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Structural and Functional Consequences of the Mutation of a Conserved Arginine Residue in αA and αB Crystallins*

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A point mutation of a highly conserved arginine residue in αA and αB crystallins was shown to cause autosomal dominant congenital cataract and desmin-related myopathy, respectively, in humans. To study the structural and functional consequences of this mutation, human αA and αB crystallin genes were cloned and the conserved arginine residue (Arg-116 in αA crystallin and Arg-120 in αB crystallin) mutated to Cys and Gly, respectively, by site-directed mutagenesis. The recombinant wild-type and mutant proteins were expressed in *Escherichia coli* and purified. The mutant and wild-type proteins were characterized by SDS-polyacrylamide gel electrophoresis, Western immunoblotting, gel permeation chromatography, fluorescence, and circular dichroism spectroscopy. Biophysical studies reveal significant differences between the wild-type and mutant proteins. The chaperone-like activity was studied by analyzing the ability of the recombinant proteins to prevent dithiothreitol-induced aggregation of insulin. The mutations R116C in αA crystallin and R120G in αB crystallin reduce the chaperone-like activity of these proteins significantly. Near UV circular dichroism and intrinsic fluorescence spectra indicate a change in tertiary structure of the mutants. Far UV circular dichroism spectra suggest altered packing of the secondary structural elements. Gel permeation chromatography reveals polydispersity for both of the mutant proteins. An appreciable increase in the molecular mass of the mutant αA crystallin is also observed. However, the change in oligomer size of the αB mutant is less significant. These results suggest that the conserved arginine of the α-crystallin domain of the small heat shock proteins is essential for their structural integrity and subsequent *in vivo* function.

Molecular chaperones facilitate the correct folding of proteins *in vivo* and are instrumental in maintaining them in a properly folded and functional state. The small heat shock protein (sHSP)[1] family is a group of closely related proteins that are induced during stress. They bind to aggregation-prone proteins and act as a reservoir for non-native folding intermediates, which subsequently are refolded by other chaperones (1). Many diseases are known to result from defective protein folding (2). However, the mechanism of "chaperoning" has not yet been completely understood. Interestingly, α-crystallin, a lens protein, was shown to prevent the thermal aggregation of proteins in a manner similar to molecular chaperones (3).

α-Crystallin is a multimer of two gene products, αA and αB, which have arisen probably because of gene duplication and which share sequence homology with other members of the small heat shock protein family (4). Like other sHSPs, they also show chaperone-like activity by preventing the aggregation of proteins. Using various non-thermal modes of aggregation, we have shown earlier that α-crystallin prevents the photoaggregation of γ-crystallin (5), the DTT-induced aggregation of insulin (6), and the refolding-induced aggregation of proteins (7). These studies demonstrated that a structural perturbation of α-crystallin beyond 30 °C leads to an increase in its chaperone-like activity (8). The mutations studied in our current investigation provide an excellent opportunity to probe structural aspects related to chaperone-like activity of α-crystallin and its enhancement by a structural perturbation. αA and αB crystallins were initially thought to be present only in the eye lens, contributing to its transparency. Recently the non-lenticular expression of αB crystallin (9) has been shown in heart, muscle, and kidney, suggesting that it might have a diverse function. The expression of αA crystallin outside of the lens is in trace amounts, but it is one of the important structural proteins in the eye lens. αB crystallin constitutes 3–5% of the soluble protein in the cardiac tissue (10). It has been demonstrated to interact with actin, desmin, and other intermediate filaments (11).

