The function of the $\beta 3$ interactive domain in the small heat shock protein and molecular chaperone, human αB crystallin

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Abstract Knowledge of the interactive domains on the surface of small heat shock proteins (sHSPs) is necessary for understanding the assembly of complexes and the activity as molecular chaperones. The primary sequences of 26 sHSP molecular chaperones were aligned and compared. In the interactive $\beta 3$ sequence, ₇₃DRFSVNLDVKHFS₈₅ of human αB crystallin, Ser-76, Asn-78, Lys-82, and His-83 were identified as nonconserved residues on the exposed surface of the α crystallin core domain. Site-directed mutagenesis produced the mutant αB crystallins: S76E, N78G, K82Q, and H83F. Domain swapping with homologous $\beta 3$ sequences, ₃₂EKFEVGLDVQFFT₄₄ from *Caenorhabditis elegans* sHSP12.2 or ₆₉DKFVIFLDVKHFS₈₁ from αA crystallin, resulted in the mutant αB crystallins, CE1 and $\alpha A1$, respectively. Decreased chaperone activity was observed with the point mutants N78G, K82Q, and H83F and with the mutant, CE1, in aggregation assays using β_L crystallin, alcohol dehydrogenase (ADH), or citrate synthase (CS). The S76E mutant had minimal effect on chaperone activity, and domain swapping with αA crystallin had no effect on chaperone activity. The mutations that resulted in altered chaperone activity, produced minimal modification to the secondary, tertiary, and quaternary structure of human αB crystallin as determined by ultraviolet circular dichroism spectroscopy, chymotrypsin proteolysis, and size exclusion chromatography. Chaperone activity was influenced by the amount of unfolding of the target proteins and independent of complex size. The results characterized the importance of the exposed side chains of Glu-78, Lys-82, and His-83 in the interactive $\beta 3$ sequence of the α crystallin core domain in αB crystallin for chaperone function.

INTRODUCTION

Human αB crystallin is a major constituent of the ocular lens, a small heat shock protein (sHSP) and molecular chaperone (de Jong et al 1989; Dubin et al 1989; Iwaki et al 1989, 1990; Piatigorsky 1989; Atomi et al 1991; Bhat et al 1991; Reddy et al 1991; Horwitz 1992; Narberhaus 2002). sHSPs are up-regulated in cells responding to abnormal stress and, as molecular chaperones, bind unfolding proteins to protect against the formation of protein aggregates (Craig 1985; Dubin et al 1989; Nagineni and Bhat 1989; Piatigorsky 1990; Wang et al 2003). The structural basis for the function of sHSPs in the inhibition of

unfolding and aggregation of the lens proteins, β/γ crystallins, and nonphysiological target proteins including alcohol dehydrogenase (ADH), and citrate synthase (CS) remains a subject of intense interest (Horwitz 1992; Plater et al 1996; Muchowski et al 1997, 1999b; Clark and Muchowski 2000; Rajaraman et al 2001; Srinivas et al 2001; Thampi et al 2002). sHSPs are characterized by the conserved α crystallin core domain of 80–100 residues, which form an immunoglobulin-like antiparallel β sandwich composed of 6–10 β strands (Kim et al 1998; van Montfort et al 2001; Guruprasad and Kumari 2003; Ghosh and Clark 2005). The crystal structures of 2 homologous sHSPs, *Methanococcus jannaschii* sHSP16.5 and wheat sHSP16.9, that form monodisperse assemblies provided basic structural information about the interactive domains used in dimerization and complex assembly in sHSPs (Kim et al 1998; van Montfort et al 2001).

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Amino acid sequence analysis of 26 sHSPs found that the primary sequences corresponding to β strands 3, 8, and 9 in human α B crystallin were conserved. Protein pin arrays identified 7 interactive sequences in the α crystallin core domain of human α B crystallin (Ghosh and Clark 2005; Ghosh et al 2005). Synthesized peptides containing the β 3 sequences of α A crystallin and α B crystallin were previously reported to have chaperone activity in vitro (Sharma et al 2000; Sreelakshmi and Sharma 2001; Ghosh et al 2005). The β 3 strand was conserved at 5 of 8 positions, and 4 of the 5 conserved residues were buried inside the β sandwich structure of the α crystallin core domain where the side chains were positioned to form intramolecular bonds. In contrast, the side chains of the nonconserved residues were surface exposed, suggesting their involvement in protein–protein interactions with homologous sHSP subunits and/or unfolded chaperone target proteins at the surface of the α crystallin core domain (Ghosh and Clark 2005; Ghosh et al 2005). These observations were consistent with previous reports that the chaperone activity in α crystallins tolerated single amino acid substitutions of conserved residues that were internal and uninvolved in recognition, binding, and maintaining solubility of unfolding proteins (Horwitz et al 1998). The current report is an analysis of the effect of selected mutations in the β 3 sequence on the secondary, tertiary, and quaternary structure of human α B crystallin and their influence on chaperone activity.

