A Positive Charge Preservation at Position 116 of αA-Crystallin Is Critical for Its Structural and Functional Integrity †

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A Positive Charge Preservation at Position 116 of αA-Crystallin Is Critical for Its Structural and Functional Integrity†

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ABSTRACT: An autosomal dominant congenital cataract associated with a missense mutation, Arg-116 to Cys (R116C), in the coding sequence of human αA-crystallin has been reported. Subsequent study of this mutant, generated by site-directed mutagenesis, showed significant changes in secondary and tertiary structures, partial loss of chaperone activity, and substantially increased oligomeric size. The study presented here aims to show whether these changes are due to the loss of a positive charge at this position or due to the presence of an extra Cys. To show this, Arg-116 in αA-crystallin was mutated to Lys (R116K), Cys (R116C), Gly (R116G), and Asp (R116D) and expressed in *Escherichia coli* cells. The wild-type (αA-wt) and mutant proteins were purified by size exclusion chromatography and characterized by measurements of circular dichroism, intrinsic tryptophan fluorescence, and TNS fluorescence and by determination of molecular masses and chaperone function which was assessed as the ability to suppress target protein aggregation or enhance target protein refolding. Mutation of Arg-116 to a Cys or Gly showed very similar changes in structure, oligomerization, and chaperone function which suggest that the presence of this Cys per se is not the cause of the changes. The R116K mutant, on the other hand, had nearly the same structure, oligomeric size, and chaperone function as αA-wt, whereas the mutant with an acidic amino acid in this position, R116D, showed drastic changes in protein structure. Thus, a positive charge must be preserved at this position for the structural and functional integrity of αA-crystallin.

α-Crystallin which constitutes 25−50% of the total protein of the lens is the largest crystallin composed of two subunit polypeptides, namely, αA- and αB-crystallins (*1*). These subunits, the sequences of which are 57% homologous, exist in αA:αB ratios of approximately 3:1−1:1, depending on the age (*2*). Although these two crystallins are expressed most abundantly in the lenticular tissue, αB in particular is widely present in other tissues such as heart, skeletal muscle, brain, retina, and lungs (*3*, *4*). αA-Crystallin on the other hand is present in small amounts in brain, liver, spleen, retina, and thymus (*5*, *6*). The sequences of both subunits of α-crystallin are similar to those of small heat-shock proteins (*7*), and this sequence homology is confined to a stretch of 100 residues in the C-terminal domain, termed the α-crystallin domain. In addition to its structural function in the maintenance of lens transparency, α-crystallin has been shown to have chaperone-like activity (chaperone activity or chaperone function), a feature which enables it to suppress nonspecific aggregation of proteins (*8*). The presence of an active molecular chaperone α-crystallin may be essential for maintaining lens transparency because in senile and diabetic cataractous lenses there is significant loss of α-crystallin chaperone activity (*9*). This belief was strengthened by studying knockout mice homologous for the αA-crystallin gene (*10*). Several weeks after birth, these mice developed lens opacification and dense inclusion bodies that contained insoluble αB-crystallin in the fiber cells.

Site-directed spin-label studies of the amino acid residues between positions 109 and 120 of αA-crystallin revealed the presence of a *β*-strand containing the presumably buried residues R112 and R116 (*11*). Subsequent studies confirmed between residues 84 and 120 the presence of antiparallel *β*-sheets consisting of three strands arranged in consecutive *β*-hairpins (*12*). This stretch of the conserved sequence became the focus again when an autosomal dominant congenital cataract associated with a missense mutation in the human αA-crystallin gene, i.e., mutation of Arg-116 to Cys (R116C) in the coding sequence, was reported (*13*). Subsequent study of this mutant αA-crystallin, generated by site-directed mutagenesis of cloned αA-crystallin, showed significant changes in secondary and tertiary structures, the partial loss of chaperone activity, and substantially increased oligomeric size (*14*, *15*). The mutant was also shown to form heteroaggregates with αB-crystallin more rapidly than the αA wild-type (αA-wt) (*16*); the heteroaggregates showed increased oligomeric size, distinctly different tertiary structure, and a small loss in chaperone activity (*16*). The R116C mutant also exhibited a reduced ability to exchange subunits with αA-wt homoaggregates (*17*) which suggests R116C is more likely to form homoaggregates than αA-wt.