A point mutation in these two crystallins is known to cause disease in humans. The conversion of arginine116 to cysteine in αA crystallin causes congenital cataract (12). The mutation of the corresponding arginine (position 120) to glycine in αB crystallin has been shown to cause desmin-related myopathy (DRM) as well as cataracts (13). Both of these inherited diseases are autosomal dominant. DRM results in weakness of the proximal and distal limb muscles and also involves the heart tissue, where it causes hypertrophic cardiomyopathy (14). This arginine is conserved throughout the small heat shock family of proteins. Modeling studies with the sHSP 16.5 of *Methanococcus janaschii* indicate that this arginine residue (position 107) is buried in the hydrophobic core of the protein and forms a salt bridge with glycine 41 (15). It has been speculated that this arginine residue in αA and αB crystallins has a role in either substrate binding or protein structure stabilization thereby influencing the chaperone-like activity. From the involvement of the conserved arginine in inherited human diseases, it is clear that it has an important role in the structure and function of sHSPs.

To study the possible structural changes caused by the mutation of this arginine in αA and αB crystallins and its influence on the chaperone-like activity, we have mutated Arg-116

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[1] The abbreviations used are: sHSP, small heat shock protein; DTT, dithiothreitol; DRM, desmin-related myopathy; wt, wild type; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

in αA crystallin to cysteine and Arg-120 in αB crystallin to glycine, by site-directed mutagenesis. We have studied the secondary, tertiary, and quaternary structures of the mutant proteins as well as their chaperone-like activity and compared them with the wild-type proteins.

EXPERIMENTAL PROCEDURES

Cloning of Human αA and αB Crystallin cDNA—RNA was isolated from human fetal lenses using Trizol reagent (Life Technologies, Inc.). The first-strand cDNA was synthesized from 1.5 μg of total RNA using oligo(dT) primer and Moloney murine leukemia virus-reverse transcriptase (Stratagene) according to the manufacturer's guidelines. The cDNA was used to amplify the αA and αB crystallin genes by polymerase chain reaction with gene-specific primers having engineered *Nde*I and *Hin*dIII sites (16). The amplified products of both αA and αB crystallins were cloned into a T-vector pCR2.1 (Invitrogen) to generate pCR2.1-αA and pCR2.1-αB plasmids.

Sequencing and Subcloning of Human αA and αB Crystallins—Sequencing was done with M13 forward and reverse primers using the Big Dye™ terminator cycle sequencing kit (Perkin-Elmer) in an Applied Biosystems automated DNA sequencer. The coding regions of both the αA and the αB genes were found to be mutationless. The coding regions of the wild-type (wt) αA and αB crystallins were removed from pCR2.1 after digestion with *Nde*I and *Hin*dIII and ligated in to *Nde*I-*Hin*dIII linearized expression vector pET21a (Novagen) to produce pET21a-αAwt and pET21a-αBwt, respectively.

Site-directed Mutagenesis—Site-directed mutagenesis was carried out by the Kunkel method (17) using the Muta-Gene *in vitro* mutagenesis kit (Bio-Rad). The αA mutant in which arginine116 is changed to cysteine (R116CαA) is made by the conversion of CGC→TGC at the 346th nucleotide of the αA crystallin gene using a non-coding oligonucleotide, which carries the mutation. Similarly, the αB mutant in which arginine 120 is changed to glycine (R120GαB) was generated by the conversion of AGG→GGG at the 358th nucleotide of the αB crystallin gene. The oligonucleotides carrying the desired mutation, which are complementary to the coding strand, were synthesized and used in the mutagenesis reaction. The sequences of the oligonucleotides used to generate mutations in αA and αB crystallins, respectively, are as follows: R116CαA, 5′-GGCGGTAGCG**GCA**GTGGAACTCACG-3′; R120GαB, 5′-TCCGGTATTT**CCC**GTGGAACTCCCT-3′.

Overexpression and Purification of Human Wild-type and Mutant αA and αB Crystallins—The expression plasmids pET21a-αAwt, pET21a-R116CαA, pET21a-αBwt, and pET21a-R120GαB were transformed into competent *Escherichia coli* BL21(DE3) cells. Growth, induction, and lysis of cells and purification of the recombinant proteins were done essentially as described by Sun *et al.* (16) except that ion exchange chromatography was done using Mono Q FPLC column (Amersham Pharmacia Biotech) in the final step of purification instead of a DEAE-Sephacel column.

