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Succinylation Is a Gain-of-Function Modification in Human Lens α B-Crystallin

Sandip K. Nandi^{†,§}, Stefan Rakete^{†,⊥,§}, Rooban B. Nahomi^{†,§}, Cole Michel[‡], Alexandra Dunbar^{†,||}, Kristofer S. Fritz[‡], Ram H. Nagaraj^{*,†,‡}

[†]Sue Anschutz-Rodgers Eye Center and Department of Ophthalmology, School of Medicine, University of Colorado, Anschutz Medical Campus, Aurora, Colorado 80045, United States

[‡]Department of Pharmaceutical Sciences, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado, Anschutz Medical Campus, Aurora, Colorado 80045, United States

Abstract

Acylation of lysine residues is a common post-translational modification of cellular proteins. Here, we show that lysine succinylation, a type of acylation, occurs in human lens proteins. All of the major crystallins exhibited *N*^ε-succinyllysine (SuccK) residues. Quantification of SuccK in human lens proteins (from donors between the ages of 20 and 73 years) by LC–MS/MS showed a range between 1.2 and 14.3 pmol/mg lens protein. The total SuccK levels were slightly reduced in aged lenses (age > 60 years) relative to young lenses (age < 30 years). Immunohistochemical analyses revealed that SuccK was present in epithelium and fiber cells. Western blotting and immunoprecipitation experiments revealed that SuccK is particularly prominent in α B-crystallin, and succinylation *in vitro* revealed that α B-crystallin is more prone to succinylation than α A-crystallin. Mass spectrometric analyses showed succinylation at K72, K90, K92, K166, K175, and potentially K174 in human lens α B-crystallin. We detected succinylation at K72, K82, K90, K92, K103, K121, K150, K166, K175, and potentially K174 by mass spectrometry in mildly succinylated α B-crystallin. Mild succinylation improved the chaperone activity of α B-crystallin along with minor perturbation in tertiary and quaternary structure of the protein. These

ASSOCIATED CONTENT

*Corresponding Author ram.nagaraj@ucdenver.edu. Phone: 303-724-5922. Fax: 303-724-5270.

[⊥]Present Addresses

S.R.: Institute and Clinic for Occupational, Social and Environmental Medicine, University Hospital LMU Munich, Ziemssenstr. 1, Munich D-80336, Germany

^{||}K.S.F.: Biomedical Engineering Department, Washington University, St. Louis, Whitaker Hall, Room 390B, St. Louis, MO 63130, USA

[§]S.K.N., S.R., and R.B.N. contributed equally to this work

Supporting Information

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Q-TOF tandem MS/MS mass spectrometric identification of SuccK residues in α A, β , and γ -crystallins and other proteins from human lenses (immunoprecipitated using SuccK antibody), MS/MS spectra of SuccK-bearing peptides from *in vitro* succinylated α B and α A from WS of human lenses (23Y and 73Y), MS/MS spectra of SuccK-bearing peptides from WS of human lens proteins (23Y and 73Y), effect of CS on the aggregation of insulin, succinylation of BSA and ADH, succinylation does not alter the inhibitory capacity of BSA and ADH against insulin and CS aggregation, and the relationship between the chaperone activity and surface hydrophobicity of succinylated α B-crystallin in (A) insulin aggregation and (B) CS aggregation assays (PDF)

The authors declare no competing financial interest.

observations imply that succinylation is beneficial to α B-crystallin by improving its chaperone activity with only mild conformational alterations.

Graphical abstract

The ocular lens is a unique avascular organ, the major function of which is to focus images onto the retina. It has elongated fiber cells that accumulate high concentrations of crystallin proteins during their differentiation from epithelial cells. Crystallins comprising α -, β -, and γ -crystallin constitute 90% of the total lens protein.¹ Whereas β - and γ -crystallin are primarily structural proteins in the lens, α -crystallin is a functional protein, and it accounts for approximately 40% of the total proteins in human lenses.² α -Crystallin is an oligomeric protein comprising two subunits, α A-crystallin and α B-crystallin, that associate in a 3:1 molar ratio in the human lens.³ Like all small heat shock proteins (sHSPs), both α A-crystallin and α B-crystallin possess a conserved “ α -crystallin domain” and are major β -sheet proteins.⁴ Through their chaperone activity, they prevent aggregation of other lens proteins. In addition, several studies of human hereditary cataract and genetically altered animals have revealed that a loss in the chaperone activity of α -crystallin results in cataract formation.^{5–7} Based on these observations, the chaperone activity of α A-crystallin and α B-crystallin is postulated to be critical for the maintenance of lens transparency.

Several studies have shown that post-translational modifications (PTMs) of α -crystallin alter its structural and functional properties. Oxidation of α -crystallin causes conformational alterations and decreases chaperone activity,¹ and deamidation perturbs its tertiary conformations, which leads to unfolding and aggregation.⁸ Phosphorylation of α -crystallin alters its surface charge, quaternary structure, and stability and reduces its solubility and chaperone activity.^{9,10} However, some studies suggest that phosphorylation improves the chaperone activity of α B-crystallin.^{12,13} In addition, modification of α A-crystallin with methylglyoxal has been shown to improve the chaperone activity.¹¹

Another related PTM is acylation,¹² which occurs on lysine residues in proteins. The acyl modifications of lysine residues include acetylation,^{13,14} propionylation,¹⁵ butyrylation,¹⁶ malonylation,¹⁷ crotonylation,¹⁸ and succinylation.^{19,20} Among all of these acyl modifications, succinylation and acetylation are found to occur predominantly in eukaryotes.^{21,22} Previously, our group demonstrated acetylation (Figure 1) in human lens α A-, α B-, and γ -crystallins.^{23–25}

Our studies have revealed that acetylation of the N-terminal glycine1 and lysine2 (K2) residues in γ D-crystallin promotes its aggregation by destabilizing its structure.²³ We also showed that K70 and K99 in α A-crystallin and K92 and K166 in α B-crystallin are acetylated in human lenses.^{24,25} Furthermore, we showed that the introduction of an acetylation mimic at K92 in α B-crystallin improved its chaperone activity.²⁴ In addition, we previously demonstrated that acetyl derivatives of the mini-chaperone peptides of α A-crystallin and α B-crystallins inhibited sodium selenite-induced lens epithelial cell apoptosis and cataract development to a slightly greater extent than the native peptides.²⁶ These observations suggest that acetylation of α -crystallin is beneficial to the lens.

Succinylation is another acyl modification that overlaps extensively with acetylation in eukaryotes.²¹ It involves conversion of the positive charge on the epsilon amino group of a lysine residue to a negatively charged carboxylic acid (Figure 1). Extensive succinylation of proteins in the mitochondria and cytoplasm of cells has been reported.²¹ Whether lens proteins are succinylated and whether succinylation affects functions of α -crystallin have not been studied yet. We report here that succinylation occurs in all of the major lens crystallins and that α B-crystallin is more prone to succinylation than α A-crystallin. We also show that succinylation improves the chaperone activity of α B-crystallin.

EXPERIMENTAL PROCEDURES

Materials.