Mchaourab et al. have speculated that substantial changes in the oligomeric structure of the R116C mutant are due to the fact that R116 exists in a buried environment, presumably forming salt bridges with residues of opposite charges, and the absence of such salt bridges could lead to protein conformational changes (*11*, *15*). Another possibility is that

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the presence of a third Cys residue in αA-crystallin could enhance the formation of protein disulfides. The purpose of this study is to show whether R116 can be replaced by another positively charged amino acid, Lys in this case, without any effect on its structure and function. Using the same reasoning, replacing R116 with an acidic amino acid could have a more adverse effect. R116 was also mutated to a Gly, another neutral amino acid, to show whether R116G will behave like R116C, i.e., to show whether the presence of a Cys at position 116 per se will have additional structural and functional consequences.

EXPERIMENTAL PROCEDURES

Cloning, Site-Directed Mutagenesis, Expression, and Purification of αA-wt and Mutants R116K, R116C, R116G, and R116D. Cloning of rat αA-crystallin and the "humanized αA" has been reported previously (*14*); the latter has been used in three previous studies (*14*, *16*, *18*). The Quick Change Site-Directed Mutagenesis Kit (Stratagene) was used to generate the mutants R116K, R116C, R116G, and R116D. Plasmid DNA from αA-wt and the mutants was sequenced by automated sequencing. αA-wt and the mutant proteins were expressed and purified as described previously (*14*). Each DNA was subcloned into the expression vector pET-23d(+) and transformed into host BL21(DE3)pLysS *Escherichia coli* cells. The expressed proteins were purified by Sephacryl S-300 HR size exclusion chromatography and their identities confirmed by SDS−PAGE[1] and Western blotting utilizing the anti-α polyclonal antibody.

Circular Dichroism (CD) Measurements. To investigate the conformation of αA-wt and the four mutants, their near-UV and far-UV spectra were recorded at 25 °C with a Jasco model 715 spectropolarimeter. Protein concentrations of 1 and 0.1 mg/mL in 50 mM phosphate buffer (pH 7.4) were used for recording near-UV and far-UV spectra, respectively. The reported CD spectra are the average of five smoothed scans. Secondary structure parameters were estimated with the program PROSEC derived from Yang et al. (*19*).

Tryptophan Fluorescence Measurements. Fluorescence measurements were taken with a Shimadzu RF-540 spectrofluorophotometer. Protein samples (0.1 mg/mL) in 50 mM phosphate buffer (pH 7.4) were used. The excitation wavelength was set at 295 nm and emission scanned between 310 and 400 nm.

TNS Fluorescence Measurements. The surface hydrophobicity of αA-wt and the mutants was studied by using the specific hydrophobic probe TNS [2-(*p*-toluidino)naphthalene-6-sulfonic acid]. To 0.1 mg of protein in 1 mL of 50 mM phosphate buffer (pH 7.4) was added 10 μL of a 10 mM methanolic solution of TNS and the mixture incubated for 2 h at room temperature. Fluorescence emission spectra were recorded between 350 and 520 nm with the excitation wavelength set at 320 nm.

Size Exclusion Chromatography for Determination of Molecular Masses. A 600 mm × 7.8 mm BIOSEP-SEC-S 4000 molecular sieve HPLC column (Phenomenex) was used with a Beckman System Gold HPLC for determination of molecular masses. The mobile phase contained 50 mM Tris, 150 mM NaCl, and 10 mM EDTA (pH 7.0). Approximately 100 μg of protein was applied to the column and developed isocratically at a flow rate of 1 mL/min. The absorbance was recorded at 280 nm. The column was calibrated with molecular mass standards (Sigma).

Table 1: Molecular Masses (kDa) of αA-wt and Its Mutants

αA-wt	645	R116G	∼2000
R116K	776	R116D	1585
R116C	∼2000		

Determination of Chaperone Activity of αA-wt and the Mutants. Chaperone activity was assayed at 37 °C by monitoring the aggregation of alcohol dehydrogenase (ADH) in the absence and presence of various α-crystallin preparations in 50 mM sodium phosphate buffer (pH 6.9) containing 60 mM NaCl and 10 mM EDTA. α-Crystallin:ADH ratios of 1:10, 1:5, and 1:1 were used in this study, although the data from only the 1:5 ratio are reported here. Light scattering was recorded at 360 nm.

