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Creatine kinase/ α -crystallin interaction functions in cataract development

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ABSTRACT

Creatine kinase (CK) is an energy storage enzyme that plays an important role in energy metabolism. CK/phosphocreatine functions as an energy buffer and links ATP production sites with ATP utilization sites. Several key mutations in the α A-crystallin (*cryaa*) and α B-crystallin (*cryab*) genes have been linked with autosomal-dominant, hereditary human cataracts. The *cryaa*-R49C mutation was identified in a four-generation Caucasian family. We previously identified an increase in the quantity of CK complexed with α -crystallin in the lenses of knock-in mice expressing the *cryaa*-R49C mutation using proteomic analyses. Increased levels of CK in postnatal cataractous lenses may indicate increased ATP requirements during early cataract development. To gain a further understanding of the relationship between CK and α -crystallin, we investigated whether α -crystallin interacts with and forms complexes with CK, *in vitro*. Isothermal titration calorimetry (ITC) showed that each CK dimer bound to 28 α -crystallin subunits, with a K_d of 3.3×10^{-7} M, and that the interaction between α -crystallin and CK was endothermic, thermodynamically favorable, and entropy-driven. High-salt concentrations did not affect the interaction between CK and α -crystallin, suggesting that the interaction between CK and α -crystallin is primarily hydrophobic. Gel permeation chromatography (GPC) detected water-soluble α -crystallin and CK complexes, as determined by increased light scattering after complex formation. In addition, CK and α -crystallin formed partially-water-insoluble, high-molecular-mass complexes. Enzyme-linked immunosorbent assay (ELISA)-based enzymatic activity analyses of lens homogenates showed a 17-fold increase in CK activity in the postnatal lenses of *cryaa*-R49C knock-in mice. These studies indicate that the interaction between α -crystallin and CK is functionally important and that increased CK levels may be necessary to meet the increased ATP demands of ATP-dependent functions in cataractous lenses.

1. Introduction

Creatine kinase (CK) is the principal regulatory enzyme involved in energy metabolism [1,2]. The enzyme catalyzes the rapid and reversible transfer of the high-energy phosphate moiety between creatine and ADP. CK is explicitly located in energy-producing cellular compartments, such as the mitochondrial intramembranous space, near glycolytic enzymes, and in compartments where energy is used, including near ATPases that are involved in ion transport, such as $\text{Na}^+ \text{K}^+$ -ATPase and Ca^{2+} -ATPase [3–6]. CK also functions as a proton buffer, preventing the local and global acidification of cells. Two primary types of cytosolic CK have been identified, brain-type CK (CKB) and muscle-type CK (CKM) [7,8], which have 80% sequence identity. The CK enzyme exists as a dimer, and CKBB, CKMM, and CKMB dimers

have been identified in various tissues. High levels of mitochondrial CK activity in oxidative tissues ensures the rapid phosphorylation of creatine in the mitochondrial compartment, which may act to maintain a high local ADP concentration near adenine nucleotide translocase sites, decreasing the apparent K_m for ADP. After uptake into cells, phosphorylated creatine is involved in the rapid and immediate production of ATP, especially under conditions with high-energy demands over short time periods. This property of mitochondrial CK appears to be very important, and CK has recently been shown to control oxidative phosphorylation *in vivo*. The levels of CKBB and CKMB isozymes have been found to be elevated during cardiomyopathy [4,9]. In contrast, in glycolytic muscles, CK is thought to act primarily as an energy buffer. High levels of cytosolic CK activity, localized near glycolytic ATP production sites, may be functionally coupled to glycolysis and serve as

Abbreviations: CK, creatine kinase; CKB, creatine kinase B; CKM, creatine kinase M; *cryaa*-R49C, α A-crystallin R49C mutant; ELISA, enzyme-linked immunosorbent assay; GPC, gel permeation chromatography; ITC, isothermal titration calorimetry; PBS, phosphate-buffered saline; RALS, right angle light scattering; RI, refractive index; WT, wild-type

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temporal buffers for high-energy phosphates, helping to maintain steady ATP and ADP concentrations during short periods of elevated muscular contractile activity, when ATP breakdown increases. Furthermore, under these conditions, CK reactions consume protons, ensuring that ATP hydrolysis and the activation of glycolysis in working skeletal muscle does not result in intracellular acidification.

Analysis of CK expression in rat lenses showed that the expression level of the CKBB isoform increases in lenses from birth to adulthood [10]. The CKBB isoform localizes to the anterior epithelium in adult rat lenses. CK is detectable in human lenses; however, no studies have examined the role played by CK during cataract formation. α -Crystallin is an abundant structural lens protein that functions as a chaperone and has been shown to protect several enzymes from heat-induced precipitation and restore the activity of other enzymes [11]. Using a proteomics approach, we recently discovered that the amount of CKB associated with α -crystallin increased 16–22-fold in the cryaa-R49C model of human hereditary cataracts compared with control lenses [12]. However, the functional relationship between α -crystallin and CK remains unclear, especially during cataract formation. To investigate the role played by CK during cataract formation, we examined the *in vitro* interaction between α -crystallin and CK, using isothermal titration calorimetry (ITC) and gel permeation chromatography (GPC). We also utilized a mouse model of human hereditary cataracts that expresses the R49C mutation in α A-crystallin. Our data showed that CK strongly interacts with α -crystallin in an endothermic reaction that is thermodynamically favorable. Furthermore, CK activity increased 17-fold in cryaa-R49C knock-in homozygous mouse lenses, which have cataracts at birth, indicating that CK may be required in cataracts due to an increased demand for ATP and suggesting the existence of a functional relationship between α -crystallin and CK.

