin’s Membrane Binding Affinity**

To confirm that the Alexa350® moiety does not affect or participate in the membrane binding event, we performed control binding experiments in which equal amounts of either conjugated or nonconjugated α-crystallin homopolymeric complexes were allowed to bind an equal amount of membrane sample. After the incubation was complete, one-third of each pellet, containing membranes and any bound α-crystallin, was run on an SDS-polyacrylamide gel, stained with Coomassie Blue, then analyzed using digital image analysis (ImageQuant version 5.0) to compare the amounts of α-crystallin bound with or without the Alexa350® attached. Fig. 1 shows a representative gel that demonstrates no measurable difference in binding between the conjugated and nonconjugated α-crystallin proteins (compare lane 4 with 5 and 6 with 7), although a slight blurring effect of the protein band is seen with the Alexa350® conjugates.

**General Membrane Binding Characterization**

To understand fully the mechanism by which α-crystallin binds to the fiber cell plasma membranes, it is important to elucidate the solution conditions that affect binding. To this end,
characterization of the membrane binding event under a variety of conditions was carried out using αA- and αB-crystallin homopolymers as well as a reconstituted 3:1 heteromeric complex.

To examine whether membrane association is a saturable event, binding assays were performed where the amount of membrane was held constant at 500 μg, and the amount of α-crystallin complex was varied. In all cases, saturable binding was observed, which then allowed calculation of the binding capacity for these membranes (Fig. 2A). For αA-crystallin, a capacity of 6.59 ± 0.13 ng of αA/μg of membrane was observed, whereas αB-crystallin had a lower capacity at 4.23 ± 0.17 ng of αB/μg of membrane. Interestingly, the binding capacity of the 3:1 heteromeric complex (3.45 ± 0.11 ng of α/μg of membrane) was lower than either of the homopolymeric complexes. In addition, control binding assays were performed in the presence of either BSA or human γD-crystallin and showed no change in the binding capacities of either αA- or αB-crystallin (Fig. 2B). These results agree with estimates made by quantitative gel analysis, indicating that the quantum yield of the fluorescent probe was not affected significantly by the local environment.

Next, the time dependence of association was examined by incubating 8 μg of Alexa350®-conjugated α-crystallin protein with a fixed amount of plasma membrane at 37 °C for up to 16 h. All three protein complexes bind with similar rates, with the calculated $t_{1/2}$ values (time to reach 50% completion) of 50 ± 5 min for αA-crystallin, 34 ± 5 min for αB-crystallin, and 31 ± 4 min for the 3:1 heteromeric complex (Fig. 3A). Although αA-crystallin binding does appear slightly slower than the other two, it is considered not significantly different based on 95% confidence analysis of each curve fit.

Dependence upon ionic strength was then determined using a similar series of binding assays for each of the three protein complexes as described above, using NaCl concentrations ranging from 0 to 1 M (Fig. 3B). Our data, normalized with no salt as the reference, show essentially no effect of salt on the membrane association of all three α-crystallin complexes, even at 1 M NaCl.

The pH dependence was also analyzed for each protein complex with a series of assays containing 8 μg of α-crystallin complex and a fixed amount of plasma membranes in the Tri-buffered system at various pH values, with neutral pH serving as the reference. We show that binding is increased markedly at low pH values (Fig. 3C). For the αA-crystallin homopolymer, the binding was increased nearly 2-fold, whereas the αB-crystallin homopolymer was increased nearly 3-fold. Interestingly, the 3:1 heteromeric complex was affected by low pH to a greater extent than either homopolymeric complex.

Finally, membrane binding was assayed for each complex at a variety of temperatures between 4 and 45 °C (Fig. 3D). Binding by both αA- and αB-crystallin homopolymers shows a high sensitivity to temperature, such that a change from room temperature to 37 °C caused a 4-fold increase in binding. The change in binding increases another 50% by raising the temperature to 45 °C for the two homopolymers. The 3:1 heteromeric complex, however, shows a much larger increase between 37 and 45 °C, similar to the additive effect seen with the pH sensitivity.

Membrane-free control experiments confirmed that the Alexa350® conjugates remained soluble at all times in all experiments described above (data not shown).