SDS-PAGE and Western Immunoblot Analysis of the Wild-type and Mutant Proteins—Proteins were analyzed on 12% SDS-polyacrylamide gels (18) under reducing conditions and stained with Coomassie Brilliant Blue R250 (Sigma). The recombinant proteins were electrotransferred to a nitrocellulose membrane using a Milliblot electroblotter apparatus (Millipore) at 0.8 mA/cm^2 for 2 h. Primary antibodies for αA and αB were raised in rabbit. The secondary antibody used was anti-rabbit IgG alkaline phosphatase conjugate (Roche Molecular Biochemicals). The blot was developed using *p*-nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

FPLC Gel Permeation Chromatography—Multimeric sizes of the wild-type and mutant proteins were evaluated on a Superose-6 HR 10/30 prepacked column (dimensions 10 × 300 mm, bed volume 24 ml; Amersham Pharmacia Biotech) with reference to high molecular mass standards (Sigma). Standards used were thyroglobulin (669 kDa), ferritin (440 kDa), and catalase (232 kDa).

Fluorescence Measurements—Intrinsic fluorescence spectra were recorded using a Hitachi F-4010 fluorescence spectrophotometer with the excitation wavelength of 295 nm. The excitation and emission band passes were set at 5 and 1.5 nm, respectively.

Circular Dichroism Studies—Circular dichroism spectra were recorded using a JASCO J-715 spectropolarimeter. All spectra reported are the average of 3 accumulations. Far and near UV-CD spectra were recorded using 0.05 and 1 cm pathlength cuvettes, respectively.

Assay for Protein Aggregation—Chaperone-like activity of the wild-type and mutant proteins was studied by the insulin aggregation assay. The aggregation of insulin (0.2 mg/ml) in 10 mM phosphate buffer, pH 7.4, containing 100 mM NaCl, was initiated by the addition of 25 μl of 1 M dithiothreitol (DTT) to 1.2 ml of insulin at 37 °C. The extent of aggregation was measured as a function of time by monitoring the scattering at 465 nm in a Hitachi F-4000 fluorescence spectrophotometer. The extent of protection by the wild-type and mutant αA and αB crystallins was studied by incubating insulin with the required concentrations of the αA and αB crystallin samples for 10 min at 37 °C. Aggregation was initiated by the addition of 25 μl of 1 M DTT.

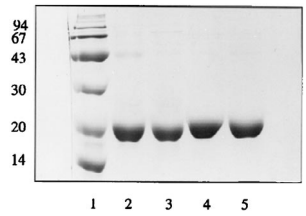

FIG. 1. **SDS-PAGE of wild-type and mutant αA and αB crystallins.** *Lane 1*, low molecular weight protein standards; *lane 2,* purified αA wild-type crystallin; *lane 3*, purified R116CαA mutant; *lane 4*, purified αB wild-type crystallin; *lane 5*, purified R120GαB mutant.

RESULTS AND DISCUSSION

Cloning of Human αA and αB Crystallin cDNA—The coding regions of αA and αB crystallins amplified by polymerase chain reaction from human fetal lens first-strand cDNA product were ligated into the plasmid pCR2.1. Double-stranded sequencing showed that the coding regions were identical to the reported exon sequences from earlier work (19, 20). Both the αA and αB crystallin genes were subcloned into the expression vector pET21a to generate pET21a-αA and pET21a-αB.

Site-directed Mutagenesis—pET21a expression plasmid is also a phagemid, and hence it was used to make the single-stranded plasmid DNA having the coding region for both the genes. Site-directed mutagenesis was used to produce both αA Arg-116→Cys (R116CαA) and αB Arg-120→Gly (R120GαA) (see "Experimental Procedures"). The presence of the mutation and the absence of any other changes in the coding region of either the αA or the αB crystallin genes was confirmed by DNA sequencing.