Insulin, citrate synthase, protease inhibitor cocktail, dithiothreitol, trinitrobenzenesulfonic acid (TNBS), 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid (bis-ANS), dipotassium salt, and succinyl coenzyme A (SuccCoA) were obtained from Sigma-Aldrich (St. Louis, MO). *N*^ε-Acetyllysine was obtained from Chem-Impex International Inc. (Wood Dale, IL). All other chemicals were of analytical grade.

Cloning, expression, and purification of human α A and α B were performed as previously described.^{24,25}

Western Blot Detection of SuccK in Lens Proteins.

Human lenses were obtained from Saving Sight, Kansas City, MO, and stored at -80°C until use. Water-soluble protein (WS) and water-insoluble protein (WIS) subjected to sonication and centrifugation were prepared as previously described.²⁵ For Western blotting, protein (30 μg) was separated on 12% SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane, and probed with a SuccK antibody (diluted 1:2000, cat. no. PTM-419, PTM Biolabs, Chicago, IL). The membrane was stripped and reprobed for α B-crystallin (diluted 1:500 000, Developmental Studies Hybridoma Bank; we obtained a hybridoma culture from this vendor and purified the antibody ourselves). The secondary antibody was horseradish peroxidase (HRP)-conjugated antirabbit IgG (diluted 1:5000, Cell Signaling Technology, Danvers, MA, cat. no. 7076S). To show protein loading in the Western blots, membranes were stained with Ponceau S stain.

Immunoprecipitation to Deplete α B-Crystallin in Human Lens WS.

We immunoprecipitated α B-crystallin from 100 μ g of WS protein (human 9-year-old lens) using 100 μ g of the α B-crystallin monoclonal antibody and treated with Protein A-Agarose (40 μ L, Santa Cruz Biotechnology), centrifuged at 1000g for 5 min and used the supernatant in Western blotting for α B-crystallin or SuccK. The sample was processed similarly but without the antibody served as a control.

Immunohistochemistry.

Paraffin-embedded human eye sections after deparafinization rehydration and high-temperature antigen retrieval were blocked with 5% normal goat serum in PBS for 1 h. Sections were incubated overnight at 4 °C with a monoclonal antibody for SuccK (diluted 1:100) followed by 1 h of incubation at 37 °C with Alexa Fluor 488 conjugated goat antimouse IgG (1:250 dilution, cat no. A11001, Life Technologies, Carlsbad, CA). Sections were mounted with DAPI/Vectashield for observations of the nuclei. Images were taken using a fluorescence microscope (Nikon Eclipse 80i, Nikon) at 20 $\times$ magnification and Nikon NIS software. The background fluorescence from the secondary antibody was verified by processing sections as above but eliminating incubation with the primary antibody.

Determination of Reactive Amino Groups in α -Crystallin.

The TNBS assay was adopted as previously described.²⁷ Briefly, α A or α B (0.2 mg/mL) was incubated with TNBS (0.02%) in 100 mM NaHCO₃ buffer, pH 8, at 40 °C for 3 h. The absorbance was measured at 335 nm against a reagent blank.

Succinylation of α A, α B, and WS *in Vitro*.

SuccCoA was prepared at a 10 mM concentration in PBS and added to 300 μ g of α B (in 50 mM phosphate buffer, pH 7.4) such that the final molar ratios of lysine residues in α B to SuccCoA reached 1:0.01, 1:0.007, 1:0.003, and 1:0.001. The pH of each mixture was adjusted to between 7.5 and 8 by adding 0.5 M NaOH, and the mixture was incubated for 3 h at 4 °C. Control samples were processed similarly except for the absence of SuccCoA. The samples were then dialyzed overnight against 50 mM phosphate buffer, pH 7.4. Succinylated α B prepared at the 1:0.001 molar ratio is referred to as Succ- α B hereafter.

To determine the relative succinylation capacity of α A and α B (each 1 mg/mL), the two proteins were succinylated under identical conditions using 0.01 mM SuccCoA and a pH between 7.5 and 8, as above. The samples were then analyzed by electrophoresis and Western blots, and the SuccK was detected using the monoclonal antibody described above diluted 1:2,000. Water-soluble 25-, 30-, and 31-year-old lenses were succinylated using 0.01 mM SuccCoA under the conditions as above and analyzed in Western blots for differential succinylation of α A-crystallin and α B-crystallin.

Q-TOF Tandem MS/MS Mass Spectrometric Identification of SuccK Residues.

Succ- α B (47 μ g) and human lens proteins (5 mg) were digested using either a modified filter-aided sample preparation method,²⁸ or the urea digest protocol provided by cell signaling technologies. Succ- α B samples were denatured, reduced, and alkylated using 4%

SDS in 100 mM Tris-HCl buffer at pH 8.5, tris(2-carboxyethyl)phosphine (TCEP), and iodoacetamide, respectively. SDS was removed using an Amicon Ultra 30 kDa molecular weight cutoff spin filter by sequentially washing with 100 mM Tris-HCl, pH 8.5, containing 8 M urea followed by 2 M urea and finally with 5% trifluoroethanol (TFE) in 50 mM ammonium bicarbonate (ABC). The protein was then digested at room temperature for 4 h with a ratio of 1:50 Lys-C (Wako chemicals, Osaka, Japan) to substrate and then overnight at 37 °C with a ratio of 1:40 trypsin to substrate. The resulting peptide solutions were evaporated to dryness in a Speed Vac concentrator at 45 °C. Human lens samples were digested with trypsin and C18 cleaned according to the manufacturer's protocol provided with the SuccK Motif Kit no. 13764 (Cell Signaling Technology). Briefly, human lens protein was solubilized and denatured using a urea lysis buffer with protease inhibitors before being reduced and alkylated with dithiothreitol (DTT) and iodoacetamide. Samples were diluted to a final urea concentration of 2 M in 20 mM HEPES buffer before digesting overnight at room temperature with 1:40 trypsin to substrate on a platform rocker set to 30 rpm. The resulting peptide solution was desalted using a 0.7 mL C18 Sep-Pak column (Waters Corporation, Milford, MA), and the eluted peptides were lyophilized. An immunoprecipitation for peptides bearing a SuccK modification was performed on both digested Succ- α B and human lens samples using the SuccK Motif Kit no. 13764 according to the manufacturer's protocol. Briefly, the beads were washed with PBS four times before incubation with the sample for 2 h at 4 °C on a shaker. After incubation, the supernatant was removed, and the beads were washed twice with ice-cold 1× IAP buffer and then three times with ice-cold water (Honeywell Inc. Burdick and Jackson, Morris Plains, NJ). The peptides were eluted by two washes with 0.15% TFA, pooled, and purified with a Pierce C18 spin column (Thermo Fisher Scientific Inc., Waltham, MA) before being evaporated to dryness in a Speed Vac concentrator at 25 °C. The peptides were resuspended in 3% acetonitrile in 0.1% formic acid for MS analysis. The enriched SuccK-bearing peptides were loaded onto a nano ProntoSIL 5u 200A C18AQ trapping column, and chromatography was performed on a 0.1 mm × 150 mm 3u 200A ProntoSIL C18AQ reverse phase nano column (nano LCMS Solutions LLC, Gold River, CA) using a nano-Advance UPLC (Bruker Daltonics, Inc., Billerica, MA). The mobile phase consisted of water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B). Peptides of Succ- α B were separated at a flow rate of 800 nL/min using a gradient of 2–50% B over 30 min. Peptides from human lens proteins were separated at a flow rate of 800 nL/min using a gradient of 5–50% B for 60 min followed by a column wash at 90% B for 5 min. Data were collected on an Impact HD Q-TOF equipped with a captive spray source (Bruker Daltonics Inc., Billerica, MA) operated using intensity-dependent CID MS/MS. Proteinscape software (Bruker Daltonics Inc.) was used to submit the data to Mascot v.2.4 for database searching. The parent ion mass tolerance was 10 ppm. Peptides were searched against the *Homo sapiens* database on SwissProt allowing up to 4 missed tryptic cleavages with variable carbamidomethyl (C), deamidated (NQ), oxidation (M), and succinyl (K) modifications. Peptides with a minimum ion score of 20 were accepted.^{29,30}

Synthesis of *N*²-(*tert*-Butoxycarbonyl)-*N*⁶-(3-carboxypropanoyl)lysine.

