Cloning, Expression, and Chaperone-like Activity of Human αA-Crystallin*

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The major protein components of the ocular lens, α-crystallin, is composed of αA and αB chain subunits that have structural homology to the family of mammalian small heat shock proteins. Like other small heat shock proteins, α-crystallin subunits associate to form large oligomeric aggregates that express chaperone-like activity, as defined by the ability to suppress nonspecific aggregation of proteins destabilized by treatment with a variety of denaturants including heat, UV irradiation, and chemical modification. It has been proposed that age-related loss of sequences at the C terminus of the αA chain subunit may be a factor in the pathogenesis of cataract due to diminished capacity of the truncated crystallin to protect against nonspecific aggregation of lens proteins. To evaluate the functional consequences of α-crystallin modification, two mutant forms of αA subunits were prepared by site-directed mutagenesis. Like wild type (WT), aggregates of ~540 kDa were formed from a tryptophan-free αA mutant (W9F). When added in stoichiometric amounts, both WT and W9F subunits completely suppressed the heat-induced aggregation of aldose reductase. In contrast, subunits encoded by a truncation mutant in which the C-terminal 17 residues were deleted (R157STOP), despite having spectroscopic properties similar to WT, formed much larger aggregates with a marked reduction in chaperone-like activity. Similar results were observed when the chaperone-like activity was assessed through inhibition of γ-crystallin aggregation induced by singlet oxygen. These results demonstrate that the structurally conservative substitution of Phe for Trp-9 has a negligible effect on the functional interaction of αA subunits, and that deletion of C-terminal sequences from the αA subunit results in substantial loss of chaperone-like activity, despite overall preservation of secondary structure.

The major components of the mammalian lens fiber cells are the α-, β-, and γ-crystallins, which constitute an estimated 35% wet weight of the lens. The crystallins contribute to the transparency and refractive power of the lens by short range interactions among themselves and cytoskeletal elements in a highly concentrated matrix (1–3). α-Crystallin is one of the most abundant of the crystallins in mature lens fiber cells. It is a $M_w \approx 0.6 \times 10^6$ complex composed of two structurally related subunits, designated αA and αB, which are encoded by genes localized to chromosomes 21 and 11, respectively (4, 5). While expression of the αA gene appears to be preferentially but not exclusively restricted to lens cells (6), the αB gene product has been described in a broad number of tissues and organs including the brain, skeletal muscle, heart, and kidney (7, 8). Elevated expression of the αB gene has been correlated with several neurological diseases (9). Since the original finding that the C-terminal region of αB-crystallin and Drosophila small heat shock proteins share striking sequence homology (10), many investigators have noted structural and functional homologies between α-crystallin and various members of the mammalian small heat shock proteins (sHSP) family. Like other sHSPs, α-crystallin is a large (0.35 to >1 MDa) oligomeric complex and has the ability to suppress nonspecific aggregation of various proteins and enzymes denatured by heat (11), UV irradiation (12), and chemical modification (13). Unlike the classical bacterial chaperonin GroEL, α-crystallin does not participate in folding or reassociation of denatured proteins (14, 15).

Exposure of the lens to various environmental stresses may result in aggregation of proteins and alteration of the refractive index gradient necessary for lens transparency. Several endogenous lens chromophores such as N-formylkynurenine, riboflavin, and age-related yellow compounds absorb light present in the environment and have the capacity to act as photosensitizers generating reactive oxygen species such as singlet oxygen (16). Singlet oxygen has been implicated in the cataractogenic process as a highly reactive species that can cross-link lens proteins and produce high molecular weight aggregates (17). When illuminated, rose bengal is a classic singlet oxygen generator (18). The effects of singlet oxygen on lens crystallin structure and cross-linking have been relatively well studied (17, 19, 20).

Since crystallins exist for the lifespan of the host due to the virtual absence of protein turnover in mature lens fiber cells, it seems likely that mechanisms must exist to maintain these proteins in their native conformations throughout decades of exposure to metabolic and environmental insult. Whether α-crystallin functions as a molecular chaperone to suppress protein aggregation in lens cells is the subject of intensive study.