2. Materials and methods

Materials: CKBB (CKB) and CKMM (CKM) were obtained from Sigma-Aldrich (St. Louis, MO, USA; catalog numbers C9983 and 10127566001, respectively). Enzyme-linked immunosorbent assay (ELISA) kits for CKB (catalog number OKEH06448) and CKM (catalog number OKCD01481) detection were obtained from Aviva Systems Biology (San Diego, CA, USA). α -Crystallin was purified from porcine lenses, as described previously [13]. Briefly, porcine lenses were homogenized and centrifuged, and then the water-soluble lens supernatant was separated by gel permeation chromatography to isolate α -crystallin, β _H-crystallin, β _L-crystallin and γ -crystallin. The α -crystallin fraction containing α A-crystallin and α B-crystallin was used in these studies.

3. Methods

3.1. Animals and lenses

Wild type C57BL/6J and α A-R49C knock-in mice generated by our laboratory were used in this study. The knock-in mice were on a C57BL/6J background. The knock-in mice express the C to T single point mutation in codon 49 of mouse α A-crystallin gene (*cryaa*). Mice were genotyped by PCR-based methods as described previously [12]. Animal maintenance was carried out by trained veterinary staff at the Mouse Genetics Core in the Division of Comparative Medicine at Washington University. Mice were euthanized by CO₂ inhalation and all animal protocols adhered to the Washington University Institutional Animal Care and Use Policy. Lenses were dissected from 1 to 4 month old mice.

3.2. ITC

Protein-protein interactions between CKM and α -crystallin were examined on a nano-ITC instrument (TA Instruments, New castle, DE,

USA), as described previously [14]. Protein solutions were prepared in Dulbecco's modified phosphate-buffered saline (PBS), without Ca²⁺ and Mg²⁺. The ITC instrument was validated using a nano-ITC validation kit, according to the manufacturer's instructions (TA Instruments). The reference cell was washed three times and then filled with 300 μ l deionized and degassed water. The calorimeter was equilibrated to a baseline drift of less than 100 nW over 10 min. After running several initial experiments, the experiment was determined to provide optimal ITC curves when CK was added by syringe to α -crystallin in the chamber. The ITC software provided the optimal protein concentrations necessary to obtain interaction data. The optimal concentration ratio of CKM to α -crystallin, which was determined during preliminary studies, was 0.075 mM (3 mg/ml) CKM to 0.15 mM (3 mg/ml) α -crystallin. The sample cell was washed three times and filled with the α -crystallin solution. Twenty sequential injections of 2.50 μ l 0.075 mM CKM (3 mg/ml) were made every 300 s, with a stirring speed of 350 rpm. Temperature was maintained at 37 °C. Data were obtained in the presence or absence of 1 mM ATP, which was added to both α -crystallin and CKM solutions. To calculate the molar concentrations of α -crystallin and CKM, we used monomer subunit molecular masses of 20 kDa for α -crystallin and 40 kDa for CKM. The binding of α -crystallin to CKM was examined 2 h after the preparation of the CKM solution. The interaction between CKM and α -crystallin was also examined at a high-salt concentration, in PBS containing 0.5 N NaCl. Additionally, control ITC titrations were performed in the absence of CK.

The titration data were analyzed using NanoAnalyze software (TA Instruments), in which downward peaks correspond to endothermic reactions. The enthalpy (ΔH), dissociation constant (K_d), and the stoichiometry of the interaction (n) were calculated using directly measured heat exchange values between α -crystallin and CK. The entropy (ΔS) and binding free energy (ΔG) of the interaction were calculated using NanoAnalyze software.

3.3. GPC

A concentration of 2 mg/ml α -crystallin (0.1 mM) was incubated with 0.1, 0.5, 1.0, and 2.0 mg/ml (2.5, 12.5, 25 and 50 μ M, respectively) concentrations of CKM at 25 °C for 2 h. The CKM- α -crystallin mixtures were then centrifuged (10,200 \times g for 30 min) to remove any insoluble material. The water-soluble proteins were separated, in succession, on G3000 PW and G5000 PW size-exclusion chromatography columns (Tosoh Bioscience LLC, Prussia, PA), in line with a Viscotek TDA 302 triple-detector array system (Viscotek/Malvern, Westborough, MA, USA) equipped with a VE-1122 pump and a VE-7510 degasser to measure ultraviolet (UV) absorption, refractive index, right-angle light scattering (RALS), and viscosity. The molecular weight range of the GPC columns was 10,000 to 7 million Daltons. Viscotek OmniSEC software was used to calculate the molecular weights of the α -crystallin proteins, using bovine serum albumin and the 92-kDa Pullulan Malvern standards. The protein samples (100 μ l) were injected into the columns using 0.5 \times Dulbecco's modified PBS as the mobile phase, at a flow rate of 0.8 ml/min at 37 °C. Peak areas under the curves were calculated using the OmniSEC software (Malvern). The protein concentrations were calculated based on the refractive index, using a dn/dc of 0.185 for both the bovine serum albumin standard and the α -crystallin proteins [15].