Protein Refolding Assay for αA-wt and the Mutants. α-Glucosidase was used as the target protein for the refolding assay. α-Glucosidase was denatured at a concentration of 10 μM in 8 M urea, 0.1 M potassium phosphate, 2 mM EDTA, and 20 mM dithiothreitol (pH 7.0) at 20 °C. Renaturation was initiated by a 20-fold dilution with 40 mM HEPES buffer (pH 7.5) and incubation for 30 min at 20 °C in the presence and absence of different proportions of αA-wt and the mutants (α-crystallin:α-glucosidase values of 0.5−4.0). The enzymatic activity of α-glucosidase was measured as described by Jakob et al. (*20*). In short, the change in absorption observed upon release of *p*-nitrophenyl α-D-glucopyranoside at 40 °C was monitored at 420 nm.

RESULTS

Molecular Masses of αA-wt and Its Mutants. αA-wt and the mutants R116K, R116C, R116G, and R116D were purified on Sephacryl-S-300 HR columns. Molecular masses of these proteins were determined by molecular sieve HPLC; the values in kilodaltons are given in Table 1. As expected from earlier studies (*14*, *15*), the molecular mass of αA-wt was 610 kDa and that of R116C was ∼3-fold higher (∼2000 kDa). The molecular mass of R116G was essentially the same as that of R116C; the mass of R116K was only slightly higher than that of αA-wt, and R116D had a molecular mass between 2- and 3-fold higher.

Conformational Studies of αA-wt and the Mutants. Far- and near-UV CD spectra are shown in Figures 1 and 2, respectively. The levels of secondary structure were estimated with PROSEC and are provided in Table 2. The content of α-helix, β-sheet, β-turn, and random coil in R116K was similar to that in αA-wt. Likewise, R116C and R116G had similar secondary structure. R116D, on the other hand, had a relatively high level (∼70%) of β-sheet and significantly decreased levels of α-helix, β-turn, and random coil. The near-UV CD spectrum of a protein offers insight into the tertiary structure, i.e., the aromatic environments in the tertiary structure, formed by folding of secondary structural elements as well as their subsequent packing to form a compact three-dimensional structure. As reported in previous communications (*14*, *16*), the near-UV CD spectrum of αA-

[1] Abbreviations: CD, circular dichroism; TNS, 2-(*p*-toluidino)-naphthalene-6-sulfonic acid; ADH, alcohol dehydrogenase; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

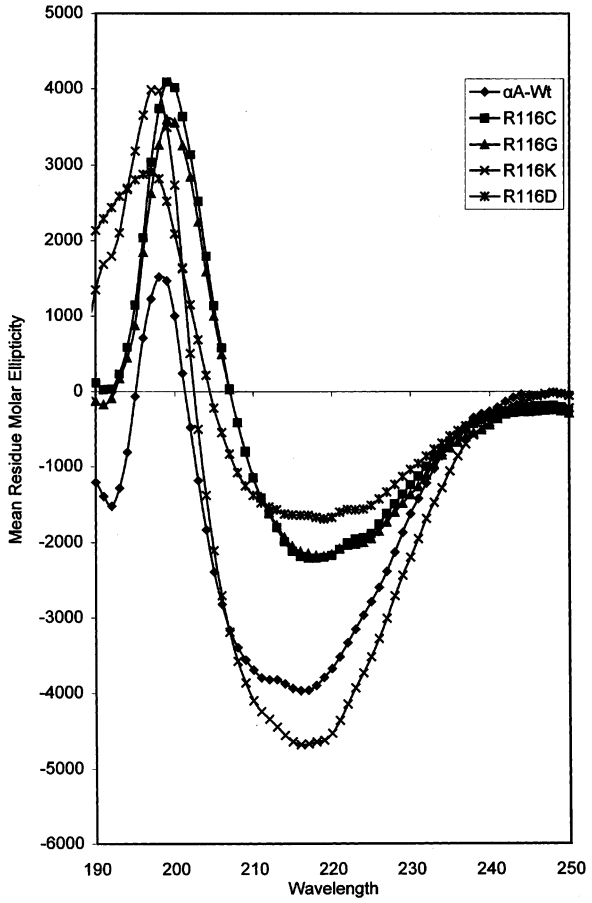

FIGURE 1: Far-UV circular dichroism spectra of αA-wt and its mutants R116K, R116C, R116G, and R116D in 50 mM phosphate buffer (pH 7.4) at 25 °C. The protein concentration was 0.1 mg/mL, and a cylindrical quartz cell with a path length of 1 mm was used.

