Effects of Site-directed Mutations on the Chaperone-like Activity of αB-Crystallin*

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Recombinant αB-crystallin has been shown to exhibit chaperone-like activity, suppressing the thermal aggregation of γ-crystallin and aggregation of the reduced insulin B chain conferring thermostolerance to Escherichia coli BL21(DE3) cells. Mutations were made in three specific areas of the αB-crystallin, the N terminus D2G, the conserved phenylalanine-rich region, F24R, F27R, F27A, and the two C-terminal lysines K174L/ K175L. Biophysical characterization of the mutant αB-crystallins using far-UV CD revealed no change in secondary structural elements. Tryptophan fluorescence demonstrated global structural changes. Heat stability of the mutant αB-crystallins was not significantly affected as indicated by tryptophan fluorescence of heat-treated proteins.

Mutations within the phenylalanine-rich region abolish the chaperone-like activity as measured by both in vivo and in vitro assays. Proteins with mutations at the C terminus demonstrated no significant chaperone-like activity, failing to confer thermostolerance on E. coli and demonstrating no significant inhibition of protein aggregation in either γ-crystallin or reduced insulin B chain assays. The N-terminal mutation D2G demonstrated a significant reduction in efficiency of the chaperone-like activity although some thermostolerance was conferred in the E. coli assay. In vitro assays showed that complete inhibition of aggregation was only achieved at 10-fold higher concentrations of D2G than that required by the native αB-crystallin.

Consistent changes in the chaperone-like activity of the site-directed mutants were demonstrated by the three assays. The results suggested that both charge and hydrophobic interactions are important in protein binding by αB-crystallin and that the conserved RLFDQFF region is vital for chaperone-like activity.

The eye lens contains high concentrations of soluble proteins, the crystallins. They fall into two classes, the α-crystallin family and the β/γ-superfamily (1). There is differential expression of the crystallins during lens development (2) which leads to different mixtures of crystallins along the visual axis. Properties of individual crystallins may be important in maintaining short range order, and thus transparency, in the lens (3). As there is no protein turnover in the majority of the lens tissue, the crystallins can survive as long as the individual. Crystallin unfolding and aggregation, caused initially by post-translational modification (4, 5) or oxidative damage (6) to these long-lived proteins, play an aetiological role in the development of cataract, the largest cause of blindness in the world.

α-Crystallins differ from β- and γ-crystallins in containing both sheet and helical structure and have been found in many extralenticular tissues including brain, spinal cord, and heart (7). Overexpression of αB-crystallin may be induced in mamalian cell lines by both heat shock (8) and osmotic stress (9) and by expression of oncogene proteins such as c-Ha-Ras (10). Expression of αB-crystallin in glial cell culture has been shown to confer increased thermostolerance and adherence and to cause an increase in cytoskeletal fibers (11). Overexpression of αB-crystallin has been observed in a large number of severe neurological disorders including Creutzfeldt-Jakob disease (12), and αB-crystallin serves as an immunodominant myelin antigen to human T cells when expressed at the elevated levels found in active multiple sclerosis lesions (13).

Chemical modification of α- and γ-crystallins by cyanate and by glucose 6-phosphate causes the proteins to alter their tertiary structure, but retain native secondary structure (14). These initial changes correspond to those found in human cataract, where glycation is an important aetiological factor (15).

Many monomeric and oligomeric proteins lack the inherent ability to correctly assemble into biologically functional molecules (16). For the correct post-translational assembly of these polypeptides into their “correct” structures, a ubiquitous class of conserved proteins, termed “chaperones” or, for bacterial proteins, “chaperonins” is thought to be involved. This class of proteins includes the Escherichia coli GroES, SecB, DnaK, and DnaJ gene products, and heat shock proteins (hsp60, -70, -90) in eucaryotic cells. As well as a general class of chaperone proteins, there appears to be a class of specific chaperones that regulate the folding of a single protein (17). Recent evidence supports the idea that GroES and SecB can act as “unfoldases,” binding and unfolding stressed and aberrantly folded proteins and restoring the native structure (18).

