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Role of the C-terminal Extensions of α-Crystallins

SWAPPING THE C-TERMINAL EXTENSION OF αA-CRYSTALLIN TO αB-CRYSTALLIN RESULTS IN ENHANCED CHAPERONE ACTIVITY*

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Several small heat shock proteins contain a well conserved α-crystallin domain, flanked by an N-terminal domain and a C-terminal extension, both of which vary in length and sequence. The structural and functional role of the C-terminal extension of small heat shock proteins, particularly of αA- and αB-crystallins, is not well understood. We have swapped the C-terminal extensions between αA- and αB-crystallins and generated two novel chimeric proteins, αABc and αBAc. We have investigated the domain-swapped chimeras for structural and functional alterations. We have used thermal and non-thermal models of protein aggregation and found that the chimeric αB with the C-terminal extension of αA-crystallin, αBAc, exhibits dramatically enhanced chaperone-like activity. Interestingly, however, the chimeric αA with the C-terminal extension of αB-crystallin, αABc, has almost lost its activity. Pyrene solubilization and bis-1-anilino-8-naphthalenesulfonate binding studies show that αBAc exhibits more solvent-exposed hydrophobic pockets than αA, αB, or αABc. Significant tertiary structural changes are revealed by tryptophan fluorescence and near-UV CD studies upon swapping the C-terminal extensions. The far-UV CD spectrum of αBAc differs from that of αB-crystallin whereas that of αABc overlaps with that of αA-crystallin. Gel filtration chromatography shows alteration in the size of the proteins upon swapping the C-terminal extensions. Our study demonstrates that the unstructured C-terminal extensions play a crucial role in the structure and chaperone activity, in addition to generally believed electrostatic "solubilizer" function.

α-Crystallins are evolutionarily well conserved from bacteria to humans and belong to the family of the ubiquitous small heat shock proteins (shsps)[1] (1). These shsps have monomeric masses ranging from 12 to 43 kDa; most of them form large oligomeric assemblies of 150–800 kDa (2). αA- and αB-crystallins are multimeric proteins having a subunit molecular mass of ~20 kDa with high sequence homology (3). They are abundantly present in the eye lens as dynamic, heteromultimeric complexes and are encoded by two different genes (4). Both subunits can also form stable homomultimers (5–7). αB-crystallin is also expressed in sufficient amounts in other non-lenticular tissues such as the brain, heart, and kidney (8, 9). Levels of αB-crystallin are seen to increase dramatically in response to heat shock, ischemia, oxidation, infection, and various disease conditions (10–14). αA-crystallin, however, is not stress-inducible and is found in trace amounts in the spleen and thymus (15, 16). The presence of αA- and αB-crystallin in non-lenticular tissues, in addition to the eye lens, and the up-regulation of αB-crystallin expression under pathological/stress conditions suggests that they have other important biological functions, in addition to their specialized role in vision. Like many shsps, α-crystallins, either in their hetero- or homo-oligomeric states, exhibit molecular chaperone-like properties in preventing thermal and non-thermal protein aggregation (17–21). α-Crystallins bind partially unfolded intermediate states of proteins having molten globule-like properties via appropriately placed hydrophobic binding sites (22–27). The chaperone activity of α-crystallins is found to be temperature-dependent; a structural transition above 30 °C enhances its activity by increasing or favorably reorganizing the hydrophobic substrate binding surfaces (18–20). This has been further confirmed by later studies (21, 28–31). α-Crystallin is also known to protect some enzymes from heat-induced inactivation (32–35); its differential and reversible interaction with early unfolding, refolding-competent intermediates of target proteins decreases their partitioning into aggregation-prone late unfolding intermediates, thus offering protection against heat-induced inactivation (35). α-Crystallins/shsps are believed to thus serve as reservoirs to maintain partially unfolded target proteins in a folding-competent state (36, 37), capable of subsequent refolding by other chaperone machines (38, 39).