Isolation of *N*^ε-Succdnyl-*N*^α-*boc*-L-Lysine.—A total of 246 mg (1 mmol) of *N*^α-*boc*-L-lysine was dissolved in 5 mL of water. The pH was adjusted to 10 by adding 10 M sodium

hydroxide solution. To this solution, 100 mg (1 mmol) of succinic anhydride dissolved in 1 mL of 1,4-dioxane was added in steps of 100 μ L. Five min after every addition, the pH was adjusted to 10 before adding more succinic anhydride. The solution was diluted with 10 mL of water and lyophilized. The crude product was suspended in 2 mL of water/acetonitrile (1:1 v/v) and fractionated by flash chromatography (silica gel, ethyl acetate +1% formic acid (v/v)). Fractions were checked by TLC (silica gel, ethyl acetate + 1% formic acid (v/v), ninhydrin staining), and fractions showing a spot with R_f = 0.4 were pooled. Evaporation of the solvent yielded a white powder [100 mg (0.3 mmol), yield = 30%]. ^1H NMR (500 MHz, D_2O): δ 1.42 (m, 2H), 1.44 (s, 9H), 1.54 (m, 2H), 1.71 (m, 1H), 1.84 (m, 1H), 2.53 (t, J = 6.7 Hz, 2H), 2.67 (t, J = 6.8 Hz, 2H), 3.20 (t, J = 6.8 Hz, 2H), 4.08 (t, J = 7.1 Hz, 1H). ^{13}C NMR (125 MHz, D_2O): δ 24.8, 28.7, 29.7, 30.8, 40.4, 41.5, 44.0, 58.4, 83.9, 160.0, 177.2, 179.4, 179.5.

Isolation of SuccK.—A total of 80 mg (0.23 mmol) of *N*^ε-Succinyl-*N*^α-boc-L-lysine was deprotected with 500 μ L of 3 M hydrochloric acid for 30 min at room temperature. Afterward, the solution was neutralized and fractionated by flash chromatography (RP C18, 5% acetonitrile + 0.1% trifluoroacetic acid (v/v)). Fractions were checked by TLC (RP C18, 5% acetonitrile + 0.1% trifluoroacetic acid (v/v), ninhydrin staining), and fractions showing a spot with R_f = 0.9 were pooled and lyophilized. The SuccK was obtained as a white powder (55 mg (0.22 mmol), yield = 96%). ^1H NMR (500 MHz, D_2O): δ 1.44 (m, 2H), 1.56 (m, 2H), 1.91 (m, 1H), 1.97 (m, 1H), 2.52 (t, J = 7.3 Hz, 2H), 2.66 (t, J = 7.4 Hz, 2H), 3.20 (t, J = 7.0 Hz, 2H), 4.01 (t, J = 7.4 Hz, 1H). ^{13}C NMR (125 MHz, D_2O): δ 24.1, 31.9, 32.9, 40.2, 41.3, 42.6, 56.4, 175.2, 177.3, 179.5.

LC–MS/MS Quantification of Ack and SuccK.

Normal lenses from human donors were obtained from Saving Sight (Kansas City, MO) and stored at -80°C until used. Decapsulated lenses were weighed and homogenized in 1 mL of argon-saturated PBS containing 1 mM EDTA. Three hundred microliters of the homogenate was dialyzed against 20 mM phosphate buffer, pH 7.4, and lyophilized. Enzymatic digestion of lens proteins was performed as described previously with some minor modifications.³¹ Five hundred micrograms of freeze-dried protein was dissolved in 150 μ L of PBS, and one small crystal of thymol was added. To this solution, 0.1 units of Pronase E (two additions), 0.3 units of leucine aminopeptidase, and 0.3 units of carboxypeptidase Y were added stepwise at 24 h intervals for 96 h. Finally, the sample was filtered through a 3 kDa molecular weight cutoff filter (VWR, Radnor, PA). To determine protein digestion efficiency, we compared the *N*^ε-carboxymethyllysine (CML) levels in the enzyme digests with those in acid hydrolysates of the same sample and considered CML levels in acid-hydrolyzed samples to represent 100% efficiency of hydrolysis, as described previously.³¹ Briefly, 500 μ g of protein powder was hydrolyzed with 6 M HCl at 110°C for 24 h. The samples were dried in a Speed Vac to remove the HCl and then dissolved in water.

Sample aliquots were diluted with water prior to UPLC-MS² analysis. Chromatographic analyses were carried out on a Waters ACQUITY UPLC system (Milford, MA) connected to a Sciex 4500 QTrap mass spectrometer (Redwood City, CA). Chromatographic separations were carried out on an ACQUITY HSS T3 column (100 mm $\times$ 2.1 mm, 1.8 μ m, Waters,

Milford, MA) connected to a guard column using a flow rate of 0.6 mL/min. Water (solvent A) and 80% acetonitrile in water (solvent B, v/v) were used as eluents. To both solvents, 0.12% heptafluorobutyric acid (v/v) was added. Analyses were performed at a column temperature of 40 °C using a gradient elution: 2% B (0–2.2 min) to 8% B (3.3 min) to 34% B (7.6 min) to 100% B (7.8 to 9.5 min). The column was equilibrated at 2% B for 2.5 min prior to the next analysis. Detection of SuccK (t_R = 4.4 min), AcK (t_R = 3.9 min), and CML (t_R = 2.3 min) was achieved by using multiple reaction monitoring. The ion source was run under the following conditions: temperature, 650 °C; ion spray voltage, 2.5 kV; curtain gas, 35 mL/min; nebulizer gas, 65 mL/min; heating gas, 70 mL/min. The declustering potential for SuccK was set to 30 V and for AcK and CML to 40 V. The MRM parameters were as follows: Q1 → Q3 [m/z], collision energy [eV], cell exit potential [V]. SuccK quantifier, 247.0 → 84.0, 38, 11; qualifier 1, 247.0 → 184.0, 19, 15; qualifier 2, 247.0 → 129.9, 22, 10. AcK quantifier, 189.2 → 126.1, 18, 12; qualifier 1, 189.2 → 84.2, 31, 5; qualifier 2, 189.2 → 143.1, 14, 10. CML quantifier, 205.1 → 130.2, 17, 11; qualifier 1, 205.1 → 84.1, 25, 13; qualifier 2, 205.1 → 56.1, 50, 10. Quantitation was performed based on the standard addition method.