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¶¶ The abbreviations used are: sHSP, small heat shock protein; WT, wild type αA-crystallin; W9F, Trp-9→Phe substitution in αA-crystallin; R157STOP, deletion of amino acids from the C terminus to Glu-156 in αA-crystallin; RB, rose bengal; RNO, p-nitrosodimethylamine; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.
Chaperone-like Activity of α-A-Crystallin

Structure-function studies to probe the mechanism of chaperone-like activity of α-crystallin can be assisted with the use of recombinant α-crystallin subunits and protein substrates. The chaperone-like activity of α-crystallin is reported to decrease as a function of age (21), possibly due to proteolytic loss of sequences from the C terminus of the αA chain subunit (22, 23).

Two-dimensional 1H NMR studies showed that the 6 amino acids at the C terminus of the αA subunit comprise a highly flexible tail having no detectable role in inter- or intrasubunit interaction (24). Unfortunately, efforts to understand the consequences of C-terminal modification in a structural context have not been successful due to lack of an accepted model for the α-crystallin complex. The protein has a predominantly β-sheet secondary structure, but a universal model consistent with all the available experimental evidence has not been established (reviewed in Ref. 25).

In the absence of a confirmed structure, the α-crystallin complex can be probed using novel mutant proteins designed to enhance the resolving power of various spectroscopic probes. To this end, we constructed a mutant form of αA-crystallin (W9F), containing phenylalanine substituted for the sole Trp residue at position 9. Utilization of this tryptophan-free mutant made it possible to probe the interaction of α-crystallin with substrate proteins by taking advantage of the tryptophan fluorescence of the substrate without interference from tryptophan of α-crystallin itself (26). Similarly, construction of a truncated αA chain subunit in which the C-terminal 17 amino acids were deleted (R157STOP) now enables us to estimate the functional consequences of in vivo proteolysis, a posttranslational modification postulated to be a factor in the apparent reduction in chaperone-like activity of α-crystallin in the aged lens (22).

In the present study we have developed a system to probe the chaperone-like activity of α-crystallin using a reconstituted system derived entirely from recombinant human lens proteins. The chaperone-like activity of αA-crystallin as well as the W9F and R157STOP mutants was assessed using two independent tests involving inhibition of heat denaturation of human aldose reductase, a protein linked to the pathogenesis of sugar cataract (27) and inhibition of single-oxygen induced aggregation of human γD-crystallin. Our results show that the recombinant αA subunit has secondary and tertiary structures similar to that of native αA isolated from human lenses. The recombinant protein forms aggregates of similar size to those of native α-crystallin and completely prevents heat-induced aggregation of aldose reductase, a protein linked to the pathogenesis of sugar cataract (27) and inhibition of single-oxygen induced aggregation of γD-crystallin. Both mutated αA subunits form oligomeric complexes and display far-UV CD spectra similar to the wild type protein. Results from the two different chaperone assay procedures were concordant and showed that the chaperone-like activity of the tryptophan-free W9F mutant was virtually unaffected, while that of the R157STOP truncation mutant was markedly reduced.