Lens α-crystallin, which has some sequence homology to heat shock proteins, was suggested to be a chaperone for correct folding of γ-crystallin in the lens (19), hence maintaining lens transparency. α-Crystallin has been shown to act as a chaperone-like protein, in vitro sequestering unfolded protein, and inhibiting its subsequent aggregation and insolubilization (19–21). However, αB-crystallin has been shown to differ from true chaperones in that subsequent release of the bound protein and restoration of native structure has not been observed (22, 23). This may be because αB requires an as yet unidentified cofactor, analogous to GroES, present in vivo but not in the in vitro experiments or because αB-crystallin belongs to a more simple class of shock proteins which bind unfolded proteins in a chaperone-like manner and prevent aggregation but do not release...
Chaperone-like Activity of αB-Crystallin

A string of amino acid residues in crystallins and a number of heat-shock proteins (26) is necessary to maintain lens transparency. Lyophilization of a lens in the presence of a crystallin in the native region of lysines involved in protein binding. Modification of the C-terminal residues makes it a very useful model for determination of specific residues involved in protein binding. The conserved phenylalanine-rich region RLFDQFF in αB-crystallin is therefore now of considerable interest both as a lens protein involved in cataractogenesis and as a general mammalian shock protein with a possible role in other disorders.

The small size of the α-crystallin monomers (175 residues) make it a very useful model for determination of specific residues involved in protein binding. Modification of the C-terminal region of αB-crystallin has been shown to inhibit the in vitro chaperone-like activity (20). The C terminus contains a number of lysine residues which may be glycated in the aging lens. Protein binding may involve hydrophobic residues, and we have found a considerable sequence homology between a conserved phenylalanine-rich region RLFDQPF in αA- and αB-crystallins and a number of heat-shock proteins (26).

We therefore decided to use site-directed mutagenesis to make substitution mutations both to the C-terminal lysines (Lys174, Lys175) and to the N-terminal aspartate (Asp2). We made substitution mutations within the phenylalanine-rich region (Fig. 1), including F27A, which converts the αB-crystallin chaperone-like function is necessary and mutant αB-crystallins. The small size of the α-crystallin sequence indicates the position of the substitutions. The substituted sequence for each mutant is given below each site in the native sequence. See text for details.

**Bacterial Strains and Plasmid**—E. coli DH5α (F−, rec−, meth−) used for propagation of plasmids, were obtained from Life Technologies, Inc. (Gibco BRL, Paisley, UK) and used as described previously (27, 28). E. coli BL21(DE3) were obtained from Novagen. The murine αB-crystallin cDNA cloned on plasmid pLemz2–19, was kindly donated by Professor J. Piatigorsky of the National Institutes of Health. Expression plasmid pET 3d (29) was obtained from Novagen. Cloning vector pBlueScriptSK was obtained from Stratagene.

**Enzymes and Media**—The restriction enzymes KpnI, EcoRI, and BamHI endonucleases, Klenow polymerase, DNA kinase, and T4 DNA ligase were purchased from Life Technologies, Inc., Taq polymerase was from Perkin-Elmer Cetus (Perkin-Elmer Corp., Warrington, UK). Chemicals, including hsp27, were from Sigma and of molecular biology grade as

![Fig. 1. DNA sequence (A) and amino acid sequence (B) of the murine αB-crystallin cDNA and protein, showing loci of the site-directed mutations. Codon/residues shown in bold in the native sequence indicate the position of the substitutions. The substituted sequence for each mutant is given below each site in italics. The conserved phenylalanine-rich region thought to be important in chaperone-like activity/aggregate formation in α-crystallin is shown as underlined italicized residues in B.](image)

![Fig. 2. Western blot of crude cell lysates from freeze-thawed lysed E. coli BL21(DE3) expressing recombinant native and mutant αB-crystallins. Bovine α-crystallin was used as a control marker. A: a, bovine α-crystallin; b, native αB-crystallin; c, F27R; d, F27A; e, K174L/K175L; f, negative control pET 3d. B: a, bovine α-crystallin; b, native αB-crystallin; c, D2G; d, F24R; e, K174G/K175G; f, negative control pET 3d.](image)

![Fig. 3. Coomassie-blue stained SDS-PAGE gels of purified native and mutant αB-crystallins. Purification was by HPLC on a Hi-pel Sephacryl S-300 high resolution gel filtration column. See text for details. A: a, bovine α-crystallin control marker; b, native αB-crystallin; c, F27R; d, F27A; e, K174L/K175L; f, control pET 3d. B: a, bovine α-crystallin control marker; b, native αB-crystallin; c, D2G; d, F24R; e, K174G/K175G; f, control pET 3d.](image)
FIG. 4

Chaperone-like Activity of αB-Crystallin
appropriate, unless otherwise stated. Cells were propagated in Luria broth and recombinant bacteria were prepared using an M13 preparation kit.