To confirm the identity of SuccK in digested lens proteins, a full product ion scan of isolated SuccK was recorded. Therefore, free SuccK was isolated from digested lens proteins by UPLC. In detail, lens proteins were enzymatically digested according to the protocol described above. Aliquots of this digest were injected into the UPLC system using the aforementioned parameters. The effluent containing free SuccK (from 3.5 to 4 min) was collected. The effluents from multiple runs were combined, dried in a speed vac, and finally dissolved in a small volume of water. An aliquot of this solution was injected into the UPLC-MS² system. Instead of MRM transitions, a full product ion scan of the parent ion m/z = 247 (SuccK, $[M + H]^+$) was recorded using the ion source parameters mentioned above with the collision energy for the fragmentation of the parent ion set to 25 eV. A standard spectrum of SuccK was recorded under the same conditions using a 1 μ M solution of the reference compound (preparation described above).

Chaperone Activity Assays.

The chaperone activity of α B and Succ- α B was determined using two client proteins, insulin and citrate synthase (CS), using a UV spectrophotometer (V630 Biospectrophotometer, JASCO, Easton, MD, total volume of assay = 500 μ L) or a Spectramax 190 (Molecular Devices, San Jose, CA, total volume of assay = 200 μ L). Percent protection by the chaperone was calculated by taking the OD at 60 min.

- (i) For the DTT-induced aggregation assay of insulin, insulin at 0.2 mg/mL was incubated in the presence or absence of α B or Succ- α B (0.1 mg/mL) at 25 °C in 50 mM phosphate buffer, pH 7.4. The aggregation of insulin was initiated by adding DTT (20 mM). Light scattering was measured at 400 nm for 1 h in a kinetic mode.
- (ii) For the thermal aggregation of CS, CS at 0.12 mg/mL in 40 mM HEPES buffer, pH 7.4, was incubated with or without α B or Succ- α B (0.12 mg/mL) at 43 °C

for 1 h in a spectrophotometer. Light scattering was monitored at 400 nm in a kinetic mode for 1 h.

Separation of the α B-or Succ- α B-CS Complex by Gel Filtration.

CS (0.35 mg/mL) and α B (or Succ- α B) (1.0 mg/mL) were incubated in 40 mM HEPES buffer, pH 7.4, at 43 °C for 1 h, and the mixture was centrifuged at 10 000*g* for 10 min. The supernatant was collected and used for the separation of the α B-or Succ- α B-CS complex. α B-CS and Succ- α B-CS complexes were separated by FPLC (NGC Chromatography System, Bio-Rad, Hercules, CA), which was equipped with an Enrich SEC-650 analytical gel filtration column (10 mm $\times$ 300 mm, 24 mL). Protein (0.65 mg of protein in 500 μ L) was injected onto the column after equilibration with PBS. The column was eluted in PBS at a flow rate of 0.7 mL/min. The absorbance of the column effluent was monitored at 280 nm. α B-Crystallin and CS were detected in the eluted fractions by SDS-PAGE. We conducted this experiment in triplicate.

Surface Hydrophobicity and Tryptophan Fluorescence.

Surface hydrophobicity was measured by incubating α B or Succ- α B (0.05 mg/mL) with bis-ANS (10 μ M) in 50 mM phosphate buffer, pH 7.4, at 25 °C for 1 h. The fluorescence emission spectra were recorded as previously described.³² Intrinsic tryptophan and phenylalanine fluorescence of α B (0.1 mg/mL) in 50 mM phosphate buffer, pH 7.4, was recorded as previously described.³²

Circular Dichroism (CD) Measurements.

Far UV-CD and near UV-CD measurements were performed using a spectro-polarimeter (J 600 CD Spectrometer, JASCO) at 25 °C, as previously described.³³

Dynamic Light Scattering Study.

The hydrodynamic diameters of α B and Succ- α B were measured at 25 °C using dynamic light scattering (Zetasizer Nano S, Malvern instruments, U.K.) as previously described.³³ Protein solutions (1 mg/mL) in 50 mM phosphate buffer, pH 7.4, were passed through a 0.22 μ m membrane filter prior to these measurements. Each number recorded is an average of 36 scans.

Statistical Analysis.

The data are the means $\pm$ SD from the number of experimental replicates indicated in the figure legends. Statistical differences between the groups were analyzed by Student's *t*-test using Graphpad Prism 6 software. *p* value $\leq$ 0.05 was considered statistically significant.

RESULTS

Spatial Distribution of Succinylated Proteins in Human Lens.

Paraffin-embedded human eye sections (lens shown) were incubated with a SuccK antibody followed by an Alexa Fluor 488 conjugated secondary antibody. We found a strong

immunoreactivity for SuccK (green) in the anterior epithelial cells and mild immunoreactivity in fiber cells (Figure 2).

Effect of Age on the SuccK Content of Human Lens Proteins.

Figure 3A,B shows the results from the Western blot analyses of the WS and WIS fractions. A strong immunoreactivity was observed at approximately 20 kDa in all lenses from both the WS (Figure 3A) and WIS fractions (Figure 3B), suggesting succinylation of crystallins. However, there was no clear trend of an increase or a decrease in the SuccK levels with advancing age of the lenses. We also detected succinylation in other proteins although their levels were lower than crystallins.

α B-Crystallin Is the Major SuccK-Modified Protein in the Human Lens.

Strong immunoreactivity in a protein just above 20 kDa suggested α B-crystallin as the modified protein. When the membranes from the above experiment were reprobed with an α B-crystallin antibody, the α B-crystallin and the major SuccK-bearing protein overlapped both in the WS (Figure 3C) and the WIS (Figure 3D) fractions, suggesting that α B-crystallin is the major SuccK-modified protein in the lens. Ponceau S staining of these membranes was performed to show equal protein loading (Figure 3E,F).

To further verify that α B-crystallin is the major SuccK-bearing protein in the lens, the WS of three young lenses (25, 30, and 31 years of age) were succinylated using 0.01 mM SuccCoA and probed in Western blots for succinylation in α A-crystallin and α B-crystallins. The α A-crystallin (slightly below 20 kDa) (Figure 4A,D) and α B-crystallin (slightly above 20 kDa) (Figure 4B,E) were separated well on the gel. At the concentration of SuccCoA used, there was hardly any SuccK detected in α A, but a considerable amount was detected in α B-crystallin in all lenses (Figure 4C). Faint immunoreactivity was seen in unmodified proteins at the α -crystallin region. It should be noted here that we used 10 μ g of protein for this experiment, unlike 30 μ g for Figure 3, which could be a reason for the less apparent α B-crystallin band. To further confirm that α B-crystallin is the major SuccK-bearing protein in the lens, we immunoprecipitated α B-crystallin from the WS of a 9-year-old lens and probed the remaining supernatant for α B-crystallin and SuccK. We found that depletion of α B-crystallin (Figure 4F) significantly reduced immunoreaction in the protein just above the 20 kDa region (Figure 4G), confirming that α B-crystallin is the major SuccK-bearing protein.