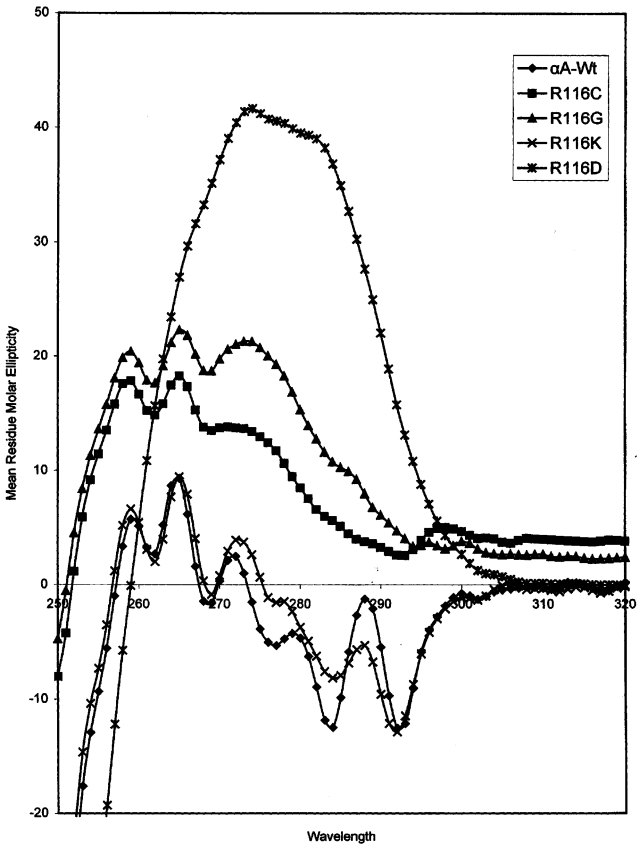

FIGURE 2: Near-UV circular dichroism spectra of αA-wt and the four mutants in 50 mM phosphate buffer (pH 7.4) at 25 °C. The protein concentration was 1 mg/mL, and a cylindrical quartz cell with a path length of 1 cm was used.

wt exhibited five distinct wavelength maxima as well as five distinct wavelength minima (Figure 2). The negative vibronic signals at ∼292 and ∼284 nm (minima) are due to Trp residues. The maxima close to 260 and 265 nm and the minima close to 268 and 262 nm are the Phe fine structure. The remaining transitions between 270 and 290 nm arise from Tyr and/or Trp. Near-UV CD spectra of αA-wt and R116K were similar. However, for R116C and R116G, there were no distinct bands due to Tyr and Trp, indicating a different Tyr and Trp microenvironment in these mutants. In the R116D mutant, no distinct bands due to Phe, Tyr, and Trp were seen; this is suggestive of substantial alteration of tertiary structure. Intrinsic fluorescence spectra (Figure 3) exhibited similar wavelength maxima at ∼336 nm for αA-wt and the four mutants. However, the intensities were different, the wild type and R116K having the highest fluorescence intensity, R116C and R116G having decreased intensities, and R116D showing a substantially lower intensity. TNS is a hydrophobic molecule that becomes highly fluorescent upon binding to protein hydrophobic sites. Figure 4 shows the fluorescence spectra of TNS bound to different αA-crystallin preparations. R116K and αA-wt had very similar fluorescence intensities; in R116C and R116G, the intensities were significantly decreased, and R116D exhibited a substantially decreased fluorescence intensity. These data imply that the relative availability of surface hydrophobic sites is as follows: R116K > αA-wt > R116G > R116C > R116D.

Table 2: Levels (%) of Secondary Structure Elements in αA-wt and Its Mutants

	αA-wt	R116K	R116C	R116G	R116D
α-helix	7	8	7	7	3
β-sheet	54	57	57	56	70
β-turn	17	16	21	21	12
random coil	22	19	15	16	15

Chaperone Activity of αA-wt and the Mutants. Chaperone activity was determined at 37 °C using ADH as the target protein. At an α:ADH ratio of 1:5, αA-wt and R116K exhibited ∼90% suppression of aggregation of ADH (Figure 5). The relative chaperone activities were nearly 50% lower for both R116C and R116G and ∼58% lower in R116D.

