Human αB-Crystallin
SMALL HEAT SHOCK PROTEIN AND MOLECULAR CHAPERONE*

(Published for publication, September 20, 1996, and in reversed form, November 6, 1996)

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The polymerase chain reaction was used to amplify a cDNA sequence encoding the human αB-crystallin. The amplified cDNA fragment was cloned into the bacterial expression vector pMAL-c2 and expressed as a soluble fusion protein coupled to maltose-binding protein (MBP). After maltose affinity chromatography and cleavage from MBP by Factor Xa, the recombinant human αB-crystallin was separated from MBP and Factor Xa by anion exchange chromatography. Recombinant αB-crystallin was characterized by SDS-polyacrylamide electrophoresis (PAGE), Western immunoblot analysis, Edman degradation, circular dichroism spectroscopy, and size exclusion chromatography. The purified crystallin migrated on SDS-PAGE to an apparent molecular weight (Mₚ = 22,000) that corresponded to total native human α-crystallin and was recognized on Western immunoblots by antiserum raised against human αB-cry stallin purified from lens homogenates. Chemical sequencing, circular dichroism spectroscopy, and size exclusion chromatography demonstrated that the recombinant crystallin had properties similar or identical to its native counterpart. Both recombinant αB-crystallin and MBP-αB fusion protein associated to form high molecular weight complexes that displayed chaperone-like function by inhibiting the aggregation of alcohol dehydrogenase at 37 °C and demonstrated the importance of the C-terminal domain of αB-crystallin for chaperone-like activity.

α-crystallins have not been elucidated, but it is possible that they share the two-domain structure found in other crystallins (5, 6). In vitro a chaperone-like activity has been described for bovine α-crystallins in suppressing the aggregation of proteins denatured at high temperature (7–13). Human αB-crystallin expression is found under normal conditions in many nonlens cells and tissues, including heart, brain, skeletal muscle, kidney, placenta, and lung (14–22) and, like the ubiquitous small heat shock proteins, is dramatically up-regulated in response to stress and under pathological conditions (23–30). αB-Crystallin has biochemical properties that result in its copurification with mammalian heat shock protein 28 from human skeletal muscle (18). The αB-crystallin gene has been shown to contain a heat shock element in its promoter that may be subject to a heat-regulated control mechanism (31).

The chemical nature of the interactions between human α-crystallins and other proteins is poorly understood because of the difficulty with isolation of sufficient quantities of unmodified protein from human lenses. For this reason, recombinant techniques have been used to characterize structure-function relationships of the individual α-crystallin subunits. The conformational properties of substrate proteins bound to recombinant human αA-crystallin was recently reported (32); however, unlike human αB-crystallin, human αA-crystallin lacks a heat shock element in its promoter, is not induced by stress or pathological conditions, and has a limited expression pattern in the body. In this article we report expression and characterization of the small heat shock protein human αB-crystallin cloned from a fetal lens cDNA library. To the best of our knowledge this is the first demonstration that human αB-crystallin displays molecular chaperone activity. The recombinant expression system described here provided an excellent source of unmodified human αB-crystallin that assembled into a high molecular weight oligomer, and displayed chaperone-like activity against protein aggregation.

EXPERIMENTAL PROCEDURES

Construction of Human Fetal Lens cDNA Library—RNA was isolated from 9–12-week human fetal lenses as described previously (33). Oligo(dT)-primed cDNA was prepared from 2 μg of total RNA using the Boehringer Mannheim cDNA synthesis kit according to the manufacturer’s instructions. The cDNA was EcoRI-linked, digested with EcoRI, and ligated to EcoRI gat11 arms (Promega, Leiden, The Netherlands). After in vitro packaging, the phages were plated on Escherichia coli Y1090 cells.