3.4. Gel electrophoresis and immunoblot analysis

Supernatants and pellets were obtained by centrifuging (10,200 \times g for 30 min) the mixtures containing α -crystallin (0, 7.5 μ M, 20 μ M, and 35 μ M) and CKB or CKM (1.5 μ M) after 1-h incubation at 37 °C in the presence of 1 mM ATP. Twenty microliters of electrophoresis sample buffer (Novex LC2676 Tris-glycine-SDS buffer; Life Technologies, Carlsbad CA, USA) was combined with 20 μ l of each sample and added to the lanes of 10–20% Tris-glycine gels (Life Technologies). Prestained

molecular weight markers (Invitrogen) were used on all gels. After electrophoresis, gels were stained with Coomassie blue or transferred to polyvinylidene difluoride membranes, stained with Revert protein stain (LI-COR Biosciences, Lincoln, NE, USA), and visualized on an Odyssey analyzer (LI-COR). Immunoblot analysis was performed using a primary antibody against CKB from R&D Systems (anti-h/m/rCKBB clone 969409 catalog number MAB9076-100) diluted to 0.5 $\mu\text{g}/\text{ml}$. The secondary antibody used was 0.5 mg IRDye 680LT donkey anti-Mouse IgG (H+L) (LI-COR catalog number 926-68022) diluted 1:20,000 in Tris-buffered saline. Supernatants obtained from mouse lens homogenates were analyzed using an antibody to CKB.

3.5. ELISA

CKB enzymatic activities in mouse lens homogenates were measured using an ELISA kit (Aviva Systems Biology). CKB standards were diluted to concentrations between 0.312 and 20 ng/ml, according to the manufacturer's recommendations, and standard curves were obtained by measuring the absorbance at 450 nm. Mouse lenses were individually homogenized, the homogenates were centrifuged at $10,200 \times g$ for 20 min, and 100 μl supernatant from wild-type, cryaa-R49C heterozygous, and cryaa-R49C homozygous mouse lenses were added to the ELISA plate. Activity was calculated in units/ μg protein, and activity of WT lenses was set to 100.

3.6. Immunohistochemical analysis

Immunohistochemical studies examining the distribution of CK in mouse lenses were performed, according to previously published procedures [16,17]. Briefly, mouse eyes were fixed and embedded in paraffin, and 3- μm sections were prepared to examine the lens epithelial and fiber cells in transverse mode. Tissue sections were treated with citrate buffer, pH 6.0, for antigen retrieval and immunostained at 4 °C overnight, using a monoclonal antibody against CKB (anti-h/m/rCKBB clone 969409-catalog number MAB9076-100 from R&D Systems at 1:200 dilution). Tissue sections were then incubated in 20% normal goat serum for 20 min and treated with the Alexa488-conjugated goat anti-rat IgG (1:250 dilution) secondary antibody. Immunostained samples were examined on an Olympus Fluoview 1000 confocal microscope.

4. Results

We examined the *in vitro* interaction between CKM and α -crystallin using ITC analysis. The use of a syringe to add CKM into an ITC chamber containing α -crystallin was found to be the optimal experimental protocol for obtaining binding isotherms. The downward peaks of the ITC titration indicated an endothermic reaction upon the addition of CKM to α -crystallin (Fig. 1A). The integrated normalized areas under the peaks were plotted against the molar ratio of CK to α -crystallin (Fig. 1B), and the nano-ITC software calculated the thermodynamic parameters. The high positive entropy (ΔS) value for the interaction (Table 1) showed that the interaction between CKM and α -crystallin was entropy-driven. The negative free energy value for the interaction showed that the interaction was thermodynamically favorable. In the absence of ATP, binding between CKM and α -crystallin was similar (data not shown).

The binding between α -crystallin and CKM was also examined at a high-salt concentration to investigate the role of ionic interactions. The binding data showed nearly identical thermodynamic parameters for the numbers of CKM subunits bound to α -crystallin subunits as those observed under normal conditions (Fig. 2 and Table 1). The results shown in Fig. 2 indicate a slight increase in both ΔH and ΔS values and an increased K_d value for α -crystallin-CK binding under high-salt conditions compared with physiological salt conditions. The ΔH value was positive, indicating that the reaction was endothermic. The

instrument was calibrated so that the downward peaks (Figs. 1A and 2A) corresponded with endothermic reactions. However, the ΔS value was large and positive, showing a large increase in entropy. As a result, the ΔG values were negative, showing that the binding process is thermodynamically favorable. The ΔG value remained very similar under high-salt conditions compared with physiological salt conditions. GPC analysis (Fig. 3) showed that the CK molecule exists as a dimer between two 40-kDa subunits and that α -crystallin exists as an oligomer containing 28–40 subunits (20-kDa subunit molecular mass). The binding stoichiometry showed that each CKM dimer bound to 28 α -crystallin subunits, with a molar ratio of binding (n) of 14, indicating that one CKM dimer binds to 28 α -crystallin subunits (molecular mass 560 kDa). This binding stoichiometry did not change under high-salt concentrations. GPC analysis also showed that the molecular mass of α -crystallin was over 1,000 kDa, suggesting that two CK dimers bind to each α -crystallin oligomer. The high binding constant suggests that the complex of CK and α -crystallin will not dissociate. It should be noted these binding parameters are based on the oligomer molecular mass of 28 subunits of α -crystallin.