Expression and Purification of Wild-type and Mutant Proteins—Wild-type and mutant αA and αB crystallin proteins were expressed in *E. coli* BL21(DE3) using the isopropyl β-D-thiogalactopyranoside-inducible pET21a expression plasmids. Induction of the plasmids resulted in the expression of the recombinant proteins with a molecular mass of 20 and 22 kDa (on SDS-PAGE) for αA (wt and mutant) and αB (wt and mutant) crystallins, respectively. Some amount of the expressed mutant proteins was seen in the insoluble fraction after cell lysis. Only the soluble fraction was used for purification and further studies. The lysate containing the expressed wild-type and mutant proteins was fractionated by ammonium sulfate precipitation (30–60% saturation) and passed through a Sephacryl-S300 HR gel filtration column, which removed most of the contaminating *E. coli* proteins. Following gel permeation chromatography, the proteins were purified to homogeneity by passing through a Mono Q FPLC ion exchange column. Fig. 1 shows the SDS-PAGE patterns for wild-type and mutant proteins. The recombinant proteins were purified to ~95% homogeneity as evident from the Coomassie Blue-stained SDS-PAGE gel. Western immunoblotting using anti-human αA and αB crystallin antisera confirmed their expression.

Superose-6 Gel Filtration Chromatography—To investigate the effect of mutation on the molecular masses of the mutant proteins, wild-type and mutant αA and αB crystallins were chromatographed on a FPLC Superose-6 gel filtration column. The aggregate sizes of the individual homo-oligomers of wild-type αA and αB crystallins were observed to be slightly smaller than reported for the α-crystallin heteroaggregate. This finding is consistent with earlier published reports (16, 21, 22). Elec-

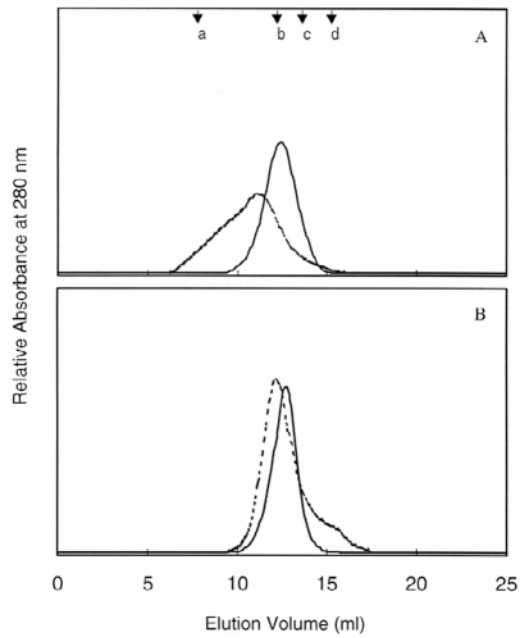

FIG. 2. **FPLC gel filtration profiles of wild-type and mutant αA and αB crystallins on a Superose-6 column.** *A*, αAwt crystallin (——) and R116CαA (····). *B*, αBwt crystallin (——) and R120GαB (– – –). The void volume (*a*) and elution positions of thyroglobulin (669 kDa) (*b*), ferritin (440 kDa) (*c*), and catalase (232 kDa) (*d*) are also indicated.

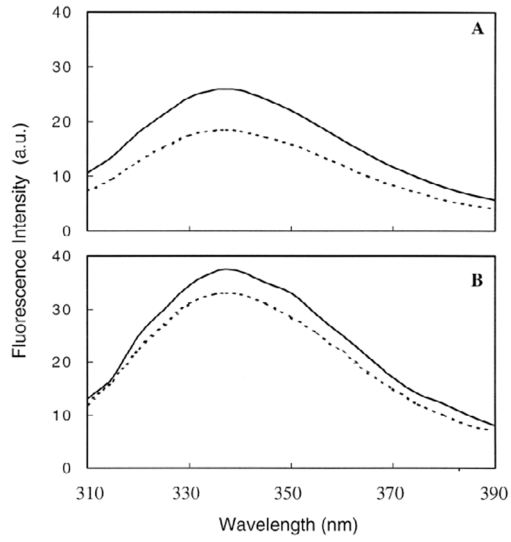

FIG. 3. **Intrinsic fluorescence spectra of wild-type and mutant αA and αB crystallins.** *A*, αAwt crystallin (——) and R116CαA (····). *B*, αBwt crystallin (——) and R120GαB (····).