EXPERIMENTAL PROCEDURES

Cloning and Overexpression of Human γ-Crystallin—Complementary DNA clones (cDNA) encoding γ-crystallins were constructed by the reverse transcription-PCR method (36). Total RNA was typically isolated from a single human lens by homogenization in Trizol (TelTest, Friendswood, TX) followed by thorough extraction with phenol/chloroform and ethanol precipitation. First strand cDNA synthesis, primed with an oligo(dT) adaptor (Table I), was catalyzed by Moloney murine leukemia virus reverse transcriptase contained in a RNA PCR kit (Perkin-Elmer). Amplification of target sequences by PCR was carried out using the oligo(dT) adaptor and upstream primers designed with reference to γC- and γD-crystallin genes, respectively (Table I). PCR products, which migrated as prominent bands at the expected position on agarose gels, were gel-purified (QIAextract, Qiagen, Palo Alto, CA), treated with T4 DNA polymerase to expose cohesive ends and subsequently cloned into the pDIRECT plasmid vector (accession no. U02449 (1993)) following the vendor’s guidelines (PCR-Direct™ cloning system, Clontech Laboratories Inc., Palo Alto, CA). Plasmid minipreps from the resulting transformants were screened for γ-crystallin clones by restriction mapping and Southern blot hybridization with a bovine γ-crystallin cDNA (28). The sequence of both strands of selected cDNA clones was determined by the dideoxy chain termination method (29) using Sequase (Amersham) or by cycle sequencing using Taq DNA polymerase contained in a sequencing kit (Promega). At least two individual clones for each amplified γ-crystallin transcript were sequenced and found to contain identical structures. For overexpression of γ-crystallins in Escherichia coli, their cDNA sequences were subcloned into an expression vector derived from pMON20,400, a derivative of the plasmid expression vector described previously (30). Their cDNA sequences were excised from pDIRECT by digestion with SacI, followed by treatment with T4 DNA polymerase to produce blunt ends. Plasmid DNA was then treated with NcoI to produce a restriction fragment containing the complete coding sequence and 3′-nontranslated region. Such fragments were purified from agarose gel slices and ligated into pMON20,400 which had been sequentially treated with EcoRI, DNA polymerase (Klenow fragment), and NcoI. Coding regions were completely sequenced after cloning into expression plasmids to ensure that no unintended mutations had been introduced. For expression studies, plasmids were introduced into E. coli strain JM101. Cultures (1.6 liters) were grown in baffled shaker flasks as described previously (30, 31). Expression of crystallins was induced in logarithmic cultures by addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM and cultures were grown for an additional 12–18 h. Cells from a typical culture (AγA were collected by centrifugation (15,000 g, 4°C) and were extracted by treatment with DNase (Sigma) and lysozyme essentially as described (32). Extraction buffers contained a mixture of protease inhibitors (each at 1 μg/mL including antipain, bestatin, chymotrypsin, leupeptin, and pepstatin (Boehringer Mannheim).

Recombinant crystallins were purified by chromatography using Macro-Prep S cation exchange resin (Bio-Rad). Chromatography steps were conducted at 23 °C in columns shielded from ambient light. In addition to other components as specified, all chromatography buffers contained 0.5 mM diethiothreitol and 1 mM EDTA. The crude lysate was dialyzed (Mw 12,000–14,000 cut-off) at 4 °C first against 70 volumes of 10 mM Tris-HCl (pH 8.0) and was applied at 1 ml/min to a column (2.5 × 30 cm) packed with Macro-Prep S previously equilibrated with 20 mM Tris acetate (pH 6.0). After a wash to remove unadsorbed material, bound proteins were eluted with a linear gradient of sodium acetate (0–500 mM) contained in the column buffer. The presence of γ-crystallin in elution fractions was estimated by determination on SDS-PAGE of recombinant crystallins with native γ-crystallin purified from human and/or bovine lenses (see below). Western blotting of selected fractions using antibodies against native bovine γ-crystallin (31) was also used to verify the identity of putative γ-crystallin protein bands on SDS gels. Selected peak fractions containing γ-crystallin were pooled and concentrated by pressure filtration (Amicon) using Mw 10,000 cut-off membranes (YM10, Amicon, MA). Where indicated, γ-crystallins were further purified by chromatography using a 1.0 × 100-cm FPLC column packed with Superose 200 gel permeation chromatography support (Pharmacia Biotech Inc.). Purified materials were stored at −20 °C. γ-Crystallins were quantified by Bradford assays (33) using bovine serum albumin as standard. Alternatively, homogeneous preparations of γC and γD were quantified by measurements of A280 assuming ε = 41.4 mM−1 cm−1 calculated on the basis of amino acid composition (34).

