Published in final edited form as:

Biochemistry. 2013 November 12; 52(45): 8126–8138. doi:10.1021/bi400638s.

Acetylation of Lysine92 Improves the Chaperone and Anti-apoptotic Activities of Human α B-Crystallin

Rooban B. Nahomi[‡], Rong Huang[§], Sandip K. Nandi[¶], Benlian Wang^{‡‡}, Smitha Padmanabha[‡], Puttur Santhoshkumar^{§§}, Slawomir Filipek[†], Ashis Biswas^{¶*}, and Ram H. Nagaraj^{‡*}

[‡]Department of Ophthalmology and Visual Sciences, Case Western Reserve University School of Medicine, Cleveland, OH 44106 ^{‡‡}Center for Proteomics and Bioinformatics, Case Western Reserve University School of Medicine, Cleveland, OH 44106 [§]Department of Medicinal Chemistry, School of Pharmacy, Virginia Commonwealth University, Richmond, VA 23298 [¶]School of Basic Sciences, Indian Institute of Technology Bhubaneswar, Bhubaneswar-751013, India ^{§§}Department of Ophthalmology, University of Missouri-Columbia, MO 65212 [†]University of Warsaw, Faculty of Chemistry, PL02093, Warsaw, Poland

Abstract

α B-Crystallin is a chaperone and an anti-apoptotic protein that is highly expressed in many tissues, including the lens, retina, heart and kidney. In the human lens, several lysine residues in α B-crystallin are acetylated. We have previously shown that such acetylation is predominant at lysine92 (K92) and K166. We have investigated the effect of lysine acetylation on the structure and functions of α B-crystallin by the specific introduction of an N^ε-acetyllysine (AcK) mimic at K92. The introduction of AcK slightly altered the secondary and tertiary structures of the protein. AcK introduction also resulted in an increase in the molar mass and hydrodynamic radius of the protein, and the protein became structurally more open and more stable than the native protein. The acetyl protein acquired higher surface hydrophobicity and exhibited 25-55% higher chaperone activity than the native protein. The acetyl protein had higher client protein binding per subunit of the protein and higher binding affinity relative to the native protein. The acetyl protein was at least 20% more effective in inhibiting chemically induced apoptosis than the native protein. Molecular modeling suggests that acetylation of K92 makes the ‘ α -crystallin domain’ more hydrophobic. Together, our results reveal that the acetylation of a single lysine residue in α B-crystallin makes the protein structurally more stable and improves its chaperone and anti-apoptotic activities. Our findings suggest that lysine acetylation of α B-crystallin is an important chemical modification to enhance α B-crystallin’s protective functions in the eye.

Keywords

Chaperone; α B-crystallin; lysine acetylation; apoptosis; lens; retina

*Correspondence: Ram H. Nagaraj, Ph.D., Department of Ophthalmology and Visual Sciences, Case Western Reserve University School of Medicine, Pathology Building, Room 301, 2085 Adelbert Road, Cleveland, OH 44106; ram.nagaraj@case.edu, Phone (216)368-2089 or Ashis Biswas, Ph.D., School of Basic Sciences, Indian Institute of Technology Bhubaneswar, Bhubaneswar-751013; abiswas@iitbbs.ac.in.

Notes: The authors declare no competing financial interest.

Supporting information: Detection of acetyl α B-crystallin in human and mouse retinas, amino acid sequence alignment to show conservation of K92 in small heat shock proteins, effect of MTCA on α B-crystallin’s structure and function, chaperone activity of acetyl α B-crystallin at different client protein to chaperone ratios, effect of time and α B-crystallin’s concentration on the thermal aggregation of HeLa cell proteins. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

α -Crystallin is a major protein in the human lens and consists of two subunits, α A and α B. These proteins belong to the family of small heat shock proteins that contain a common central core domain of approximately 90 amino acids, known as the 'alpha-crystallin domain' (ACD). The two subunits share significant sequence homology and exist in the lens as hetero-oligomers of varying sizes with an average molecular weight of 800 kDa.^{1, 2} In the lens, these two proteins function as molecular chaperones.³ Through direct interaction, these two proteins prevent the aggregation of β - and γ -crystallins, the major proteins of the lens, during aging.⁴ α -Crystallin binds to the cytoskeletal proteins actin and vimentin and to filament assembly intermediates,⁵⁻⁷ and it prevents filament disassembly during cell stress. α -Crystallin also binds to the plasma membrane in lens fiber cells, although the significance of this binding is not known.⁸ In addition, α -crystallin binds to microtubules and facilitates microtubule assembly in the lens,⁹ and α B-crystallin associates with the centrosome¹⁰ and may participate in cytokinesis during mitosis.¹¹ Deletion of α A-crystallin or α A/ α B-crystallin together causes cataracts, which are accompanied by the aggregation of proteins.^{12, 13} Forced expression of chaperone-compromised α -crystallin also leads to cataracts,^{14, 15} further underlying the importance of α -crystallin's chaperone activity for lens transparency. Outside of the lens, α -crystallin has been shown to bind to β -amyloid and synuclein and prevent their aggregation, which have implications for neurological diseases.^{16, 17} In addition, the loss of the chaperone activity of α B-crystallin leads to desmin-related cardiomyopathy.¹⁸ Furthermore, α B-crystallin binds to pro-inflammatory cytokines,¹⁹ an immune response against α B-crystallin enhances inflammation in multiple sclerosis,²⁰ and administration of α B-crystallin reduces paralysis in experimental autoimmune encephalomyelitis,²¹ which is dependent on its chaperone function.

In addition to its role as a chaperone, α -crystallin is an anti-apoptotic protein²² that prevents the activation of procaspase-3, binds to Bax, activates PI3-kinase and inhibits phosphatase and tensin homolog (PTEN) by directly interacting with those proteins^{23, 24} and promoting cell survival. There is evidence for such interactions in cells exposed to external stresses.²²

In the retina, α B-crystallin is present in retinal pigmented epithelial (RPE) cells, Muller cells, retinal capillary cells and photoreceptors.²² RPE cells secrete α B-crystallin through an exosomal pathway.^{25, 26} Although the exact function of the secreted α B-crystallin is not known, α B-crystallin is known to protect RPE cells from oxidative damage.²⁷ α B-Crystallin could play a role in diabetic retinopathy. Its neuroprotective function decreases in diabetic retinas,²⁸ which could partly explain early apoptosis in the neural retina during diabetes. α -Crystallin translocates from the cytoplasm to the nucleus when cells are stressed.²⁹ Whether this nuclear localization is a requirement for its anti-apoptotic function is not known. α B-Crystallin is also present in mitochondria, where it inhibits cytochrome C oxidation.³⁰ Taken together, it is evident that α B-crystallin is a multifaceted protein and plays an important role in preventing superfluous protein denaturation and apoptosis in stressed cells.

Lysine acetylation is a widespread protein modification that occurs in cells. The reaction is catalyzed by lysine acetyl transferases (KATs) in the cytoplasm (and by histone acetyl transferases (HATs) for histones in the nucleus), and deacetylation is regulated by lysine deacetylases (KDACs) (or histone deacetylases (HDACs)).^{31, 32} While histone acetylation and deacetylation regulate gene expression, lysine acetylation of cytosolic proteins regulates cellular functions and metabolism.^{33, 34} In the human lens, acetylation of both α A- and α B-crystallin was first reported by Smith's laboratory.^{35, 36} We have recently identified K70 and K99 in α A-crystallin and K92 and K166 in α B-crystallin to be acetylated in the human lens.³⁷ We now extend this study to show that α B-crystallin is acetylated in the mouse and

human retina (Supplemental Fig. S1). We found that lysine acetylation in α -crystallin improves its chaperone function³⁷ and that prior lysine acetylation inhibits protein modification by glycation.³⁸

Among many lysine residues in human small heat shock proteins (Hsp20, α A- and α B-crystallin and Hsp27), K92 is highly conserved (Supplemental Fig. S2). Our aim in this study was to determine the specific effects of acetylation at this site on α B-crystallin. To study the effects of acetylation at specific lysine residues on the function of proteins, lysine residues have been mutated to glutamine to mimic acetylation.^{39, 40} While this mutation replicates the neutralization of the positive charge on the protonated lysine residue, it is not fully structurally compatible with AcK. To circumvent this problem, Huang et al. first described the use of methylthiocarbonyl-aziridine (MTCA) to introduce site-specifically an acetylation mimic into proteins.⁴¹ MTCA reacts with cysteine residues (replacing lysine residues by mutation) in proteins and generates an acetylation mimic, methylthiocarbonyl thialysine (MTCTK). Proteins carrying MTCTK modifications are recognized by the AcK antibody. Furthermore, Huang et al, validated their approach by demonstrating that the introduction of the MTCTK group at K102 of the CK2 protein kinase stimulates its catalytic activity, similar to enzymatic acetylation at these lysine residues.⁴¹ Using the same approach, we have studied the site-specific effect of lysine acetylation at K92 in α B-crystallin. First, we converted K92 to cysteine92 (C92) by site-directed mutagenesis and introduced an acetylation mimic using MTCA. We report below the impact of acetylation on the structure and function of α B-crystallin.