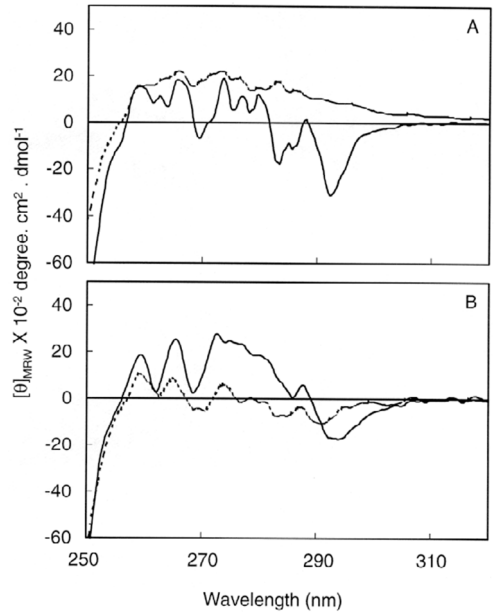

FIG. 4. **Near UV-CD spectra of wild-type and mutant αA crystallins.** *A*, αAwt crystallin (——), R116CαA (····). *B*, αBwt crystallin (——) and R120GαB (····). The samples were prepared in 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl and 1 mM EDTA.

tron microscopy has revealed that the recombinant α-crystallin complexes are characterized by a polydisperse morphology (23). The sizes of the complexes depend on the physicochemical conditions (24). In the present study, the average molecular masses of wild-type αA and αB crystallins were observed to be ~640 and ~620 kDa, respectively. The arginine mutation altered the oligomer size in R116CαA and R120GαB (Fig. 2). The R116CαA homoaggregate was observed to be highly polydisperse in nature. The experiment was repeated several times and at different concentrations, confirming the polydispersity. Berengian *et al.* (25) also observed a significant change in the elution volume for the R116CαA mutant. They have suggested that this change may occur either because of an increase in the number of subunits in the oligomer or because of structural rearrangement in the subunits. We have observed that R116CαA forms a wide range of large aggregates, with larger aggregates exceeding 2000 kDa, as shown in Fig. 2*A*. It is possible that this large increase in size is due to the increase in the number of subunits in the oligomer. The elution pattern of R120GαB protein is shown in Fig. 2*B*. The R120GαB mutant shows an apparent molecular mass of ~720 kDa. The profile suggests the presence of species larger than the wild-type αB crystallin. We have also observed some species having lower molecular masses than the wild-type protein.

Intrinsic Fluorescence and Circular Dichroism Measurements of Wild-type and Mutant αA and αB Crystallins—Intrinsic fluorescence spectra (Fig. 3, *A* and *B*) show that the emission maxima of the R116CαA and R120GαB crystallins remain unaltered (337 nm) relative to the wild-type αA and αB crystallins. This finding indicates that the mutations did not lead to an alteration in the solvent accessibility of tryptophan residues. They remain relatively inaccessible to solvent in both the wild-type and the mutant proteins. However, there is a significant decrease in fluorescence intensity, suggesting that the tryptophan residue is in a more flexible environment. Near UV-CD spectra of the R116CαA mutant show significant loss of resolution and change in the 275–295 nm region of the spectrum (Fig. 4*A*). The loss of resolution is probably due to scattering caused by the larger oligomer size. The changes in the near UV-CD spectra are observed in the region contributed by tryptophan residues. The near UV-CD spectrum of the R120GαB mutant also shows an alteration in the 270–290 nm region (Fig. 4*B*). Taken together the results of the fluorescence and near UV-CD spectroscopy suggest increased mobility of tryptophan residues and significant changes in tertiary structures of the mutant proteins. Far UV-CD spectra of wild-type and mutant proteins are shown in Fig. 5, *A* and *B*, respectively. The spectra of R116CαA and R120GαB showed distinct changes in the region around 208 nm. Such alterations in this spectral region were observed by Wynn and Richards (26) in core mutants of thioredoxin and by Mchaourab *et al.* (27) in T4 lysozyme. They attributed these changes to an alteration in the packing of secondary structural elements. An interesting observation is the change in the cross-over position of the spectra from 204 nm for αAwt to 207 nm for R116CαA (Fig. 5*A*).