During the GPC analysis, mixing CKM and α -crystallin was found to produce turbidity, which was dependent on the α -crystallin concentration. When we mixed 3 mg/ml α -crystallin with 1 mg/ml CK, we found that turbidity forms instantly. Due to the degree of turbidity observed after mixing, we chose to centrifuge CKM and α -crystallin mixtures at $10,200 \times g$ for 30 min to prevent high-molecular-weight aggregates from blocking the GPC column filter. However, this step also eliminated any very high-molecular-weight, water-soluble materials, along with any water-insoluble materials. Fig. 3 shows the refractive index (RI) and the right-angle light scattering (RALS) traces for CK alone, α -crystallin alone, and CK + α -crystallin mixtures, in the presence of ATP. The red line shows the RI (Fig. 3A) of CKM at 2 mg/ml, whereas the green line shows the RI of CKM at 2 mg/ml in the presence of α -crystallin at 2 mg/ml. A comparison between these two lines indicated that the centrifugation process likely removed a significant amount of CKM from the α -crystallin-CKM mixture. The RI peak area for the CKM peak at a retention volume of 19 ml decreased from 433, for CKM alone, to 327 for the α -crystallin-CKM mixture, a 25% decrease. In contrast, the RALS peak area for CKM increased from 122 for CKM alone to 321 for the α -crystallin-CKM mixture, a 180% increase (Fig. 3B). Similarly, the RALS peak area for α -crystallin decreased by 15% when α -crystallin was mixed with CKM (from 240 to 216). These results suggested that the centrifugation process removed some high-molecular-weight, water-soluble materials.

Fig. 3B shows the RALS traces for CK alone, α -crystallin alone and CK + α -crystallin mixtures, in the presence of ATP. The light scattering peaks for α -crystallin followed similar patterns to those observed for the RI traces, and it is likely that the centrifugation process removed the very-high-molecular-weight and insoluble α -crystallin and CKM complexes from the samples. The CKM RALS traces showed a significant increase in light scattering for the α -crystallin-CK mixtures, compared with either α -crystallin alone or CKM alone. This result indicates that some α -crystallin subunits migrated with CKM at the retention volumes between 19 and 23 ml. Combined with the result showing that the RI peak is smaller (25% decrease) for the α -crystallin + 2 mg/ml CKM mixture than for 2 mg/ml CKM alone, the observed shift in the RALS peak for CKM to a later retention volume 20.5 ml suggests that the complex formed between CKM and α -crystallin is more compact than CKM alone.

We also examined the polypeptide composition of the peak fractions from GPC column by SDS-PAGE and Coomassie blue staining of the protein bands. The first peak in the mixture separated by GPC contained α -crystallin (Fig. 4). The second peak obtained from the GPC column labeled creatine kinase showed the presence of a small amount of α -crystallin associated with CKM. This suggests that some CKM is binding to α -crystallin.

Next, we examined the ratio of the water-soluble and water-

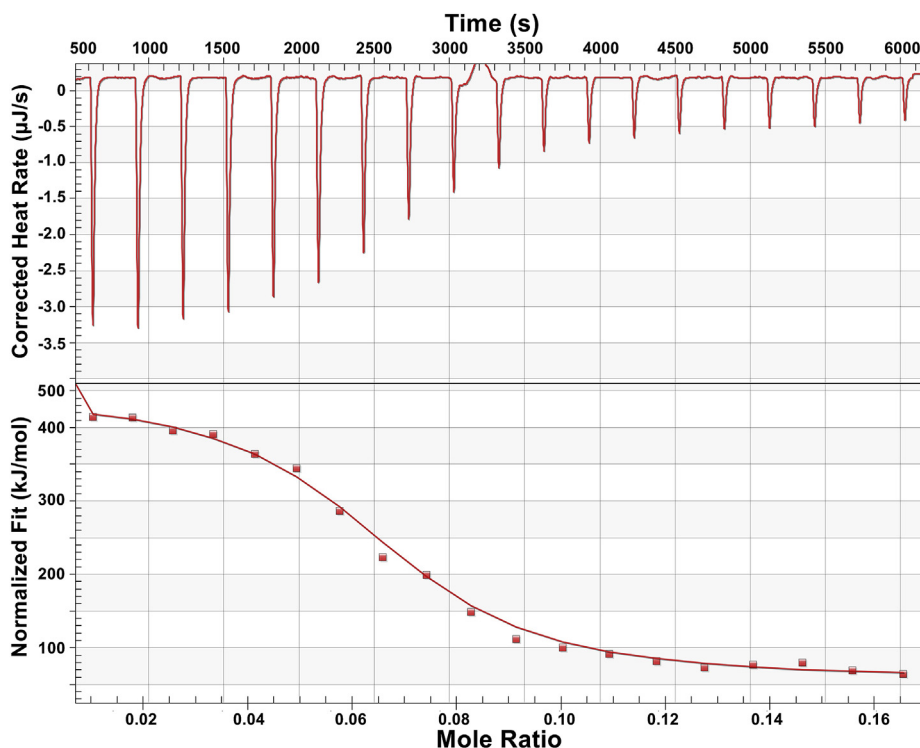


Fig. 1. Isothermal titration (ITC) analysis of the interaction between creatine kinase (CKM) and α -crystallin in the presence of ATP. (A) Changes in current were recorded after sequential injections of 0.075 mM (3 mg/ml) CKM into a solution of 0.15 mM (3 mg/ml) α -crystallin, in the presence of 1 mM ATP (red). (B) Integrated and normalized areas under each peak were plotted against the molar ratio (CK/ α -crystallin), using the NanoAnalyze program.

insoluble protein in the incubation mixture. Mixing 2 mg/ml CKM was mixed with 2 mg/ml α -crystallin results in a small amount of insoluble pellet. We separated the water-soluble proteins from the pellet by centrifugation. Protein assay of the supernatant and pellet showed that 8.4% of the total protein was in the pellet. The amount of insoluble protein did not change significantly with time of incubation. We analyzed the composition of the supernatant and pellet by SDS-PAGE and Coomassie blue staining (Fig. 4). This analysis showed the presence of both α -crystallin and CK in the insoluble fraction.

We next examined the effects of α -crystallin on CKB by immunoblot analysis. CKB appeared as a 38-kDa band, with a minor band at a slightly lower molecular weight that may represent degradation products (Supplementary Figure 1). The intensity of the immunoblot quantified by Odyssey analyzer showed a trend toward a decreasing amount of soluble CKB in the sample, suggesting that some of the soluble CKB became insoluble in the presence of α -crystallin.