MATERIALS AND METHODS

Citrate synthase (CS), lysozyme (Lyso), malate dehydrogenase (MDH), protease inhibitor cocktail, methyl chlorothioformate, staurosporine and etoposide were obtained from Sigma-Aldrich Chemical Co. LLC (St. Louis, MO, USA). Aziridine was purchased from ChemService, Inc. (West Chester, PA). All other chemicals were of analytical grade. γ -Crystallin (GC) from bovine lenses was purified as previously described.⁴²

Cloning and purification of wild type (WT) and mutant α B-crystallin

The α B-crystallin (K92C) mutant was amplified using the following forward and reverse primers for cloning:

F: 5'-GGAACTCAAAGTTTGCCTGTTGGGAGATGTG-3'

R: 5'-CACATCTCCCAACACGCAAACCTTTGAGTTCC-3'

The amplified PCR product was cloned, expressed in *E. coli* and purified as previously described.³⁷ The WT protein was similarly purified.³⁸

Introduction of the AcK mimic into the K92 position in α B-crystallin

MTCA was synthesized, and the acetylation mimic was introduced at K92 using previously described procedures.⁴¹ Briefly, we added 500 μ l and 200 μ l, respectively, of MTCA (1.0 M solution in acetonitrile) to 5.0 ml of the K92C mutant of α B-crystallin (5.0 mg/ml) and to 2.0 ml of WT α B-crystallin (4.0 mg/ml) in 100 mM ammonium bicarbonate. The mixtures were incubated for 3 hrs at room temperature. The samples were then dialyzed against 50 mM phosphate buffer (pH 7.5) for 24 hrs.

Western blotting for K92 acetylation in α B-crystallin

The proteins were separated on a 12% denaturing gel, transferred to a nitrocellulose membrane and probed with a monoclonal antibody to AcK (Cell signaling Technologies,

Danvers, MA; dilution, 1:50,000) and an HRP-conjugated goat anti-mouse IgG (Promega, Madison, WI; dilution, 1:5,000). Immunoreactivity was detected using the Enhanced Chemiluminescence Detection Kit (Thermo Scientific).

Protein thiol assay

The protein thiol assay was performed using the Thiol Quantification Assay Kit from Abcam (Cambridge, MA) and 100 µg protein sample.

Mass spectrometric confirmation of K92 acetylation in αB-crystallin

The MTCA-derivatized K92C αB-crystallin was subjected to SDS-PAGE on a 12% reducing gel. Gel pieces containing the protein were de-stained with 50% acetonitrile in 100 mM ammonium bicarbonate followed by 100% acetonitrile for mass spectrometric detection of the AcK mimic, MTCTK. Similarly, MTCA-treated WT-αB-crystallin was subjected to electrophoresis and processed. The samples were then treated with 20 mM DTT at room temperature for 60 min followed by treatment with 50 mM iodoacetamide for 30 min in the dark. The reagents were removed and the gel pieces were washed with 100 mM ammonium bicarbonate and dehydrated in acetonitrile. The gel pieces were then dried in a Speed Vac concentrator (Savant Speed Vac, Thermo Scientific, Rockford, IL), rehydrated in 50 mM ammonium bicarbonate containing sequencing grade modified trypsin and left for overnight digestion. Proteolytic peptides extracted from the gels with 50% acetonitrile in 5% formic acid were completely dried in a Speed Vac concentrator and then re-suspended in 10 µl of 0.1% formic acid. Three microliters of the peptides was injected into an Orbitrap Elite Hybrid Mass Spectrometer (Thermo Electron, San Jose, CA) equipped with a Waters nanoAcquity UPLC system (Waters, Milford, MA). Liquid chromatography was performed using the mobile phase A=0.1% formic acid in water and B=0.1% formic acid in acetonitrile with a linear gradient of B at an increment of 1% per min at a flow rate of 300 nl/min for 90 min. All spectra were recorded in a positive ion mode using data-dependent methods consisting of a full MS scan (m/z 300–1800) in a high-resolution Orbitrap (120,000 resolution) followed by MS/MS scans of the 15 most abundant precursor ions determined from the full MS scan. The MS/MS spectra were generated in an ion trap with relatively low resolution by collision-induced dissociation of the peptide ions at a normalized collision energy of 35% to generate a series of b- and y-ions as major fragments. Raw LC-MS/MS data were subjected to a database search using the Mascot search engine (version 2.2.0, Matrix Science) against a protein database containing αB-crystallin sequences and K92Cys-mutated sequences. Oxidized methionine and MTCTK were included as variable modifications. The mass tolerance was set as 10 ppm for the precursor ions and 0.8 Da for the product ions. The significance threshold was $p < 0.05$.

Circular dichroism spectroscopy

The far-UV CD spectra were measured at 25°C using a Chirascan Plus (Applied Photophysics, UK). The spectra were collected from 190–260 nm using a rectangular quartz cell with a 1-mm path length. The proteins (0.2 mg/ml) were dissolved in 50 mM phosphate buffer (pH 7.5). The spectra were analyzed for secondary structure content using CDNN CD spectra deconvolution software (Applied Photophysics). The near-UV CD spectra were measured at 25°C using the same spectropolarimeter. The spectra were measured with a 1.0 mg/ml protein solution in 50 mM phosphate buffer (pH 7.5). The reported spectra are the average of 5 scans.

Surface hydrophobicity and tryptophan fluorescence

The surface hydrophobicity of the protein (0.05 mg/ml) was measured using 10 µM 2-p-toluidinonaphthalene-6-sulphonate (TNS) (emission: 350–520 nm; excitation: 320 nm). The

intrinsic tryptophan fluorescence spectra of the proteins (0.05 mg/ml) in 50 mM phosphate buffer (pH 7.5) at 25°C were recorded using a Fluoromax 4P spectrofluorometer (Horiba Scientific, Edison, NJ). The excitation wavelength was set to 295 nm, and the emission spectra were recorded between 310 and 400 nm. The data were collected at 0.5-nm wavelength resolution.

Determination of structural stability

The structural stabilities of native α B-crystallin and acetyl α B-crystallin were determined using an equilibrium chemical denaturation experiment. Protein (0.05 mg/ml in 50 mM phosphate buffer, pH 7.5) was incubated with 0 to 7 M urea for 18 hrs at 25°C. The intrinsic tryptophan fluorescence spectrum of the protein solution was recorded in the 310–400 nm region at an excitation wavelength of 295 nm. The equilibrium unfolding profile was fit according to a three-state model, as described previously.⁴³

DLS-MALS analysis

The samples were injected into a TSK-5000PW_{XL} (Tosoh Bioscience, Japan) gel filtration column equilibrated with 50 mM phosphate buffer with 150 mM NaCl buffer (pH 7.2) and connected to an HPLC (Shimadzu, Columbia, MD) coupled to multi-angle light-scattering and dynamic light-scattering detectors (Wyatt Technology, Santa Barbara, CA). The flow rate was set to 0.75 ml/min. The average molar mass and hydrodynamic radius of the oligomers were determined using ASTRA software (Wyatt Technology).