Refolding of α-Glucosidase in the Presence of αA-wt and the Mutants. α-Glucosidase unfolded in the presence of 8 M urea and was allowed to refold upon dilution of the denaturant, in the absence or presence of added αA-wt and the mutants. The refolding yield (α-glucosidase reactivation) of α-glucosidase increased from 2 to 4% in the absence of α-crystallin to a maximum of ∼50% in the presence of αA-wt and R116K (at an αA-crystallin:α-glucosidase ratio of 4), 35−40% in the presence of R116C and R116G, and 15% in the presence of R116D (Figure 6).

DISCUSSION

This study confirms the importance of having residue 116 in αA-crystallin be a basic amino acid. Most noteworthy is the observation that R116 which is conserved in this position can be replaced by another basic amino acid, lysine, without

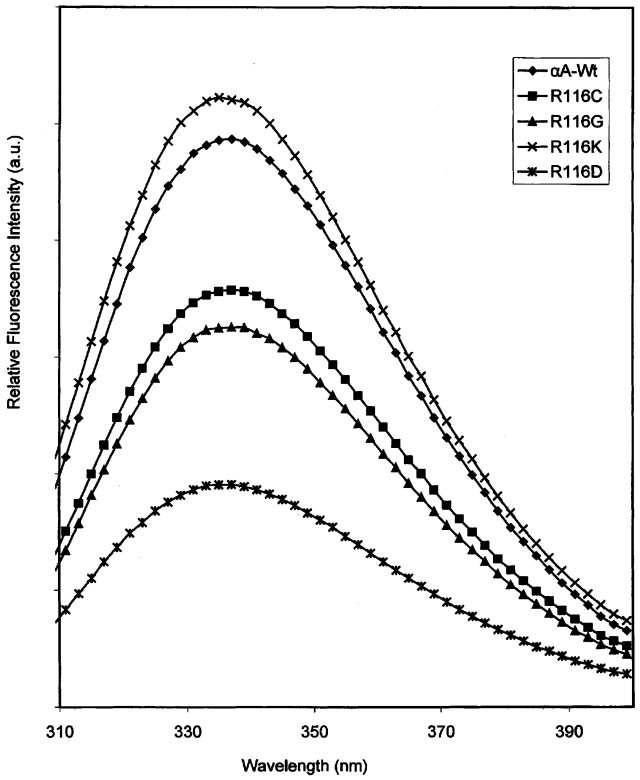

FIGURE 3: Intrinsic fluorescence spectra of αA-wt and the mutants R116K, R116C, R116G, and R116D. Protein samples (0.1 mg/mL) in 50 mM phosphate buffer (pH 7.4) were used. Excitation was fixed at 295 nm and emission scanned between 310 and 400 nm.

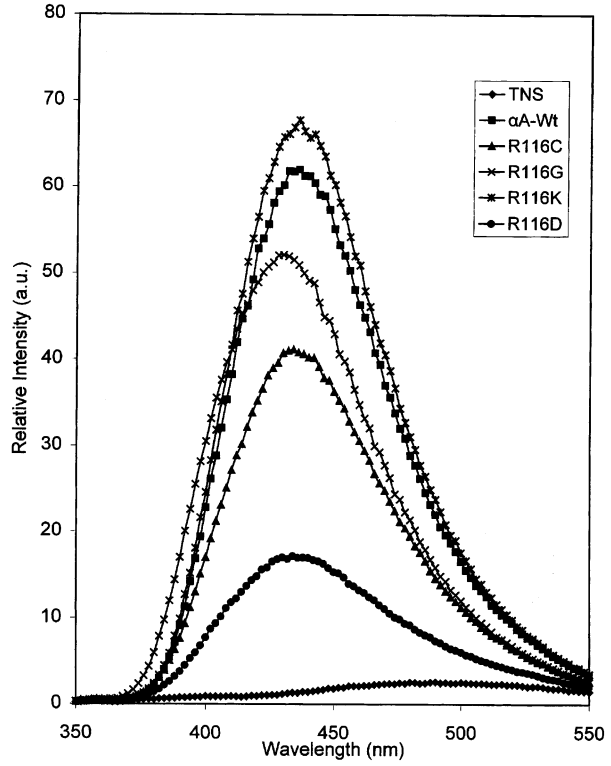

FIGURE 4: Fluorescence measurements of TNS binding with αA-wt and its mutants. Protein samples (0.1 mg/mL) in 50 mM phosphate buffer (pH 7.4) were used. Excitation was fixed at 320 nm and emission scanned between 350 and 550 nm.