Subcloning of Murine αB-Crystallin cDNA and Preparation of Plasmids—PCR amplification (31) using primers containing NcoI sites was used to prepare native and mutant amplicons of murine αB-crystallin. After PCR amplification and subsequent purification, the αB-crystallin amplicons were blunt-ended with Klenow fragment DNA polymerase, phosphorylated with DNA kinase, and then cloned into EcoRV-cut pBluescript SK(-) with the EcoRV site of pET 3d. Recombinant plasmids were identified and ori-entated by BamHI digests. Plasmid DNA was purified and pro-duced using the standard methods (30).

Site-directed Mutagenesis—Site-directed mutagenesis was carried out using PCR. C-terminal substitutions were made by incorporating mismatches in the C-terminal PCR primers. Substitutions in the RLFDQFF region were made using overlap extension PCR mutagenesis (32). PCR may introduce occasional random mutations; therefore, DNA sequences of native and mutant amplicons were verified using the standard Sequenase deoxyxynucleotide chain termination method (33, 34).

Expression, Purification, and Quantitation of Native and Mutant Recombinant αB-Crystallins—E. coli BL21(DE3) cells were trans-formed by the standard E. coli transformation procedure (30). Trans-fermants were grown at 37 °C in Luria broth to A600 = 0.6, and αB-crystallin expression was then induced by addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM, then the culture was incubated at 37 °C for 12 h.

Cells from 500-mL cultures were collected by centrifugation at 3,000 × g for 5 min at 4 °C and resuspended in 20 ml of lysis buffer (100 mM Tris-HCl, 0.05% aprotinin, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 mM dithiothreitol, pH 7.5). The suspensions of cells were disrupted using 2 passages through a French pressure cell at 12,000 p.s.i. The bacterial lysates were then centrifuged at 12,000 × g for 5 min at 4 °C, and the supernatant was assayed for the presence of αB-crystallin by SDS-PAGE and Western blotting using rabbit anti-murine α-crystallin antibodies.

Nucleotides which would interfere with spectrophotometric estima-tion of protein concentration were precipitated from the soluble fraction by addition of polyethyleneimine and dithiothreitol to final concentrations of 0.12% and 10 mM, respectively. Incubation at room temperature for 10 min was followed by centrifugation at 15000 × g for 10 min. The supernatant containing the proteins was then removed and the recom-binant αB-crystallin was purified by HPLC gel filtration in 100 mM sodium phosphate buffer (pH 7.4) on a Pharmacia Hi-Prep Sphero-phyll S-300 high resolution column. Fractions containing the αB-crystallin were identified by immuno dot-blotting. The elution volume of the αB-crystallin peaks were used to estimate the size of the recombinant protein aggregates. Purity of the αB-crystallin in the positive fractions was assessed by SDS-PAGE. The purified protein concentration was then estimated by A280 determination using a Beckman DU-70 spectrophotometer.

N-terminal Sequencing of Recombinant αB-Crystallin—Mammalian α-crystallins have blocked N termini and cannot be sequenced; how-ever, proteins expressed in E. coli frequently have unblocked N termini. Samples of recombinant αB-crystallins were therefore sent to Dr. A. Willis at the MRC Immunochimistry Unit, Oxford, for N-terminal sequencing, as described previously (27).

Aggregate M, Tryptophan Fluorescence, and Circular Dichroism—Mutations could affect the ability of the αB-crystallin monomers to form aggregates. Nondenaturing PAGE was used to compare the size of the αB-crystallin aggregates formed by each of the recombinant proteins.

The three-dimensional structures of native and mutant purified recom-binant αB-crystallins were investigated by both circular dichroism and tryptophan fluorescence using HPLC buffer as a blank and control samples of purified recombinant αB-crystallin.