Chaperone activity assays

Chaperone activity assays were carried out as previously described.³⁷ We compared the chaperone activity of WT and MTCA-treated α B-crystallin. The ratios of α B-crystallin to client proteins (w/w) are given in figure legends. The chaperone activity of α B-crystallin was also assessed in cells transferred with α B-crystallin as previously described.⁴⁴ HeLa cells (70% confluent) were transferred with α B-crystallin (4 μ g) with the aid of a cationic lipid, BioPORTER (Polyplus transfection reagent, Illkirch, France). A batch of cells treated with BioPORTER alone served as a control. After 4 hrs of incubation, cell lysate was prepared using M-PER containing a protease inhibitor cocktail (1:100). Western blotting for α B-crystallin was used to confirm that an equal amount of protein was transferred into the cells. Protein corresponding to 50 μ g from cell lysates was either incubated at 55°C for 2 hrs or kept at RT for 2 hrs (control). After incubation, the samples were centrifuged at 14,000 $\times$ g for 10 min. The protein concentration in the supernatant was measured using the BCA Protein Assay Kit.

Determination of protein refolding ability

MDH (1.0 μ M) was denatured in 6 M guanidine hydrochloride (Gu-HCl) for 8 hrs at 25°C. Refolding of MDH was initiated by a 100-fold dilution with a refolding buffer (50 mM phosphate buffer, 10 mM magnesium acetate, 5 mM DTT, pH 7.5) in the presence or absence of 20 μ M native and acetyl- α B-crystallin. The MDH concentration was 10 nM during refolding. The activity of the refolded MDH was measured by adding 20 μ l of the refolding mixture to 580 μ l of refolding buffer containing 0.1 mM NADH and 0.4 mM oxaloacetic acid (pre-incubated at 25°C) and measuring the absorbance at 340 nm.

Equilibrium binding study

The chaperone-substrate binding study was performed using the membrane filtration method.⁴⁵ Briefly, acetyl and native α B-crystallin (12.5 μ M) were incubated at 60°C for 1 hr with 2–18 μ M GC in 50 mM phosphate buffer (pH 7.5). After equilibration at 25°C for 1 hr, the incubation mixture was spun through a Microcon centrifugal filter device (Millipore,

Billerica, MA) (4,000 xg) fitted with a 100-kDa cutoff membrane filter to separate the unbound substrate. The number of binding sites (n) and the dissociation constant (K_d) were determined as previously described.^{45, 46}

Apoptosis measurements

α B-Crystallin was transferred into HeLa cells with the aid of BioPORTER according to the manufacturer's instructions. The cells were cultured in DMEM media supplemented with 10% FBS. When the cells were 70–80% confluent, the adherent cells were treated with BioPORTER containing proteins in serum-free media. Cells treated with BioPORTER alone served as a control. The cells were incubated for 4 hrs at 37°C in a humidified 5% CO₂ atmosphere and then washed with PBS. To induce apoptosis, the cells were treated with either 100 nM staurosporine or 20 μ M etoposide at 37°C for 24 hrs. Early apoptotic cells were detected by staining cells with annexin V-FITC (BD Biosciences, San Jose, CA).

Measurement of caspase-3 activity

HeLa cells treated as above were lysed using M-PER reagent. An equal volume of fluorogenic substrate solution [2X reaction buffer: 10 mM DTT and 50 μ M Ac-DEVD-AFC] was added to the cell lysate, which was incubated for 1 hr at 37°C in the dark. The samples were read in a Spectramax Gemini XPS spectrofluorometer (Molecular Devices, Sunnyvale, CA) at excitation/emission wavelengths of 400/505 nm.

Molecular modeling

The crystal structure of the ACD (which contains K92) was taken from the Protein Data Bank (PDB id: 2WJ7).⁴⁷ The file contains the coordinates of residues 67–157, which are numbered starting at number 4. The structure was renumbered for analysis. Acetylated K92 was built and optimized in the YASARA program (YASARA Biosciences Inc., Vienna, Austria) employing an Amber03 force field. To visualize the effect of K92 acetylation, the electrostatic potential isosurfaces were calculated at the 25 kcal/mol level. The electrostatic potential was also mapped on the molecular surfaces of the wild-type and K92-acetylated α B-crystallin. All of the calculations and the generation of images were performed in YASARA.

Statistics

The results are presented as the means $\pm$ S.D. Significant differences among the groups were determined using analysis of variance, and $p < 0.05$ was considered significant.

RESULTS

MTCA-mediated introduction of the AcK mimic, MTCTK, occurs specifically on cysteine residues in proteins.⁴¹ The absence of cysteine residues in human α B-crystallin worked to our advantage. We introduced a cysteine residue at K92 and treated the resulting K92C protein with MTCA (Fig. 1). SDS-PAGE of the MTCA-treated WT and K92C protein showed single protein bands similar to the unmodified WT and K92C α B-crystallin (Fig. 2A). Western blotting using an AcK monoclonal antibody showed reaction with MTCA-modified K92C protein (Fig. 2B) but not with untreated WT or K92C protein. MTCA-treated WT protein did not show a reaction. Our procedure resulted in nearly 98% conversion of the cysteine to MTCTK, as revealed using a protein thiol assay (Fig. 2C). Mass spectrometric analysis of the peptides derived from the gel band covered approximately 97% of α B-crystallin's sequence. For C92-containing peptides, both unmodified (C92) and modified peptide (AcK mimic at C92) were identified in several trypsin miscut peptides, such as peptides HFSPEELKVCVLGDVIEVHGK (83–103),

HFSPEELKVCVLGDVIEVHGKHEER (83–107) and VCVLGDVIEVHGK (91–103). As an example, the tandem mass spectrum of ion m/z of 495.5900(3+) of the peptide VCVLGDVIEVHGK (91–103) with the AcK mimic is shown in Fig. 2D. Compared with the unmodified peptide, there is no mass change for the y series ions from y2 to y11. However, a mass shift of 117 Da was observed for y12, b series ions from b2 to b9 and the precursor ion, which indicates the modification of the C92 residue to MTCTK. Together, these data confirm the specific introduction of the AcK mimic at K92 in α B-crystallin.

Next, we determined whether MTCA treatment alone changes the structure and function of α B-crystallin. WT protein was treated with MTCA (conditions similar to those used for the K92C protein). This treatment changed neither the tryptophan fluorescence (Supplemental Fig. S3A) nor the surface hydrophobicity (Supplemental Fig. S3B) of the protein and did not alter the secondary structure of α B-crystallin (data not shown). Next, we determined whether MTCA treatment affected the chaperone activity of α B-crystallin. The chaperone activity was assessed using four client proteins, CS, Lyso, MDH and GC. The client proteins were subjected to thermal (CS, MDH and GC) or chemical (Lyso) stress to induce protein denaturation. The light scattering from the denatured proteins was monitored spectrophotometrically in the presence or absence of MTCA-treated or untreated α B-crystallin. Both proteins were equally effective against all four client proteins (Supplemental Fig. S3C). These results suggest that MTCA treatment alone does not affect the structure or the chaperone activity of α B-crystallin. Because MTCA treatment had no effect on the structure and chaperone activity and because MTCA-mediated conversion of K92C to MTCTK was close to 100%, in all of the subsequent experiments, we used only MTCA-treated K92C and MTCA-treated WT protein for comparison, hereafter referred to as acetyl and native proteins, respectively.

The far UV-CD spectra for the acetyl and native proteins were nearly identical (Fig. 3A). The secondary structural elements of the two proteins are shown in Table 1. The acetyl protein showed marginally higher α -helicity with a slight decrease in β -sheet structure relative to the native protein. Near UV-CD spectra of these two proteins revealed that the microenvironments of the aromatic amino acid residues were altered (Fig. 3B). The tryptophan fluorescence of the acetyl protein was 33% higher than that of the native protein (Fig. 3C), further implying that acetylation perturbed the microenvironment of the tryptophan residues. Taken together, these data show that K92 acetylation had a marginal effect on the secondary structure, but had a greater effect on the tertiary structure of the protein.