Cloning, Mutagenesis, and Overexpression of Human α-A-Crystallin—Complementary DNA clones encoding human αA-crystallin were constructed using the reverse transcriptase-PCR method following the general approach described above for the γ-crystallins. First strand cDNA synthesis was primed with a downstream primer/adaptor designed to anneal in the 5′-untranslated region approximately 350 base pairs downstream from the translational termination codon (Table I). An upstream primer overlapping to the translational initiation codon was designed with reference to the published sequence of human αA-crystallin gene (35). The upstream primer was also designed to create an NcoI recognition site precisely at the initiation codon to aid in subsequent transfer of the cloned cDNA into our expression vector. A prominent band corresponding to the expected ~800-base pair PCR product was extracted from agarose gel slices and cloned into pDIRECT as described above. Transformants in E. coli strain DH5α were identified by PCR screening using primers designed to anneal at vector sites.
flanking the cloned cDNA (T5 and T7 primer binding sites, respectively). Nucleotide sequence analysis of both strands of selected clones confirmed the co-linearity of the published sequence of the human α-crystallin gene and our cloned cDNA. The cloned cDNA was then excised from pDIRECT vector by digestion with NcoI and HinII and transferred into pMON20,400.

Mutants of α-crystallin was produced by site-directed mutagenesis (Life Technologies, Inc.). The W9F mutant was created by replacing Trp-9 with Phe (W9F). A truncation mutant in which the C-terminal 17 amino acids were deleted was constructed by converting the codon for Arg-157 to CCA (CGC terminator codon) (U). In all cases, the encoding sequences in both wild type and mutant expression constructs were completely sequenced to verify the structures of their encoded proteins. Mutagenesis primers are shown in Table I.

Overexpression of human α-crystallin was achieved in E. coli strain JM101. Recombinant proteins were extracted from host cells by the DNase/lysozyme method as described above for the γ-crystallin. When necessary, insoluble proteins present in the initial crude cell extract were purified by treatment for 30 min at 23 °C with cracking buffer consisting of 10 mM sodium phosphate, pH 7.2, 6 mM urea, 142 mM β-mercaptoethanol, and protease inhibitors. Materials solubilized in cracking buffer, as with buffer-soluble materials, were dialyzed (A, 12,000–14,000 cut-off tubing) against 70 volumes of 10 mM Tris-HCl, pH 8.0. After centrifugation to remove precipitated materials, lysates were dialyzed in chromatography buffer (50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 0.5 mM EDTA) and applied at 1 ml/min to a column packed with Macro-Prep Q anion exchange resin (Bio-Rad). Bound proteins were eluted with a 0–0.5 M NaCl linear salt gradient. Column fractions were continuously monitored by measuring the A280, which revealed a single predominant peak. The corresponding fractions were pooled and α-crystallin further purified by gel permeation chromatography using Sephacryl S400 HR (Pharmacia Biotech Inc.). Quantification of homogenous preparations of α-crystallins was carried out by measuring A280 using extinction coefficients calculated as described previously (34).-Crystallin aggregation with the addition of a quencher, sodium azide, to the lens protein solutions.

**TABLE I**

| Primer Application | Gene specific sequence | Reference |
|--------------------|-----------------------|-----------|
| pD5CRYGC γC upstream primer | GGTGCAACCTGACGCC-3’ | K03003 |
| pD5CRYGD γD upstream primer | GCCGCAAGGCAACCA-3’ | K03005 |
| pD5CRYAA αA upstream primer | CATTGACCTTGGACCTCAG-3’ | X14789 |
| pD3CRYAA αA downstream primer | GGCGTGCTATCTAA-3’ | X14789 |
| W9F upstream | AUC CAG CAC CCC UUU UUC AAG CCA GCC CUG-3’ |
| W9F downstream | CUC AAA GGA GGG GUG CAG GAU GCC CAC CUG-3’ |
| R157STOP upstream | CCA CGC UGC GGG AGC CAU CCC GGT GTG-3’ |
| R157STOP downstream | GGA UGG CUC AUC AAC CAG GGG CGG TGG CAA-3’ |

a Gene sequences on which primers were designed are referenced according to their GenBank accession numbers.

Underlined bases represent an addition to the 5’ extension to create an NcoI site in the PCR product which facilitated its cloning into the expression vector.