Far-UV circular dichroism spectra of each recombinant αB-crystallin were determined using an ISA JOBIN YVON CD6 Dichrograph with a 10-μm cell. Five repeat spectra were obtained for each sample and averaged out to minimize noise in the final spectrum. CD spectra were analyzed using Contin software (35) to estimate the secondary structure content of each mutant. Tryptophan fluorescence spectra (exciting at 295 nm) were determined using a Perkin Elmer L550 spectrofluorimeter.

Assays of In Vitro Chaperone-like Activity—The chaperone-like activity of the purified recombinant αB-crystallins was assayed by both the heat aggregation method (19) using γ-crystallin as substrate and the reduced insulin B chain method (36) at varying concentrations of the αB-crystallins. For the reduced insulin assay, we also included the small heat shock protein hsp27. (All spectrophotometry was carried out using a Beckman DU-70 spectrophotometer with a water-heated cuvette holder.)

Measurement of Death Rate in E. coli—Potential in vivo heat shock protein activity of the native and mutant recombinant αB-crystallins was determined by comparing the thermal death curves at 50 °C of stationary phase E. coli BL21(DE3) cells expressing the different recom-binant αB-crystallins with that of E. coli BL21(DE3) containing only pET 3d without a cDNA insert. Bacteria were grown and induced with isopropyl-1-thio-β-D-galactopyranoside as for protein production (see above), and then 50-ml cultures were placed in 50 °C water baths for 12 h. Samples were removed at 1–2 h intervals and the numbers of surviving colony-forming units/ml were determined by the standard spread plate method on Luria/ampicillin plates.

RESULTS

Expression and Purification of Native and Mutant αB-Crys-tallin in E. coli Cells—Western blots of soluble fractions from E. coli BL21(DE3) transformed with pET 3d/αB-crystallin am-plison recombinants identified an expressed protein (absent in nonexpressing control cells) which co-migrated with control α-crystallin and cross-reacted with anti-α-crystallin antibodies (Fig. 2, A and B). Tryptophan fluorescence of this protein demonstrated that the N terminal was not blocked and identified the first 10 residues as identical to the known se-quence of murine αB-crystallin: MDIAIIHHWLI. Gel filtration chromatography succeeded in purifying the expressed αB-crystallin to a purity of 85–90% (Fig. 3, A and B) with a yield of 3–4 mg/ml.

Site-directed Mutagenesis—Site-directed mutagenesis of αB-crys-tallin was used to produce the six mutants shown in Fig. 1. DNA sequencing was used to verify the authenticity of native and mutant recombinant αB-crystallin amplicons. All of these mutants were expressed in E. coli BL21(DE3) as soluble proteins with monomer size and antigenic reactions identical to that of the native and native recombinant proteins (Fig. 2).

Native Aggregate Size, CD, and Tryptophan Fluorescence of Recombinant αB-Crystallins—The native aggregate size of α-crystallins is very high (approximately 800 kDa) and difficult to estimate by PAGE or HPLC. However, comparison of the immuno dot-plot-positive fractions from the Sephacryl S-300 column demonstrated that control bovine α-crystallin, native recombinant αB-crystallin, and all 6 mutants eluted in the 94–97-mL fractions with no lower molecular weight α-crystallin species detected in later fractions. This was confirmed by the nondenaturing PAGE (Fig. 4C) which demonstrated no observ-able low molecular weight species in the purified αB-crystallin samples. In all samples (native and mutant), the protein was

1The abbreviations used are: PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography.