The chaperone activity of α-crystallin is crucial and appears to be one of the important functions of the protein *in vivo*. Mutation of a conserved arginine residue has been shown to result in disease in humans; the R120G mutation in αB-crystallin leads to desmin-related myopathy, and R116C in αA-crystallin leads to congenital cataract (40, 41). These mutant molecules have been shown to exhibit poor chaperone activity *in vitro* (42, 43) indicating a molecular basis for pathology. Recently, it has been shown that the R116C mutant of αA-crystallin offers reduced protection to apoptosis in lens epithelial cells subjected to UV stress (44). Although growing evidences appear, implicating the functional importance of α-crystallins in many cellular processes (45), our knowledge about its structure-function relationship remains elusive largely because of the lack of an x-ray crystal structure or NMR solution structure. As of today, only two members of the shsp family, namely *Methanococcus jannaschii* hsp16.5 (46) and wheat hsp16.9 (47), have been crystallized successfully, and

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[1] The abbreviations used are: shsp, small heat shock protein; ANS, 1-anilino-8-naphthalenesulfonate; DTT, dithiothreitol; FPLC, fast protein liquid chromatography.

the three-dimensional has been structure determined. α-Crystallin-like small heat shock proteins from prokaryotes and plants differ significantly from their mammalian counterparts in structure and function (45). One notable feature of these proteins (*e.g.* hsp16.5 and hsp16.9) is that they form monodisperse oligomers with discreet numbers of subunits in the assembly, whereas α-crystallins exhibit a dynamic, polydisperse oligomeric assembly. The polydispersity makes it difficult to crystallize α-crystallin.

The α-crystallins are evolutionarily related by the presence of a conserved sequence of about 80 to 100 amino acids, called the "α-crystallin domain" (48). This domain is rich in β-strands and is proposed to form the classical seven-β-strand Ig-like fold; it has therefore been hypothesized that the shsp family of proteins could belong to the immunoglobulin super family (49). Sequence analysis of shsps, including α-crystallins, shows that though these molecules possess a highly conserved α-crystallin domain, they differ considerably in both sequence and length of the N- and C-terminal regions flanking this domain (16). In general, the N-terminal region forms an independent folding structural domain, whereas the C-terminal region is largely unstructured and is referred to as the C-terminal extension. These differences in the N-terminal domains and the C-terminal extensions of shsps may result in their differing oligomeric assembly, size, dynamics, and functions.

The chaperone-like activity of the α-crystallins is attributed to the ability of these large, functional oligomeric complexes to bind aggregation-prone target proteins and keep them from precipitating. Binding sites for target proteins and hydrophobic probes such as bis-ANS map to regions in the N-terminal and the α-crystallin domains (50–52). These domains are also involved in subunit contacts, forming large and small molecular mass aggregates, respectively (53, 54). The role of the C-terminal extension in both structure and chaperone activity of α-crystallin is not understood. It is believed to function as solubilizer of the α-crystallin-target protein complex as it has a preponderance of charged amino acid residues (55). Either deletion or introduction of hydrophobic residues within this domain was found to decrease the chaperone activity (55). As mentioned earlier, the length and the sequence of the C-terminal extensions of shsps differ considerably. However, a conserved motif, I*X*(I/V), has been shown to be present toward the C-terminal end (16). Though the C-terminal extensions of αA- and αB-crystallins share more homology compared with the rest of the shsps, they differ significantly as shown in Fig. 1*A*. We therefore set out to investigate the role of the C-terminal extensions of αA- and αB-crystallins by swapping these regions between them. An *Hpa*I site engineered by silent mutagenesis was inserted at the end of the α-crystallin domain. This unique site has been used to create the chimeric proteins, αA-crystallin with the swapped C-terminal of αB-crystallin, $\alpha ABc_{(144-175)}$, and αB crystallin with the swapped C-terminal of αA crystallin, $\alpha BAc_{(140-173)}$ (see Fig. 1*B*). For the sake of simplicity we refer to the chimeras as αABc and αBAc from here onwards. The chimeras were then studied with respect to their wild type counterparts for their structure and chaperone activities.