We then verified the relative propensity of α A and α B for succinylation by first measuring the reactive amino groups followed by succinylation of the two proteins under identical conditions. The TNBS assay showed that recombinant human α B-crystallin (α B) had significantly more reactive amino groups (~2-fold) than recombinant human α A-crystallin (α A) (Figure 5A). The Western blot results showed that α B was more prone to succinylation than α A (Figure 5B). There were two additional protein bands at a higher molecular weight; the identity of those is not known. Coomassie staining of the gel confirmed equal loading of proteins (Figure 5C). There were some minor proteins in the high molecular weight region that were succinylated but were not visible in the Coomassie stained gel. Densitometric scans of the monomeric protein revealed that α B had ~4-fold more SuccK than α A (Figure

5D). In summary, these experiments demonstrated that α B is more prone to succinylation than α A.

Q-TOF Tandem MS/MS Mass Spectrometric Identification of SuccK Sites in Human Lens Proteins.

Mass spectrometric analyses of the WS fraction from 23- and 73-year-old human lenses indicated that K72, K90, K92, K166, K175, and potentially K174 of α B-crystallin were succinylated (Table 1). Based on the mass spectra data, SuccK at K174 could not be localized but is a potential modification site. It should be noted that succinylated K72 was identified only in the 23-year-old lens. The 73-year-old lens had an additional SuccK-modified lysine residue at K92. Mass spectrometry also revealed SuccK in α A, β -, and γ -crystallins (Table S1). These data confirm that succinylation occurs in all of the crystallins in the human lens.

LC-MS/MS Quantification of SuccK and N ϵ -Acetyllysine (AcK) in Human Lens Proteins.

SuccK and AcK were quantified in protein digests of 23 human donor lenses. The age of those lenses ranged from 20 to 73 years. For unambiguous identification, the product ion mass spectra of an authentic SuccK standard and a lens workup are shown in Figure 6A,B. Due to matrix interferences, SuccK had to be isolated from enzymatically digested lens proteins. Consequently, a clean product ion mass spectrum was obtained that was identical to the mass spectrum observed for the standard. The levels of SuccK and AcK in human lens proteins in relation to the age of individual lenses are shown in Figure 6C,D. The SuccK levels ranged from 1.2 to 5.3 pmol/mg protein. One 37-year-old lens showed a much higher level (14.3 pmol/mg protein), and it was considered an outlier; the levels in other lenses showed a weak but significant negative correlation with the lens age. The AcK levels, in comparison, were significantly higher than the SuccK levels; the levels ranged from 1610 to 6200 pmol/mg protein, but there was no significant correlation with age. Together, these data suggest that succinylation is a relatively minor modification compared with acetylation in lens proteins. However, even at those levels, succinylation impacts the function of α B-crystallin (see below).

Succinylation *in Vitro* of α B to Levels Found in the Crystallins of Human Lenses.

To understand the implications of succinylation on α B-crystallin, it was necessary to succinylate α B-crystallin to an extent similar to what is found in human lenses. Quantification of α B in human lens WS fraction was performed by Western blotting. Densitometric analysis of the Western blot revealed that 20 μ g of WS contained approximately 2 μ g of α B (Figure 7A). Recombinant α B was then succinylated with various concentrations of SuccCoA to match SuccK levels to those in α B in WS fraction of human lenses. We used 2 μ g of the succinylated α B (Succ- α B) and 20 μ g of lens WS for Western blot analysis to compare the SuccK levels in human lens α B and recombinant α B. Western blots in Figure 7B demonstrated similar levels of α B in human lens samples and the recombinant nonsuccinylated or succinylated proteins. Western blot in Figure 7C showed that at a molar ratio of 1:0.001 (the lowest SuccCoA concentration used) the SuccK level in *in vitro* succinylated α B was comparable to that found in the *in vivo* succinylated α B-crystallin in human lenses. To further confirm that α B was succinylated at a 1:0.001 ratio,

we digested the sample with proteolytic enzymes (as above), immunoprecipitated SuccK-bearing peptides and analyzed these SuccK-enriched peptide by mass spectrometry. We found K72, K82, K90, K92, K103, K121, K150, K166, and K175 (and potentially K174) to be succinylated (Table 1). A point to note here is that succinylated α B-crystallin in human lenses showed a discordant migration in SDS-PAGE when compared to recombinant α B-crystallin. This could be due to additional modification in the protein.

Succinylation of α B Enhances Its Chaperone Activity.

Even though the 1:0.001 Succ- α B had slightly lower amounts of SuccK than α B-crystallin in human lenses, we used this preparation in all subsequent experiments to confirm that the structural and functional changes observed in recombinant α B succinylated *in vitro* are relevant to human lenses. We also investigated how succinylation alters the structure and chaperone activity of α B-crystallin.

In the chemically induced aggregation assay, α B inhibited insulin aggregation by 19.9%. However, at a similar protein concentration, the Succ- α B inhibited the aggregation of insulin by 56.8% (Figure 8A,B). Similarly, in the citrate synthase (CS) thermal aggregation assay, α B inhibited the aggregation by 18%, whereas Succ- α B inhibited the aggregation by 35% (Figure 8C,D). In both the aggregation assays, when succinylation in α B was increased to 1:0.01 (M/M), the chaperone activity against insulin and CS aggregation was further increased to ~73% and ~59% (Figure 8A–D). These results imply that mild succinylation of α B (1:0.001 M/M, lysine residues in α B/succinyl CoA) at levels comparable to those found *in vivo* improves its chaperone activity. Even greater succinylation of α B (1:0.01 M/M, lysine residues in α B/succinyl CoA) is beneficial and causes further improvement in its chaperone function.

To establish that the improved chaperone activity in Succ- α B was due to the exposure of additional client protein binding sites, α B or Succ- α B (0.12 mg/mL) was first incubated with CS (0.12 mg/mL) at 43 °C for 2 h. This was done to ensure that all of the CS binding sites in α B were occupied. The assay mixture was then centrifuged at 10 000g for 15 min to remove the aggregated CS, and the supernatant containing the soluble complex of α B and CS was used in the insulin aggregation assay. Figure 8E,F shows that α B preincubated with CS lost its ability to prevent insulin aggregation, indicating that CS and insulin bound to the same site in α B. This was not the case for Succ- α B; Succ- α B was able to inhibit insulin aggregation by 13.8%. To ascertain that insulin has no affinity toward CS or CS itself does not aggregate in the presence of DTT, we monitored DTT-induced aggregation of insulin in the absence or presence of CS. Figure S3 clearly demonstrates that CS does not aggregate in the presence of DTT. Together, these observations suggest that succinylation exposes additional client protein binding sites in α B.

To determine whether the improved chaperone activity of α B was due to just the succinyl moiety in lysine residues, we investigated effects of succinylation in BSA and ADH. Succinylation of BSA and ADH was performed at a ratio of lysine/Succ CoA of 1:0.001 and 1:01 (M/M) and under conditions similar to those used for α B. Western blots in Figure S4 demonstrate succinylation of both BSA and ADH. We used DTT-induced aggregation of insulin and thermal aggregation of CS to test effects of succinylation in BSA and ADH.

Figure S5 shows that succinylation of BSA and ADH had no effect on the aggregation of either insulin or CS. These results confirm that SuccK modification by itself does not bestow chaperone activity in proteins and strengthen our hypothesis that succinylation exposes additional client protein binding sites in α B, which makes it a better chaperone.