In its native state, α B-crystallin exists as a polydisperse oligomer that ranges from 4–40 subunits. Whether K92 acetylation affected the oligomeric structure (i.e., quaternary structure) of the protein was investigated using DLS-MALS. As shown in Fig. 4A, the acetyl protein had a broader peak compared with the native protein, with an average molar mass of 9.24×10^5 (g/mol) compared with 5.73×10^5 (g/mol) for the native protein. The hydrodynamic radii (R_h) of the native and acetyl proteins were 8.2 and 10 nm, respectively. Together, these data suggest that K92 acetylation led to an increase in the average oligomeric size, consisting of ~28 subunits in the native protein to ~46 subunits in the acetyl protein.

To determine whether an increase in the oligomeric size and the subtle changes in the tertiary structure had any effect on the overall structural stability of the acetyl protein, the thermodynamic stabilities of the native and acetyl proteins were compared. Equilibrium urea unfolding was measured by following the tryptophan fluorescence of these two proteins at various urea concentrations. The $\lambda_{\max}$ values for the native and unfolded WT α B-crystallin were recorded at 340 and 354 nm, and for the acetyl protein at 340 and 352 nm. We plotted

the ratio of the intensities in the native and unfolded states as a function of the urea concentration (Fig. 4B). An estimate of the transition midpoint ($C_{1/2}$) from the sigmoidal analysis of the denaturation profiles indicated that the $C_{1/2}$ value increased slightly from 2.41 M urea for the native protein to 2.51 M urea for the acetyl protein (Fig. 4B and Table 2). To measure the stability against chemical denaturation, all of the profiles were analyzed with the aid of a global three-state fitting procedure according to the following equation:

$$F = \frac{F_N + F_I \cdot \exp(-\Delta G_1^0 + m_1 \cdot [\text{urea}]) / RT + F_U \cdot \exp(-\Delta G_2^0 + m_2 \cdot [\text{urea}]) / RT}{1 + \exp(-\Delta G_1^0 + m_1 \cdot [\text{urea}]) / RT + \exp(-\Delta G_2^0 + m_2 \cdot [\text{urea}]) / RT}$$

where F_N , F_I and F_U are the fluorescence intensities for the 100% native, 100% intermediate and 100% unfolded forms, respectively. The term ΔG_1^0 refers to the standard free energy change between the native and intermediate forms, and ΔG_2^0 refers to the standard free energy change between the intermediate and unfolded forms. The term ΔG^0 is the sum of ΔG_1^0 and ΔG_2^0 and refers to the standard free energy change of unfolding (between the native and unfolded forms) at a urea concentration of zero. The fitted parameters are listed in Table 2. The standard free energy change of the unfolding of the native protein at 25°C was 22.94 kJ/mol. The ΔG^0 value for the acetyl protein increased to 29.95 kJ/mol, indicating an increase in thermodynamic stability by approximately 7 kJ/mol.

We then measured the chaperone activity of the protein using the same four client proteins used in the assessment of the effect of MTCA on WT protein (Supplemental Fig. S3C). The chaperone activity against CS, Lyso, MDH and GC was 38, 25, 75 and 66% more effective, respectively, with the acetyl protein than with the native protein (Fig. 5A). When tested against CS, the chaperone activity of the acetyl protein was consistently higher than WT protein across various α B-crystallin to client protein ratios (Supplemental Fig. S4). Since only a fraction of K92 residue in α B-crystallin is acetylated in the human lens,³⁶ we determined whether a mixture of acetyl and native proteins, wherein acetyl protein constituted 1 and 5% of the total protein (1% acetyl + 99% native, and 5% acetyl + 95% native) would show an improvement in the chaperone activity (over native protein). Remarkably, both mixtures showed significantly higher activity than the native protein against CS. When tested against GC, only the 5% acetyl + 95% native protein mixture showed a significant higher activity (Fig. 5B and C). Together, these data show that acetylation of K92 improves the chaperone activity of α B-crystallin and such an improvement in the chaperone activity is maintained even when the acetyl protein makes up a small portion of the total protein.

Next, we compared the ability of the native and acetyl protein to inhibit protein aggregation in HeLa cells. α B-Crystallin was transferred into cells with the aid of BioPORTER. This technique resulted in an equal amount of acetyl and native protein transferred into the cells, as revealed by western blotting (Fig. 6A). The proteins in the cell lysate aggregated and precipitated as insoluble pellets when incubated at 55°C. We then measured the concentration of recovered soluble protein in the supernatant. We found a significant difference between the acetyl and native protein in preventing protein aggregation when we used 4 μ g α B-crystallin and 2 hrs of incubation time (Supplemental Fig. S5). In the controls (without α B-crystallin), there was a 64% loss of protein in the supernatant; however, this value was reduced to 53% with the transfer of the native protein ($p < 0.05$ compared to control) and further reduced to 41% with the transfer of the acetyl protein $p < 0.005$ compared to control) (Fig. 6B). Protein precipitation was not observed in any of the samples maintained at room temperature. Together, these data imply that the acetyl protein maintained stronger chaperone activity than the native protein.

We also determined the ability of the native and acetyl protein to refold denatured proteins, using MDH as the client protein. When fully denatured, MDH was allowed to refold by diluting it in the appropriate refolding buffer (in the absence of α B-crystallin), a nominal 1% of the enzyme's activity was recovered (Fig. 7, trace 1). However, in the presence of 20 μ M native α B-crystallin, approximately 19% of the activity of MDH was regained (trace 2). At the same protein concentration, the recovery of the enzyme activity with acetyl α B-crystallin was 14% higher than with the native protein (trace 3). To rule out non-specific effects, we used BSA in the place of α B-crystallin, which had no effect on MDH refolding (trace 4). Thus, both the protein aggregation and the refolding assays showed that the acetylation of K92 improves the chaperone activity of α B-crystallin.

We measured the surface hydrophobicity using a hydrophobic probe, TNS, to determine whether an increase in the surface hydrophobicity resulted in higher chaperone activity of acetyl protein. The chaperone assays with Lyso, CS, MDH and GC were carried out at 25°C, 43°C, 50°C and 60°C, respectively; therefore, we measured the surface hydrophobicity at these temperatures. As shown in Fig. 8A, the fluorescence intensity of TNS bound to the acetyl protein was 11 to 20% higher compared with that of the native protein. These results suggest that K92 acetylation led to an increase in the hydrophobicity of the protein, which improved the chaperone activity of the protein.

To assess whether the improved chaperone activity is a result of higher binding of the client protein to the acetyl protein, we performed an equilibrium binding study using GC as the client protein (Fig. 8B). We determined the dissociation constant (K_d) using the Scatchard equation:

$$\tilde{v}/S = n/K_d - 1/K_d \cdot \tilde{v}$$

where $\tilde{v}$ is the number of moles of substrate bound per mole of chaperone, n is the number of binding sites and K_d is the dissociation constant. We noted that the number of binding sites (n) per subunit of acetylated protein increased to 3.34 from 2.33 in the native protein and that the K_d (dissociation constant) value decreased slightly from 2.81 μ M in the native protein to 2.73 μ M in the acetyl protein (Table 3). From these data, we conclude that the acetylation of K92 in α B-crystallin provides a higher affinity towards denatured proteins. However, in these experiments, we cannot rule out the client protein aggregation/association before binding to α B-crystallin at 60°C, which could result in a higher number of 'n' per molecule of α B-crystallin.

Next, we investigated the effect of K92 acetylation on the second important function of α B-crystallin, its ability to prevent apoptosis in stressed cells. We used HeLa cells for these experiments because these cells contain low levels of endogenous α B-crystallin. The native and acetyl proteins were transferred into cells using BioPORTER. We induced apoptosis by treating the cells with either 100 nM staurosporine or 20 μ M etoposide. As shown in Fig. 9A and B, both proteins significantly inhibited apoptosis. However, the acetyl protein was 18–20% more effective than the native protein in both treatments. The inhibition of apoptosis was accompanied by significant inhibition of caspase-3 activity in both treatments (Fig. 9C and D). Again, the acetyl protein was at least 5–10% more effective than the native protein. Taken together, these results show that the acetylation of K92 makes α B-crystallin a more effective anti-apoptotic protein through greater inhibition of caspase-3.