Construction of Human αB-Crystallin Expression Vector—The coding region for human αB-crystallin was isolated from the fetal lens cDNA library by polymerase chain reaction and inserted into the cloning vector pCR™II (Invitrogen, San Diego, CA). The following primers, which correspond to the 5’- and 3’-ends of the coding region for human αB-crystallin (16, 36), were synthesized and used in the polymerase chain reactions: 5’-CCAGAATTCATGGACATCGGATCCATCCACCCAC-3’ (forward) and 5’-CCATCGATATTTGGGCTGGGCTGGGT-3’ (reverse). EcoRI and XbaI sites were attached to the 5’-ends of the forward

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* This work was supported by National Institutes of Health Grant EY04542 and Vision Training Grant Research Training in Biotechnology Grant T32 GM 08437, EY07031. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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and reverse primers, respectively. The coding region for the human αB-crystallin was then removed from the pCR®-II vector at its flanking restriction sites and ligated into EcoRI-XbaI-digested pMAL™-c2 (New England Biolabs, Beverly, MA) to produce pMAL-c2-αB. The coding region was inserted downstream of the maltE gene of E. coli, which encodes MBP, resulting in the expression of a MBP-αB fusion protein. The coding region of this expression construct was confirmed by DNA sequence analysis using the dyeode chain termination method (34).

Expression and Purification of Human MBP-αB Fusion Protein—The pMAL-c2-αB3 expression plasmid was used to transform competent E. coli cells (Stratagene, San Diego, CA). One liter of cells containing 100 μg ampicillin was inoculated with 10 ml of an overnight culture of the transformed E. coli cells, and cells were grown with vigorous shaking at 37 °C. The cells were induced until the culture reached an optical density of ~0.5 at A = 600 nm, at which point protein expression was induced by addition of isopropyl-β-d-thiogalactopyranoside to a final concentration of 0.3 mM (Sigma). Four hours after induction, cells were harvested by sedimentation and resuspended in 50 ml of column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, and 1 mM EDTA). Cells were stored overnight at ~20 °C. After thawing, the cells were disrupted by sonication on ice by eight 30-s cycles at 70 watts on a Branson (Plainview, NY) Ultrasonics power sonifier. Insoluble cellular debris was removed by sedimentation at 9,000 × g for 30 min at 4 °C. Soluble protein present in the supernatant was purified by adsorption to a 10 ml amyllose resin affinity column (New England Biolabs) for 1 h at 25 °C. After washing with 10 column volumes of column buffer, bound fusion protein was eluted using column buffer that contained 10 mM maltose. Preparations of fusion protein were concentrated using Centriplus-10 microconcentrators (Amicon, Beverly, MA). Preparations of fusion protein contained <5% contaminating proteins as assessed by SDS-PAGE and Coomassie Blue staining. Concentrations of purified fusion protein were determined by a protein assay (Bio-Rad).

Purification of Recombinant Human αB-Crystallin—MBP was cleaved from human αB using the protease Factor Xa, the recognition sequence of which is encoded in the pMAL-c2-αB3 vector within the fourth and fifth codons 5′ from the coding region for αB-crystallin. Recombinant human αB-crystallin was purified by anion exchange chromatography in the presence of 8 M urea. Briefly, cleaved fusion protein was dialyzed against ion exchange buffer 20 mM Tris-HCl, pH 8.0, 25 mM NaCl, 1 mM EDTA, and 8 M urea. MBP and Factor Xa were separated from human αB-crystallin by absorption to a 10 ml column of Q-Sepharose anion exchange resin (Pharmacia Biotech Inc.). In the presence of ion exchange buffer, recombinant human αB-crystallin had no affinity for the Q-Sepharose anion exchange resin and hence could easily be separated from MBP and Factor Xa by this method. Preparations of recombinant human αB-crystallin were dialyzed overnight against ion exchange buffer that lacked 8 M urea to promote renaturation and then concentrated on Centriplus-10 microconcentrators. Preparations of recombinant human αB-crystallin contained <5% contaminating proteins as assessed by SDS-PAGE and Coomassie Blue staining. Concentrations of purified recombinant human αB-crystallin were determined by a protein assay (Bio-Rad).

SDS-PAGE and Western Immunoblot Analyses of Recombinant Human αB-Crystallin—Proteins were analyzed on 4–20% polyacrylamide electrophoretic gels in the presence of 0.1% SDS (Noves, San Diego, CA) and were stained with Coomassie Blue R-250 (Pharmacia). Proteins were electrophoretically transferred to a polyvinylidene difluoride membrane using a Blot Module II (Novex). Antiserum raised against αB-crystallin purified directly from human lens homogenates was used as a primary antibody (35). For immunodetection, alkaline phosphatase conjugated to goat anti-rabbit IgG antibody and 5-bromo-4-chloro-3'-indolyphosphate and p-nitro blue tetrazolium chloride (Bio-Rad) were used.