**pH Dependence of Ionic Strength Sensitivity**

To investigate further the increased binding seen at low pH, assays were performed at pH 5.0 using the αB-crystallin homopolymeric complex with varied NaCl (0–1 M) and compared with similar assays performed at pH 7.3 (Fig. 4). At pH values >6.5, binding of αB-crystallin is
Insensitive to NaCl. However, at pH 5.0, binding is reduced nearly 2-fold in the presence of 1.0 M NaCl.

Reversibility of Membrane Association

To assess the reversibility of membrane binding, we set up a series of identical binding assays using the standard protocol, then challenged the bound Alexa350®-conjugated protein using a range of concentrations of nonconjugated α-crystallin with and without control proteins in a second incubation. As shown in the inset of Fig. 5, virtually no α-crystallin is released into the soluble phase when the membrane-α-crystallin complex (the pellet) is resuspended in binding buffer alone (the y axis), but addition of nonconjugated α-crystallin to the incubation effectively removed a portion of the membrane-associated α-crystallin. However, even at >50 fold molar excess of nonconjugated α-crystallin, only about 55% of the bound Alexa350®-conjugated αA-crystallin could be removed by competition with nonconjugated αA-crystallin, whereas 75% of the bound αB-crystallin could be removed with nonconjugated αB-crystallin. Addition of control proteins (BSA or γD-crystallin) in the second incubation had no effect on release of bound α-crystallin (Fig. 5).

α-Crystallin Membrane Binding Affinity

To calculate the binding constants (K) for each α-crystallin complex, we defined the plasma membrane to be the ligand. This facilitated the mathematical treatment of membrane binding as a function of membrane concentration. Therefore, binding assays were performed using a range of membrane amounts (~50 μg to greater than 8,000 μg) at 37 °C. Fig. 6 shows the data obtained in these assays transformed into an affinity plot to illustrate best the binding constant for each complex. The plot for the αA-crystallin homopolymer data is horizontal and linear, indicative of noncooperative binding with a K value of $5.9 \pm 0.3 \times 10^{-4} \mu g^{-1}$ of membrane. The 3:1 heterocomplex data also indicate noncooperative binding with a binding affinity of $4.6 \pm 0.5 \times 10^{-4} \mu g^{-1}$ of membrane, which is essentially the same as obtained for the αA-crystallin homopolymer. However, with the αB-crystallin homopolymer the affinity plot is characterized by a sigmoidal curve with positive slope, which indicates apparent positive cooperativity in the binding to plasma membranes. As membrane concentration increases, the affinity, or binding constant, increases approximately 3-fold. Based on the two horizontal asymptotes of this affinity curve, a binding constant, $K_{low}$, of $5.6 \pm 0.5 \times 10^{-4} \mu g^{-1}$ of membrane is seen at low membrane concentration, but at high membrane concentrations this changes to $15 \pm 3 \times 10^{-4} \mu g^{-1}$ of membranes ($K_{high}$).

The calculated binding constants for all three proteins are summarized in Table II. This table illustrates that the binding affinity is essentially the same at low concentrations of lens plasma membrane for both homopolymers as well as the heteromeric complex. However, a considerable difference is seen in the binding affinity of αB-crystallin at high membrane concentrations.

To determine if the high affinity observed for αB-crystallin ($K_{high}$) at high membrane concentration correlated with a change in the observed binding capacity (B), assays were performed as before with constant membrane concentration and varied αB-crystallin concentration. However, in these experiments, 5,000 μg of membrane was used in each sample rather than 500 μg (curve not shown). The measured capacity at high membrane concentration (3.1 ± 0.09 ng/μg of membrane) was reduced marginally compared with low membrane concentration, 4.2 ± 0.2 ng/μg of membrane (Table II).

Control experiments were performed to assess the effect of membranes on the fluorescence measurements to ensure accuracy. By measuring the specific activity of Alexa350® conjugates
in the presence of a wide range of membrane concentrations added just prior to each fluorescence reading, a standard curve was created and curve fitted (data not shown). These experiments verified that at membrane concentrations less than 1,000 μg (a majority of the reported measurements fall in this category), the fluorescence was not affected significantly by either light scattering or inner filter effects (<1% error).

