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Changes in function but not oligomeric size are associated with αB-crystallin lysine substitution☆

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ABSTRACT

αB-Crystallin, ubiquitously expressed in many tissues including the ocular lens, is a small heat shock protein that can prevent protein aggregation. A number of post-translation modifications are reported to modify αB-crystallin function. Recent studies have identified αB-crystallin lysine residues are modified by acetylation and ubiquitination. Therefore, we sought to determine the effects of lysine to alanine substitution on αB-crystallin functions including chaperone activity and modulation of actin polymerization. Analysis of the ten substitution mutants as recombinant proteins indicated all the proteins were soluble and formed oligomeric complexes similar to wildtype protein. Lysozyme aggregation induced by chemical treatment indicated that K82, K90, K121, K166 and K174/K175 were required for efficient chaperone activity. Thermal induction of γ-crystallin aggregation could be prevented by all αB-crystallin substitution mutants. These αB-crystallin mutants also were able to mediate wildtype levels of actin polymerization. Further analysis of two clones with either enhanced or reduced chaperone activity on individual client substrates or actin polymerization indicated both retained broad chaperone activity and anti-apoptotic activity. Collectively, these studies show the requirements for lysine residues in αB-crystallin function.

1. Introduction

αB-Crystallin, along with αA-crystallin, make up about ~ 40% of the proteins found in the ocular lens [1]. These proteins are involved in the refractive properties of the lens [1]. Additionally, α-crystallins have chaperone activity to prevent protein aggregation which allows for maintaining the lens transparency [2]. These proteins also function in protein kinase signaling cascades [3,4]. It is also well documented that α-crystallin proteins undergo post-translationally modifications (PTM), some of which are reported to affect function [5–8]. Moreover, mutations to α-crystallins often result in cataract indicating changes to function have detrimental effect on the ocular lens [9,10].

The requirements for many of the PTM that occur on αB-crystallin are unknown. Some studies have reported changes in cellular localization as a result of αB-crystallin phosphorylation [11]. Other studies have reported increased anti-apoptotic activity and chaperone function using phospho-mimics at serine 19, 45, and 59 [5]. Moreover, known cataract mutations are reported to affect these activities suggesting the importance of phosphorylation in αB-crystallin function in vivo [12,13]. While phosphorylation of αB-crystallin has been characterized by a number of studies, other PTMs have only been limited. One amino acid that is a target of PTM in multiple proteins including αB-crystallin is lysine. Previous studies looking at αB-crystallin lysine modifications have characterized K92 and K166 both of which undergo acetylation [14,15]. Moreover, acetylation at K92 is reported to have improved chaperone activity and anti-apoptotic activity indicating the importance of this lysine modification. However, eight additional lysines are present in αB-crystallin, but have not been characterized. Since studies with mutant forms of αB-crystallin still detect changes in its ubiquitination state, suggesting that other lysines may be modified [16]. Herein, we set out to determine how alanine substitutions of the ten lysines effect αB-crystallin oligomeric complex formation, chaperone activity, its ability to modulate actin polymerization, and if these changes impact antiapoptotic activity.

2. Materials and methods

2.1. Construction of αB-crystallin substitution mutant plasmids

The wildtype αB-crystallin (WT-αB) construct has been previously

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Table 1
Oligonucleotides sequences used for PCR and cloning.

Name	Sequence (5′–3′)[a]
αB Forward	TTTCCATGGACATCGCCATC
αB Reverse	AACAAGCTTTCATTTCTTGGGGGCTGC
K72A For	CTGGAGGCGGACAGGTTCTCTGTC
K72A Rev	CCTGTCCGCCTCCAGGCGCATCTC
K82A For	GATGTGGCGCACTTCTCCCCAGAG
K82A Rev	GAAGTGCGCCACATCCAGGTTGAC
K90A For	GAACTCGCAGTTAAGGTGTTGGGAG
K90A Rev	CTTAACTGCGAGTTCCTCTGGGGAG
K92A For	AAAGTTGCGGTGTTGGGAGATGTG
K92A Rev	CAACACCGCAACTTTGAGTTCCTC
K103A For	CATGGAGCACATGAAGAGCGCCAG
K103A Rev	TTCATGTGCTCCATGCACCTCAATC
K121A For	CACAGGGCATACCGGATCCCAGCTG
K121A Rev	CCGGTATGCCCTGTGGAACTCCCTG
K150A For	CCAAGGGCACAGGTCTCTGGCCCTG
K150A Rev	GACCTGTGCCCTTGGTCCATTCAC
K166A For	AAGAGGCGCCTGCTGTCACCGCAG
K166 Rev	AGCAGGCGCCTCTTCACGGGTGATG
K174/175A Reverse	TATAAGCTTCTATGCCGCGGGGGCTGCGGTGAC

[a] Restriction endonuclease recognition sites are underlined.

reported [17]. The αB-crystallin substitution mutants were designed for cloning into the pET23d vector for expression in *E. coli.* For lysines 72, 82, 90,92, 103, 121,150 and 166 sequence overlap extension PCR (SOE PCR)was performed. In the first step of PCR the αB forward primer was mixed with reverse (rev) primers for each of the eight alanine substitution mutants (Table 1). Additionally, αB reverse primer was mixed with forward (for) primers for each of the eight alanine substitution mutants (Table 1). The wildtype αB-crystallin plasmid was used as a template. PCR was performed by 5 min at 95 °C for *Taq* polymerase activation followed by 30 cycles of 30 s at 95 °C for denaturing, 30 s at 55 °C for annealing, and 30 s at 72 °C amplification. PCR products were separated on a 1% Tris-acetate-EDTA (TAE) gel and DNA fragments were extracted using Qiaquick gel extraction kit (Qiagen, location). Second step SOE PCR was performed under similar conditions, except using first step PCR DNA fragments as templates. Purified PCR products were gel purified as before, digested with *NcoI* and *HindIII* and cloned into the same sites in pET23d using the quick ligation kit (New England Biolabs, Ipswich, MA). Plasmids were confirmed by DNA sequencing and transformed into BL-21 *E. coli* cells for expression. For amino acid substitution of K174 and K175 with alanine, a single reverse primer K174/175A (Table 1) was used with αB Forward and amplified using the same conditions as first step SOE PCR. The DNA fragment was separated on a 1% TAE agarose gel, gel purified as other DNA fragments, digested with *NcoI* and *HindIII* and cloned and sequenced as above.