Edman Degradation of Human αB-Crystallin—N-terminal sequencing of the purified human αB-crystallin was performed by sequential Edman degradation (15 cycles) using an Applied Biosystems 477A liquid phase protein sequencer with an on-line 120A phenylthiodyanin analyzer after immobilization on a polyvinylidene difluoride membrane.

Circular Dichroism Spectroscopy of Human αB-Crystallin—Circular dichroism spectra were obtained using a Jasco 720 spectropolarimeter. A 0.5-mm path length cell was used. Sixteen scans were averaged per sample, and spectra of the buffers were subtracted from the spectra of the protein samples.

Reassociation of Human MBP-αB and αB-Crystallin—Reassociation human MBP-αB and αB-crystallin were fractionated by size exclusion chromatography on a Macrophere GPC 300-5, 7 μm, 250 × 4.6-mm size exchange column (Altech, Deerfield, IL) using a Hewlett-Packard (Palo Alto, CA) 1090 high performance liquid chromatography with the following mobile phase: 0.1 mM KH2PO4 (pH 7.0) and 0.2 mM NaCl, at a flow rate of 0.1 ml min⁻¹. High molecular weight protein standards (Pharmacia) were used to calibrate the column.

Aggregation Measurements—The effect of human αB-crystallin on protein aggregation was measured as described previously (7). Briefly, the aggregation of ADH at 37 °C was measured as the apparent optical density at A = 360 nm using a Shimadzu (Kyoto, Japan) UV-160 UV-visible recording spectrophotometer equipped with a temperature-controlled cuvette holder. In a total reaction volume of 400 μl, 5 μM equine liver ADH (Sigma) was incubated at 37 °C with varying amounts of purified human MBP-αB or αB-crystallin. All reagents were diluted into the following reaction buffer: 50 mM sodium phosphate buffer (pH 7.0), 0.1 mM NaCl, and 2 mM EDTA. Stock solutions were stored on ice until they were mixed at room temperature and quickly placed in the temperature-controlled sample chamber of the spectrophotometer. The temperature in the cuvette was monitored using a bead thermistor installed in a cuvette within the sample chamber. The optical density in each cell was recorded every 60 s.

RESULTS

Characterization of Human αB-Crystallin cDNA Clone—The cDNA-coding region of αB-crystallin, isolated by polymerase chain reaction from a human fetal lens cDNA library, was ligated into the plasmid pMAL™-c2 to produce pMAL-c2-αB3. Double-stranded dyeide sequencing of both strands encoding pMAL-c2-αB3 demonstrated that the αB sequence was 100% identical to the corresponding exon sequence of the αB-crystallin gene and to the coding regions of partial- and full-length αB-crystallin cDNA clones (16, 36, 37). The N terminus of the recombinant human αB-crystallin contained 4 additional amino acids: isoleucine, serine, glutamic acid, and phenylalnine, all of which are derived from the insertion of the human αB-coding region into the EcoRI and XbaI sites in the pliymolinker of pMAL™-c2. Hence, pMAL-c2-αB3 contained an open reading frame of 537 base pairs, predicted to encode a polypeptide of 179 amino acids after cleavage and separation from MBP.