A comparison of the β 3 sequence of human α B crystallin with the β 3 sequence of *Caenorhabditis elegans* sHSP12.2, a sHSP that lacks molecular chaperone activity (Kokke et al 1998, 2001), indicated that amino acid differences at positions 76, 78, 82, and 83 in the β 3 sequence could account for the difference in chaperone activities of the 2 proteins. To evaluate experimentally the function of the nonconserved, surface-exposed β 3 residues in the chaperone activity of human α B crystallin, these residues were replaced with the corresponding nonconserved residues from the β 3 motifs of human α A crystallin and *C. elegans* sHSP12.2. In the assays used to evaluate chaperone activity, 3 target proteins, β _L crystallin, ADH, and CS, which vary in unfolding and aggregation characteristics, were used to determine the selectivity of the β 3 sequence. Minimal effect on the secondary, tertiary, and quaternary structure of α B crystallin was observed by near- and far-ultraviolet circular dichroism spectroscopy (UVCD), chymotrypsin proteolytic analysis, and size exclusion chromatography. In contrast, chaperone activity decreased with 3 of the 4 single-site mutants, N78G, K82Q, H83F, and with the CE1 chimera. Little or no effect on chaperone activity and structure was observed with the S76E mutant and with the α A1 chimera in which the β 3 motif of α B crystallin was domain swapped with the corresponding sequence from α A crystallin. The β 3 mutations

had a larger effect on the chaperone activity for target proteins that were partially (ADH) or completely (CS) unfolded. This report established the functional importance of the exposed side chains of the β 3 sequence, ₇₃DRFSVNLDVKHFS₈₅, in the interface of the human α B crystallin that is necessary for chaperone activity.

MATERIALS AND METHODS

Multiple sequence alignment and homology modeling of human α B crystallin

The primary sequence of human α B crystallin was aligned with the primary sequences of *C. elegans* sHSP12.2 and human α A crystallin using ClustalX (Jeanmougin et al 1998). The 3-dimensional (3D) model of human α B crystallin was constructed using the wheat sHSP16.9 crystal structure as a template as described previously (Ghosh and Clark 2005). Briefly, the program Molecular Operating Environment (MOE) (Chemical Computing Group, Montreal, Quebec, Canada) was used to first create a sequence alignment of the primary sequence of human α B crystallin and wheat sHSP16.9. Electron spin resonance data was incorporated into this alignment, and a model was constructed using MOE (Koteiche and McHaourab 1999). When the 3D homology model of human α B crystallin was superimposed on the crystal structures of wheat sHSP16.9 and *M. jannaschii* sHSP16.5, the C α root mean square deviation of the fit was 3.25 Å. Superimposition of the conserved α crystallin core domains of wheat sHSP16.9, *M. jannaschii* sHSP16.5, and human α B crystallin resulted in a C α root mean square deviation of 2.06 Å.

Site-directed mutagenesis

Single-site and multisite-directed mutagenesis of the β 3 motif in human α B crystallin was performed using the QuikChange site-directed mutagenesis kit (Qiagen, Valencia, CA, USA). Custom primers with the appropriate substitutions were procured from Qiagen (Table 1). A pET16b plasmid containing full-length wild-type (wt) human α B crystallin was used for the mutagenesis. After polymerase chain reaction (PCR) and subsequent digestion by Dpn1 to remove template parental DNA, plasmids were transformed into *Escherichia coli* XL-10 Gold competent cells (Stratagene, La Jolla, CA, USA) and plated on a Luria–Bertani (LB) agar plate containing carbenicillin. Antibiotic-resistant colonies containing mutated plasmids were extracted and purified using Midiprep plasmid extraction kits (Qiagen). Plasmids were sequenced by standard ABI gene sequencing to verify that only desired mutations were introduced. Plasmids containing the verified sequence were transformed into *E. coli* BL21 (DE3) cells (Stratagene) for protein expression and purification.

Table 1 Primers used for the site-directed mutagenesis of the $\beta 3$ motif of αB crystallin

Mutation	Primer sequence (5'→3')
Single residue substitutions	
S76E (forward)	GC CTG GAG AAG GAC AGG TTC GAG GTC AAC CTG GAT GTG AAG CAC
S76E (reverse)	GTG CTT CAC ATC CAG GTT GAC CTC GAA CCT GTC CTT CTC CAG GC
N78G (forward)	GAC AGG TTC TCT GTC GGT TTA GAT GTG AAA CAT
N78G (reverse)	ATG TTT CAC ATC TAA ACC AGA GAA CCT GTC
K82Q (forward)	AAC TTA GAT GTG CAA CAT TTC TCT CCA GAG GAG
K82Q (reverse)	CTC CTC TGG AGA GAA ATG TTG CAC ATC TAA GTT
H83F (forward)	AAT CTG GAT GTG AAG TTT TTC TCC CCA GAG
H83F (reverse)	CTC TGG GGA GAA GAA CTT CAC ATC CAG ATT
Multiple residue substitutions	
CE1 (forward)	CAC CTT AAC TTT GAG TTC CTC TGG GGT AAA GAA CTG CAC ATC CAG TCC GAC CTC GAA CTT CTC CAG GCG CAT CTC
CE1 (reverse)	GAG ATG CGC CTG GAG AAG GAG AAG TTC GAG GTC GGA CTG GAT GTG CAG TTC TTT ACC CTC AAA GTT AAG GTG
$\alpha A1$ (forward)	CAC CTT AAC TTT GAG TTC CTC TGG GGA GAA GTG CTT CAC ATC CAG GAA AAT GAC GAA CTT GTC CAG GCG CAT CTC
$\alpha A1$ (reverse)	GAG ATG CGC CTG GAG AAG GAC AAG TTC GTC ATT TTC CTG GAT GTG AAG CAC TTC TCC CCA GAG GAA GTT AAG GTG