However, above 1,000 μg of membranes, the measured fluorescence was increased by approximately 2–10%, depending on the amount of membranes (data not shown). To correct for this, each fluorescence measurement containing more than 1,000 μg of membranes was adjusted according to the standard curve equation

\[ F_c = F \times \left( 1 - \frac{M}{47,619} \right) \]

(Eq. 6)

where \( F \) is the measured fluorescence, \( M \) is the amount of membranes (μg), and \( F_c \) is the corrected fluorescence.

**Effects of Membrane Digestion by Trypsin on Membrane Binding**

The role of membrane proteins in mediating α-crystallin association with the plasma membrane is uncertain. To evaluate the effects of membrane-associated proteins on α-crystallin binding, fractionated plasma membranes were digested with trypsin. Once the digestion was complete, the binding affinity (\( K_t \)) and binding capacity (\( B_t \)) of both homopolymers and the 3:1 complex for trypsinized membranes were measured.

Saturation curves obtained for trypsinized and non-trypsinized membranes using \( \alpha A \), \( \alpha B \), and the 3:1 heterocomplex, respectively, are shown in Fig. 7. In the case of \( \alpha A \)-crystallin (Fig. 7A), little or no significant change in capacity is observed when using the trypsinized membranes (\( p = 0.813 \)). However, for the \( \alpha B \)-crystallin homopolymer (\( p = 0.015 \)) (Fig. 7B) and especially the heteromeric complex (\( p = 0.016 \)) (Fig. 7C), the binding capacity is changed significantly (summarized in Table II). In fact, the 3:1 complex binding capacity increases by 2-fold when using trypsinized membranes.

The binding affinities (\( K_t \)) for trypsinized membranes were determined by holding the amount of α-crystallin constant at 8 μg and varying the amount of membranes added. In all cases, the binding constants were reduced when using trypsinized membranes (Fig. 8 and summarized in Table II). For the \( \alpha A \) homopolymer complex and the 3:1 heteromeric complex, the affinity is reduced by about 50%, from 5.9 ± 0.03 × 10^{-4} μg^{-1} to 2.9 ± 0.7 × 10^{-4} μg^{-1} and 4.6 ± 0.5 × 10^{-4} μg^{-1} to 1.8 ± 0.3 × 10^{-4} μg^{-1} membrane, respectively (Fig. 8, A and C). However, the most striking result is seen with the \( \alpha B \)-crystallin homopolymer (Fig. 8B). Without trypsin treatment, the binding affinity increases from 5.6 ± 0.5 × 10^{-4} to 15 ± 3 × 10^{-4} μg^{-1} of membrane with increasing membrane concentration. However, the apparent positive cooperativity observed with native membrane preparations was lost when the trypsinized membranes were used, giving one binding constant of 2.7 ± 0.2 × 10^{-4} μg^{-1} of membrane.

**DISCUSSION**

α-Crystallin is known to associate with lenticular plasma membranes in vivo as well as in vitro, and the amount bound increases with both age and the onset of cataract (29,30). α-Crystallin also shows an increasing tendency to become insoluble during aging and/or at the onset of cataract (24–28). Given this correlation between cataract formation and increased binding of α-crystallin to the plasma membrane complex, it is important to understand how...
this process is regulated in lens cells. In the present report, we have examined some fundamental binding properties of lens αA- and αB-crystallins.

Human αA- and αB-crystallin as homopolymers and in a reconstituted 3:1 heteromeric complex show saturable binding to lenticular fiber cell plasma membranes (Fig. 2). These observations are in contrast to previous reports that suggested that αA-crystallin but not αB-crystallin was the only subunit involved with membrane association (20,23). Our results also show that this interaction is sensitive to time and temperature, collectively suggesting that binding is specific in nature (Fig. 3, A and D). In fact, we observed a significant enhancement of binding at temperatures above 37 °C. It is unclear whether this increase is due to α-crystallin’s strong affinity for heat-stressed proteins, presumably including intrinsic membrane proteins, or if it results from a phase transition of lens lipids at high temperatures.