EXPERIMENTAL PROCEDURES

Materials—pET-21a(+), T7 promoter, and terminator primers were obtained from Novagen, pBS(II)SK was from Stratagene, insulin, bis-ANS, pyrene, and butyl-Sepharose were from Sigma, and rabbit muscle aldolase from Roche Molecular Biochemicals. Gel filtration supports, Bio-Gel A-1.5m, and Bio-Gel A-5m were purchased from Bio-Rad Laboratories, and Superose-6 HR 10/30 and a high molecular weight calibration kit were from Amersham Biosciences. DTT was obtained from Sisco Research Laboratories, Mumbai, India. β_L- and ζ-crystallin were purified as described earlier (3, 56).

Swapping the C-terminal Extensions of αA- and αB-crystallins—Recombinant human αA- and αB-crystallin genes were cloned in pET-21a(+) as described earlier (42). These constructs were used as templates to generate a *Hpa*I site by silent mutagenesis using the PCR at the region coding the C-terminal end of the α-crystallin domain. For αA-crystallin, two independent PCRs were performed using T7 promoter primer and the mutagenic primer bearing the *Hpa*I site (underlined), 5′-CACAGAAGGTTAACATGCCATC-3′, as one primer pair and 5′-GATG GCAGTTAACCTTCTGTG-3′ and T7 terminator primer as the second primer pair. Fragments of 515 and 362 bp generated in the PCR, having a partial overlap, were cloned separately in pBS(II)SK. The same strategy was employed for αB-crystallin using T7 promoter primer and 5′-CATTCACAGTTAACACCCCATA-3′ and 5′-GATGGGGTGTTAACTGTGAATG-3′ and the T7 terminator primer to generate 527- and 300-bp fragments, respectively. These fragments, having partial overlap at the *Hpa*I site, were also cloned separately in pBS(II)SK. To construct the gene encoding the chimeric protein, αABc, the 417-bp *Nde*I-*Hpa*I fragment of αA-crystallin and the 199-bp *Hpa*I-*Hin*dIII of αB-crystallin were excised from pBS(II)SK, ligated, and cloned into the *Nde*I and *Hin*dIII sites of pET-21a(+) expression vector. αBAc was generated similarly by ligating the 429-bp *Nde*I-*Hpa*I fragment of αB-crystallin to the 261-bp *Hpa*I-*Hin*dIII of αA-crystallin and cloning into the *Nde*I and *Hin*dIII sites of pET-21a(+). These constructs were verified by sequencing using a 3700 ABI automated DNA sequencer.

Expression and Purification of the Recombinant Wild Type and Chimeric Proteins—The wild type and the chimeric recombinant proteins were over expressed in *Escherichia coli* BL21(DE3) cells. All the purification processes were performed using TNE (50 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl, 1 mM EDTA, and 0.02% sodium azide). Bacterial cells were lysed in TNE buffer using lysozyme and sodium deoxycholate. The purification procedures for the wild type proteins were followed as described by Sun *et al.* (7). αABc was purified from the soluble fraction of the bacterial lysate as follows. The protein precipitated with 30–60% saturated ammonium sulfate was dissolved in TNE buffer and loaded onto a Bio-Gel A-1.5m gel filtration column (1.8 × 130 cm). The fractions containing αABc were pooled and subjected to hydrophobic interaction chromatography. The protein in TNE buffer containing 20% ammonium sulfate was loaded onto a butyl-Sepharose (C4) column (1 × 5 cm). The protein was eluted using the same buffer with decreasing ammonium sulfate gradient. It elutes at 15% of ammonium sulfate. The other chimeric protein, αBAc, was precipitated with 10–35% saturated ammonium sulfate, dissolved in TNE buffer, and loaded onto a Bio-Gel A-1.5m gel filtration column. The fractions containing the protein were pooled and concentrated using an Amicon ultra filtration unit and loaded onto a Bio-Gel A-5m gel filtration column (1.8 × 145 cm). The purified proteins were dialyzed against the TNE buffer and concentrated by ultra filtration. The purity of wild type and chimeric proteins was checked by SDS-polyacrylamide gel electrophoresis and were found to be homogeneous. The concentrations of the protein samples were determined by the method described by Pace *et al.* (57). The samples were stored at 4 °C.