Fig. 4. Structural characterization of recombinant αB-crystallins. A and B, tryptophan fluorescence spectra of purified native and mutant recombinant αB-crystallins. Expressed α-crystallins were excited at 295 nm. All gave identical emission maxima at 340–341 nm. Bovine α-crystallins were used as a control. Intensities were all identical and have been vertically displaced for clarity. C, Coomassie blue-stained nondenaturing PAGE gel of native and mutant αB-crystallins. The break in each lane is the junction between stacking and separating gels. a, bovine α-crystallin control marker; b, native αB-crystallin; c, F23T; d, F27A; e, K144L/K155L; f, F24R; g, D2G; h, K174G/K175G; i, bovine α-crystallin control marker. D, circular dichroism of recombinant native αB-crystallin. All mutant recombinant αB-crystallins showed identical CD spectra. E, changes in tryptophan fluorescence emission spectra of purified recombinant αB-crystallins and γ-crystallin at 66 °C.
Fig. 5. Inhibition of γ-crystallin aggregation assay in the presence and absence of variable amounts of recombinant native and mutant αB-crystallin at 66 °C. A, native recombinant αB-crystallins; B, mutant K174L/K175L αB-crystallins; C, mutant F27R αB-crystallins; D, mutant D2G αB-crystallins.
Aggregation of the insulin B chain by reduction with dithiothreitol in the presence and the absence of variable amounts of recombinant native and mutant αB-crystallin. A, native recombinant αB-crystallins; B, mutant F27R; C, mutant K174L/K175L; D, mutant D2G αB-crystallins.
found as a high $M_r$ species which sat on the top of the 5% separating gel. Thus, it appears that all of the mutations studied did not alter the aggregation behavior of the αB-crystallin in such a way as to produce monomers, tetramers, or other low molecular weight species.

Circular dichroism studies revealed that the recombinant native and mutant αB-crystallins contained 42% β structure ± 3–5% and 12% α helix ± 1–2%, which represent no significant differences in secondary structure (Fig. 4D). Tryptophan fluorescence emission spectra from native and mutant recombinant α-crystallins all gave emission maxima at 341 nm (Fig. 4, A and B). This was identical with emission maxima of the positive control of bovine α-crystallin. The emission maxima were consistent with previously documented spectra (37). Thus, both circular dichroism and tryptophan fluorescence studies show that the mutations had not produced any gross significant structural alteration.

Tryptophan fluorescence emission maxima were also observed during prolonged incubation at 66 °C to evaluate any possible structural changes that could occur at elevated temperatures similar to those that exist in the γ-crystallin aggregation assay. A control sample of bovine γ-crystallin was observed to undergo significant unfolding as demonstrated by a shift in the emission maximum from 337 to 346 nm. There were no changes in the emission maxima after a 1-h incubation at 66 °C indicating no heat-induced structural changes in any of the α-crystallins (Fig. 4E).

**In Vitro Assessment of Chaperone-like Activity of Native and Mutant αB-Crystallins**—Native recombinant αB-crystallin was shown to possess chaperone-like activity. 0.1 mg of native αB-crystallin successfully inhibited the thermal aggregation of γ-crystallin. 0.05 mg of αB-crystallin reduced the chaperone-like activity by 50% (Fig. 5A). Mutant αB-crystallins showed no significant chaperone-like activity (Fig. 5, B and C), apart from the D2G mutant, which demonstrated a reduced efficiency in chaperone-like activity (Fig. 5D).

A similar pattern of chaperone-like activity was obtained for each recombinant protein in the room temperature reduced insulin B chain aggregation assay (Fig. 6).

In contrast, 0.1 mg of hsp27 failed to exhibit any chaperone-like activity in the reduced insulin assay, in a fashion similar to F27R (Fig. 5C).

**In Vivo Assessment of Chaperone-like Activity of Native and Mutant αB-Crystallins**—Native αB-crystallin was shown to confer thermotolerance on and preserve longevity of *E. coli* BL21(DE3), when compared to pET 3d control cells. Mutant αB-crystallins failed to confer thermotolerance to *E. coli* BL21(DE3) apart from the D2G mutant which conferred a similar but reduced thermotolerance to that conferred by native αB-crystallin (Fig. 7 and Table I).

**Discussion**

Recombinant αB-crystallin lacks the blocked N terminus found in the native lens protein but appears to be similar in all other respects (tryptophan fluorescence, circular dichroism, oligomer size, and chaperone-like activity) to the lens protein. This implies that the presence of a free positive charge on the N terminus does not influence gross α-crystallin structure or inhibit protein binding.

We have produced mutants of this protein in three specific areas: substituting neutral or charged residues for hydrophobic residues in the conserved phenylalanine-rich region, substituting neutral or hydrophobic residues for the C-terminal lysine residues, and substituting glycine for the N-terminal aspartate Asp2.