In addition, we show that binding is not affected by high ionic strength at physiological pH. This suggests that binding is mediated mostly by hydrophobic interactions presumably involving either the fatty acid core of the membrane bilayer or the buried hydrophobic regions of the intrinsic membrane proteins still present in the membrane preparation (Fig. 3B). We also demonstrate that binding increases at pH values below pH 7.0, which is in contrast to a previous study in which native α-crystallin was reported to have optimum binding at pH 7.5 (Fig. 3C) (22). We observed a significant enhancement of binding, especially with the reconstituted 3:1 heteromeric complex, at pH 5.0, and this increased binding can be eliminated by increasing the salt concentration. This suggests that the salt sensitivity at low pH is due to either the introduction of electrostatic interactions or the elimination of electrostatic repulsion with some part of the membrane. The degree of enhancement is different for the three α-crystallin complexes, which further implies that the ionizable group is within the protein complex itself rather than on the membrane. In addition, the point at which pH sensitivity is observed for aA- and aB-crystallin can be predicted by their theoretical isoelectric pH values of 5.77 and 6.76, respectively. Despite the sensitivity to ionic strength at low pH, we believe that the interaction between α-crystallin and the plasma membrane is mediated mostly through hydrophobic interactions rather than electrostatic interactions in vivo.

Our data also suggest that intact α-crystallin complexes are not in equilibrium between the membrane-bound and soluble phases. If this were the case, >99% removal of the bound fluorescent α-crystallin should be possible when competed with a large excess of nonconjugated α-crystallin. Rather, it appears that once on the membrane, some fraction of the subunits is not exchangeable with subunits comprising soluble α-crystallin complexes (Fig. 5). We propose that the conjugated α-crystallin removed by competition with unlabeled α-crystallin reflects exchange of subunits between Alexa350®-conjugated complexes associated with the membrane and nonconjugated complexes in solution. According to this model, the nonexchangeable subunits become buried in the membrane bilayer such that they are irreversibly associated. Another interesting observation is that different percentages of aA- and aB-crystallin are removable. If subunit exchange is the mechanism by which fluorescent α-crystallin is being released from the membrane, then these results suggest that bound aA- and aB-crystallin have a different number of subunits in direct contact with the membrane. This could occur if either the quaternary structures of the complexes are significantly different while bound or if αA-crystallin simply embeds itself deeper into the membrane than αB-crystallin, resulting in fewer exchangeable subunits.

It is difficult to compare the calculated binding capacities of each homopolymer and the reconstituted 3:1 heteromeric complex for lens plasma membrane in the present study with those reported previously. In protein-free synthetic vesicles containing 50 mol % cholesterol, the binding capacity was approximately 40 ng of native α-crystallin/μg of lipid (33). In addition, Ifeanyi and Takemoto (20) measured a binding capacity of 44 ng/μg of membrane protein using...
purified lens αA-crystallin and lens membranes prepared in a manner similar to that used in the present study but quantified by the Bradford dye binding assay rather than total dry weight. Using the Bradford assay on the membranes used in the present study, we calculate a binding capacity of 87 ng of αA-crystallin/μg of membrane protein (data not shown). Because previous studies utilized α-crystallins purified from adult cow lenses, differences in binding capacity could reflect post-translational changes known to accumulate in lens proteins which would not be represented in the recombinant human proteins used in the present study (43–45).

Trypsinization of the membranes was performed to determine the relative contribution of exposed protein domains on the binding of α-crystallin to the membrane. Previous studies have shown that brief treatment of lens membrane preparations with trypsin results in cleavage of the presumed cytoplasmic loops of MP26, the major intrinsic membrane protein (46). With both αB-crystallin and especially with the 3:1 heteromeric complex, the binding capacity to trypsinized membranes was increased markedly (Fig. 7, B and C), suggesting that exposed intrinsic membrane protein domains actually decrease the amount of α-crystallin that can bind to the membrane. As membrane proteins in lens fiber cells are thought to contribute a large percentage of the lens membrane mass, we consider it likely that the apparent increase in binding capacity of trypsinized compared with untreated membranes reflects enhanced access of α-crystallin complexes to the lipid bilayer, although we cannot rule out the possibility that trypsinization creates damaged proteins to which α-crystallin could potentially bind. Given the insensitivity of α-crystallin binding to increased ionic strength at physiologic pH, the apparent enhancement of binding capacity following trypsinization of membranes, and the published reports on the saturable association of α-crystallin with protein-free synthetic phospholipid vesicles, we postulate that α-crystallin binding to the membrane occurs predominantly through hydrophobic interactions rather than through protein-protein interactions as proposed previously (22,31–33,47).