Because CK levels are known to increase in damaged muscle, we next sought to examine the effects of the cryaa-R49C mutation, which is associated with cataract formation, on CKB levels in the lens. Our previous work showed a 16–22-fold increase in the size of protein spots containing CKB when cryaa-R49C knock-in mutant mouse lens samples were separated on two-dimensional gels [12]. To examine whether CKB protein levels increased in adult cryaa-R49C knock-in lenses, we examined CKB levels using SDS-PAGE and immunoblot analysis. Wild-type lenses showed very low levels of CKB, whereas cryaa-R49C homozygous mutant lenses showed increased CKB levels (Fig. 5). The results of immunoblot analysis, using an antibody specific to CKB,

Table 1
Thermodynamic parameters of α -crystallin binding to CKM^a.

Samples	K_d (M)	n	ΔH (kJ/mol)	ΔS (J/mol-K)	ΔG (kJ/mol)	K_a (M^{-1})	-TAS (kJ/mol)
CKM into α -crystallin	3.33E-07	0.068	362.5	1293	-38.46	3.00E+06	-400.9
CKM into α -crystallin (high-salt)	8.16E-07	0.068	428.1	1497	-36.15	1.23E+06	-464.3
CKM into α -crystallin (no ATP)	2.28E-07	0.063	544.5	1954	-37.60	4.37E+06	-582.4

^a CKM concentration was 0.075 mM, α -crystallin concentration was 0.15 mM, and ATP was 1 mM unless otherwise indicated. All incubations were performed in PBS (0.15 N NaCl), except the high-salt incubation, which was performed in the presence of 0.5 N NaCl.

Table 2
Molecular mass (Mn) of α -crystallin and CKM peaks by GPC analysis^a.

Sample	Mn α -crystallin peak (kD)	Mn CKM peak (kD)
α -crystallin	1,042	-
CKM	-	86
α -crystallin + CKM	915	341

^a α -crystallin (100 μ M; 2 mg/ml) and CKM (50 μ M; 2 mg/ml) were incubated in PBS, in the presence of 1 mM ATP. Samples were centrifuged, and supernatants analyzed by GPC (see Methods for details). Molecular mass was determined from RALS tracings, as shown in Fig. 3.

demonstrated a 7-fold increase in the amount of protein migrating at approximately 40 kDa, which corresponds with the expected size of the CKB monomer. The CKB band intensity was similar between wild-type and cryaa-R49C heterozygous mouse lens samples. These results indicated that CKB expression levels increased in the absence of wild-type α -crystallin in the cryaa-R49C mouse model of cataract formation.

Next, we examined the expression levels of CK in mouse lenses by immunohistochemical analysis. CKB can be visualized in the nuclei and the basal lens epithelia, near the capsule, in both wild-type (Fig. 6A) and cryaa-R49C homozygous lenses (Fig. 6B), and increased CKB expression levels were observed in the apical lens epithelia of cryaa-R49C homozygous lenses compared to wild-type lenses (Fig. 6C and D).

To determine whether increased CKB protein levels in cryaa-R49C mouse lenses corresponded with increased CKB enzymatic activity, we

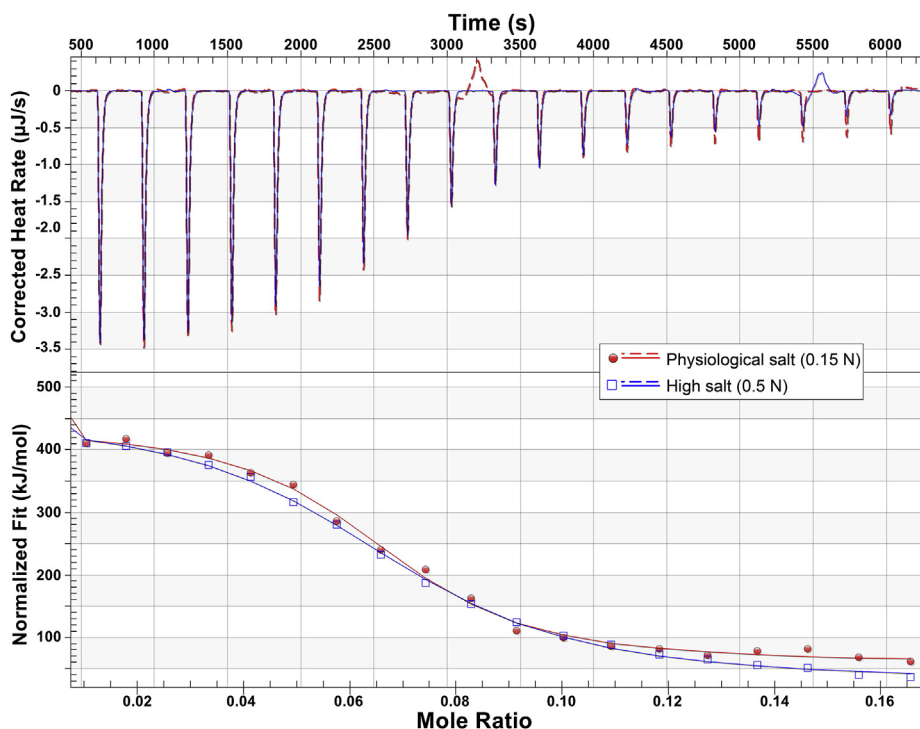


Fig. 2. High NaCl concentration does not affect the interaction between CKM and α -crystallin. ITC analysis in the presence of a high-salt concentration (0.5 N NaCl) showed very similar titration curves to those observed in PBS (0.15 N NaCl). CKM (0.075 mM) was added into a solution of 0.15 mM α -crystallin. ATP concentration was 1 mM.