Chaperone Assays—The chaperone-like activity of the wild type and the chimeric α-crystallins was studied using various model systems where the aggregation of the target proteins is induced either by thermal or non-thermal means as described below. The aggregation of the target proteins was monitored as right angle light scattering using a Hitachi F-4000 fluorescence spectrophotometer. The samples were placed in the thermostated cuvette holder, and the light scattering was measured as a function of time by setting the excitation and emission monochromators at 465 nm with the excitation and emission band passes set at 3 nm.

DTT-induced Aggregation—Insulin (0.2 mg/ml) in 10 mM phosphate buffer (pH 7.4) containing 100 mM NaCl in the absence or the presence of chaperones (at the different concentrations mentioned in figure legends to obtain different chaperone to target protein ratios) was incubated at 37 °C. Aggregation was initiated by adding DTT to a final concentration of 20 mM.

Thermal Aggregation—Citrate synthase (0.02 mg/ml) in 50 mM phosphate buffer (pH 7.8) containing 150 mM NaCl and 2 mM EDTA or ζ-crystallin (0.1 mg/ml) in 20 mM phosphate buffer (pH 7.4) containing 100 mM NaCl was incubated at 43 °C in the absence or the presence of the required concentrations of the wild type or the chimeric proteins. Aldolase (0.2 mg/ml) in 10 mM phosphate buffer (pH 7.4) containing 100 mM NaCl in the absence or the presence of the required concentrations of the chaperones was incubated at 60 °C, and the aggregation as a function of time was measured as light scattering at 465 nm. Similarly, 0.1 mg/ml β_L-crystallin in the same buffer was incubated at 63 °C in the absence or the presence of the mentioned concentrations of the chaperone proteins, and the aggregation was measured as mentioned above.