To further confirm the presence of additional client binding sites, we subjected α B-CS and Succ- α B-CS incubation mixtures to gel filtration. Figure 9 shows the elution profiles. Both peak 1 and peak 2 showed α B and CS in SDS-PAGE (Figure 10A), indicating they contained complexes. It is likely peak 2 also had the free form of α B (or Succ- α B). We analyzed the chaperone activity in both peaks. The components in peak 1 did not show chaperone activity against insulin aggregation (Figure 10B), but rather they promoted aggregation of insulin. Components in peak 1 did not aggregate when incubated in the presence of DTT (Figure 10B) in the absence of insulin or when incubated with insulin in the absence of DTT (Figure 10C). These results demonstrate that protein aggregation observed in the chaperone assay was due to aggregation of insulin. In contrast to peak 1, components in peak 2 prevented insulin aggregation; the chaperone activity of peak 2 obtained from Succ- α B-CS mixture was higher (18.9% protection) than that from the α B-CS mixture (6.3% protection) (Figure 10B,D,E). Together, these data suggest that succinylation exposes additional client binding sites in α B.

The Structure of Succ- α B Is Mildly Perturbed.

To ascertain whether the improvement in the chaperone activity of Succ- α B was accompanied by any alteration in its conformation, we analyzed the protein for secondary, tertiary, and quaternary structures. The far UV-CD spectra of α B and Succ- α B (0.001 or 1:0.01 succinylated α B) were similar (Figure 11A), indicating that the secondary structure of α B was unaltered upon succinylation. The near UV-CD results in Figure 11B confirmed mild perturbation in the tertiary conformation of α B upon succinylation. These results suggest that succinylation mildly alters the tertiary structure of α B. The tryptophan (Figure 11C) and phenylalanine fluorescence (Figure 11D) spectra showed alterations in the intensity by ~5 and ~13%, respectively, in Succ- α B relative to α B. In 0.01 succinylated α B, similar trends in alterations of tryptophan and phenylalanine fluorescence were observed. In the dynamic light scattering (DLS) experiment, α B showed a hydrodynamic diameter of ~19.26 nm, which corresponded to a molecular weight of 674 kDa (Figure 11E). However, Succ- α B and 0.01 succinylated α B (succinylated recombinant α B generated at a α B/SuccCoA molar ratio of 1:0.01) showed a reduced hydrodynamic diameter of ~16.34 and ~16.16 nm that corresponded to a molecular weight of 458 and 447 kDa. Thus, succinylation of α B appears to cause a mild dissociation of the oligomeric assembly of the protein. Additionally, the surface hydrophobicity, measured using bis-ANS, was ~5–6% higher in Succ- α B or 0.01 succinylated α B than α B (Figure 11F). However, we did not observe a direct relationship between the chaperone activity and bis-ANS binding (Figure S6), similar to observations in other studies.^{33–35}

DISCUSSION

The purpose of this study was to determine (1) whether succinylation of lysine residues occurs in human lens proteins, (2) the effect of age on succinylation in lens proteins, (3) whether succinylation occurs in α -crystallin, and (4) the effect of succinylation on the structure and chaperone activity of α B-crystallin.

Our study clearly established that succinylation occurs in both the WS and the WIS of human lenses and that it occurs at very early age. The LC-MS/MS results indicated that the extent of succinylation in lens proteins slightly decreased with advancing age, but such a trend was not detected by Western blotting. The fact that Western blotting is less quantitative than LC-MS/MS could be a reason for the discrepancy. Theoretical analyses using the “SuccFind” software³⁶ predicted that K72 and K92 are most likely to be succinylated in α B. Our observation of SuccK at K72 and K92 agreed with the predicted sites. However, we also found SuccK at K90, K166, K175, and possibly K174. Some of these lysine residues are also acetylation sites as observed in our previous study,²⁵ reiterating the notion that acetylation and succinylation sites overlap in proteins. Previous studies that have shown acetylation and succinylation to overlap in many proteins^{21,37} support this view.

Whether succinylation is enzymatic or nonenzymatic in the human lens is unclear. Succinylation from SuccCoA has been shown to occur nonenzymatically and is favored by a slightly alkaline pH,³⁸ a condition likely to be present in mitochondria; however, it is also known to occur, albeit at a much slower rate, at neutral pH,³⁸ a condition present in the cytosol of cells. Succinylation, similar to other acylations, is a two-way modification in that it can be reversed by protein deacylases. Deacylation in proteins other than histones mainly occurs through sirtuins, which are NAD⁺-dependent deacylases.^{39,40} Whether sirtuins, especially sirtuin5, which is known to desuccinylate proteins,^{41,42} has any role in the regulation of the succinylation levels in human lens proteins remains to be established.

Immunohistochemical experiments with human lenses showed that succinylation occurs in epithelial and fiber cells. Mass spectrometric measurements showed a gradual reduction in the SuccK content in aging lenses. These observations suggest that succinylation of proteins occurs in epithelial cells and that, as they are differentiated into fiber, proteins carrying SuccK are retained in fiber cells. A gradual reduction of SuccK content in aging lenses and reduced immunoreactivity in the cortical region (compared to the epithelium) suggest the possibility that, at least to some extent, succinylation may be reversed.

The MS analysis revealed SuccK in all of the major crystallins and several other proteins in the lens. However, α B-crystallin was found to be the major succinylated protein in the lens. Why is α B-crystallin more susceptible to succinylation than other crystallins? The difference between α A-crystallin and α B-crystallin is more understandable than the differences between α B-crystallin and other crystallins. First, the lysine content in α B is higher than α A; the former contains 10 and the latter 7 lysine residues per protein molecule. Second, the TNBS assay showed that the surface-exposed lysine content is higher in α B than α A. This is not surprising because lysine is a hydrophilic amino acid and tends to be surface-exposed in proteins. Furthermore, the reversal of succinylation, if it occurs, may be

inefficient in α B-crystallin compared to other crystallins in the lens. It is also possible that α B-crystallin, unlike other crystallins, is stress-inducible; preferential succinylation could be a strategy to enhance its chaperone activity for better protection of lens cells.

The chaperone activity of α B-crystallin has been considered to be important for preventing protein aggregation in aging lenses.⁴³ To study the effect of succinylation on the structure and function of α B in the physiological context, we succinylated the α B to levels relevant to human lenses. To accomplish this, we incubated α B with SuccCoA (corresponding to a 1:0.001 molar ratio of lysine in α B to succinyl CoA) at a pH between 7.5 and 8.0. The α B-crystallin in human lenses migrated slightly slower than the recombinant α B. Other PTMs that can occur in α B-crystallin early in life, including acetylation,⁴⁴ could be the reason for this difference.