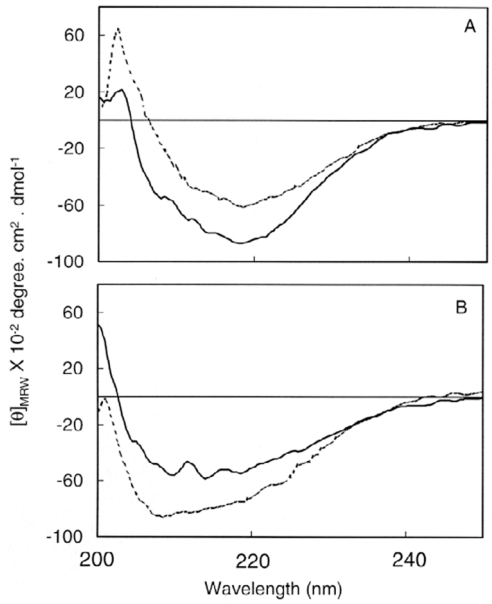

FIG. 5. **Far UV-CD spectra of wild-type and mutant αA crystallins.** *A*, αAwt crystallin (——) and R116CαA (····). *B*, αBwt crystallin (——) and R120GαB (····). The samples were prepared in 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl and 1 mM EDTA.

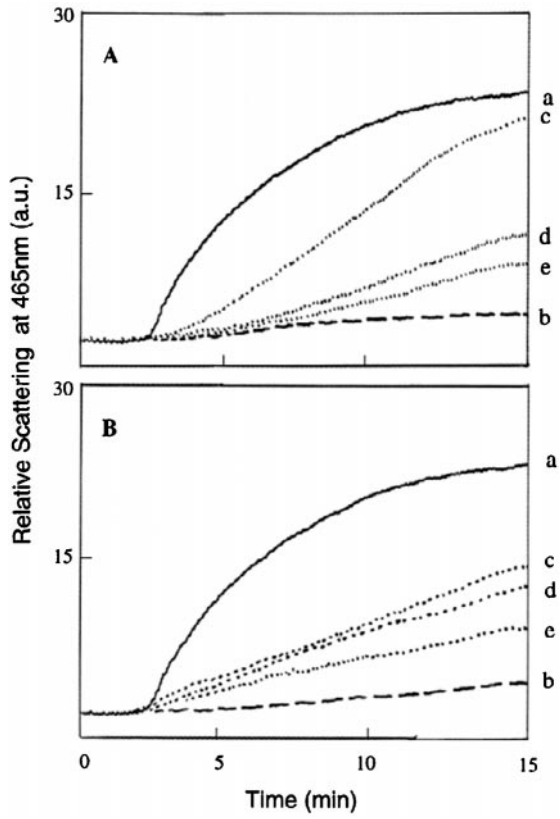

FIG. 6. **Chaperone-like activity of wild-type and mutant αA and αB crystallins.** *A*, Effect of αA crystallin. DTT induced aggregation of 0.2 mg/ml insulin alone (*a*) and in the presence of 0.2 mg/ml (1:1 w/w) αAwt (*b*). *Curves c*, *d*, and *e* represent the aggregation of insulin at ratios of 1:1, 1.5:1, and 2:1 R116CαA:insulin, respectively. *B*, effect of αB crystallin. DTT induced aggregation of 0.2 mg/ml insulin alone (*a*) and in the presence of 0.2 mg/ml (1:1 w/w) αBwt (*b*). *Curves c*, *d*, and *e* represent the aggregation of insulin at ratios of 1:1, 1.5:1, and 2:1 R120GαB:insulin, respectively.