Column 1 lists the substitution, and column 2 lists the nucleotide sequence of the primer used, with the actual substitution in boldface.

Protein expression and purification

For each mutant, 12 flasks containing 250 mL of Luria broth (+0.25 mL of 50 mg/mL carbenicillin) were inoculated with 2.5 mL of an overnight culture. Cells were grown to an optical density >0.5 at 595 nm in a 37°C incubator while shaking at 200 rpm. Expression was induced with 0.25 mL of 1 M isopropyl- β -D-thiogalactopyranoside (IPTG) per flask to a final concentration of 1 mM. Three hours after induction, cells were pelleted, the supernatant was discarded, and the cell pellet was frozen at -20°C until further use. Cell pellets were thawed and resuspended in 50 mL of lysis buffer (20 mM Tris-Cl, pH 8.0). One tablet of Complete Protease Inhibitor (Roche, Indianapolis, IN, USA) was added to inhibit proteolysis. One milliliter of a 25 mg/mL lysozyme stock solution (Acros Organics/Fisher Chemicals, Fairlawn, NJ, USA) was added to the suspension while stirring on ice. After a 30-minute incubation, 1 mL of a 100 mg/mL deoxycholic acid stock solution was added and kept stirring on ice for 30 minutes. Subsequently, 2000 U of DNase (Sigma, St Louis, MO, USA) were added to the suspension and heated at 37°C for 15 minutes. The lysate was then stirred at 23°C for another 15 minutes. The lysate was then sonicated on ice for 10 minutes, and centrifuged at 17 500 rpm for 30 minutes. Two milliliters of 5% polyethylimine and 0.8 mL of 1 M dithiothreitol (DTT) were added to the supernatant and stirred for 10 minutes at 22°C. The solution was centrifuged at 17 500 rpm for 10 minutes, and then filtered through a 0.22- μm syringe filter. The filtered 50-mL lysate was loaded onto a pre-equilibrated XK 50/20 column filled with 200 mL of Q Sepharose Fast Flow resin (Amersham Biosciences, Piscataway, NJ, USA). The column was pre-equilibrated with 20 mM Tris-Cl, pH 8.0. Elution fractions were collected over a 3-column volume linear gradient of 0→2 M NaCl in 20 mM Tris-Cl, pH 8.0. Elution was monitored at 280 nm, and fractions with absorbance peaks were collected and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing αB crystallin were combined and concentrated to a final volume of 2 mL. A pre-equilibrated Superdex 200 HR 10/30 (Amersham Biosciences) was loaded with 0.5 mL of the filtered concentrated samples. Elution fractions were collected over a 1-column volume of 20 mM Tris-Cl, pH 8.0, buffer. Elution fractions were analyzed by SDS-PAGE and pure fractions were pooled. Protein concentrations of the pooled fractions were determined using the BCA Protein Assay kit (Pierce, Rockford, IL, USA). The purity of the samples was determined by electrospray ionization mass spectrometry and densitometry of SDS-PAGE to be $>97\%$ pure (see Purification supplemental figure). Proteins were dialyzed into 5 mM PBS, pH 7.0, and stored at -80°C for further analysis.

Purification supplemental figure. SDS-PAGE analysis of purified wt α B crystallin and 6 α B crystallin β 3 mutants. Lane 1 is purified wt α B crystallin, lane 2 is the molecular weight standard, lanes 3–8 are purified β 3 mutants S76E, N78G, K82Q, H83F, CE1, and α A1, respectively. All proteins were determined to be >97% pure by densitometric analysis.

Circular dichroism

The secondary and tertiary structures of wt α B crystallin and each of the 6 β 3 mutants were determined by ultraviolet circular dichroism at 37°C and 50°C using a Jasco 720 circular dichroism spectrophotometer. Far-UVCD experiments were performed using samples at a concentration of 0.1 mg/mL in 5 mM PBS, pH 7.0, with a 1-mm pathlength cuvette, whereas near-UVCD experiments were performed with samples that were at 5 mg/mL concentration in 5 mM PBS, pH 7.0, with a 1-mm pathlength cuvette. Five accumulations were collected for each sample at each temperature. The near- and far-UVCD spectra of the buffer (5 mM PBS, pH 7.0) were used for baseline correction. Raw far-UVCD ellipticities were converted to mean residue molar ellipticities and expressed as $\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$ to normalize for slight molecular weight differences between wt α B crystallin and the β 3 mutants. Raw near-UVCD ellipticities were converted to molar ellipticity expressed in $\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$ to normalize for slight molecular weight differences between wt α B crystallin and the β 3 mutants.