Biophysical characterization of the expressed proteins demonstrates no global structural changes in any of the mutants studied. Oligomer size, far UV CD spectra, and tryptophan emission maxima were not significantly altered by any of the mutations. Das and Sureau (38) have observed changes in α-crystallin structure at elevated temperatures; however, our mutations do not appear to have influenced the sensitivity of the recombinant α-crystallins to temperature. All of the recombinant α-crystallins demonstrated no shift in tryptophan emission maximum after a 1-h incubation at 66 °C, suggesting that the heat stabilities of the recombinant mutant proteins were also unaffected by the mutations. Furthermore, the results of the room temperature assay demonstrates no observable differences in mutant behavior from the 66 °C assay. Thus, observed changes in chaperone-like behavior of these mutants is likely to be a direct result of substituting key residues in the peptide binding site(s).

It is of interest that in the three functional assays we used, two *in vitro* (heat aggregation of γ-crystallin and aggregation of insulin at room temperature) and one *in vivo* (*E. coli* heat tolerance), the effects of the mutations were identical, and followed the pattern: native > D2G > K174G/K175G = K174/ K175L >= F24R = F27A = F27R. Substitution of the either of the phenylalanines Phe24 or Phe27 appears to completely abolish chaperone-like activity, as measured by the γ-crystallin, insulin, and *in vivo* assays. This suggests that these highly conserved hydrophobic residues play a vital role in the chaperone-like activity of αB-crystallin.

Unexpectedly, F27A, which converted the αB-crystallin sequence in the phenylalanine-rich region to that of the functional small heat shock protein hsp27, produced a protein which failed to exhibit chaperone-like activity. When hsp27 was itself used as a control in the reduced insulin assay, no chaperone-like activity was demonstrated. This suggests that the conditions of even the least aggressive *in vitro* assay were too extreme for demonstration of heat shock protein functionality. The observation that native αB-crystallin can exhibit chaperone-like activity under these conditions suggests that it may be more efficient than hsp27 in binding unfolded protein. F27A appears to abolish this increased efficiency, suggesting that phenylalanine 27 plays a key role in binding unfolded protein.

Smulders et al. (39) suggested that the hydrophobic residues Phe24, Leu27, and Val22 were not involved in αA-crystallin function; however, they did not investigate the RLDQFF region. Removal of the N terminus from αA-crystallin removes a very hydrophobic region αA/32–37 and may be responsible for the chaperone-like activity (40). This is in accord with our experiments on the interactions of α-crystallin with chymosin; binding only occurs with unfolded chymosin or with prochymosin (which contains a hydrophobic N-terminal region), not with correctly folded chymosin (41).

Mutations to the C-terminal lysines greatly reduced the chaperone-like activity of the αB-crystallin such that it failed to confer thermotolerance on *E. coli* and *in vitro* inhibition of both γ-crystallin and insulin B chain aggregation was incomplete even at very high concentrations of α-crystallin (partial protection was observed only at concentrations 15-fold greater than that required for protection by the native recombinant). This is consistent with the observations of Boyle and Takemoto (25), who suggested that the C terminus of α-crystallin monomers were located in the central region where they had previously demonstrated γ-crystallin binding and were therefore likely to be involved in protein binding. It is of interest that modeling studies (42) show the C-terminal amino acids (KK) form a strong electropositive region, which is preceded by an electro-negative region. The C-terminal arm could then act like a charged “fishhook” to interact with unfolded proteins via char-
ge-charge interactions and then link the substrate proteins further via hydrophobic interactions with the exposed phenylalanine-rich domain near the N terminus. Thus, unless there were significant exposed hydrophobic residues on the surface of the substrate protein, there would not be a stable interaction with αB-crystallin and its substrate protein. Thus, even if the lysine residues had been deleted or glycated, there would be sufficient charge-charge interactions with the remainder of the C-terminal arm to ensure efficient chaperone-like activity. This is in agreement with experiments where proteolytic removal of the C-terminal (Thr$^{171}$ → Lys$^{175}$) region did not significantly affect αB-crystallin chaperone-like function (25). In addition, it may be that the mutations we have made resulted in a largely intact but less mobile C terminus. The absence of a strong hydrophilic positive charge at the end of the highly flexible C-terminal extension may result in the C terminus folding back on itself, losing its flexibility, and sterically hindering protein binding.