Chaperone-like Activity—The mutations R116C and R120G decrease the chaperone-like activity of both the αA and αB crystallins, respectively. A 1:1 (w/w) ratio of chaperone to insulin prevents DTT-induced aggregation of insulin completely when wild-type αA and αB crystallins are used. On the other hand, much reduced chaperone-like activity is observed in the presence of mutant proteins. Similar ratios show decreases in the protective ability to different extents for the two mutants. In the case of R116CαA, a 1:1 (w/w) ratio shows only 15% protection. Increasing the chaperone concentration to 1.5 and 2 times the insulin concentration does not give complete protection. As shown in Fig. 6*A*, a 1.5:1 ratio gives only 60% protection from aggregation, and a 2:1 ratio of chaperone to insulin shows 75% protection. The initial scatter value for the R116CαA alone without insulin was very high. The large initial scatter value suggests a large molecular size of the R116CαA mutant. The data were normalized to determine the protective effect of the mutant protein. The protection ability of R120GαB also decreased compared with the wild-type protein (Fig. 6*B*). At a 1:1 ratio of the mutant protein to insulin, the observed protection is 40%, whereas the wild-type protein exhibits complete protection. Increasing the R120GαB concentration 2-fold shows only 70% protection. These results suggest that the arginine mutation impaired the protective ability of both αA and αB crystallins. They also indicate that this arginine residue has an important role in maintaining the structure and hence the chaperone-like activity of αA and αB crystallins. It is interesting to note that this arginine residue is conserved in 28 species of mammals and other vertebrates such as chicken and frog (28). In an earlier site-directed spin labeling study, Arg-116 was shown to be present in a β-strand located near a subunit interface forming a buried salt bridge (25). Other site-directed mutation studies with both αA and αB crystallins resulted in the loss of chaperone activity whenever charged residues were mutated (29, 30). These studies demonstrate the need for α-crystallins to conserve their net charge through evolution (28). The present study also shows that disturbing the net charge of the α-crystallins alters the structure and reduces the chaperone-like activity. The α-crystallin domain in particular is highly invariant across different species signifying its functional importance.

αB crystallin is known to interact with intermediate filaments under stress (31). It exhibits a weak interaction with desmin in physiological conditions. Under conditions of ischemic stress where there is acidification in the cytosol, the binding affinity of αB crystallin to desmin increases considerably (11). In DRM, aggregates of desmin and mutant αB crystallin could be seen in patient muscle biopsies. From the protein aggregation assay, it is clear that R120GαB has reduced chaperone-like activity when compared with the wild-type protein.

αA crystallin is present mainly in the eye lens and is found in traces in non-lenticular tissues. The mutation R116C in αA crystallin causes congenital cataracts. Because αB crystallin is present in the eye lens as well as other tissues such as heart, kidney, and muscle, the mutation R120G is involved in causing DRM along with cataract. Even though αA and αB crystallins have 55% amino acid identity (32), the mutation of the conserved arginine residue in the two proteins shows structural and functional changes in the mutants to different extents, signifying differences in these two proteins. Further studies on the mutants should prove useful in understanding the molecular mechanism of the chaperone-like activity of α-crystallin and in designing strategies to prevent or postpone several complications where α-crystallins appear to play a role.[2]

[2] While this manuscript was under review, two reports from an annual meeting (33, 34) and a paper (35) appeared that gave similar results concerning mutations leading to altered aggregate size and decreased chaperone-like activity, with minor differences in aggregate

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weight. These minor variations in aggregate weight could be because of the differences in distribution of aggregates and/or the buffer conditions.

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