Molecular modeling based on the x-ray crystal structure of the ACD⁴⁷ shows that the removal of the positive charge on K92 by acetylation diminishes the electrostatic potential, changing this region from hydrophilic to hydrophobic. The resulting electrostatic potential

in this area is neutral due to compensation by ionic interactions between neighboring residues glutamine 99 (Q99) and K90. The shape of the electrostatic potential changed from a quadrupole to a dipole (Fig. 10A and B), which can facilitate the formation of large oligomers of ACD. Changes are also visible on the molecular surface, mapped with electrostatic potential (Fig. 10C and D). Thus, the molecular modeling supports the finding that K92 acetylation makes α B-crystallin more hydrophobic and consequently a better chaperone.

DISCUSSION

Lysine acetylation is a dynamic process, regulated through KATs and KDACs. Several recent studies have shown that this process plays an important role in the regulation of metabolism and cell functions.^{33, 34} We previously found that α A and α B-crystallin are acetylated at specific lysine residues in the lens.³⁷ The purpose of this study was to investigate the effects of lysine acetylation at K92 on the structure and functions of human α B-crystallin. This site was chosen based on our previous finding that K92 is acetylated in the human lens³⁷ and because it is a highly conserved residue among several major small heat shock proteins.

Our previous *in vitro* lysine acetylation using a chemical method showed improvement in the chaperone and anti-apoptotic activity of α -crystallin.^{37, 38} However, in these studies, lysine acetylation was performed by reacting α A-crystallin with acetic anhydride. This method indiscriminately acetylates lysine residues in α A-crystallin even at low acetic anhydride concentrations. Thus, the method does not permit investigation of the effects of specific AcK residues in α -crystallin. To circumvent this problem in this study, we used the novel chemical method of Huang et al.⁴¹ in which we used MTCA to introduce an AcK mimic at K92 by first mutating the lysine to cysteine and then converting the cysteine to MTCTK. Our mass spectrometric and western blotting data confirmed that we have achieved this goal. The fact that MTCA had no effect on either the structure or function of WT α B-crystallin and that C92 was almost entirely converted into the AcK mimic allowed us to compare only native and acetyl α B-crystallin in subsequent experiments.

The alteration in the near UV-CD spectra and the greater tryptophan fluorescence intensity of the acetyl protein relative to the native protein (in light of the same emission maxima) suggest that K92 acetylation perturbs only the tertiary structure of α B-crystallin. This finding is analogous to our previous observation that lysine acetylation in α A-crystallin resulted in increased tryptophan fluorescence.³⁷ The two tryptophan (W) residues (W9 and W60) in human α B-crystallin are located in the N-terminus of the protein, which is thought to be involved in monomeric interactions.⁴⁸ We speculate that the N-terminus is more relaxed in the acetyl protein, which results in the exposure of W9 and W60 to a more polar environment. Thus, the protein exhibits greater tryptophan fluorescence. Similarly, a previous study has shown changes in tryptophan fluorescence by subtle modifications in the ACD.⁴⁹

The DLS-MALS data revealed that K92 acetylation led to an increase in the molecular mass and the hydrodynamic radius of the protein. The average molar mass of the acetyl protein almost doubled to 975 kDa from 573 kDa for the native protein. This result implies that the acetyl protein exists in a larger oligomeric structure than the native protein. In α -crystallin, the core ACD is thought to be responsible for monomer interactions that lead to protein oligomerization.⁵⁰ The most favored model, based on solid-state NMR spectroscopy and cryo-electron microscopy, is that the dimeric building blocks of α B-crystallin assemble into hexameric rings through ACD interactions and that four such hexamers assemble through N-terminal interactions to form a 24-mer oligomer (one of many possible

oligomeric structures) of α B-crystallin.⁵¹ K92 is present within the ACD. Thus, its acetylation might enhance the oligomerization of the protein. However, monomeric interaction sites identified in previous studies do not contain K92.^{48, 52} Thus, the effect of K92 acetylation on the oligomerization could be a distal, secondary effect.

The increase in the chaperone activity of the acetyl protein is complemented by an increase in the surface hydrophobicity at four different temperatures. This finding indicates that the K92 acetylation that led to a larger oligomer of the protein might have contributed to the overall increase in surface hydrophobicity and, consequently, higher chaperone activity. The increased ability of the acetyl protein to refold a Gu-HCl-denatured protein (MDH) and the higher binding of a client protein (GC) supports this notion. K92 is located in the β 2 strand of the ACD in α B-crystallin. The results of the molecular modeling experiments are fully compatible with the surface hydrophobicity (TNS) and DLS-MALS data.

Previous studies did not establish a direct relationship between the chaperone activity and the oligomeric size of α -crystallin. In some studies, a lower molecular mass led to an improvement in the chaperone activity,^{53, 54} but other studies have shown the opposite⁵⁵ or no relationship.⁵⁶ In this study, we found that subtle changes in surface hydrophobic patches and in the tertiary structure caused by K92 acetylation increased the oligomeric size of α B-crystallin. We also observed that such acetylation increased the overall structural stability by 7 kJ/mol. We believe that the larger oligomeric assembly and greater structural stability contributed to the improvement of the chaperone activity of acetyl α B-crystallin. This conclusion is similar to observations in other previous studies, where it has been noted that increased chaperone activity of α -crystallin is associated with greater structural stability of the protein.^{45, 46}

The higher anti-apoptotic activity of the acetyl protein relative to the native protein could stem from its higher chaperone activity. We previously showed that the anti-apoptotic activity of α -crystallin is intricately linked to its chaperone function.²³ The higher anti-apoptotic activity of the acetyl protein could be due to its greater ability to block Bax translocation to mitochondria, release of cytochrome C into the cytoplasm and prevent caspase-8 and caspase-9 mediated activation of caspase-3. Further work is needed to verify these possibilities. The acetyl protein may more effectively preserve the cytoskeletal network, as α B-crystallin is known to bind cytoskeletal proteins and prevent their destabilization when cells are experiencing stress.⁵⁷ Whether lysine acetylation in α B-crystallin is reversed by lysine deacetylase in eye tissues is not known. Investigation into the enzymatic regulation of lysine acetylation should provide further insight into the role of this chemical modification on the functions of α B-crystallin.

What is the significance of our findings to lens and retinal biology? In the lens, negligible turnover of protein occurs; therefore, proteins have to be preserved in their native state to maintain transparency despite being exposed to sustained and chronic stresses. Acetylation of α B-crystallin could be a mechanism to enhance the chaperone activity and reduce the aggregation of proteins. In addition, apoptosis of lens epithelial cells is a common occurrence during cataract formation,^{58, 59} which may be inhibited to a greater extent by the acetyl protein than by native α B-crystallin. Similarly, acetylation of α B-crystallin in the retina could help in preventing apoptosis of cells, which occurs in diseases such as, macular degeneration, glaucoma and diabetic retinopathy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

FUNDING

This study was supported by NIH grants R01EY-022061, R01EY-023286 (RHN) and P30EY-11373 (Visual Sciences Research Center of CWRU); Research to Prevent Blindness, NY (to CWRU and UM-Columbia), The Ohio Lions Eye Research Foundation, CSIR, India grant 37(1535)/12/EMR-II (AB). SKN acknowledges the receipt of Institute Fellowship from the IIT Bhubaneswar. This work was also supported by the grant no N N401 557840 from Polish Ministry of Science and Higher Education (SF).

We thank Dr. Irina Pikuleva for the human retinal specimen and Dr. Scott Howell for help with immunohistochemistry.