For each homopolymer and the 3:1 heteropolymer, binding constants were determined to measure the affinity of the interaction. All three complexes show similar affinity at low membrane concentration; however, the αB homopolymer shows a marked increase in apparent affinity at high membrane concentrations (Fig. 6 and Table II). It is possible that this apparent positive cooperativity results from polymeric linkage, whereby the binding of a ligand influences the aggregation state of the macromolecule (48). In the case of αB-crystallin, it could be that binding to the membrane alters its quaternary structure such that additional αB subunits can insert into the existing bound complexes. Another possible explanation for the positive cooperativity behavior is that at high membrane concentrations, interactions between αB-crystallin and some other intrinsic membrane protein begin to add to the interactions between αB and the lipid bilayer. Consistent with this idea, we observed that the affinity plot of αB-crystallin measured with trypsinized membranes did not show the same positive cooperativity as seen with the nontreated membranes (Fig. 8B). Therefore we believe that the apparent increase in binding affinity seen with αB-crystallin at high membrane concentrations likely reflects the additive effects of protein-lipid and protein-protein interactions with MP26 or other membrane proteins.

One unexpected trend in our data was the properties of the reconstituted 3:1 heteromeric complex. In the cases of the pH and temperature dependence (Fig. 3, C and D), this heteropolymer did not behave as an average of the component subunits, as would be expected if they were functionally and structurally equivalent. Indeed, the heteromeric complex had the lowest binding capacity measured with the fractionated lens membranes, and this capacity was the most dramatically affected by trypsinization of the membrane (2-fold increase) (Fig. 7C). These results support the hypothesis that the mixed aggregate may hold particular functional significance in the lens. Based on the observation that enhanced membrane association is correlated with aging and the onset of cataract, it is reasonable to suggest that irreversible
membrane binding is an event that negatively affects the transparency of the lens. By virtue of its relative resistance to membrane binding, the heteromeric complex may represent a more favorable state than homomeric complexes derived from either αA- and αB-crystallin subunits. The formation of cataract in the αA-crystallin knockout mouse lens adds support to this hypothesis (8).

Our studies suggest that α-crystallin is able to bind membranes through hydrophobic interactions in such a way as to embed a portion of the complex into the hydrophobic fatty acid core of the bilayer. This interaction appears to be irreversible for the subunits in direct contact with the membrane, but it does not appear to prevent subunit exchange between the exposed subunits with soluble α-crystallin aggregates. The present data suggest particular biological significance for the heteromeric complex comprised of both αA- and αB-crystallin, as found in the lens.

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Fig. 1. SDS-PAGE of membrane and protein preparations
Fractionated plasma membranes (100 μg, lane 1) as well as the αA- and αB-crystallins (0.8 μg, lanes 2 and 3) were resolved by SDS-PAGE. The membrane sample mainly shows MP26, whereas the amount of endogenous α-crystallin is essentially undetectable (labeled arrows indicated along gel). Both αA- and αB-crystallin subunits were essentially homogeneous. Lanes 4 and 6 show 33% of a pellet resulting from binding assays in which 500 μg of membrane was incubated with 16 μg of either nonconjugated homopolymeric complex. Lanes 5 and 7 show an equivalent fraction of the pellet resulting from similar binding assays containing Alexa350®-conjugated homopolymers. Based on digital image analysis of multiple samples, the amount of α-crystallin bound was not affected significantly by Alexa350® conjugation, although the protein bands for each conjugate are somewhat more diffuse.
Fig. 2. Binding capacity for α-crystallin membrane binding

Panel A, binding capacity curves. Assays were performed using 500 μg of membranes and a varied amount of α-crystallin. The amount bound was calculated using the fluorescence of the membrane pellet and the specific activity for each α-crystallin complex. The binding capacity (B) was calculated using the asymptote of each curve and was 6.59 ± 0.13 ng/μg of membrane for αA-crystallin (●), 4.23 ± 0.17 ng/μg of membrane for αB-crystallin (○), and 3.45 ± 0.11 ng/μg of membrane for the 3:1 heteropolymer (∗). (n = 3)