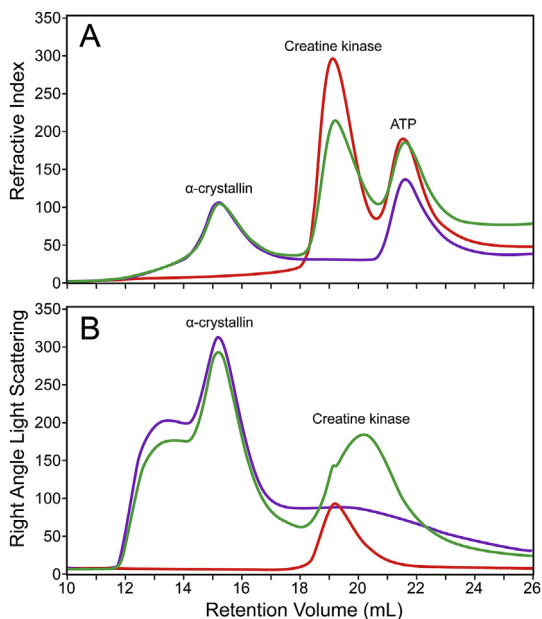


Fig. 3. GPC of α -crystallin incubated with CKM. α -Crystallin (100 μ M; 2 mg/ml) was incubated with increasing concentrations of CKM. (A) RI traces of α -crystallin–CKM mixtures in the filtered samples. The peak observed at the 15-ml retention volume represents α -crystallin, and the peak observed at the 19-ml retention volume represents CKM (50 μ M; 2 mg/ml). ATP alone also generated a signal in the RI trace at 21 ml retention volume. The incubation between CKM and α -crystallin was performed in the presence of 1 mM ATP. CKM alone (red), α -crystallin alone (purple), CKM + α -crystallin (green). (B) Right-angle light scattering (RALS) of the soluble proteins shown in (A). Water-soluble α -crystallin isolated from 6-month-old porcine lenses usually contains both low molecular weight and water-soluble high molecular weight α -crystallin. This can be visualized as a shoulder at 12–14 ml (Fig. 3B) in the light scattering trace of the water-soluble proteins separated on the GPC column (purple). Molecular weights of the α -crystallin and CKM peaks calculated from RALS are shown in Table 2.

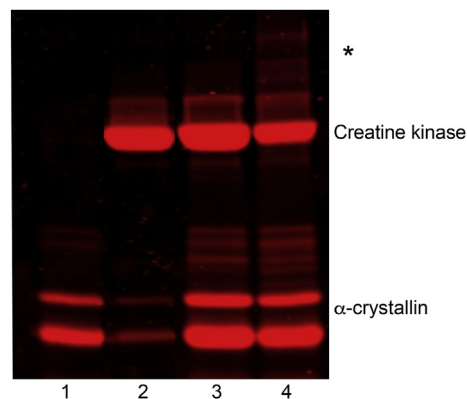


Fig. 4. SDS-PAGE and Coomassie blue staining of the protein fractions obtained from GPC analysis. α -crystallin (2 mg/ml) and CKM (2 mg/ml) were incubated for 2 h at room temperature, centrifuged, and supernatants were run on a GPC column. Fractions (0.5 ml) were collected under the peaks similar to the first and second peaks in Fig. 3. Lane 1, proteins in the first peak labeled α -crystallin. 25 μ l of the fraction was applied to the lane. Lane 2, proteins in the second peak labeled creatine kinase. 25 μ l of the fraction was applied to the lane. Note the presence of a small amount of α -crystallin in the creatine kinase peak. Lane 3, water-soluble proteins obtained from the mixture of α -crystallin and CKM. 10 μ g protein was applied to the lane. Lane 4, pellet obtained by centrifugation of the mixture of α -crystallin and CKM. 10 μ g protein was applied to the lane. Note the presence of high molecular weight proteins in the pellet (*). The minor bands may be other crystallins.

next measured CKB enzymatic activity by ELISA. We found a 7-fold increase in CKB enzymatic activity in cryaa-R49C homozygous mouse lenses compared with that in wild-type lenses, indicating that increased enzymatic activity accompanied the increased CKB levels observed during cataract formation (Fig. 7). We also examined the effects of α -crystallin on CKB activity, *in vitro*. We added increasing amounts of α -crystallin (0, 7.5 μ M, 20 μ M and 35 μ M) to 1.5 μ M CKB or CKM, *in vitro*, and determined the enzymatic activities of CKB and CKM by ELISA. The activity levels of both CKB and CKM were reduced by two to five-fold in the presence of α -crystallin (data not shown). The observed reductions