Abbreviations

AcK	N ^ε -acetyllysine
MTCA	methylthiocarbonyl-aziridine
MTCTK	methylthiocarbonyl thialysine
CS	citrate synthase
MDH	malate dehydrogenase
GC	γ-crystallin
Lyso	lysozyme
TNS	2-(p-toluidinyl) naphthalene-6-sulfonic acid sodium salt
Gu-HCl	guanidine hydrochloride
DLS-MALS	dynamic light scattering-multi-angle light scattering
STS	staurosporine
ETP	etoposide
KAT	lysine acetyltransferase
PTEN	phosphatase and tensin homolog
RPE cells	retinal pigmented epithelial cells

References

1. Clark AR, Lubsen NH, Slingsby C. sHSP in the eye lens: crystallin mutations, cataract and proteostasis. *Int J Biochem Cell Biol.* 2012; 44:1687–1697. [PubMed: 22405853]
2. Horwitz J. Alpha-crystallin. *Exp Eye Res.* 2003; 76:145–153. [PubMed: 12565801]
3. Basha E, O'Neill H, Vierling E. Small heat shock proteins and alpha-crystallins: dynamic proteins with flexible functions. *Trends Biochem Sci.* 2012; 37:106–117. [PubMed: 22177323]
4. Peterson J, Radke G, Takemoto L. Interaction of lens alpha and gamma crystallins during aging of the bovine lens. *Exp Eye Res.* 2005; 81:680–689. [PubMed: 15967431]
5. Acunzo J, Katsogiannou M, Rocchi P. Small heat shock proteins HSP27 (HspB1), alphaB-crystallin (HspB5) and HSP22 (HspB8) as regulators of cell death. *Int J Biochem Cell Biol.* 2012; 44:1622–1631. [PubMed: 22521623]
6. Song S, Hanson MJ, Liu BF, Chylack LT, Liang JJ. Protein-protein interactions between lens vimentin and alphaB-crystallin using FRET acceptor photobleaching. *Mol Vis.* 2008; 14:1282–1287. [PubMed: 18618007]
7. Nicholl ID, Quinlan RA. Chaperone activity of alpha-crystallins modulates intermediate filament assembly. *EMBO J.* 1994; 13:945–953. [PubMed: 7906647]

8. Truscott RJ, Comte-Walters S, Ablonczy Z, Schwacke JH, Berry Y, Korlimbinis A, Friedrich MG, Schey KL. Tight binding of proteins to membranes from older human cells. *Age*. 2011; 33:543–554. [PubMed: 21181282]
9. Xi JH, Bai F, McGaha R, Andley UP. Alpha-crystallin expression affects microtubule assembly and prevents their aggregation. *FASEB J*. 2006; 20:846–857. [PubMed: 16675842]
10. Inaguma Y, Ito H, Iwamoto I, Saga S, Kato K. AlphaB-crystallin phosphorylated at Ser-59 is localized in centrosomes and midbodies during mitosis. *Eur J Cell Biol*. 2001; 80:741–748. [PubMed: 11831387]
11. Gangalum RK, Schibler MJ, Bhat SP. Small heat shock protein alphaB-crystallin is part of cell cycle-dependent Golgi reorganization. *J Biol Chem*. 2004; 279:43374–43377. [PubMed: 15339919]
12. Boyle DL, Takemoto L, Brady JP, Wawrousek EF. Morphological characterization of the Alpha A- and Alpha B-crystallin double knockout mouse lens. *BMC Ophthalmol*. 2003; 3:3. [PubMed: 12546709]
13. Brady JP, Garland D, Douglas-Tabor Y, Robison WG Jr, Groome A, Wawrousek EF. Targeted disruption of the mouse alpha A-crystallin gene induces cataract and cytoplasmic inclusion bodies containing the small heat shock protein alpha B-crystallin. *Proc Natl Acad Sci U S A*. 1997; 94:884–889. [PubMed: 9023351]
14. Andley UP, Hamilton PD, Ravi N, Weihl CC. A knock-in mouse model for the R120G mutation of alphaB-crystallin recapitulates human hereditary myopathy and cataracts. *PLoS One*. 2011; 6:e17671. [PubMed: 21445271]
15. Hsu CD, Kymes S, Petrash JM. A transgenic mouse model for human autosomal dominant cataract. *Invest Ophthalmol Vis Sci*. 2006; 47:2036–2044. [PubMed: 16639013]
16. Rekas A, Adda CG, Andrew Aquilina J, Barnham KJ, Sunde M, Galatis D, Williamson NA, Masters CL, Anders RF, Robinson CV, Cappai R, Carver JA. Interaction of the molecular chaperone alphaB-crystallin with alpha-synuclein: effects on amyloid fibril formation and chaperone activity. *J Mol Biol*. 2004; 340:1167–1183. [PubMed: 15236975]
17. Ecroyd H, Carver JA. Crystallin proteins and amyloid fibrils. *Cell Mol Life Sci*. 2009; 66:62–81. [PubMed: 18810322]
18. Wang X, Osinska H, Klevitsky R, Gerdes AM, Nieman M, Lorenz J, Hewett T, Robbins J. Expression of R120G-alphaB-crystallin causes aberrant desmin and alphaB-crystallin aggregation and cardiomyopathy in mice. *Circ Res*. 2001; 89:84–91. [PubMed: 11440982]
19. Rothbard JB, Kurnellas MP, Brownell S, Adams CM, Su L, Axtell RC, Chen R, Fathman CG, Robinson WH, Steinman L. Therapeutic effects of systemic administration of chaperone alphaB-crystallin associated with binding proinflammatory plasma proteins. *J Biol Chem*. 2012; 287:9708–9721. [PubMed: 22308023]
20. Ousman SS, Tomooka BH, van Noort JM, Wawrousek EF, O'Connor KC, Hafler DA, Sobel RA, Robinson WH, Steinman L. Protective and therapeutic role for alphaB-crystallin in autoimmune demyelination. *Nature*. 2007; 448:474–479. [PubMed: 17568699]
21. Kurnellas MP, Brownell SE, Su L, Malkovskiy AV, Rajadas J, Dolganov G, Chopra S, Schoolnik GK, Sobel RA, Webster J, Ousman SS, Becker RA, Steinman L, Rothbard JB. Chaperone activity of small heat shock proteins underlies therapeutic efficacy in experimental autoimmune encephalomyelitis. *J Biol Chem*. 2012; 287:36423–36434. [PubMed: 22955287]
22. Fort PE, Lampi KJ. New focus on alpha-crystallins in retinal neurodegenerative diseases. *Exp Eye Res*. 2011; 92:98–103. [PubMed: 21115004]
23. Pasupuleti N, Matsuyama S, Voss O, Doseff AI, Song K, Danielpour D, Nagaraj RH. The anti-apoptotic function of human alphaA-crystallin is directly related to its chaperone activity. *Cell Death Dis*. 2010; 1:e31. [PubMed: 21364639]
24. Kamradt MC, Chen F, Cryns VL. The small heat shock protein alpha B-crystallin negatively regulates cytochrome c- and caspase-8-dependent activation of caspase-3 by inhibiting its autoproteolytic maturation. *J Biol Chem*. 2001; 276:16059–16063. [PubMed: 11274139]
25. Sreekumar PG, Kannan R, Kitamura M, Spee C, Barron E, Ryan SJ, Hinton DR. alphaB crystallin is apically secreted within exosomes by polarized human retinal pigment epithelium and provides neuroprotection to adjacent cells. *PLoS One*. 2010; 5:e12578. [PubMed: 20949024]