Expression and Purification of Human MBP-αB and αB-Crystallin—Fig. 1 contains SDS-PAGE (A) and Western immunoblot analysis (B) of the expression and purification of recombinant human MBP-αB and αB-crystallin. FIG. 1A is a Coomassie Blue stain of a polyacrylamide electrophoretic gel run in the presence of 0.1% SDS. Control of induction of MBP-αB fusion protein expression was apparent in crude cell lysates of bacterial cultures transformed with the human αB-crystallin expression construct before and after the addition of isopropyl-β-d-thio galactopyranoside (Fig. 1A, lanes 1 and 2, respectively). Treatment of the affinity-purified fusion protein (Fig. 1A, lane 3) with the serine protease Factor Xa demonstrated that this fusion protein was cleaved into two distinct polypeptides (Fig. 1A, lane 4) that migrate to molecular weights corresponding to native MBP (~45,000) and native human αB-crystallin (~22,000). The recombinant human αB-crystallin was isolated by anion exchange chromatography in the presence of 8 M urea and was found to contain <5% contaminating proteins as assessed by SDS-PAGE (Fig. 1A, lane 5). Further confirmation of the expression and purification of recombinant human αB-crystallin was demonstrated by Western immunoblot analysis using anti-human αB-crystallin antiserum (Fig. 1B). One predominant immunoreactive band was observed in the purified uncleaved fusion protein (Fig. 1B, lane 1), in the mixture of cleaved fusion protein (Fig. 1B, lane 2), and with purified human αB-crystallin (Fig. 1B, lane 3).
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Chemical Sequencing Results of Human αB-Crystallin—To verify that the $M_r \sim 22,000$ band corresponded to human αB-crystallin, the cleaved fusion protein mixture was immobilized on a polyvinylidene difluoride membrane, and the crystallin, the cleaved fusion protein mixture was immobilized from 1 to 100 nM (Fig. 4). The spectrum of recombinant human αB-crystallin in the absence and presence of 1% SDS are presented. Measurements were made at a concentration of 0.18 mg/ml using a path length of 0.5 mm in 20 mM Tris-HCl (pH 8.0), 25 mM NaCl, and 1 mM EDTA. Spectra of buffers were subtracted from the spectra of protein samples. All circular dichroism data are expressed as molar ellipticity (θ) in millidegrees cm$^2$ dmol$^{-1}$. Each spectrum represents the average of 16 scans.

Secondary Structure of Human αB-Crystallin—In Fig. 2 the far UV circular dichroism spectra of recombinant human αB-crystallin in the absence and presence of 1% SDS are presented. The spectrum of recombinant human αB-crystallin in the presence of buffer alone (Fig. 2, solid line) resembles published spectra for total native bovine α-crystallin as well as recombinant and native bovine αA-crystallin and is typical of a spectrum for a predominantly β-pleated structure (12, 38–40). On addition of SDS to a final concentration of 1%, an increase in molar ellipticity was observed at all wavelengths, indicative of major structural changes (Fig. 2, dashed line) in the spectrum, as reported previously for total native bovine α-crystallin (38).

Reassociation of Human MBP-αB and αB-Crystallin—Fractionation of the recombinant MBP-αB fusion protein and the αB-crystallin alone by chromatography on a size exclusion column under nondenaturing conditions demonstrated that they formed high molecular weight oligomers between 2.32 × 10$^5$ and 4.40 × 10$^5$ in size (Fig. 3). Complete Inhibition of the Thermally Induced Aggregation of ADH by Human MBP-αB and αB-Crystallin—The apparent optical density was a direct measure of the aggregation of ADH at 37 °C over 60 min. Human αB-crystallin and MBP-αB suppressed the aggregation of ADH over a range of concentrations from 1 to 100 nM (Fig. 4, A and B, respectively). We assumed molecular weights of 8.0 × 10$^4$ for ADH, 1.3 × 10$^5$ for MBP-αB-crystallin, and 4.4 × 10$^5$ for αB-crystallin. At a molar ratio of 200:1 (ADH:human MBP-αB/αB-crystallin), partial inhibition of ADH aggregation was observed. At a molar ratio of 50:1 (ADH:human MBP-αB/αB-crystallin), complete suppression of ADH aggregation was observed. At a molar ratio of 5,000:1 (ADH:human MBP-αB/αB-crystallin), no effect on the aggregation of ADH was observed. As a control, MBP alone was tested at a molar ratio of 1:1 or lower (ADH:MBP), and no effect on the aggregation of ADH was observed (data not shown).

DISCUSSION

Human lens crystallins undergo extensive posttranslational modifications during the aging process (41–44). These modifications lead to protein heterogeneity, which has precluded the successful purification of large amounts of homogenous human crystallins to be used for biophysical, functional, and structural analyses. Here we report for the first time expression and characterization of functional human αB-crystallin in E. coli.