The Succ- α B we chose for further studies was an order of magnitude less succinylated than the α B-crystallin in human lenses. Even at this level of succinylation, α B showed improvement in the chaperone activity. The improvement in the chaperone activity with 1:0.001 molar ratio of lysine residues (in α B) to succinyl CoA translates into modification of one lysine residue for every 1000 lysine residues. With 10 lysine residues in each α B subunit, this would mean that 1 out of every 100 α B subunits are modified. Since each α B homooligomer contains about 20 subunits, 1 in 5 α B homooligomer would therefore be expected to contain a succinylation modification. Further work is needed to understand how this extremely low medication improved the chaperone activity of α B. The improvement in the chaperone activity of Succ- α B was accompanied by a mild increase in its surface hydrophobicity. Whether the mildly increased surface hydrophobicity was the reason for the improvement in the chaperone activity is unclear, as many studies failed to establish a clear relationship between surface hydrophobicity and chaperone activity in α -crystallin.^{45,46} However, our observation that CS saturated Succ- α B was still able to prevent insulin aggregation, unlike α B, pointing to the exposure of additional chaperone sites, which could have resulted in a higher hydrophobicity and chaperone activity of Succ- α B. Despite the improvement in the chaperone activity, the secondary structure was unchanged. Only very subtle changes were detected in the tertiary or quaternary structure. Despite Succ- α B displaying a higher chaperone activity when compared to α B, the results in Figure 9 indicated that the client protein binding is similar between Succ- α B and α B. It is possible that Succ- α B has a higher affinity to unfolding protein, such as, CS compared to the affinity of α B. In addition, recent studies have shown transient interactions of α B with client proteins.^{47,48} It is therefore possible that the transient interaction of Succ- α B with CS is higher than that of α B.

A large number of studies have demonstrated a link between the chaperone activity and oligomeric assembly of α -crystallin.^{49–51} It has been shown that dissociation of the quaternary structure of α -crystallin results in an enhanced chaperone activity.⁴⁹ Analogous to these observations, succinylation of α B caused a mild dissociation of the oligomeric assembly of the protein. The protein quaternary structure analyzed by DLS suggested that mild succinylation reduced the oligomeric size by approximately 216 kDa or ~11 subunits. Future studies could shed light on the reason for this reduction in oligomeric size. The sequence ₁₅₅PERTIPITREEK₁₆₆ in α B-crystallin is considered to be the subunit–subunit

interaction site.⁵² This sequence also possesses the “IXI” motif that links with the $\beta 4$ - $\beta 8$ groove of neighboring dimers to form a higher order oligomeric assembly in αB .⁵² Any mutation in this sequence results in its altered oligomeric assembly.⁵³ Therefore, the succinylation at K166, which we observed in Succ- αB , might have weakened the subunit–subunit interaction, triggered a mild dissociation of the oligomeric assembly, and exposed additional hydrophobic sites.

In summary, the present study demonstrated that succinylation of human lens αB -crystallin occurs early in life and continues to be present at an advanced age in the human lens. Our study showed that succinylation improves the chaperone activity of αB -crystallin. Furthermore, it is tempting to speculate that succinylation could inhibit proteolytic cleavage of proteins and modification by glycation (by competing for the same lysine residues). Thus, succinylation could be an adaptive, beneficial modification to prevent protein damage in aging human lenses and help the lens remain transparent. It is possible that succinylation of αB -crystallin in the heart, kidney, and skeletal muscle may have a similar effect on its chaperone activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

SuccK	N^{ϵ} -succinyllysine
AcK	N^{ϵ} -acetyllysine
SuccCoA	succinyl CoA
αA	recombinant human αA -crystallin
αB	recombinant human αB -crystallin
Succ-αB	αB -crystallin succinylated with 1:0.001 M/M of lysine residues in αB -crystallin to succinyl CoA
WS	water-soluble lens protein
WIS	sonicated and centrifuged water-insoluble lens protein
sHSPs	small heat shock proteins
PTMs	post-translational modifications

CML	N ^ε -carboxymethyllysine
DLS	dynamic light scattering
IP	immunoprecipitation
CS	citrate synthase
DTT	dithiothreitol
TNBS	trinitrobenzenesulfonic acid
bis-ANS	4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid, dipotassium salt
TCEP	tris(2-carboxyethyl)phosphine
TFE	trifluoroethanol
ABC	ammonium bicarbonate

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Figure 1.

Acetylation and succinylation of proteins. The epsilon amino group of lysine residues in proteins is acylated by acetyl CoA or succinyl CoA via enzymatic (by acyl transferase) or nonenzymatic reaction. Acylation of lysine can be reversed by the NAD⁺-dependent sirtuins.

Figure 2.

SuccK-modified proteins are present in the human lens. Immunohistochemical analyses of a human lens (age: 20 years) transverse section showed a strong immunoreactivity for SuccK (green) in the anterior epithelium. The cortical region also showed immunoreactivity, but the intensity was less than that in the epithelium. Omission of the primary antibody resulted in no significant immunoreactivity (negative control, bottom three panels). The nucleus of the epithelial cells was stained with DAPI (blue). The scale bar =100 μ m.

Figure 3.

The effect of age on SuccK-modified proteins in the human lens. Western blotting of the WS (A) and WIS (B) ($30 \mu\text{g}$ protein from each) was performed using a monoclonal antibody against SuccK. The membranes were reprobed with a monoclonal antibody against α B-crystallin (C and D). Comparison of the blots in panels A and C as well as B and D suggests that α B-crystallin (indicated by arrows) is the major SuccK-bearing protein in the lens. M, molecular weight markers. Ponceau S staining of the membranes (E and F, respectively) showing equal protein loading.

Figure 4.

α B-Crystallin is the dominant succinylated protein in the lens. WS from 25, 30, and 31 old human lenses (10 μ g of protein from each) was Western blotted using a polyclonal α A-crystallin antibody (1:2000 dilution, A) or a monoclonal α B-crystallin antibody (1:500 000 dilution, B). The arrows indicate the position of α A-crystallin and α B-crystallin in panels A and B, respectively. Western blot for unsuccinylated and succinylated WS (with 0.01 mM SuccCoA) is shown in panel C and the corresponding Westerns for α A-crystallin and α B-crystallin are shown in panels D and E. Preferential succinylation of α B-crystallin was further confirmed by an immunoprecipitation experiment. One hundred μ g of human lens WS (from a 9-year-old donor) was treated with 100 μ g of α B-crystallin antibody followed by 40 μ L of Protein A-Agarose. After centrifugation, the supernatant was used for Western blotting. Western blot for α B-crystallin is shown in panel F and the same membrane reprobed for SuccK, which is shown in panel G. The arrows indicate the position of α B-

crystallin and SuccK in panels F and G, respectively. Ab, antibody; HC, heavy chain of the free antibody; LC, light chain of the free antibody; M, molecular weight markers.

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Figure 5. Comparison of reactive amino groups and SuccK levels in αA and αB succinylated *in vitro*. The TNBS assay determined the reactive amino groups in αA and αB (A). A Western blot of unsuccinylated and succinylated αA and αB (with 0.01 mM SuccCoA) was performed using a SuccK antibody (B). Coomassie blue staining of the gel (shown are after protein transfer) showed equal loading of the proteins in the samples (C). A densitometric plot for the Western blot is shown in panel D. The bar graphs indicate the means $\pm$ SD of triplicate measurements. M, molecular weight markers. *** $p < 0.0005$.

Figure 6.

LC-MS/MS quantification of SuccK and AcKin human lens proteins. A product ion scan of a 1 μ M authentic SuccK ($m/z = 247.1$) standard is shown in panel A. A product ion scan of a human lens protein workup is shown in panel B. The levels of SuccK in the enzyme digested human lens protein as a function of age is shown in panel C; the data point in parentheses was excluded from the statistical analysis. The levels of AcKin in the enzyme digested human lens protein as a function of age is shown in panel D.