**RESULTS AND DISCUSSION**

**Cloning and Overexpression of Human γ-Crystallins**—While mRNAs encoding a variety of crystallins have been cloned from lenses of many experimental animal models (reviewed in Ref. 38), it is surprising that most of the orthologous human crystallin gene transcripts have not been characterized. Given the practical difficulties in obtaining adequate human lens material to support traditional cloning studies, we sought to apply PCR-based cloning technology for rapid amplification of target gene sequences from limited quantities of lens tissue. In all cases, a poly(dT) downstream primer was used to initiate first strand cDNA synthesis mediated by reverse transcriptase. Quantities of this material were then combined with gene-specific upstream primers to selectively amplify the individual target sequences. The utility of this approach was confirmed by the demonstration that transcripts derived from the highly similar γC and γD genes were readily amplified with high fidelity, as was the mRNA encoding α-crystallin.

With respect to γ-crystallin sequences, our efforts were focused on cloning and overexpression of γC and γD transcripts...
because these genes correspond to the major $\gamma$-crystallin species in the human lens (39). Sequence analysis of the cloned sequences confirmed the structures of the mRNAs predicted from mRNA splice sites observed at the boundaries of each of the three exons (40) and localized the site of polyadenylation for each of the $\gamma$-crystallin gene transcripts (Fig. 2).

Two exceptions to the previously reported $\gamma$-crystallin gene sequences were found in our study. In the $\gamma$C sequence, assignment of codon 135 as 5'-CTG-3' abolishes a SacI recognition site in the original sequence (accession no. K03004 (1986)) that would otherwise have represented a convenient endonuclease cleavage site for distinguishing $\gamma$C and $\gamma$D gene sequences. The $\gamma$C gene sequences reported previously (accession no. M11973 (1985)) are concordant with our sequence at this position (39, 41). In comparison to the original $\gamma$D sequence report (accession no. K03006 (1985)), we observed differences at positions corresponding to codons 100 and 101. While the sequence difference at codon 100 is translationally neutral, the other specifies incorporation of Met at codon 101 rather than Val as reported previously (40). All other reported human $\gamma$-crystallin gene sequences encode Met at this position (39, 40).

Overexpression of $\gamma$C and $\gamma$D in bacterial host cells gave rise to abundant quantities of the corresponding proteins, which were readily purified by ion exchange and gel permeation chromatography. SDS-PAGE and Western blotting demonstrated that each co-migrated with native $\gamma$-crystallin isolated from human lenses and cross-reacted with antibodies raised against the $\gamma$-crystallin fraction isolated from bovine lenses (Fig. 3A). Recombinant $\gamma$C- and $\gamma$D-crystallins purified by FPLC Superdex chromatography exhibited absorption and fluorescence properties similar to the $\gamma$-crystallin fraction isolated from young lenses (42). Upon excitation of tryptophan residues at 295 nm, the fluorescence maxima of recombinant $\gamma$C- and $\gamma$D-crystallins were at 331 and 329 nm, and the fluorescence quantum yields were 0.17 and 0.06, respectively (data not shown). As reported for the native human $\gamma$-crystallins, the far-UV CD spectra of recombinant $\gamma$C- and $\gamma$D-crystallins were characteristic of predominantly $\beta$-sheet secondary structures (Fig. 4A).

**Fig. 1.** Production of singlet oxygen by a photodynamic system. The time course of formation of singlet oxygen by irradiation of rose bengal at 548.6 nm was measured by the bleaching of RNO at 440 nm.

**Fig. 2.** Sequences of human $\gamma$C- and $\gamma$D-crystallin cDNAs and their encoded proteins. Numbers along right side refer to nucleotide and deduced amino acid sequences (underlined), respectively. Sequences underlined at 5' end of cDNAs were obtained from genomic sequences of human $\gamma$C and $\gamma$D genes (accession nos. K03004 and K03006, respectively) and represent binding sites for upstream PCR primers. Asterisk represents the translational termination codon. Not shown are poly(A) tails at the 3' ends of each cDNA. GenBank accession numbers for $\gamma$C and $\gamma$D sequences are U66582 and U66583, respectively.
tallins probed with antisera against bovine a Crystallin (Fig. 4A) and Western immunoblot analysis of recombinant aA-crystallin (lane 4), with maxima centered near 270 nm and minima between 285 and 300 nm, were similar to that typical of native a-crystallins (43). Immune complexes corresponding to bovine lens a-crystallin (lane 2) and recombinant a-crystallin (lane 3) were visualized after treatment with 125I-protein A and autoradiography. B, SDS-PAGE and Western immunoblot analysis of recombinant aA-crystallin and its mutants. Lanes containing Coomassie Blue-stained samples include molecular weight standards (MW, lane 1) and purified WT aA-crystallin (lane 1), W9F (lane 2), and R157STOP (lane 3). A Western immunoblot of recombinant aA-crystallins probed with antibodies against bovine a-crystallin is shown for WT aA-crystallin (lane 4), W9F (lane 5), and R157STOP (lane 6).