significant structural and functional changes being caused. Thus, a positive charge must be preserved in this position

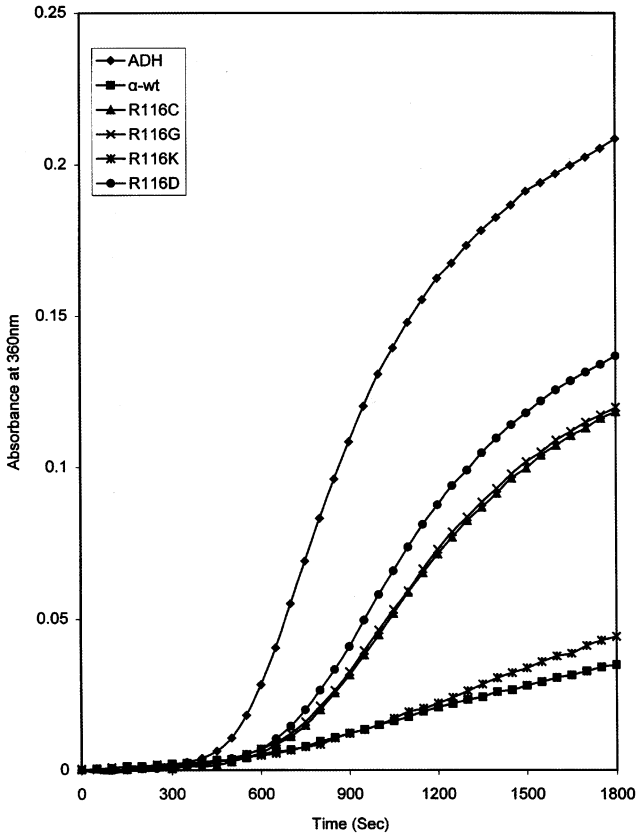

FIGURE 5: Chaperone activity of αA-wt and the mutants R116K, R116C, R116G, and R116D. Assays were carried out at 37 °C with ADH as the target protein and a 1:5 α-crystallin:ADH ratio.

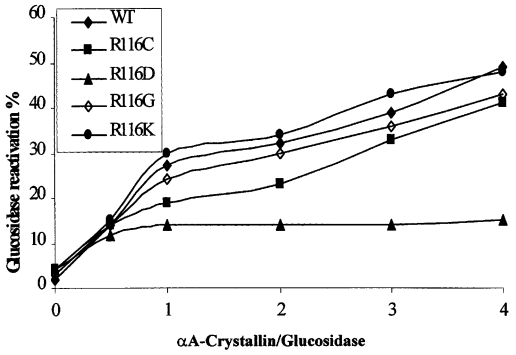

FIGURE 6: Influence of αA-wt and its mutants on the refolding and reactivation of urea-denatured α-glucosidase. α-Glucosidase was denatured in 8 M urea and subsequently renatured by dilution of the denaturant in the presence and absence of αA-wt and its mutants at the indicated ratios.

to have the normal oligomeric structure and the chaperone function. This conclusion is strengthened by the observation that an acidic amino acid, aspartic acid, in this position has a devastating effect on the secondary and tertiary structures. Another important point which is emphasized in this study is whether the mutation of R116 to Cys per se is the cause of the structural and functional abnormalities in the R116C mutant, which causes congenital cataract. In fact, when R116 was mutated to Gly, the effect was very similar. This suggests that another neutral amino acid would have the same effect as a Cys in this position.