Figure 7.

Succinylation of αB *in vitro* compared to the level of succinylation of αB -crystallin in human lenses. Quantitative comparison of αB levels in the WS from 9-, 23-, 46-, and 71-year-old lenses with the recombinant αB (A). M, molecular weight markers; lanes 1–4, 20 μ g of WS from 9-, 23-, 46-, and 71-year-old lenses; lanes 5–9, recombinant αB at 1, 1.5, 2, 2.5, and 3 μ g of protein. Densitometric analysis in the right panel shows that 20 μ g of WS contains ~ 2 μ g of αB (red bar). Recombinant αB was then succinylated *in vitro* with increasing concentrations of SuccCoA and then compared with succinylated αB in human

lenses. Western blot of WS and succinylated recombinant α B was performed and probed with a monoclonal antibody against α B-crystallin (B) and then reprobed for SuccK (C). The succinylation of α B *in vitro* was performed using various molar concentrations of SuccCoA relative to the lysine content in α B (see below). The red bar indicates succinylation of α B to an almost similar extent to that of α B-crystallin in human lenses. M, molecular weight markers; lanes 1–4, WS from 9-, 23-, 46-, and 71-year-old lenses; 5, recombinant α B control; 6–9, succinylated recombinant α B generated at α B/SuccCoA molar ratios of 1:0.001, 1:0.003, 1:0.007, and 1:0.01, respectively. Right panels are the densitometric analysis of the respective Western Blots in the left panels. The dashed line in panel C indicates the density of Succ- α B (red) (1:0.001) relative to the succinylated α B-crystallin in human lenses.

Figure 8.

The effect of succinylation on the chaperone activity of α B. DTT-induced aggregation assay of insulin at 25 °C (A) and heat-induced aggregation assay of CS at 43 °C (C) in the absence and presence of α B and succinylated α B (generated at α B/SuccCoA molar ratios of 1:0.001, 1:0.003, 1:0.007, and 1:0.01). Total volume of assay mixture = 500 μ L. Percent protection by α B and succinylated α B against insulin aggregation (B) and CS aggregation (D). DTT-induced aggregation assay of insulin in the absence or presence of α B and Succ- α B precomplexed with CS (E). Percent protection by α B and Succ- α B (generated at α B/

SuccCoA molar ratios of 1:0.001) precomplexed with CS against insulin aggregation (F). The bar graphs indicate the means $\pm$ SD of triplicate measurements. $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$.

Figure 9.

Isolation of the α B-CS complex by gel filtration. An Enrich SEC-650 analytical gel filtration column (10 mm $\times$ 300 mm, 24 mL) was used for separation of proteins. The column was equilibrated in PBS and eluted in the same buffer at a flow rate of 0.7 mL/min. The elution profiles for CS (0.35 mg/mL), α B (1 mg/mL), α B-CS complex, Succ- α B (1 mg/mL), and Succ- α B-CS complex are shown.

Figure 10.

Isolation of the α B-CS complex and determination of chaperone activity. SDS-PAGE (12% gel) stained with Coomassie of FPLC-isolated α B-CS and Succ- α B-CS complexes (A). 1, α B-crystallin; 2, CS; 3, α B-CS (peak 1); 4, Succ- α B-CS (peak 1); 5, α B-CS (peak 2); 6, Succ- α B-CS (peak 2). DTT-induced aggregation of insulin in the presence or absence of complexes from peak 1 (B) and peak 2 (D). Insulin incubated as above (without DTT) in the presence or absence of the α B-CS or Succ- α B-CS complex did not show aggregation (C). Total volume of assay mixture = 200 μ L. In the chaperone assay in panel C, mean $\pm$ SD values at 6.6 min intervals are shown. The assay was performed in 50 mM phosphate buffer, pH 7.4, at 25 $^{\circ}$ C. We used equal concentrations of α B (determined by SDS-PAGE) from each peak in these assays. Percent protection against insulin aggregation by α B-CS and

Succ- α B-CS complexes in peak 2 is shown in panel E. The bar graphs indicate the means $\pm$ SD of three independent experiments. $*p < 0.05$.

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Figure 11.

The effect of succinylation on the conformation of α B. Far UV-CD spectra (A) and near UV-CD spectra of α B and Succ- α B (B). Tryptophan fluorescence spectra at the excitation wavelength of 295 nm (C). Phenylalanine fluorescence spectra at the excitation wavelength of 257 nm (D). The excitation and emission slit widths for the tryptophan and phenylalanine fluorescence spectra were 5 nm each. The protein concentration for both fluorescence spectra was 0.1 mg/mL in 50 mM phosphate buffer, pH 7.4. The intensity particle size distribution spectra (E) of α B and Succ- α B α B (1 mg/mL) were recorded using dynamic light scattering at 25 °C in 50 mM phosphate buffer, pH 7.5. The spectra shown are the averages of 36 scans. The surface hydrophobicity of α B and succinylated α B was measured using bis-ANS (F); the excitation wavelength used was 390 nm.

Q-TOF Tandem MS/MS Mass Spectrometric Identification of SuccK Residues in α B-Crystallin of Human Lenses and in α B-Crystallin Succinylated *in Vitro* (Immunoprecipitated Using a SuccK Antibody)^a

peptides	SuccK site	<i>in vitro</i> mass (da)	human lens age 23 mass (da)	human lens age 73 mass (da)	theoretical mass (da)	mass error inv, 23, 73 (ppm)
LEKDRFSVNLVDK	K72	1661.8569	1661.8656	NF	1661.8625	-3.37, 1.87, NF
FSVNLVDKHFSPPEELKVK	K82	2215.1728	NF	NF	2215.1525	9.16, NF, NF
HFSPEELKVK	K90	1312.6558	1312.6686	1312.6728	1312.6663	-8.00, 1.75, 4.95
VKVLGDVIEVHGK	K92	1491.8217	NF	1491.8304	1491.8297	-5.36, NF, 0.27
VLGDVIEVHGKHEER	K103	1815.9040	NF	NF	1815.9115	-4.13, NF, NF
KYRIPADVDPILTITSSDGVLTNGPR	K121	3170.6451	NF	NF	3170.6459	-0.25, NF, NF
KQVSGPER	K150	999.4992	NF	NF	999.4985	0.70, NF, NF
TIPITREEKPAVTAAPK	K166	1921.0455	1921.0533	1921.0470	1921.0520	-3.38, 0.68, -2.60
TIPITREEKPAVTAAPKK	K174 or 175	2049.1569	NF	2049.1456	2049.1470	4.83, NF, -0.68
EEKPAVTAAPKK	K174 or 175	1367.7201	1367.7348	1367.7237	1367.7296	-6.95, 3.80, -4.31
TIPITREEKPAVTAAPKK	K175	2049.1551	NF	NF	2049.1470	3.95, NF, NF
EEKPAVTAAPKK	K175	1367.7334	NF	1367.7282	1367.7296	2.78, NF, -1.02

^a NF = no spectrum matched for a specific peptide sequence and SuccK modification. K's shown in bold are succinylated residues. K174 or K175 denotes an ambiguous site identification and either lysine could be succinylated. See Figure S1A–L for the MS/MS spectra associated with each peptide.