Chymotrypsin digestion

For each digestion, 50 μg of protein was diluted in 200 μL of 5 mM PBS buffer, pH 7.0, for a final concentration of 0.25 mg/mL. Four microliters of 0.17 mg/mL α -chymotrypsin (Calbiochem, San Diego, CA, USA) was added to each sample, and the samples were heated on a 37°C heating block. After 10 minutes of digestion, 40 μL was removed from each sample. Four microliters of 50 mM phenylmethylsulfonyl fluoride (Alexis Biochemicals, San Diego, CA, USA) was added to each removed sample to inhibit further proteolysis by α -chymotrypsin. Twenty-microliter samples were loaded on a 10-well 16% Tris-glycine gel for SDS-PAGE analysis.

Size exclusion chromatography

The quaternary structure of wt α B crystallin and the 6 β 3 mutants were determined using a Biosep SEC-S4000 column with a molecular weight range of 15–2000 kDa (Phenomenex, Torrance, CA, USA) and an AKTA FPLC Purifier (Amersham Biosciences). In 3 separate runs, 30- μL samples (2.5 mg/mL) were loaded on a pre-equilibrated column and chromatographed at a flow rate of 1.0 mL/min in 5 mM PBS, pH 7.0. Peaks were recorded at 280 nm and analyzed using Unicorn 4.12 (Amersham Biosciences). A high-molecular-weight protein calibration kit (Amersham Biosciences) was used to calibrate the column and to determine the apparent molecular weight of wt α B crystallin and all 6 β 3 mutants. High-molecular-weight calibration proteins aldolase (146 kDa), catalase (226 kDa), ferritin (398 kDa), and blue dextran (2000 kDa) eluted with retention times of 9.86, 9.56, 8.53, and 6.43 minutes, respectively. The apparent molecular weights of the complex assemblies formed by wt α B crystallin and the β 3 mutants were calculated from a plot of the elution time vs $\log(\text{apparent molecular weight})$ of the high-molecular-weight calibration proteins. The average number of subunits per assembly for wt α B crystallin and each of

Table 2 Analysis of the assembly size of wt α B crystallin and the 6 β 3 mutants

Protein	Molecular weight of subunit (kDa)	Elution time (minutes)	Polydispersity (minutes)	Apparent molecular weight (kDa)	Number of subunits/assembly
Wt	20.16	8.5	0.74	485 $\pm$ 22	24 $\pm$ 1
S76E	20.20	8.3	0.87	531 $\pm$ 12	26 $\pm$ 1
N78G	20.10	8.4	0.73	495 $\pm$ 6	25 $\pm$ 0
K82Q	20.16	8.4	0.91	516 $\pm$ 31	26 $\pm$ 2
H83F	20.17	8.2	0.94	590 $\pm$ 17	29 $\pm$ 1
α A1	20.16	8.3	0.79	536 $\pm$ 10	27 $\pm$ 1
CE1	20.11	8.3	0.79	546 $\pm$ 36	27 $\pm$ 2

Column 1 lists the protein. Column 2 lists the molecular weight of an α B crystallin subunit (wt or mutant) calculated from its primary sequence. Column 3 lists the average elution time of the protein on a Biosep SEC-S4000 column, and its standard deviation calculated from 3 separate chromatographs. Column 4 lists the average polydispersity of the elution peaks, which is defined as the width of the elution peak at half-peak height. Column 5 lists the average apparent molecular weight calculated from the average elution time. Column 6 lists the average number of subunits/assembly and its standard deviation calculated from the apparent molecular weight (see Materials and Methods).

Table 3 Summarized results for the chaperone assays of wt αB crystallin and the 6 $\beta 3$ mutants

	β_1 crystallin		ADH		CS	
	Aggregation	Chaperone activity (%)	Aggregation	Chaperone activity (%)	Aggregation	Chaperone activity (%)
Target	1.00 $\pm$ 0.00	0	1.00 $\pm$ 0.00	0	1.00 $\pm$ 0.00	0
wt αB crystallin	0.16 $\pm$ 0.00	100	0.13 $\pm$ 0.00	100	0.59 $\pm$ 0.06	100
S76E	0.18 $\pm$ 0.00	97	0.10 $\pm$ 0.00	103	0.59 $\pm$ 0.13	100
N78G	0.17 $\pm$ 0.02	98	0.45 $\pm$ 0.04	63	0.94 $\pm$ 0.06	14
K82Q	0.24 $\pm$ 0.01	90	1.22 $\pm$ 0.03	0	0.91 $\pm$ 0.02	22
H83F	0.39 $\pm$ 0.01	73	0.53 $\pm$ 0.04	53	1.02 $\pm$ 0.04	0
CE1	0.49 $\pm$ 0.02	60	0.42 $\pm$ 0.02	67	0.69 $\pm$ 0.02	77
$\alpha A1$	0.25 $\pm$ 0.01	90	0.22 $\pm$ 0.01	89	0.51 $\pm$ 0.02	120