Substitution of the N-terminal aspartate (D2G) resulted in a greatly reduced efficiency of chaperone-like activity. Some thermostolerance was conferred on E. coli by expression of the D2G mutant, but this was significantly less than that conferred by the native recombinant protein. Similarly, in the in vitro assays, complete aggregation inhibition was demonstrated by the D2G mutant but only at concentrations 10-fold greater than the native recombinant protein. This suggests that while the Asp$^2$ residue is not vital for binding of unfolded proteins, it does play some role in the chaperone-like activity. Boyle and Takemoto (25) have suggested that the N terminus of the α monomers may also be located in the binding site, and it is possible the the Asp$^2$ is involved in a salt bridge with the C-terminal lysines. However, in that case, one might have expected similar efficiency on in vitro and in vitro chaperone-like activity for the D2G and K174G/K175G which was not the case.

We have used three different assays to demonstrate consistent changes in chaperone-like behavior produced by specific mutations in recombinant murine αB-crystallin. The results of these assays suggest that charge-charge interactions involving the C-terminal lysines and possibly Asp$^2$ are important in binding of unfolded protein but also that hydrophobic interaction involving the conserved phenylalanine-rich region plays a vital role in the chaperone-like activity. This region is conserved in a number of heat shock proteins, suggesting that similar hydrophobic interactions may be involved in the activities of all small heat shock proteins, although subtle sequence modifications, such as F27A in human hsp27 (26) appear to modify the relative efficiency of binding to unfolded proteins. It is apparent that in vitro assays for functionality of hsp2 and αB-crystallin are as yet rather insensitive in recognizing cellular function(s), and investigations on novel cellular assays are under way in our laboratory.

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REFERENCES

1. Wistow G., and Piatigorsky J. (1988) Annu. Rev. Biochem. 57, 479–504
2. Luhsen N. H., Aarts H. J. M., and Schoenmakers J. G. G. (1988) Prog. Biophys. Mol. Biol. 51, 47–76
3. Delaye M., and Tardieu A. (1983) Nature 302, 415–417
4. Harding J. J., and Crabbe M. J. C. (1984) in Cataract—Biochemistry and Epidemiology, (Davson H., ed) Vol. 1B, pp. 207–492, Academic Press, New York
5. Harding J. J., and Crabbe M. J. C. (1984) in The Eye (Dawson, H., ed) Vol. 1B, pp. 207–492, Academic Press, New York
6. Harding J. J., and Crabbe M. C. (1985) Biochim. Biophys. Acta 826, 625–630
7. Iwaki, T., Kume-Iwaki, A., Liem, R. K. H., and Goldman, J. E. (1989) Cell 57, 71–78
8. Klemenz, R., Frohli, E., Steiger, R. H., Schafer, R., and Ayoama, A. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 3652–3656
9. DasGupta, S., Hohman, T. C., and Carper, D. (1992) Exp. Eye Res. 54, 461–470
10. Klemenz, R., Frohli, E., Ayoama, A., Hoffman, S., Simpson, R. J., Moritz, R. L., and Schafer, R. (1991) Mol. Cell Biol. 11, 803–812
11. Iwaki, T., Iwaki, A., Tateishi, J., and Goldman, J. E. (1994) J. Cell Biol. 125, 1385–1393
12. Renkawek, K., De Jong, W. V., Merck, K. B., Frenken, C. W. G. M., van Workum, F. P. A., and Bosman, G. J. G. M. (1992) Acta Neuropathol. 83, 323–327
13. van Noort, J. M., van Seel, A. C., Bajamovic, J. J., El Ouagmari, M., Polman, C. H., Laschmann, H., and Reiv, R. (1995) Nature 375, 788–801
14. Goode, D., and Crabb, M. J. C. (1995) Comput. Chem. 19, 65–74
15. Harding, J. (1991) Cataract—Biochemistry and Epidemiology, Chapman and Hall, London.

**FIG. 7. Thermal death rates of E. coli BL21(DE3) at 50 °C, represented as CFU (colony-forming units)/100 μl.** Native expressed αB-crystallins reduced the thermal death rate of E. coli BL21(DE3) at 50 °C compared to that observed in pET 3d control cells and in E. coli expressing mutant K174G/K175G under the identical conditions. Similar results were obtained with all other recombinant mutant αB-crystallins.