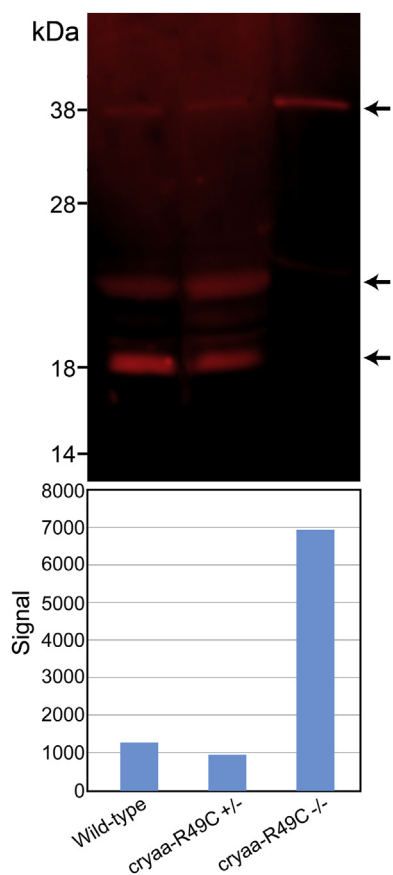


Fig. 5. SDS-PAGE and immunoblot analysis using an antibody against CKB in mouse lenses. (A) Immunoblot analysis of CKB in wild-type (WT), *cryaa*-R49C-heterozygous, and *cryaa*-R49C-homozygous mutant lenses. CKB immunoreactivity is indicated by the *top arrow*. The lower molecular weight bands were apparently due to the cross reactivity of the secondary antibody with crystallins (*lower arrows*). (B) Quantification of the CKB band observed at approximately 40 kDa in (A). Equal amount of protein was loaded in each lane. Note the increase in expression of CKB in the *cryaa*-R49C homozygous lenses.

in CKB and CKM activity levels in the presence of α -crystallin are consistent with the reduced levels of CKB observed in the water-soluble fractions from CKB and α -crystallin mixtures (Supplementary Figure 1).

In the present study, analysis of the *in vitro* interaction of the *cryaa*-R49C mutant protein as compared with wild type α -crystallin with CK were not feasible due to the high insolubility of the mutant protein in aqueous buffer [18], making it difficult to uniquely identify interactions of CK associated with the mutant *in vitro*.

5. Discussion

Glycolysis plays a predominant role in the energy production necessary to maintain lens integrity and continued growth, and glycolytic activity is primarily active in the lens epithelium and superficial cortex. Declined glycolytic activity is associated with cataract formation but not normal aging [19,20]. Coordinated control of glycolytic enzymes such as hexokinase, phosphofructokinase, and pyruvate kinase regulates total ATP output in the lens [21], and CK is known to be coupled with glycolytic enzymes in tissues, such as the skeletal muscle [22]. Although CK is an important enzyme associated with energy metabolism, very little information is available regarding the role played by CK in the lens and during cataract development. The R49C mutation in α -crystallin is associated with human hereditary cataracts [23]. Knock-in mice homozygous for this mutation present with cataracts at birth, and heterozygous mice develop early-onset cataracts [23]. Other studies

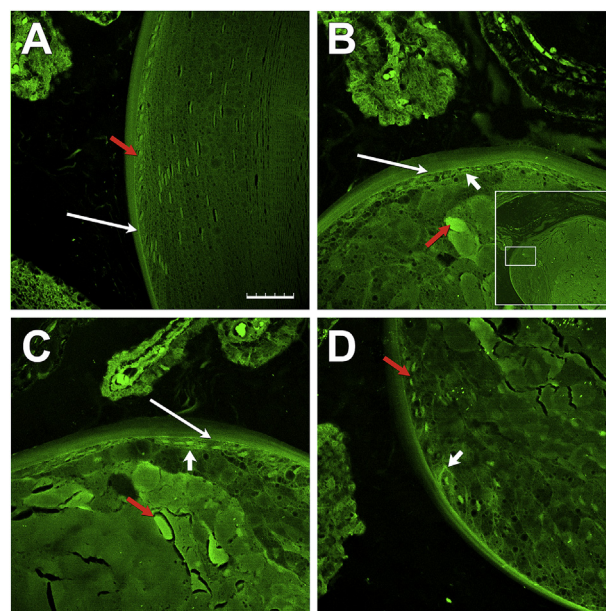


Fig. 6. Immunohistochemical staining of the lens epithelial layer in mouse lenses, using an antibody against CKB. (A) Wild-type (WT) mouse lens. Example of the lens epithelium in the equatorial region. *Long white arrows*, basal lens epithelium; *Red arrow*, nuclei. (B–D) α A-crystallin-R49C-homozygous mice. Lens epithelium in the equatorial region of three different mice. The inset in (B) shows a low magnification image of the lens. *Long white arrows*, basal lens epithelium; *Short white arrows*, apical lens epithelium; *Red arrows*, nuclei. Mice were 3–4 months old. Scale bar = 50 μ m (A–D) and 200 μ m for the inset in (B).

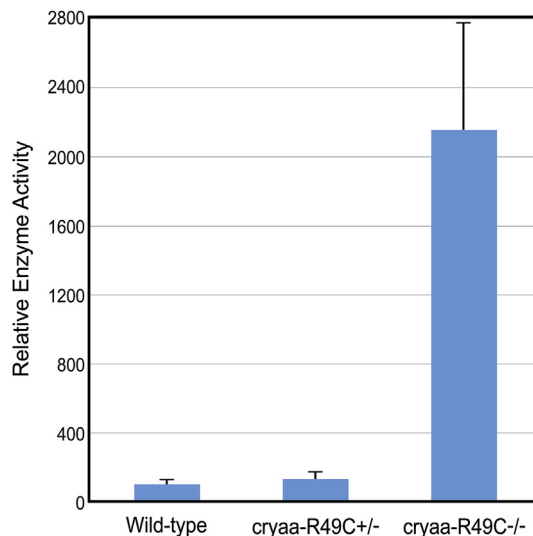


Fig. 7. ELISA assay for CKB activity in mouse lenses. (A) CKB activity in wild-type (WT) and R49C mutant mouse lenses. 1–2-month-old mice were used. The enzyme activity was normalized to a value of 100% for WT lenses. Data from three or more lenses of each genotype is shown. WT vs. *cryaa*-R49C $^{-/-}$, $p < 0.05$.

have shown that the arginine 49 residue in the α A-crystallin amino acid sequence is highly conserved, and the mutation of this residue to an uncharged amino acid, such as cysteine or alanine, results in protein aggregation and the loss of chaperone activity [24,25].