Column 1 lists the αB crystallin mutant. Columns 2, 4, and 6 list the aggregation (normalized light scattering at $\lambda = 340$ nm) of β_1 crystallin, ADH, and CS, respectively, in the absence or presence of wt αB crystallin or the $\beta 3$ mutants. Columns 3, 5, and 7 list the chaperone activity of wt αB crystallin and the $\beta 3$ mutants expressed as percent protection. The aggregation of β_1 crystallin/ADH/CS after heating at 50°C for 60 minutes in the absence of a chaperone corresponds to 0% protection, and the chaperone activity of wt αB crystallin corresponds to 100% protection. Aggregation values higher than the aggregation values of β_1 crystallin/ADH/CS in the absence of a chaperone were set to 0% protection.

the 6 $\beta 3$ mutants listed in Table 2 were calculated from 3 separate chromatographs using the formula: number of subunits per assembly = apparent molecular weight calculated from elution time/molecular weight of a single wt or mutant αB crystallin subunit.

Polydispersity of the elution peaks was calculated as peak width at half-peak height.

Chaperone assays

Chaperone assays of wt αB crystallin and the 6 $\beta 3$ mutants were performed using previously established methods with minor modifications (Muchowski et al 1999c). Bovine βL crystallin (Sigma), equine alcohol dehydrogenase (Sigma), and porcine citrate synthase (Roche) were used as chaperone target proteins. Chaperone assays were performed at concentrations that would enable detection of small changes in the chaperone activity of the $\beta 3$ mutants. Initially, chaperone assays were performed at a 10:1, 5:1, 1:1, 1:5, and 1:10 of chaperone:target protein to determine optimal conditions. A 1:1 monomeric molar ratio of chaperone:target protein was determined to be the optimum ratio, and all subsequent chaperone assays of the $\beta 3$ mutants were performed in triplicate and at a 1:1 monomeric molar ratio of chaperone:target protein in a 96-well enzyme-linked immunosorbent assay (ELISA) microtiter plate. For the single-site mutants, 0.1 mmol, and for the chimeric mutants, 0.125 mmol of the chaperone and target protein were mixed in a total volume of 200 μ L buffer (5 mM PBS, pH 7.0). Light scattering at $\lambda = 340$ nm was measured using a Multiskan MCC/340 plate reader before heating (time = 0). After the first reading, the plate was heated at 50°C, and readings were taken at 15-minute intervals for 1 hour. Raw light scattering data was normalized as follows: light scattering of target protein at $\lambda = 340$ nm in the absence or presence of chaperone after 60 minutes/light scattering of target

protein alone at $\lambda = 340$ nm after 60 minutes. Chaperone activity listed in Table 3 was calculated as follows: chaperone activity (%) = (aggregation of target protein in the absence of chaperone – aggregation of target protein in the presence of chaperone)/aggregation of target protein in the presence of wt αB crystallin.

RESULTS

Site-directed mutagenesis of the $\beta 3$ motif of human αB crystallin

Based on primary sequence and 3D structural analysis (Fig 1A), residues Ser-76, Asn-78, Lys-82, and His-83 in the $\beta 3$ strand of the α crystallin core domain of human αB crystallin were substituted with homologous residues, Glu-35, Gly-37, Gln-41, and Phe-42 from the corresponding $\beta 3$ strand of *C. elegans* sHSP12.2. In separate mutations, the entire $\beta 3$ sequence, $_{73}\text{DRFSVNLDVKHFS}_{85}$ of αB crystallin was substituted with the $\beta 3$ sequence of *C. elegans* sHSP12.2, $_{32}\text{EKFEVGLDVQFFT}_{44}$ (CE1) and αA crystallin, $_{69}\text{DKFVIFLDVKHFS}_{81}$ ($\alpha A1$) (Fig 1A). Structural analysis using a homology model of αB crystallin computed on the basis of the crystal structure of wheat sHSP 16.9 indicated that the 4 residues selected for mutagenesis were surface exposed and not involved in intramolecular interactions (Fig 1B). The 4 single-site and 2 multisite chimeric $\beta 3$ mutants of αB crystallin were synthesized, expressed, and purified as described (Muchowski et al 1999c). The purity of each protein was determined to be >97% by SDS-PAGE and densitometric analysis (see Purification SDS-PAGE supplemental figure).