Panel B, effects of BSA and γD-crystallin. Control assays were performed with 500 μg of plasma membranes and 25 μg of conjugated α-crystallin with and without 25 μg of control protein (either BSA or γD-crystallin). Samples A–C show the effect of the control proteins on the binding of αA-crystallin; samples D–F show their effects on αB-crystallin binding. Data were normalized to the “no addition” data. (n = 3).
Fig. 3. Characterization of α-crystallin membrane binding
All binding assays were incubated with an equivalent amount of membranes (500 μg) and α-crystallin (8 μg) for 6 h at 37 °C in PBS supplemented with 5 mM MgCl₂, except as indicated. For the bar graphs ■, αA-crystallin; □, αB-crystallin; and △, the 3:1 heteropolymer. For the line graph ●, αA-crystallin; ○, αB-crystallin; and ×, the 3:1 heteropolymer. All data points shown are the average of at least three replicates. Panel A, time dependence of membrane association. Assays were incubated for a range of time points between 0 and 16 h at 37 °C. For all assays presented each data point is the average of at least three replicates. Panel B, salt dependence of membrane association. All assays were carried out in phosphate buffer, pH 7.3, containing a range of NaCl concentrations from 0 to 1 M, with 0 M NaCl serving as the reference. Panel C, pH dependence of membrane association. Assays were incubated for 6 h in Tri-buffer at a pH range of 5.0–9.0, with pH 7.0 as the reference binding activity. Panel D, temperature dependence of membrane association. Assays were performed in the standard buffer at a range of temperatures from 4 to 45 °C for 6 h, with the 37 °C measurements serving as the reference.
Assays were performed with 8 μg of αB-crystallin and 500 μg of membranes in Tri-buffer at either pH 5.0 (□) or 7.3 (■) for 6 h with a range of NaCl concentrations between 0 and 1 M, with 0 M NaCl serving as the reference. All points are the average of at least three repetitions.
Fig. 5. Reversibility of α-crystallin membrane binding

Binding assays were performed using a constant membrane concentration and 8 μg of Alexa350®-conjugated α-crystallin protein under standard conditions. Once formed, the α-crystallin-membrane complexes were resuspended in binding buffer. For competition experiments, membrane-bound α-crystallin was incubated for >24 h at 37 °C with 100 μg of nonconjugated α-crystallin and 100 μg of control protein (BSA or γD-crystallin) or buffer alone (n = 3). After centrifugation, the α-crystallin-membrane complex (pellet) and supernatant were analyzed for fluorescence and compared with the amount bound during the first incubation. There was no observable effect of either BSA or γD-crystallin on αA-(samples A–C) and αB-(samples D–F) crystallin reversibility. In titration experiments (inset), a maximum of 44 ± 2%
and 25 ± 1% of bound αA- (●) and αB- (○) crystallins, respectively, could not be removed after treatment of membranes with up to a 50-fold molar excess of the corresponding nonconjugated protein subunit (n = 2).
Fig. 6. Affinity plots of α-crystallin membrane binding

Binding assays were performed using 8 μg of conjugated α-crystallin with a varied amount of membranes under standard conditions. The apparent binding constant $K$ at each point was calculated as described under “Experimental Procedures.” For αA-crystallin (●), a linear horizontal plot was observed with a binding constant of $5.89 \pm 0.27 \times 10^{-4}$ μg$^{-1}$ of membrane ($n = 5$). For αB-crystallin (○), a sigmoidal curve was found, with two binding constants, $K_{\text{high}} = 14.9 \pm 2.5 \times 10^{-4}$ μg$^{-1}$ of membrane and $K_{\text{low}} = 5.60 \pm 0.50 \times 10^{-4}$ μg$^{-1}$ of membrane ($n = 5$). The 3:1 heteropolymer (×) has a linear horizontal plot and a binding constant of $4.57 \pm 0.50 \times 10^{-4}$ μg$^{-1}$ of membrane ($n = 3$).
Fig. 7. Effect of membrane trypsinization on α-crystallin binding capacity
Plasma membranes were treated with trypsin for 3 h at 37 °C. These membranes were then used in binding assays in which the amount of membrane was held constant at 500 μg, and the α-crystallin concentration was varied from 0 to 64 μg. For all three graphs, n = 3; ○, trypsinized membranes; and ●, non-treated membranes. Panel A, αA-crystallin; panel B, αB-crystallin; panel C, 3:1 heteropolymer.
Fig. 8. Effects of membrane trypsinization on α-crystallin binding affinity