DNA primers designed against the 5’- and 3’-ends of the αB coding region successfully amplified a ~525-base pair cDNA from a human fetal lens cDNA library, confirming that this gene is transcriptionally active in the human lens (data not...
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5 μM ADH + 0 nM Alpha B
5 μM ADH + 1 nM Alpha B
5 μM ADH + 25 nM Alpha B
5 μM ADH + 100 nM Alpha B

A

O.D. (A = 280 nm)

Time (min)

0.05

0.25

0.5

0.75

1.0

2.0

3.0

4.0

5.0

60

B

O.D. (A = 280 nm)

Time (min)

0.05

0.25

0.5

0.75

1.0

2.0

3.0

4.0

5.0

60

FIG. 4. Complete suppression of ADH aggregation at 37 °C by recombinant human MBP-αB and αB-crystallin. A, influence of 1, 25, and 100 nM recombinant human αB-crystallin on the thermal aggregation of ADH. The apparent optical density (O.D.) was a direct measure of the aggregation of ADH at 37 °C over 60 min. The final concentration of ADH was 5 μM in all samples. B, influence of 1, 25, and 100 nM recombinant MBP-αB on the thermal aggregation of ADH at 37 °C over 60 min. The final concentration of ADH was 5 μM in all samples. Values are means ± S.D. (bars) calculated from three independent experiments.

shown). The amplified coding region for this crystallin was subcloned into a bacterial expression plasmid and expressed in the bacterial cytoplasm as a soluble fusion protein coupled to MBP. After cleavage and separation from MBP, SDS-PAGE demonstrated that the secondary structure of the reassociated αB-crystallin was 100% identical to the deduced amino acid sequence predicted from the exon sequence of the αB-crystallin gene (36). Although the N-terminal methionine of the recombinant αB-crystallin is preceded by four additional residues (isoleucine, serine, glutamic acid, and phenylalanine), circular dichroism spectroscopic analysis indicated that the secondary structure of the reassociated αB-resembled the secondary structures previously reported for total purified bovine α-crystallin as well as recombinant and purified bovine α-crystallin (12, 38–40).

On a size exclusion chromatography column recombinant human MBP-αB and αB-crystallin eluted as high molecular weight oligomers similar in size (between 2.3 × 10^5 and 4.4 × 10^5) to total α-crystallin purified directly from human eye lenses (45). Interestingly, MBP-αB fusion protein was able to associate into a high molecular weight oligomer despite the presence of the Mr, 42,700 N-terminal fusion partner MBP. Assuming molecular weights of 1.3 × 10^6 for MBP-αB and 4.4 × 10^5 for αB-crystallin, both the human MBP-αB and αB-crystallin displayed molecular chaperone activity, as demonstrated by their complete suppression of the aggregation of ADH at a molar ratio of approximately 50:1 (ADH:crystallin). When the molecular weights of the subunits of MBP-αB and αB-crystallin were used (6.5 × 10^4 and 2.2 × 10^4, respectively), complete suppression of the aggregation of ADH was observed at a molar ratio of approximately 2.5:1 (ADH:crystallin). A systematic evaluation of the precise stoichiometry involved in the suppression of ADH aggregation by MBP-αB and αB-crystallin will need to be addressed in the future.

It was recently demonstrated using site-directed mutagenesis that specific residues at the C terminus of recombinant bovine αA-crystallin influenced its reassociation and chaperone activity (46). To date, no mutants of human αB-crystallin have been characterized; however, in the studies presented here the presence of four extra N-terminal residues had no observable effect on the secondary structure, reassociation, and chaperone activity of recombinant human αB-crystallin. Strikingly, human MBP-αB formed a large functional structure and was able to completely suppress the aggregation of ADH at concentrations identical to those used with recombinant human αB-crystallin alone. Taken together, these results contribute to the evidence that the well conserved C-terminal domain of the α-crystallins is responsible for the proper assembly into large functional oligomers (5, 6).

At the amino acid level αB-crystallin is remarkably well conserved among mammalian species. This high degree of conservation among species may indicate a critical function for αB-crystallin in nonlens cells, in which expression occurs in response to environmental and pathological stress. The successful expression of functional recombinant human αB-crystallin creates the first opportunity to characterize the chemical basis of the interactions between αB and other proteins in lens and nonlens cells under normal and pathological conditions.

Acknowledgments—We thank Dr. Larry Takemoto for the kind gift of anti-human αB-crystallin antiserum and Chris Ganders, Dr. Hiro Matsushima, Alireza Milaninia, and Melissa Valdez for technical support. DNA sequencing reactions were performed by the Seattle Biomedical Research Institute, and chemical protein sequencing was performed by the Molecular Pharmacology Facility at the University of Washington.

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