Structural characterization of the $\beta 3$ mutants

The effect of the $\beta 3$ mutations on the secondary structure of αB crystallin at 37°C and 50°C was determined by far-

Fig 1. Sequence analysis of the β 3 sequences of 26 sHSPs. (A) Sequence alignment of human α B crystallin and 25 homologous small heat shock proteins using ClustalX. β 3 sequences of human α B crystallin, human α A crystallin, and *C. elegans* HSP12.2 are enclosed in a box, and a detailed view is shown below the full-length sequence alignment. Conserved residues have shaded backgrounds. The primary sequence of the β 3 sequence is conserved at 9 of 13 positions. Point mutations substituted Glu-35, Gly-37, Gln-41, and Phe-42 from *C. elegans* sHSP12.2 (underlined residues) for the corresponding residues, Ser-76, Asn-78, Lys-82, and His-83 in the β 3 strand of human α B crystallin. The chimeric mutants substituted residues 73–85 in human α B crystallin with residues 69–81 from human α A crystallin (α A1) and residues 32–44 from *C. elegans* sHSP12.2 (CE1). (B) 3D modeling of the β 3 sequence in the α crystallin core domain of human α B crystallin. Surface and ribbon representations of the computed homology model of human α B crystallin (beige) are shown (Ghosh and Clark 2005; Ghosh et al 2005). Surface-exposed residues of the β 3 sequence are colored by amino acid type (orange, polar; red, acidic; blue, basic).

Fig 2. Far-UVCD spectroscopy of wt αB crystallin and the 6 $\beta 3$ mutants at 37°C and 50°C. For each protein, the average of 3 UVCD spectra acquired between $\lambda = 200$ and 250 nm expressed in $\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$ were plotted. At both 37°C and 50°C, the far-UVCD spectra of wt human αB crystallin contained single broad negative ellipticity minima between 210 to 218 nm. The far-UVCD spectra of the $\beta 3$ mutants S76E, N78G, K82Q, H83F, CE1, and $\alpha A1$ contained single broad minima between 210 and 218 nm, resembling the far-UVCD spectra of wt human αB crystallin. The shape of the far-UVCD spectra and the value of the negative ellipticity minima of wt αB crystallin and the 6 $\beta 3$ mutants were similar at 50°C, indicating thermal stability of wt αB crystallin and the 6 $\beta 3$ mutants at temperatures up to 50°C.

Fig 3. Near-UVCD spectroscopy of wt αB crystallin and the 6 $\beta 3$ mutants at 37°C and 50°C. For each protein, the average of 5 UVCD spectra acquired between $\lambda = 245$ and 315 nm were plotted. At both 37°C and 50°C, the near-UVCD spectra of wt αB crystallin contained peaks for phenylalanine, tryptophan, and tyrosine residues. The magnitude of the absorption peaks in the near-UVCD spectra of wt and the 6 $\beta 3$ mutants were observed to be temperature dependent and decreased with increasing temperature (molar ellipticity $[\theta]$: 37°C > 50°C). The similarity of the near-UVCD spectra of all 6 $\beta 3$ mutants with wt αB crystallin at each of the 3 experimental temperatures indicated stable tertiary structure.

UVCD spectroscopy (Fig 2). The far-UVCD spectrum of wt α B crystallin contained a single broad negative ellipticity minimum between 210 and 218 nm at 37°C, a measure of the characteristic β sheet- β turn rich structure with little helical content found in crystallins. The far-UVCD spectrum of wt α B crystallin at 50°C was similar to the spectrum at 37°C. The far-UVCD spectra of the 6 β 3 mutants were similar to wt α B crystallin and contained single broad negative ellipticity minima between 210 and 218 nm at both 37°C and 50°C. A comparison of the spectra collected at the 2 different temperatures indicated that there were no significant differences in the negative ellipticity minima of the β 3 mutants and that wt α B crystallin and the β 3 mutants were thermostable up to 50°C. Overall, the single-site and multisite substitutions involving the β 3 motif of α B crystallin had no measurable effect on the secondary structure of α B crystallin as determined by far-UVCD spectroscopy.

The tertiary structures of wt α B crystallin and the 6 β 3 mutants at 37°C and 50°C were analyzed by near-UVCD spectroscopy (Fig 3). The near-UVCD spectrum of wt α B crystallin at 37°C contained 3 positive molar ellipticity peaks at approximately 259, 267, and 277 nm and a single negative peak at 291 nm corresponding to tryptophan, phenylalanine, and tyrosine residues. As the temperature increased from 37°C to 50°C, the magnitude of the positive peaks decreased while the magnitude of the negative peak at 291 nm remained unchanged. The same response to temperature was observed for all 6 β 3 mutants, indicating that the tertiary structures and thermal stabilities of the β 3 mutants were similar to wt α B crystallin. Overall, near- and far-UVCD spectroscopy of the β 3 mutants indicated that mutations to the surface-exposed residues, Asp-73, Arg-74, Ser-76, Asn-78, Lys-82, His-83, and Ser-85 of the β 3 sequence, in α B crystallin had no measurable effect on the secondary and tertiary structure of α B crystallin.