Mutations in crystallin genes associated with hereditary cataracts have been reported in humans and other species (*13*, *21*−*28*). Litt et al (*13*) have reported an autosomal

dominant congenital cataract in humans associated with a missense mutation, R116C, in αA-crystallin. These authors have speculated that the mutant R116C could show a greater tendency toward destabilization of the native αA-crystallin. This is a strong possibility because Arg-116 is believed to be in a buried environment, presumably forming salt bridges either in homoaggregates or in heteroaggregates (*11*). Another possibility which has been suggested is the formation of additional disulfide bonds which could also destabilize αA-crystallin structure (*13*). Subsequent studies in other laboratories have demonstrated that mutation of R116 to Cys results in highly oligomerized αA-crystallin having altered secondary and tertiary structures and decreased chaperone actvity (*14*, *15*). αA-Crystallin is predominantly (>50%) in the *β*-conformation (Figure 1 and Table 2) (*14*−*16*). Mutation of R116 to Cys or Gly resulted in a nearly unchanged *β*-sheet content, a significant increase in the level of *β*-turns, and a significant decrease in the level of the random coil conformation (Table 2). Some distinct changes in the region between 200 and 210 nm in the far-UV spectra (Figure 1) could be due to changes in the packing of secondary structural elements (*15*, *29*). The most striking change in the secondary structure occurred in the R116D mutant in which the *β*-sheet content increased to ∼70% from ∼54% in αA-wt. The near-UV CD spectrum of a protein offers insight into the tertiary structure, i.e., the aromatic environments in the tertiary structure, formed by folding of secondary structural elements as well as their subsequent packing to form a compact three-dimensional structure. Since α-crystallin subunit polypeptides rapidly aggregate, the near-UV CD spectrum cannot be considered that coming from single subunits. The individual polypeptides have already suffered a perturbation during the homologous and/or heterologous subunit packing. The near-UV spectrum of R116K is similar to that of αA-wt which suggests a similar tertiary folding for both proteins (Figure 2). However, close examination of the spectra reveals a minor, but interesting, difference in the R116K mutant between 270 and 290 nm. The wavelength maxima and minima within this wavelength range of R116K are not as sharply divided as in the wild type. This may indicate the presence of minor changes in the Trp/Tyr environment and the overall tertiary structure. This is consistent with an ∼12% increase in oligomeric size (Table 1) in this mutant without any effect on chaperone activity (Figure 5). The unchanged fluorescence emission maxima of 336 nm (Figure 3) in all these mutants as compared to that of αA-wt indicate similar hydrophobic environments of Trp residues. However, the differential fluorescence intensities observed among these proteins may be due to different extents of quenching from structural perturbation caused by mutations. This structural perturbation is also reflected in the near-UV CD spectra. Thus, both the CD and Trp fluorescence studies indicate substantial changes in secondary and tertiary structures due to replacement of the positively charged amino acid residue (Arg-116) with a neutral (Cys/Gly) or a negatively charged amino acid residue (Asp) and retention of the same structural features with another positively charged amino acid residue (Lys). Like the guanidinium group of Arg, the α-amino group of Lys will be protonated carrying a positive charge at physiological pH, thus playing the role of Arg at position 116 of αA-crystallin. The structural changes of the mutated proteins are also reflected in the TNS binding study (Figure 4) which gives the overall surface hydrophobicity of the proteins in the following order: R116K > αA-wt > R116G > R116C > R116D. Although it is difficult to interpret the observed differences in the TNS binding results in these mutants, it is customary to correlate the increase in the bound TNS fluorescence with the increase in the surface hydrophobicity of the proteins. In this line of argument, the observed differences in TNS binding data may be interpreted in terms of changes in the overall surface hydrophobicity of the proteins.

An earlier electron microscopy study has revealed that the recombinant α-crystallin complexes have a polydisperse morphology (*30*) and the oligomeric sizes depend on the physicochemical conditions (*14*, *31*). The mutant R116C homoaggregates were shown to be highly polydisperse in nature (*14*, *15*). The molecular mass of this mutant was shown to decrease significantly with increasing temperatures (*14*). The molecular mass, obtained at 25 °C, of αA-wt was 645 kDa, and those of R116C and R116G were more than 3-fold higher. This increase in size could have been due to an increase in the number of subunits in the oligomer or due to structural rearrangements in the subunits (*11*, *15*). It is possible that large aggregates with molecular masses of ∼2000 kDa are formed due to an increase in the number of subunits in the oligomer (*11*, *15*). The finding that the molecular mass of R116K was only slightly higher than that of αA-wt clearly shows that a positive charge at this position stabilizes the oligomeric structure irrespective of the fact that the charge comes from a lysine rather than an arginine. R116D was more polydisperse than R116C and R116G (data shown), although the average size was slightly smaller than that of the latter two mutants (Table 2).