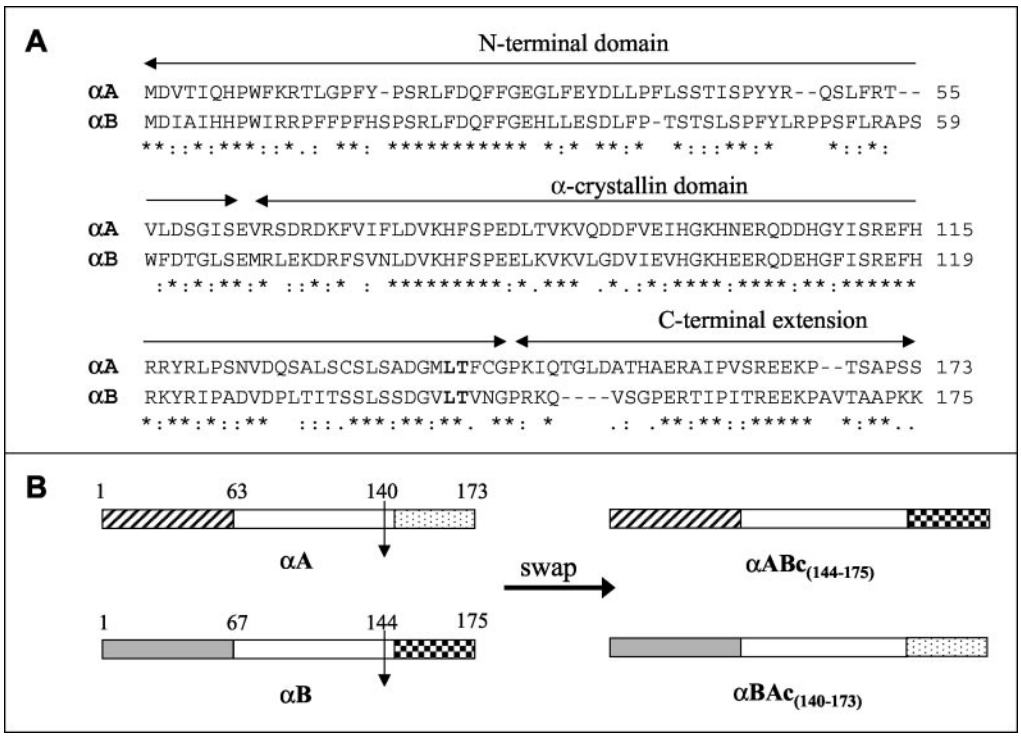

FIG. 1. *A*, sequence alignment of αA- and αB-crystallins performed using the program ClustalW showing the N-terminal domain, α-crystallin domain, and the C-terminal extension. The symbols (*, :, and .) in the ClustalW alignment format represent identical residue, conserved substitution, and semi-conserved substitution, respectively. *B*, schematic diagram showing the swapping of C-terminal extensions between αA- and αB-crystallins performed in this study. The conserved leucine and threonine residues, toward the end of the α-crystallin domain, where the C-terminal extensions are exchanged between αA- and αB-crystallin, are shown in *bold*.

The process of aggregation was monitored in all these cases by means of light scattering at 465 nm, keeping the excitation and emission band-passes at 3 nm.

Fluorescence Studies—All fluorescence spectra were recorded using a Hitachi F-4010 fluorescence spectrophotometer with excitation and emission band passes set at 5 and 3 nm, respectively. All spectra were recorded in corrected spectrum mode. Intrinsic tryptophan fluorescence spectra of the wild type and the chimeric α-crystallins (0.15 mg/ml) in 10 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl were recorded by exciting the sample with 295 nm of light.

To study binding of the hydrophobic probe, bis-ANS, 0.15 mg/ml of the wild type or chimeric proteins in 10 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl were used. To each of the protein samples (1.2 ml), 15 μl of 0.8 mM methanolic solution of bis-ANS was added, and the samples were incubated for 10 min at 37 °C. Fluorescence spectra of the samples were recorded from 400 to 600 nm with the excitation wavelength set at 390 nm.

Pyrene Solubilization—A 1.2-ml sample of the wild type or the chimeric α-crystallins (0.3 mg/ml) in 10 mM phosphate buffer (pH 7.4) containing 100 mM NaCl was incubated with 1 mM pyrene suspension (12 μl of 100 mM pyrene in Me_2SO_4) at 37 °C for 30 min with constant stirring. The mixture was then centrifuged at 14,000 rpm for 20 min, and optical density of the clear supernatant was measured at 338 nm using a Hitachi 200–20 UV-visible spectrophotometer.

Circular Dichroism Studies—Near- and far-UV CD spectra of the wild type and chimeric α-crystallins were recorded using a JASCO J-715 spectropolarimeter. Experiments were performed with 1.0 mg/ml of protein in TNE buffer using a 1-cm path length cell for near-UV region and 0.01-cm path length cell for far-UV region. All spectra reported are the average of five accumulations.

Gel Permeation Chromatography—Oligomeric sizes of the wild type and chimeric proteins were estimated by gel filtration chromatography using a Superose-6 HR 10/30 FPLC column. 100 μl of 1.2 mg/ml α-crystallins in TNE buffer were applied to the column equilibrated with the same buffer and eluted at a flow rate of 0.3 ml/min. High molecular mass standards comprising aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa) were used for calibration.