Plasma membranes were treated with trypsin for 3 h at 37 °C. These membranes were then used in binding assays in which the amount of membrane was varied between 80 and 5,000 μg, and the α-crystallin concentration was held constant at 8 μg. For all three graphs n = at least 3; ○, trypsinized membranes; and ●, nontreated membranes. Panel A, αA-crystallin. Both the trypsinized and nontryptsinized affinity plots are roughly linear and horizontal and indicate binding constants of $5.89 \pm 0.27 \times 10^{-4} \mu g^{-1}$ and $2.88 \pm 0.75 \times 10^{-4} \mu g^{-1}$ of membrane, respectively, as well as an overall 2-fold loss of affinity. Panel B, αB-crystallin. With nontreated membranes αB-crystallin shows marked positive cooperativity, with a $K_{low}$ of $5.60 \pm 0.50 \times 10^{-4} \mu g^{-1}$ of membrane, but with trypsinized membranes, a linear plot was observed with a binding constant of $2.69 \pm 0.24 \times 10^{-4} \mu g^{-1}$ of membrane, which is a 2-fold reduction. Panel C, 3:1 heteromeric complex. The binding constant with trypsinized membranes, $1.78 \pm 0.26 \times 10^{-4} \mu g^{-1}$ of membrane, versus nontreated membranes, $4.57 \pm 0.50 \times 10^{-4} \mu g^{-1}$ of membrane, represents roughly a 2-fold reduction in affinity.
Table I
Characterization of Alexa350®-crystallin conjugates

Values are shown for conjugation efficiency, fluorescence specific activities, molecular masses, and CLA of Alexa350® conjugates and nonconjugated controls. Conjugation efficiencies and specific activities are average values obtained from six separate preparations of Alexa350®-conjugated proteins. The molecular mass of each complex was estimated from the elution volume of each protein using Superose 6 gel permeation chromatography. CLA measurements were obtained using the insulin reduction assay and then normalized using the nonconjugated α-crystallin protein as the reference. NA, not applicable.

| Protein            | Conjugation efficiency<sup>a</sup> | Specific activity<sup>b</sup> | Molecular mass | CLA<sup>c</sup> |
|--------------------|-------------------------------------|-------------------------------|----------------|-----------------|
| αA-crystallin      | NA                                  | NA                            | 540 ± 5        | 100             |
| αA-Alexa350®-crystallin | 0.7 ± 0.1                         | 37 ± 3                       | 526 ± 4        | 97 ± 3          |
| αB-crystallin      | NA                                  | NA                            | 541 ± 5        | 100             |
| αB-Alexa350®-crystallin | 1.0 ± 0.1                         | 49 ± 5                       | 534 ± 4        | 99 ± 2          |

<sup>a</sup> In mol of dye/mol of α-crystallin subunit.

<sup>b</sup> In fluorescence units/μg of protein.

<sup>c</sup> Chaperone-like activity.
Table II

Binding constants and binding capacities for lens membranes

All values are the average of at least three replicates. For the binding constants of the homopolymers, seven replicates were used.

|                | $K^a$            | $K^b$              | $B^c$   | $B^d$   |
|----------------|------------------|--------------------|---------|---------|
| $\alpha$-Crystallin | 5.9 ± 0.3 × 10$^{-4}$ | 2.9 ± 0.8 × 10$^{-4}$ | 6.6 ± 0.1 | 8.0 ± 0.6 |
| $\alpha\beta$-Crystallin | $K_{low}$ = 5.6 ± 0.5 × 10$^{-4}$ | $K_{high}$ = 15 ± 3 × 10$^{-4}$ | 2.7 ± 0.2 × 10$^{-4}$ | 4.2 ± 0.2 |
|                | At $K_{low}$ = 5.6 ± 0.5 × 10$^{-4}$ | At $K_{high}$ = 3.1 ± 0.09 | 5.2 ± 0.3 |
| 3:1 complex    | 4.6 ± 0.5 × 10$^{-4}$ μg$^{-1}$ | 1.8 ± 0.3 × 10$^{-4}$ | 3.5 ± 0.1 | 6.9 ± 0.2 |

$^a$ Binding affinity for nontreated membranes in μg of membrane.

$^b$ Binding affinity for trypsinized membranes in μg membrane.

$^c$ Binding capacity of non-treated membranes in ng of α-crystallin/μg of membrane.

$^d$ Binding capacity of trypsinized membranes in ng of α-crystallin/μg of membrane.