Chaperone activities of αA-wt and the four mutants were determined at physiological temperature using ADH as the target protein. Three different αA:ADH ratios (1:1, 1:5, and 1:10) were used, although the data given (Figure 5) are from only the 1:5 ratio. This ratio was chosen because, at this ratio, αA-wt and R116K were effective chaperones while the mutants R116C, R116G, and R116D showed significant losses in chaperone activity. At a 1:10 ratio, αA-wt and R116K exhibited nearly 50% lower chaperone activity while the other mutants showed an almost 90% loss of chaperone activity. At a 1:1 ratio, on the other hand, there was only a slight improvement in the chaperone activity of αA-wt and R116K, whereas the difference between αA-wt and the other mutants narrowed. We have also investigated the chaperoning ability of αA-wt and the mutants by assessing their ability to refold and reactivate fully denatured and inactivated α-glucosidase. The extents of refolding or reactivation of α-glucosidase were in the following order: αA-wt > R116K > R116G > R116C > R116D [which is nearly the same order as the chaperone activities, defined as the ability to suppress aggregation of a target protein (Figures 5 and 6)]. It should be pointed out that the maximum level of refolding or reactivation of glucosidase was ∼50%; this level of refolding is comparable to what has been reported for αB-crystallin and citrate synthase in the presence of ATP (*32*). These results suggest that mutation of R116 with a neutral amino acid or with an acidic amino acid impaired the ability of αA-crystallin to suppress target protein aggregation and enhance target protein refolding. It is worth pointing out that

the chaperone activity loss can be correlated to protein conformational changes and increased oligomeric size (Figures 1−4 and Table 1). The most striking correlation exists between the surface hydrophobicity and chaperone activity; with decreasing surface hydrophobicity, there is a decrease in chaperone activity (Figures 4 and 5). This supports the general belief that binding of the chaperone to the target protein is facilitated by specific hydrophobic interactions.

Arg-116 is situated in a conserved region which is also a β-strand located near a subunit interface having no accessibility to aqueous solvent. Buried charged amino acids are believed to be paired with a residue of opposite charge, and this may have structural and functional consequences. This conserved region is a part of the α-crystallin domain which is also highly invariant across different species. It should be pointed out that in addition to R116 there are three other arginines (R112, R117, and R119) in this region. Our recent study had shown that R112C had structural and functional changes similar to that of R116C, but the changes were much smaller in magnitude (*33*). R117C and R119C, on the other hand, were similar to αA-wt. This is consistent with the finding that R116 and R112 are buried residues (*11*). Thus, it seems that R116 is uniquely positioned. Another excellent example of a conserved arginine mutated to a neutral amino acid in one of the α-crystallin subunits is a missense mutation in αB-crystallin, R120G, which cosegregates with a desmin-related myopathy in a French family (*28*). This is an adult-onset muscular disease characterized by the accumulation of large aggregates of cytoplasmic desmin in conjunction with other proteins. In the French family, there were spheroid inclusion bodies containing large amounts of R120G with desmin. This mutant has been characterized by Bova et al. (*34*), who showed altered secondary and tertiary structures, an increased level of oligomerization to nearly 1400 kDa, and a substantial loss in chaperone activity. Thus, there is significant similarity between this mutant and the R116G and R116C mutants of αA-crystallin, except for a decreased β-sheet content in the R120G mutant as compared to αB-wt. In fact, all the αA-crystallin mutants that were investigated here showed either no significant change or an increase in the level of β-sheet (Table 2). So, it is irrelevant whether R116 of αA-crystallin is replaced with Cys, Gly, or Asp; loss of positive charge at this position is the key factor for modified structure and function. Moreover, the third Cys in the R116 mutant probably does not form any inter- and/or intrapolypeptide disulfide bonds. These studies conclude that the conserved positive charge at position 116 of αA-crystallin is needed for the integrity of the protein, the folding of the subunit, the correct mode of subunit assembly, and its function.

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