26. Bhat SP, Gangalum RK. Secretion of alphaB-Crystallin via exosomes: New clues to the function of human retinal pigment epithelium. *Commun Integr Biol.* 2011; 4:739–741. [PubMed: 22446542]
27. Kannan R, Sreekumar PG, Hinton DR. Novel roles for alpha-crystallins in retinal function and disease. *Prog Retin Eye Res.* 2012; 31:576–604. [PubMed: 22721717]
28. Losiewicz MK, Fort PE. Diabetes impairs the neuroprotective properties of retinal alpha-crystallins. *Invest Ophthalmol Vis Sci.* 2011; 52:5034–5042. [PubMed: 21467180]
29. Adhikari AS, Sridhar Rao K, Nagaraj R, Parnaik VK, Mohan Rao C. Heat stress-induced localization of small heat shock proteins in mouse myoblasts: intranuclear lamin A/C speckles as target for alphaB-crystallin and Hsp25. *Exp Cell Res.* 2004; 299:393–403. [PubMed: 15350538]
30. McGreal RS, Kantorow WL, Chauss DC, Wei J, Brennan LA, Kantorow M. alphaB-crystallin/sHSP protects cytochrome c and mitochondrial function against oxidative stress in lens and retinal cells. *Biochim Biophys Acta.* 2012; 1820:921–930. [PubMed: 22521365]
31. Sadoul K, Wang J, Diagouraga B, Khochbin S. The tale of protein lysine acetylation in the cytoplasm. *J Biomed Biotechnol.* 2011; 2011:970382. [PubMed: 21151618]
32. Yang XJ, Seto E. Lysine acetylation: codified crosstalk with other posttranslational modifications. *Mol Cell.* 2008; 31:449–461. [PubMed: 18722172]
33. Zhao S, Xu W, Jiang W, Yu W, Lin Y, Zhang T, Yao J, Zhou L, Zeng Y, Li H, Li Y, Shi J, An W, Hancock SM, He F, Qin L, Chin J, Yang P, Chen X, Lei Q, Xiong Y, Guan KL. Regulation of cellular metabolism by protein lysine acetylation. *Science.* 2010; 327:1000–1004. [PubMed: 20167786]
34. Choudhary C, Kumar C, Gnad F, Nielsen ML, Rehman M, Walther TC, Olsen JV, Mann M. Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science.* 2009; 325:834–840. [PubMed: 19608861]
35. Lin PP, Barry RC, Smith DL, Smith JB. In vivo acetylation identified at lysine 70 of human lens alphaA-crystallin. *Prot Sci.* 1998; 7:1451–1457.
36. Lapko VN, Smith DL, Smith JB. In vivo carbamylation and acetylation of water-soluble human lens alphaB-crystallin lysine 92. *Prot Sci.* 2001; 10:1130–1136.
37. Nagaraj RH, Nahomi RB, Shanthakumar S, Linetsky M, Padmanabha S, Pasupuleti N, Wang B, Santhoshkumar P, Panda AK, Biswas A. Acetylation of alphaA-crystallin in the human lens: effects on structure and chaperone function. *Biochim Biophys Acta.* 2012; 1822:120–129. [PubMed: 22120592]
38. Nahomi RB, Oya-Ito T, Nagaraj RH. The combined effect of acetylation and glycation on the chaperone and anti-apoptotic functions of human alpha-crystallin. *Biochim Biophys Acta.* 2013; 1832:195–203. [PubMed: 22982407]
39. Wang X, Hayes JJ. Acetylation mimics within individual core histone tail domains indicate distinct roles in regulating the stability of higher-order chromatin structure. *Mol Cell Biol.* 2008; 28:227–236. [PubMed: 17938198]
40. Scroggins BT, Robzyk K, Wang D, Marcu MG, Tsutsumi S, Beebe K, Cotter RJ, Felts S, Toft D, Karnitz L, Rosen N, Neckers L. An acetylation site in the middle domain of Hsp90 regulates chaperone function. *Mol Cell.* 2007; 25:151–159. [PubMed: 17218278]
41. Huang R, Holbert MA, Tarrant MK, Curtet S, Colquhoun DR, Dancy BM, Dancy BC, Hwang Y, Tang Y, Meeth K, Marmorstein R, Cole RN, Khochbin S, Cole PA. Site-specific introduction of an acetyl-lysine mimic into peptides and proteins by cysteine alkylation. *J Am Chem Soc.* 2010; 132:9986–9987. [PubMed: 20608637]
42. Biswas A, Das KP. Differential recognition of natural and nonnatural substrate by molecular chaperone alpha-crystallin-A subunit exchange study. *Biopolymers.* 2007; 85:189–197. [PubMed: 17103422]
43. Nagaraj RH, Panda AK, Shanthakumar S, Santhoshkumar P, Pasupuleti N, Wang B, Biswas A. Hydroimidazolone modification of the conserved Arg12 in small heat shock proteins: studies on the structure and chaperone function using mutant mimics. *PLoS One.* 2012; 7:e30257. [PubMed: 22272318]
44. Pasupuleti N, Gangadhariah M, Padmanabha S, Santhoshkumar P, Nagaraj RH. The role of the cysteine residue in the chaperone and anti-apoptotic functions of human Hsp27. *J Cell Biochem.* 2010; 110:408–419. [PubMed: 20225272]

45. Biswas A, Goshe J, Miller A, Santhoshkumar P, Luckey C, Bhat MB, Nagaraj RH. Paradoxical effects of substitution and deletion mutation of Arg56 on the structure and chaperone function of human alphaB-crystallin. *Biochemistry*. 2007; 46:1117–1127. [PubMed: 17260942]
46. Biswas A, Das KP. Role of ATP on the interaction of alpha-crystallin with its substrates and its implications for the molecular chaperone function. *J Biol Chem*. 2004; 279:42648–42657. [PubMed: 15292216]
47. Bagneris C, Bateman OA, Naylor CE, Cronin N, Boelens WC, Keep NH, Slingsby C. Crystal structures of alpha-crystallin domain dimers of alphaB-crystallin and Hsp20. *J Mol Biol*. 2009; 392:1242–1252. [PubMed: 19646995]
48. Ghosh JG, Clark JI. Insights into the domains required for dimerization and assembly of human alphaB crystallin. *Prot Sci*. 2005; 14:684–695.
49. Liu Y, Zhang X, Luo L, Wu M, Zeng R, Cheng G, Hu B, Liu B, Liang JJ, Shang F. A novel alphaB-crystallin mutation associated with autosomal dominant congenital lamellar cataract. *Invest Ophthalmol Vis Sci*. 2006; 47:1069–1075. [PubMed: 16505043]
50. Berengian AR, Parfenova M, McHaourab HS. Site-directed spin labeling study of subunit interactions in the alpha-crystallin domain of small heat-shock proteins. Comparison of the oligomer symmetry in alphaA-crystallin, HSP 27, and HSP 16.3. *J Biol Chem*. 1999; 274:6305–6314. [PubMed: 10037719]
51. Delbecq SP, Klevit RE. One size does not fit all: The oligomeric states of alphaB crystallin. *FEBS Lett*. 2013; 587:1073–1080. [PubMed: 23340341]
52. Sreelakshmi Y, Sharma KK. Recognition sequence 2 (residues 60–71) plays a role in oligomerization and exchange dynamics of alphaB-crystallin. *Biochemistry*. 2005; 44:12245–12252. [PubMed: 16142923]
53. Santhoshkumar P, Murugesan R, Sharma KK. Deletion of (54)FLRAPSWF(61) residues decreases the oligomeric size and enhances the chaperone function of alphaB-crystallin. *Biochemistry*. 2009; 48:5066–5073. [PubMed: 19388699]
54. Pasta SY, Raman B, Ramakrishna T, Rao Ch M. Role of the conserved SRLFDQFFG region of alpha-crystallin, a small heat shock protein. Effect on oligomeric size, subunit exchange, and chaperone-like activity. *The Journal of biological chemistry*. 2003; 278:51159–51166. [PubMed: 14532291]
55. Kumar LV, Rao CM. Domain swapping in human alpha A and alpha B crystallins affects oligomerization and enhances chaperone-like activity. *J Biol Chem*. 2000; 275:22009–22013. [PubMed: 10896951]
56. Saha S, Das KP. Unfolding and refolding of bovine alpha-crystallin in urea and its chaperone activity. *Prot J*. 2007; 26:315–326.
57. Wettstein G, Bellaye PS, Micheau O, Bonniaud P. Small heat shock proteins and the cytoskeleton: an essential interplay for cell integrity? *Int J Biochem Cell Biol*. 2012; 44:1680–1686. [PubMed: 22683760]
58. Li WC, Kuszak JR, Dunn K, Wang RR, Ma W, Wang GM, Spector A, Leib M, Cotliar AM, Weiss M, et al. Lens epithelial cell apoptosis appears to be a common cellular basis for non-congenital cataract development in humans and animals. *J Cell Biol*. 1995; 130:169–181. [PubMed: 7790371]
59. Takamura Y, Kubo E, Tsuzuki S, Akagi Y. Apoptotic cell death in the lens epithelium of rat sugar cataract. *Exp Eye Res*. 2003; 77:51–57. [PubMed: 12823987]

Figure 1. A schematic illustration of the site-specific introduction of an AcK mimic into αB-crystallin
 αB-Crystallin mutant K92Cys was treated with MTCA to generate an AcK mimic at K92. The absence of cysteine residues in αB-crystallin allowed us to introduce an AcK mimic at K92 by first mutating K92 to Cys⁹².