The near-UV CD spectra (Fig. 4B), with maxima centered near 270 nm and minima between 285 and 300 nm, were similar to that typical of native a-crystallins (43). In all respects examined thus far, recombinant a-crystallins have structural properties indistinguishable from the corresponding proteins present in native tissues and appear to be excellent tools to support in vitro studies of lens protein interaction.

Cloning, Overexpression, and Aggregation Behavior of Human aA-Crystallin and Its Mutants—Sequence analysis of the cloned human aA-crystallin cDNA produced by RNA PCR from human lens total RNA confirmed the colinearity of the mRNA transcript with that predicted by splicing of the three exon sequences published previously (Fig. 5) (35). When subcloned into the expression plasmid, the cDNA directed synthesis of abundant quantities of aM &sim; 20,000 band that comigrated with aA-crystallin purified from human lenses. Similarly, sequences encoding the W9F and R157STOP mutants directed synthesis of abundant quantities of each protein. Both WT and W9F forms of aA were recovered predominantly in the soluble fraction when host cells were extracted by lysozyme treatment. In contrast, the R157STOP mutant protein partitioned with the insoluble phase following lysozyme treatment. Mutant protein in the insoluble pellet was easily recovered by solubilizing in cracking buffer containing 6 M urea.

In comparison to the WT protein, the R157STOP mutant protein appeared to have reduced solubility following removal of urea by dialysis. When tested at 23 °C in the presence of 50 mM Tris-HCl, 50 mM NaCl, and 1 mM EDTA at pH 7.6, concentrations greater than 0.3 mg/ml of the purified R157STOP could not be achieved by pressure filtration without formation of a grainy white precipitate. Sequential chromatography over ion exchange and gel permeation columns was sufficient to purify both WT and mutant aA-crystallins to apparent homogeneity. Western blots probed with antisera directed against bovine a-crystallin confirmed the identity of the purified materials (Fig. 3B).

When applied to an FPLC column packed with Superdex 200, the WT and W9F forms of aA-crystallin eluted as broad symmetrical peaks at a volume corresponding to a molecular mass of approximately 540 kDa (Fig. 6A). This size estimate is somewhat smaller than the aggregate size of 640 kDa reported previously for WT aA-crystallin aggregates (32). As noted previously (26), it appears that replacement of Trp-9 with Phe does not substantially alter the subunit interactions since the W9F mutant elutes at a position similar to that of WT. In contrast, the elution volume and peak profile observed with purified R157STOP mutant were distinctly different. The R157STOP mutant eluted as an asymmetric peak near the column exclusion volume with a trailing descending shoulder (Fig. 6B). Since purification of the R157STOP mutant required solubilization from the host cell lysate using a denaturing buffer containing 6 M urea, we questioned whether differences in its elution profile relative to WT could be due to altered subunit renaturation during dialysis. When denatured and dialyzed under the same conditions as the R157STOP mutant, WT aA gave rise to an elution pattern similar to that observed with the R157STOP mutant (Fig. 6B). Factors influencing the aggregation behavior of a-crystallin complexes are not well understood although the concentration of salts, chaotropic agents, and temperature have been reported to play important roles (44–46). Redistribution of sHSPs into large molecular weight aggregates in cells subjected to heat shock has also been reported (47, 48). Since the present studies examined the chromatographic behavior of aggregates composed exclusively of aA chain subunits, we question whether the aB subunit might play an important role in remodeling of a-crystallin complexes following denaturation with chaotropic agents. Future studies are planned to examine this possibility.