2.2. Expression and purification of recombinant αB-crystallin proteins

Expression and purification of αB-crystallin mutant proteins was performed similar to wildtype αB-crystallin over a Macro-S column (Bio-Rad, Hercules, CA) followed by gel filtration on Sephacryl S400-HR as previously reported [17–20]. All purified αB-crystallins were stored at − 80 °C.

2.3. Analysis of oligomeric complexes (OC)

Similar to previous analyses, purified modified αB-crystallins were injected onto a Superose 6 gel filtration column using an AKTA FPLC (GE Healthcare Bio-Sciences, Pittsburgh, PA) [17]. αB-Crystallin proteins were eluted in PBS into 1 mL fractions. The elution chromatograms of αB-crystallins mutants were detected by absorbance (280 nm) and plotted against wildtype αB-crystallin.

2.4. Individual substrate chaperone activity assays

Chemical and thermal chaperone activity assays were performed as previously described with either 1:1 or 1:2 M ratio of client substrate to αB-crystallin [17,21]. Briefly, for thermal chaperone assays, 125 μg/mL of γD-crystallin was incubated in the presence or absence of 6.25 μg/mL αB-crystallin proteins. Protein samples were incubated in 50 mM Phosphate buffer pH [7.4] for 1 h at 65 °C in a Cary 1E UV/vis spectrophotometer fitted with a Peltier controlled sample carrier. Samples were constantly monitored for light scattering at 360 nm. Similarly, for chemical chaperone assays, 10 μM lysozyme (EMD Millipore, Philadelphia, PA) was mixed with 2 mM DTT in the presence or absence of 1 μM αB-crystallin protein. Reactions were performed in PBS as a total volume of 1 mL PBS. Samples were monitored as above for 1 h at 37 °C.

2.5. Actin Polymerization assays

Actin Polymerization Assays were performed using a modified assay with the actin polymerization kit from Cytoskeleton Inc. (Denver, CO). The modified assay used G-buffer by combining 10 mL of General Actin Buffer with 40 μL ATP. Actin buffer (AB) was prepared by adding 50 μL of 20 μg/μL actin with 2.25 mL of G-buffer. Actin oligomers were depolymerized by incubating AB on ice for 60 min and centrifuged at 20,000 × *g* for 30 min at 4 °C. Reactions were setup in black 96-well plates (corning) using 65 μLG-buffer, 10 μL AB, and 25 μL of 1 μM αB-crystallin protein or PBS control. Assays were started by adding 12 μL of actin polymerization buffer (500 mM KCl, 20 mM $MgCl_2$, 50 mM guanidine carbonate, and 10 mM ATP). Wells were monitored for 60 min at excitation (λ 350 nm) and emission (λ 407 nm) on a Synergy 4 Multi-Mode Microplate Reader and Gen5 Reader Control and Data Analysis Software (BioTek, Winooski, VT). Data were plotted and analyzed statistically by ANOVA on repeated measure with Tukey's multiple comparison with GraphPad Prism (La Jolla, CA).

2.6. Cell lysate aggregation assay

Assays were performed similar to those previously described [22]. Briefly, Human embryonic kidney (HEK293) cells from were grown in DMEM (4.5 g/L glucose) with 10% fetal calf serum plus Penicillin/Streptomycin (standard media). At 90% confluency, cells were washed in PBS, scraped from the plate and centrifuged down in PBS containing 1 mM DTT and protease inhibitor cocktail (ThermoFisher). Cell membranes were disrupted by passage through a 27 gauge needle and sonicated before pelleting insoluble debris. The soluble fraction was incubated with 20 U/mL of T4 kinase (New England Biosciences) and 2 mM $MgCl_2$ for 30 min at 37 °C. Lysated were subsequently quantified by BCA (ThermoFisher) and frozen in aliquots at − 80 °C. Cell lysates (1 mg/mL) were mixed with a range of concentrations (0–8 μM) of wildtype or mutant αB-crystallin or control human aldose reductase and incubated at 45 °C for 90 min. Following incubation samples were pelleted, washed in PBS and pellets were suspended in SDS-PAGE loading buffer with 1% 2-mercaptoethanol. Samples were heated at 95 °C for 5 min, loaded and run onto 4–20% mini protean TGX stain free gels (Bio-Rad) at 200 V for 30 min. Gels were imaged using ChemiDoc XRS + system (Bio- Rad). Densitometry was determined using ImageJ software with background subtracted from each lane. Each assay was performed at least three times. The IC_{50} values were determined as concentration of protein at which half-maximum aggregation was suppressed, normalized to the concentration of total cell lysate protein and significance determined using Graphpad Prism software.

2.7. Apoptosis assays

HEK293 cells were plated at a density of 8×10^5 cells per well in a 6-well plate overnight in standard media. Cells were transfected with 5 μg of αB-crystallin, mutant α-crystallin, or β-galactosidsase as a