In addition to near- and far-UVCD, structural modifications due to mutations in the β 3 sequence containing a key structural β strand in the α crystallin core domain, were analyzed using chymotrypsin digestion of the β 3 mutants (Fig 4). After 10 minutes of chymotrypsin digestion, approximately half of the full-length intact wt α B crystallin remained as a protein band at approximately 20 kDa, and new protein fragments ranging in molecular weight from 3 to 20 kDa were observed. After 30 minutes of digestion, only low-molecular-weight protein fragments (<5 kDa) were observed. The chymotrypsin digestion pattern of the β 3 mutants was similar to wt α B crystallin after 10 minutes of chymotrypsin digestion. The patterns of protein bands in H83F and CE1, 2 mutants in which an additional chymotrypsin cleavage site (phenylalanine) was introduced, were similar to wt α B crystallin, which indicated that substituting His-83 with a phenyl-

Fig 4. SDS-PAGE analysis of the effect of the β 3 mutations on the structure of α B crystallin by chymotrypsin digestion. At $t = -5$ minutes, each protein is a single band on SDS-PAGE, prior to the addition of chymotrypsin (top). After 10 minutes of digestion with chymotrypsin (bottom), the proteolytic fragments appear at molecular weights below α B crystallin. Lanes 1–7 show the products of the chymotrypsin digestion of wt α B crystallin S76E, N78G, K82Q, H83F, CE1, and α A1. After 10 minutes of digestion, the new protein bands that appeared had molecular weights ranging from full-length α B crystallin (20 kDa) to fragments at approximately 3 kDa. The number and molecular weights of the peptide fragments obtained from the chymotrypsin digestion of the β 3 mutants were similar in all lanes. Exposure of additional chymotrypsin cleavage was not observed in the β 3 mutants, consistent with the near- and far-UVCD analysis of the β 3 mutants.

alanine residue did not modify the β strand secondary structure of the β 3 motif. The results of the chymotrypsin digestion of the β 3 mutants were consistent with the UVCD analysis of the β 3 mutants, and no major structural modifications resulting from the β 3 mutations were produced by the mutations.

The effect of substituting the nonconserved surface-exposed residues of the β 3 motif on the complex assembly of α B crystallin was measured using size exclusion chromatography (Fig 5). The assembled complex of wt α B crystallin consisted of approximately 24 subunits. The single-site substitutions S76E, N78G, and K82Q had complex assemblies similar to wt α B crystallin, whereas the H83F mutant had an assembly size that was 5 subunits larger than wt α B crystallin (Table 2). Swapping the entire β 3 sequence of α B crystallin with the β 3 sequences of *C. elegans* sHSP12.2 (CE1) and human α A crystallin (α A1) resulted in assembled complexes of 27 subunits, respectively, similar to wt α B crystallin (Table 2). Similar to the UVCD and chymotrypsin proteolysis, size exclusion chromatography indicated that mutations to the β 3 motif did not have measurable effects on the secondary, tertiary, or quaternary structure of α B crystallin.

Fig 5. Size exclusion chromatography of wt αB crystallin and the 6 $\beta 3$ mutants. Elution profiles of the 6 $\beta 3$ mutants recorded on a high-performance liquid chromatography (HPLC) Biosep SEC-S4000 column were plotted and compared to the elution profile of wt αB crystallin. Wt αB crystallin had an elution time of 8.46 minutes, corresponding to an apparent mean molecular weight of 485 ± 22 kDa or 24 ± 1 subunits per assembly. Overall, single-site and multi-site mutations in the $\beta 3$ motif (except H83F) did not have a measurable effect on the complex assembly of αB crystallin.

Functional characterization of the $\beta 3$ mutants

The chaperone activities of the 4 single-site and 2 multi-site chimeric $\beta 3$ mutants of αB crystallin were measured in thermal aggregation assays using β_L crystallin, ADH, and CS as the target proteins (Fig 6). Wt αB crystallin and the $\beta 3$ mutants were thermostable, and no increase in light scattering at $\lambda = 340$ nm was observed in the absence of target proteins upon heating at 50°C for 60

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Fig 6. Chaperone assays of wt αB crystallin, 4 single-site and 2 multi-site αB crystallin $\beta 3$ mutants. The ability of wt αB crystallin and the 6 $\beta 3$ mutants to suppress the thermal aggregation of β_L crystallin, ADH, and CS was measured spectrophotometrically as light scattering at $\lambda = 340$ nm from solutions containing 1:1 monomeric molar ratio of ADH/CS: wt/mutant αB crystallin. In the absence of β_L crystallin, ADH, or CS, the light scattering at $\lambda = 340$ nm in the wells containing wt αB crystallin and the $\beta 3$ mutants was similar to that of buffer alone, indicating that the $\beta 3$ mutants did not aggregate upon heating. Three of the 4 single-site mutants, N78G, K82Q, and H83F, had diminished chaperone activity for ADH and CS, relative to wt αB crystallin. The CE1 chimeric mutant had reduced chaperone activity for all 3 target proteins. In contrast, the chaperone activities of the S76E mutant and the $\alpha A1$ chimera were relatively unmodified for all 3 target proteins.