Since the R157STOP mutant and urea-treated WT proteins...
Chaperone-like Activity of αA-Crystallin

**Fig. 5. Sequence of human α-crystallin.** Sequences corresponding to the upstream and downstream primer binding sites (underlined) were obtained from the genomic sequence (35). Location of mutation sites for substitution of phenylalanine for tryptophan 9 (W9F) and insertion of a translational termination codon corresponding to arginine 157 (R157STOP) are indicated by dashed underlining. Asterisk indicates the translational termination codon in the wild type sequence. GenBank accession number U66584 has been assigned to this sequence.

**Fig. 6. Gel permeation chromatography of α-crystallin aggregates.** Samples of purified αA-crystallin were dialyzed overnight against the column buffer consisting of 50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA prior to injection onto the column. Elution positions of gel filtration standards and their corresponding aggregate sizes are shown along the top of panel A. The void volume position (V_0) was determined by elution of blue dextran. A, absorbance trace of WT and W9F isolated from the water-soluble fraction of host cell lysates; B, absorbance tracet of WT and R157STOP treated with 6M urea and W9F isolated from the water-soluble fraction of host cell lysates; C, absorbance trace of WT protein with urea followed by dialysis. The observed changes are likely to be due to treatment of the protein with 6M urea during extraction procedure, followed by complete removal of urea by dialysis. Urea denaturation followed by dialysis may result in loss of the tyrosine/tryptophan peaks of the near-UV CD spectrum of bovine α-crystallin between 270 and 290 nm as reported by Siezen and Bindels (50). These authors noted that the phenylalanine peaks below 270 nm, and tryptophan minimum around 290 nm was completely recovered after exhaustive dialysis.

**Fig. 7. CD spectra of αA crystallins.** A, CD spectra in the far-UV region were collected for WT αA-crystallin (solid line), and W9F (dotted line) and R157STOP (dashed line) mutants of αA-crystallin. Measurements were made at a protein concentration of 0.1 mg/ml in a 0.1-cm pathlength cuvette containing 10 mM potassium phosphate buffer, pH 7.4. B, CD spectra in the near-UV region were collected for WT αA-crystallin (solid line) and W9F (dotted line) and R157STOP (dashed line) mutants of αA-crystallin. Spectra were collected at a protein concentration of 0.2 mg/ml in a 1-cm pathlength cuvette containing 10 mM potassium phosphate buffer, pH 7.4. All CD data are expressed as molar ellipticity in degrees-cm^2-dmol^-1.

**Spectroscopic Properties of Recombinant Crystallins—WT αA**

Absorption and fluorescence properties similar to the native α-crystallin (a mixture of αA and αB subunits). Upon excitation of tryptophan residues at 295 nm, the fluorescence maxima of recombinant αA was at 341 nm and the fluorescence quantum yield was 0.10. These fluorescence properties are similar to those reported for native mixtures of α-crystallins (42, 49). The far-UV CD spectrum of WT αA was characteristic of predominantly β-sheet secondary structure (Fig. 7A). The far-UV CD spectra of W9F and R157STOP mutants were similar to those of WT, indicating that the amide backbone conformation was unaffected by the mutations (Fig. 7A). The contribution of tryptophan to the near-UV CD of WT αA could be resolved by examination of the spectrum of the W9F mutant (Fig. 7B). The most obvious difference between the near-UV CD spectra of WT and W9F was the disappearance of the positive dichroism at 255–265 nm. This dichroism was previously assigned to a 1ω tryptophan transition (43). Therefore, the negative dichroism around 270 nm in W9F is most likely due to 1ω tyrosine, while that around 255 nm is due to Phe or Tyr bands.