We previously showed in a proteomic study that examined a knock-in mouse model for cataracts that the CKB protein was expressed at a 16-fold higher level in 2-day old *cryaa*-R49C heterozygous lenses and at a 22-fold higher level in *cryaa*-R49C homozygous lenses than that in wild-type lenses. In the present work, using immunoblot analysis, we

demonstrated that CKB levels were 7-fold higher in cryaa-R49C α -crystallin mutant lenses than in wild-type lenses, even in 1–2 months old cryaa-R49C homozygous lenses. In the current study, the increased levels of CKB observed in cryaa-R49C homozygous mutant lenses, as detected by immunoblot and ELISA further confirm the findings of previous proteomics studies and suggest an important function for CK during cataract development.

However, several important experimental results of this study require further discussion. There is 80% sequence identity between CKB and CKM, and both exist in rat lenses. We used CKM to perform ITC and GPC studies on the effect of α -crystallin on CK *in vitro* because CKB would have been very difficult to purify from mouse lenses because the lenses are very small. Additionally, CKM is much more readily available commercially in purified form at a reasonable cost than CKB. The limited availability of purified CKB also posed a problem for our work that required large amounts of protein, such as ITC and GPC. Thus, we used CKM for our ITC and GPC studies, but investigated both CKB and CKM in other work that required less protein, such as SDS-PAGE and immunoblot (Supplementary Figure 1) and ELISA analyses, and we found the results to be nearly identical.

An additional objective of this study was to investigate the *in vitro* interaction between CK and α -crystallin. We demonstrated using ITC analysis that CK and α -crystallin form a stable complex *in vitro* and that their interaction is thermodynamically favorable. GPC data showed changes in the compactness of CK following the addition of α -crystallin, which further supported the idea that these two proteins formed a complex and was consistent with the ITC data. CK has been shown to be constitutively upregulated during human myopathy disease and cardiomyopathy. Lenses express both CKM and CKB [10]; however CKM is more readily available in a purified form than CKB; therefore, we performed most of our *in vitro* experiments using CKM. CKB and CKM possess 80% sequence homology [26–28], and this sequence homology appears to be more relevant and important than the 20% difference observed between the ATP binding sites of CKB and CKM. Thus, the interaction properties we observed during CKM and α -crystallin ITC studies are likely to also be relevant to CKB interactions with α -crystallin.

Changes in salt concentration did not significantly affect the binding of CK and α -crystallin (Fig. 2). This finding indicates that CKM and α -crystallin likely do not bind through ionic attractions but are instead likely to bind through hydrophobic interactions.

Knock-out of the CK M-subunit results in different patterns of regulation in oxidative and glycolytic muscles [29]. CK activity has been detected in both adult rat and human lens epithelium homogenates by non-denaturing electrophoresis, but the effects of cataract formation on CK activity have not been investigated [10]. CKB immunoreactivity was reported in the epithelium of rat lenses but not in lens fiber cells. Using the CKB antibody in the present studies, we found that CKB immunoreactivity can be detected in the basal lens epithelial cells, in the pre-equatorial regions of mouse lenses, and in lens nuclei, but not in secondary lens fiber cells. We demonstrated that CKB immunostaining was more intense in the cryaa-R49C mouse cataract model than in wild-type mice.

Evidence for a possible correlation between CK activity and α -crystallin has primarily been reported by studies on CKM in muscle. Muscle CK is the primary downregulated protein in a model for dilated cardiomyopathy, in addition to downregulated levels of α -actin and α -tropomyosin [30]. The early response to these expression levels is the phosphorylation of α B-crystallin, increased levels of desmin intermediate filaments and actin depolymerization [30]. Other studies have suggested the existence of a relationship between CK, skeletal muscle disorders, and cataracts. Abnormally high levels of CK have been reported in a patient with an unusual type of muscular dystrophy and cataracts [31]. The authors hypothesized that the lens capsule-epithelium had become defective in this patient, resulting in capsular opacification and cataract formation. Other studies indicated that the

structural coupling between CK and myofibrils facilitates the rapid transfer of phosphate from phospho-creatine to ADP to generate ATP [29]. ATP is also required for actin polymerization, which could affect cytoskeletal disorganization. We have previously reported an increase in high-molecular-weight actin and increases in other cytoskeletal proteins associated with α A-crystallin such as tubulin and vimentin, in mouse models of cataracts caused via α A-crystallin mutations [12]. Thus, the increased CKB levels observed in the cryaa-R49C model of cataracts may represent an early response to the previously reported impairment of the cytoskeleton [12].

In conclusion, this is the first study to report an *in vitro* interaction between α -crystallin and CK. We demonstrated that CKB protein levels and enzymatic activities in the adult lenses of homozygous mutant mice were much higher than those in wild-type or cryaa-R49C heterozygous mutant lenses. Increased CKB protein levels were observed by both proteomic [12] and immunoblot analyses, and increased CKB enzyme activity was observed *in vivo* in the current study, suggesting that CKB is an important early player in the development of cataracts in mice. Because enhanced CK expression levels have been reported in other human diseases, such as skeletal muscle diseases and cardiomyopathies [3,9], the current work suggests that measurement of CK might be a useful parameter to assess cataract in mouse models as well as human cataracts. Further studies are required to explore this possibility.

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Transparency document

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Appendix A. Supplementary data

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