minutes. Substitution of Ser-76 with an acidic glutamate residue had no effect on the chaperone activity with β_L crystallin, ADH, and CS relative to wt αB crystallin (Table 3). Substitution of Asn-78 with a nonpolar glycine residue had minimal effect on the chaperone activity with β_L crystallin, and decreased the chaperone activity to 63% with ADH and 14% with CS. Substitution of the Lys-82 with a polar glutamine residue decreased the chaperone activity to 21% with β_L crystallin, 0% with ADH and 22% with CS. Substitution of the His-83 with a nonpolar hydrophobic phenylalanine residue decreased the chaperone activity to 73% with β_L crystallin, 53% with ADH, and 0% with CS. Domain swapping the entire $\beta 3$ sequence of αB crystallin with the $\beta 3$ sequence of *C elegans* sHSP12.2 in the CE1 mutant, decreased the chaperone activity with β_L crystallin to 60% with β_L crystallin, 67% with ADH, and 77% with CS (Table 3). The chaperone activity of the $\alpha A1$ mutant was slightly decreased to 90% with β_L crystallin, 89% with ADH, but increased to 120% with CS. Point mutations at Asn-78, Lys-82, and His-83 in the $\beta 3$ sequence of α crystallin core domain had the greatest effect on chaperone activity. The strongest effect on chaperone activity was observed with CS as the target protein, a smaller effect with ADH and the least effect with β_L crystallin as the target protein, which suggested that selectivity for unfolded target proteins varied with the specific amino acid composition of the $\beta 3$ sequence.

DISCUSSION

In this report, the contribution of the $\beta 3$ sequence, $_{73}\text{DRFSVNLDVKHFS}_{85}$, to the chaperone activity of full-length αB crystallin, the archetype of sHSPs, was evaluated using site-directed mutagenesis. The residues at positions 76, 78, 82, and 83 in the $\beta 3$ strand of the α crystallin core domain of human αB crystallin were identified as nonconserved residues in a comparative sequence alignment of 26 members of the sHSP family. Mutations at 3 of the 4 positions in the $\beta 3$ strand (N78G, K82Q, and H83F) diminished chaperone activity for the target proteins, ADH and CS, and had minimal effect on secondary, tertiary, and quaternary structure as determined by in vitro chaperone assays, UVCD, chymotrypsin proteolysis, and size exclusion chromatography. The results demonstrated that the $\beta 3$ strand in the α crystallin core domain was important for chaperone activity, and single amino acid substitutions of nonconserved, surface-exposed residues in this sequence had a dramatic effect on chaperone activity.

The α crystallin core domain is the most conserved region in sHSPs, leading to the hypothesis that the core domain contains the key functional sequences for chaperone activity in sHSPs (Muchowski et al 1999a, 1999c). Protein pin array analysis of human αB crystallin deter-

mined that the $\beta 3$ sequence, $_{73}\text{DRFSVNLDVKHFS}_{85}$, in the α crystallin core domain interacted with unfolding proteins preferentially (Ghosh et al 2005). The αA crystallin minichaperone contained the $\beta 3$ sequence (Sharma et al 2000), and a synthetic peptide containing the $\beta 3$ sequence of αB crystallin, $_{73}\text{DRFSVNLDVKHFS}_{85}$, prevented the thermal aggregation of a physiological chaperone target protein β_L crystallin and 2 model chaperone target proteins, ADH and CS, in vitro (Ghosh et al 2005). These results confirmed the importance of the $\beta 3$ sequence in the chaperone activity of sHSPs and suggested that variations in the sequence of the $\beta 3$ strand may account for functional differences observed in sHSPs, including the absence of chaperone activity in *C elegans* HSP 12.2. The observation that mutations at 3 of the 4 nonconserved, surface-exposed residues in the $\beta 3$ sequence of αB crystallin had a small effect on chaperone activity with β_L crystallin, a larger effect with ADH, and a very large effect with CS suggested selective interactions between the surface of the core domain and differentially unfolded target proteins. Analysis of the structures of native and heated β_L crystallin, ADH, and CS by near- and far-UVCD spectroscopy found no measurable unfolding of β_L crystallin, partial unfolding of ADH, and complete unfolding of CS at 50°C (Ghosh et al 2005). One interpretation of the observed differences in chaperone activity with variations in unfolding of the target proteins may be that the exposed residues of the $\beta 3$ strand distinguish between different target proteins on the basis of unfolding. In the absence of measurable differences in the near- and far-UVCD spectra or in the size exclusion chromatographs of the $\beta 3$ mutant αB crystallins, the altered chaperone activity of the $\beta 3$ mutants may be the result of changes in surface hydrophobicity. The effects of mutations in the $\beta 3$ sequence on the chaperone activity of human αB crystallin was in the order CS > ADH > β_L crystallin, which was in agreement with the order of unfolding, CS > ADH > β_L crystallin. The results established the importance of the surface-exposed, nonconserved residues, Asn-78, Lys-82, and His-83 in the $\beta 3$ sequence for chaperone activity. The possibility that target protein selectivity is regulated by Asn-78, Lys-82, and His-83 is a hypothesis that needs further investigation. In summary, the $\beta 3$ sequence in αB crystallin may be an interactive site that selects target proteins for chaperone activity on the basis of the degree of unfolding and may be a chaperone site that is selective for partially and completely unfolded proteins.

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