The positive dichroism and fine structure of the near-UV CD of WT αA-crystallin was significantly reduced in the R157STOP mutant. The observed changes are likely to be due to treatment of the protein with 6 M urea during extraction procedure, followed by complete removal of urea by dialysis. Urea denaturation followed by dialysis may result in loss of the tyrosine/tryptophan peaks of the near-UV CD spectrum of bovine α-crystallin between 270 and 290 nm as reported by Siezen and Bindels (50). These authors noted that the phenylalanine peaks below 270 nm, and tryptophan minimum around 290 nm was completely recovered after exhaustive dialysis. In spectra reported in Fig. 7B, there was a loss of overall fine structure, and a 2-nm blue shift of the 292 nm negative CD band in the R157STOP mutant. The negative dichroism of the band around 270 nm had a higher intensity for the R157STOP mutant than the WT αA-crystallin. These data suggest that urea treatment changes the local environment of the tryptophan and tyrosine residues of α-crystallin. Treatment of the WT protein with urea followed by dialysis produced parallel changes in the near-UV CD spectrum (not shown).
heat-induced aggregation using recombinant human aldose reductase as a substrate. Suppression of aggregation was observed when WT αA-crystallin was added to aldose reductase solutions, with apparently complete suppression being observed by the addition of stoichiometric amounts of αA subunits relative to aldose reductase (Fig. 8A).

As noted previously, enzyme activity was lost in enzyme samples treated at 52 °C in the presence or absence of αA-crystallin (51). A similar pattern of protection against aggregation was observed with the W9F mutant (Fig. 8B), confirming the previous report that substitution of Trp-9 with Phe resulted in virtually no alteration of chaperone-like activity (26). In contrast, the R157STOP mutant provided only partial suppression of aggregation, even with addition of an almost 2-fold molar excess of the truncated subunits (Fig. 8C). The diminished chaperone-like activity of R157STOP mutant appears not to be related to urea treatment during purification, since denaturation of wild type αA by treatment with 6 M urea buffer followed by dialysis renaturation resulted in a negligible change in chaperone-like activity in the thermal aggregation assay (data not shown). In all cases, no apparent increase in solution turbidity was observed when the wild type or mutant αA-crystallins were heated in the absence of aldose reductase.

Since estimation of chaperone-like activity of α-crystallin using the heat aggregation assay is carried out at an unphysiological temperature (52 °C), we utilized a complementary assay that depends on its ability to prevent single oxygen-induced aggregation of γD-crystallin at 23 °C. Treatment of purified recombinant γD-crystallin solutions with singlet oxygen in a photodynamic system produced a time-dependent increase in light scattering that appeared to correlate with induced changes in their near-UV CD spectra as reported previously for native γ-crystallin (52). When incubated in the singlet oxygen-generating system, aggregation of γD-crystallin was observed (Fig. 9, curves 1). We estimate that under the present conditions, approximately 20–30% of γD-crystallin was aggregated.
crystallin undergoes insolubilization. Essentially complete suppression of the singlet oxygen-induced aggregation of 8.8 μM γD-crystallin was achieved by either 2 μM WT αA-crystallin or 3.6 μM W9F mutant (Figs. 9, A and B). In contrast, the R157STOP truncation mutant was markedly less effective in suppressing the singlet oxygen-induced aggregation (Fig. 9C). The relative light scattering change of γD-crystallin shown in Fig. 9A was substantially reduced (from 1.0 to 0.1 at 120 min) by the addition of 0.1 mM sodium azide to the reaction mixture (data not shown), confirming that the increase in light scattering was mediated by singlet oxygen.

Since the crystallin proteins can persist in human lens cells for decades, they are subject to numerous posttranslational modifications including oxidation, deamidation, proteolysis, and nonenzymatic glycation (1). Indeed, past efforts to elucidate the structures and properties of lens crystallins have been complicated by the heterogeneous nature of these proteins extracted from adult human and animal lenses (53). In the present work, we demonstrate that recombinant lens crystallins provide a renewable and structurally defined set of tools to probe the interaction of the molecular chaperone α-crystallin with one major class of structural proteins in the lens, the γ-crystallins. Our results confirm previous reports that the αB subunit of α-crystallin expresses chaperone-like activity even when expressed in the absence of the the αB subunit (54) and demonstrate that deletion of sequences from the C terminus results in marked reduction in the chaperone-like activity of αA. Future mutagenesis studies, as exemplified by the W9F tryptophan-free mutant (26), should enable us to use this reconstituted chaperone system to probe in detail the interactions between α-crystallin and its substrate proteins and to better understand the mechanism and potential role of α-crystallin in regulation of protein interaction in the mammalian lens.

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