Figure 2. The confirmation of the introduction of the AcK mimic into α B-crystallin

A, SDS-PAGE of WT (lane 1), K92Cys (lane 2), WT treated with MTCA (lane 3) and K92Cys treated with MTCA (lane 4). **B**, Western blotting using an AcK antibody showed reaction with only K92Cys treated with MTCA (lane assignments are the same as in **A**). **C**, Estimation of protein thiol content showed that nearly 98% of the cysteine in the K92Cys (K92C) was modified to MTCTK. α B(a)=acetyl α B-crystallin. **D**, Mass spectrometric identification of the AcK mimic at K92 in α B-crystallin. The tandem mass spectrum of the α B-crystallin peptide VCVLGDVIEVHGK (91–103) with a precursor ion of 495.5900(3+) and K92Cys-modified MTCTK is shown.

Figure 3. The structural changes in acetyl α B-crystallin

The far (A) and near UV-CD spectra (B) for the native (α B) and acetyl α B-crystallin [α B(a)]. Tryptophan fluorescence (C) was higher for the acetyl protein than for the native protein.

Figure 4. The oligomerization and thermodynamic stability of acetyl α B-crystallin

A, DLS-MALS profiles for native and acetyl α B-crystallin. The acetyl protein showed a higher oligomeric size than the native protein. **B**, Equilibrium urea denaturation profile for 0.05 mg/ml native and acetyl α B-crystallin at 25°C. The profile is normalized to a scale of 0 to 1. The symbols represent the experimental data points, and the lines represent the best fit according to the three-state model.

Figure 5. Acetylation of K92 improves the chaperone activity of αB-crystallin

A, The chaperone activities of the native and acetyl αB-crystallins were measured using citrate synthase (CS), lysozyme (Lyso), γ-crystallin (GC) and malate dehydrogenase (MDH). The following chaperone to client protein ratios (w/w) were used: CS, 1:2; Lyso, 1:4; MDH, 1:8; and GC, 1:6. Representative traces for each assay are shown in the left panels. A mixture of 1% acetyl and 99% native (0.125 μg acetyl + 12.375 μg native, CS = 25 μg, αB-crystallin to CS ratio = 1:2) and 5% acetyl and 95% native αB-crystallin (=0.625 μg of acetyl and 11.875 μg native, CS=25 μg, αB-crystallin to CS ratio = 1:2) showed significantly improved the chaperone activity against CS (**B**) and GC (**C**). αB=native αB-crystallin. αB(a)=acetyl αB-crystallin. Bars are the means ± SD of three independent experiments. * = $p < 0.05$, ** = $p < 0.005$ and *** = $p < 0.0005$, NS= not significant.

Figure 6. Chaperone activity of acetyl αB-crystallin is better than the native protein in HeLa cells

HeLa cells were transferred with native or acetyl αB-crystallin. **A**, Western blotting and densitometry (bar graph) showed that protein transferred with the aid of BioPORTER was equal for the two proteins. Lane 1, cell lysate; lane 2, cells transferred with native αB-crystallin; lane 3, cells transferred with acetyl αB-crystallin. **B**, Cell lysates from αB-crystallin-transferred HeLa cells were incubated at 55°C for 2 hrs, and the aggregated protein was removed by centrifugation. The protein content in the supernatant was measured. αB= native αB-crystallin. αB(a)=acetyl αB-crystallin. Bars are the means $\pm$ SD of three independent experiments. * = $p < 0.05$, ** = $p < 0.005$ and *** = $p < 0.0005$.

Figure 7. The effect of K92 acetylation on the ability of α B-crystallin to refold denatured MDH
 The enzyme activity of MDH (which is directly related to protein refolding) was abolished by incubation in a 6 M Gu-HCl solution at 25°C. Trace 1, 10 nM MDH alone; trace 2, 10 nM MDH + 20 μ M native α B-crystallin; trace 3, 10 nM MDH + 20 μ M acetyl α B-crystallin; trace 4, 10 nM MDH + 20 μ M BSA. Refolding was initiated by 100-fold dilution of MDH (1 μ M) in 6 M Gu-HCl in refolding buffer (pH 7.5) containing 50 mM phosphate, 10 mM magnesium acetate, and 5 mM DTT. Each data point is the average of triplicate measurements.

Figure 8. The surface hydrophobicity of acetyl αB -crystallin and estimation of binding parameters with client proteins

A. Surface hydrophobicity was probed with TNS at different temperatures. At all of the temperatures, the acetyl protein exhibited higher surface hydrophobicity than the native protein. **B.** The dissociation constant (K_d) and the number of binding sites (n) for the interaction between GC and αB -crystallin at 60°C were estimated using the Scatchard equation shown in the Results section. αB =native αB -crystallin. $\alpha B(a)$ =acetyl αB -crystallin.

Figure 9. The inhibition of apoptosis by acetyl αB-crystallin was more effective than by native αB-crystallin

The inhibition of staurosporine-induced (STS) (A) and etoposide-induced (ETP) (B) apoptosis in HeLa cells by the acetyl and native proteins. The protein was transferred with the aid of BioPORTER, and cells were treated with staurosporine and etoposide as described in Experimental Procedures. Apoptosis was detected and quantified after staining for annexin V. The bars represent the means $\pm$ SD of three independent experiments. Acetyl αB-crystallin inhibits caspase-3 activity more aggressively than native αB-crystallin. Caspase-3 activity in staurosporine (C) and etoposide (D) was measured using a fluorogenic substrate. αB= native αB-crystallin. αB(a)=acetyl αB-crystallin. The bars represent the means $\pm$ SD of three independent experiments. * = $p < 0.05$, ** = $p < 0.005$ and *** = $p < 0.0005$.

Figure 10. Molecular modeling of K92 acetylation in the ACD of αB-crystallin

Modeling was based on the published X-ray crystal structure of the ACD of αB-crystallin (PDB id 2WJ7). The loss of the positive charge on the epsilon amino group of K92, together with the electrostatic interaction of the neighboring Lys⁹⁰ and Glu⁹⁹ residues, makes the average local charge zero in this area and changes the region from hydrophilic to hydrophobic. The electrostatic potentials for native (A) and acetyl αB-crystallin (B) are shown as isosurface blobs at a level of +25 kcal/mol (in blue) and -25 kcal/mol (in red). The molecular surface of crystallin mapped with the electrostatic potential for native (C) and acetyl αB-crystallin (D). The positive and negative electrostatic potentials on the isosurfaces and on the molecular surfaces are colored in blue and red, respectively.

Table 1Secondary structural elements in native and acetyl α B-crystallin

Protein	α -helix (%)	β -sheet (%)	β -turn (%)	Random coil (%)
α B-native	14.4	38.3	15.8	31.5
α B-acetyl	16.3	34.4	16.1	33.2

Table 2

$C_{1/2}$ and ΔG^0 values for native and acetyl α B-crystallin at 25°C

Protein	$C_{1/2}$ (M)	ΔG^0 (kJ/mol)
α B-native	2.41 ± 0.02	22.94 ± 0.92
α B-acetyl	2.51 ± 0.03	29.95 ± 1.06

Table 3

Determination of n and dissociation constant (K_d) values for the interaction of native and acetyl α B-crystallin with GC at 60°C

System studied	n	K_d (μ M)
α B-native + GC	2.33 ± 0.04	2.81 ± 0.14
α B-acetyl + GC	3.34 ± 0.06	2.73 ± 0.19