**TABLE I**

| Assay                          | CFU/100 μl/h |
|-------------------------------|-------------|
| Native αB-crystallin          | 1.9         |
| K174G/K175G                  | 2.2         |
| K174L/K175L                  | 2.4         |
| Negative control (no α-crystallin) | 2.6     |
| F27A                         | 2.2         |
| F24R                         | 2.19        |
| D2G                          | 2.9         |

**log CFU/100 μl/h**
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16. Hemmingsen, S. M., Woolford, C., van der Vies, S. M., Tilly, K., Dennis, D. T., Georgopoulos, C. P., Hendrix, J. W., and Ellis, R. J. (1988) *Nature* **333**, 330–339
17. Goloubinoff, P., Gatenby, A. A., and Lorimer, G. H. (1989) *Nature* **337**, 44–47
18. Zahn, R., Perrett, S., Stenberg, G., and Fersht, A. R. (1996) *Science* **271**, 642–645
19. Horwitz, J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 10449–10453
20. Takemoto, L., Emmons, T., and Horwitz, J. (1993) *Biochem. J.* **294**, 435–438
21. Crabbe, M. J. C., Plater, M. L., and Goode, D. (1995) *Proceedings of the International Forum on Advanced Techniques in Lens Cataract Research*, 28, Kanazawa, Japan
22. Rao, P. V., Huang, Q., Horwitz, J., and Zigler, J. S., Jr. (1995) *Biochim. Biophys. Acta* **1245**, 439–447
23. Carver, J. A., Guerreiro, N., Nicholls, K. A., and Truscott, R. J. W. (1995) *Biochim. Biophys. Acta* **1252**, 251–260
24. Bettelheim, F. A., Reid, M. B., McPhie, P., and Garland, D. (1992) *Biochem. Biophys. Res. Commun.* **187**, 39–44
25. Boyle, D., and Takemoto, L. (1994) *Exp. Eye Res.* **58**, 9–16
26. Crabbe, M. J. C., and Goode, D. (1994) *Biochem. J.* **297**, 653–654
27. Goode, D., and Crabbe, M. J. C. (1994) *Arch. Biochem. Biophys.* **315**, 104–110
28. Dilsiz, N., and Crabbe, M. J. C. (1995) *Biochem. J.* **305**, 753–759
29. Barbosa, P., Ciaikowski, M., and O'Brien, W. E. (1991) *J. Biol. Chem.* **266**, 5286–5290
30. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
31. Saiki, R. K., Gelfland, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988) *Science* **239**, 487–491
32. Horton, R. M., and Pease, L. R. (1991) in *Directed Mutagenesis: A Practical Approach* (McPherson, M. J., ed) pp. 217–248, IRL Press, Oxford
33. Sanger, F., Nicken, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463–5467
34. Dilsiz, N., and Crabbe, M. J. C. (1994) *Anal. Biochem.* **222**, 510–511
35. Provencer, S. W. (1982) *Comp. Phys. Comm.* **27**, 229–242
36. Farahbaksh, Z. Y., Huang, Q.-L., Ding, L.-L., Altenbach, C., Steinhoff, H.-J., Horwitz, J., and Hubbell, W. L. (1995) *Biochemistry* **34**, 509–516
37. Palminsa, D. V., Groth-Vaselli, B., Panisworth, P. N., and Reddy, M. C. (1995) *Biochim. Biophys. Acta* **1246**, 91–97
38. Das, K. P., and Surewicz, W. K. (1995) *FEBS Lett.* **369**, 321–325
39. Smulders, H. P. H., Merck, K. B., Aendekerk, J., Horwitz, J., Takemoto, L., Slingsby, C., Bloomendal, H., and De Jong, W. W. (1996) *Eur. J. Biochem.* **232**, 834–838
40. Smith, J. B., Liu, Y., and Smith, D. L. (1996) *Exp. Eye Res.* **6**, 125–127
41. Chitpinityol, S., Goode, D., and Crabbe, M. J. C. (1996) in *Perspectives on Protein Engineering* (Geisow, M., ed) p. 29, Montpellier, France
42. Crabbe, M. J. C., Appleyard, J. A., and Rees Lay, C. (1994) *DeskTop Molecular Modeller*, version 3.0, Oxford University Press, Oxford, UK