RESULTS AND DISCUSSION

Our knowledge of the structure and function of small heat shock proteins, especially αA- and αB-crystallins, is far from complete. Though considerable progress has been made to understand the role of the N-terminal and the α-crystallin domain in the chaperone function of these proteins, the functional and structural roles of the C-terminal extensions are not understood completely and are debatable (45, 58). The domain-swapping approach may be particularly useful to give insights into these aspects. The C-terminal extensions of αA- and αB-crystallins are so far characterized as largely unstructured, flexible, and solvent-exposed regions (59, 60). If they function as only as "charged tags" that keep the rest of the molecule and the substrate-bound complex in solution, exchanging these regions between αA- and αB-crystallins would not be expected to alter the structure and function of the molecules significantly. Whether in addition to their solubilizer role C-terminal extensions also significantly influence the structure and function of α-crystallins is an important aspect that needs to be investigated. In this context, we have swapped the C-terminal extensions of αA- and αB-crystallin and investigated the resultant chimeric proteins.

An *Hpa*I site was engineered within the αA- and αB-crystallin genes by silent mutagenesis to exchange C-terminal extensions between their conserved leucine and threonine residues toward the end of the α-crystallin domain (see Fig. 1). The resulting chimeric proteins were called αABc, (N-terminal and α-crystallin domains of αA-crystallin with the swapped C-terminal extension of αB-crystallin) and αBAc (N-terminal and α-crystallin domains of αB-crystallin with the swapped C-terminal extension of αA-crystallin) having 171 and 177 residues, respectively. The predicted pI values of αA- and αB-crystallin, αABc, and αBAc are 5.77, 6.76, 6.59, and 6.04, respectively. The proteins were expressed in *E. coli* and purified to homogeneity.

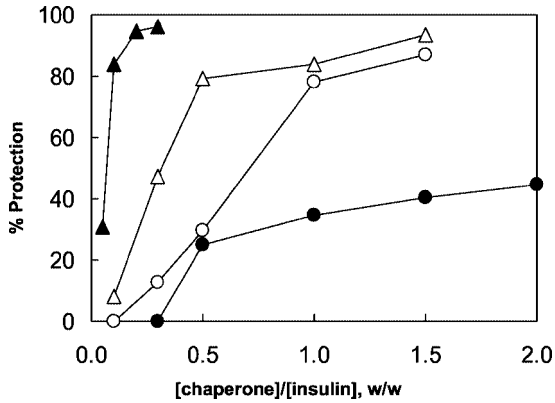

FIG. 2. **Chaperone activity of the wild type and chimeric α-crystallin toward DTT-induced aggregation of insulin at different chaperone:insulin ratios at 37 °C.** αA-crystallin (○), αABc (●), αB-crystallin (△), and αBAc (▲) are shown. The concentration of insulin was 0.2 mg/ml, and the concentrations of the chaperone molecules were varied.

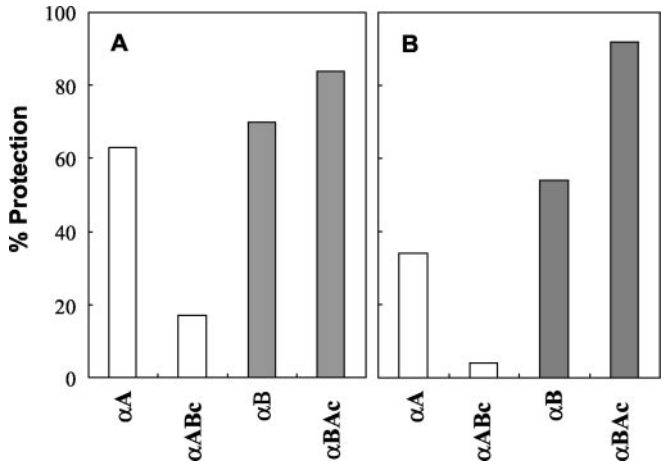

FIG. 3. **Chaperone activity of the wild type and the chimeric α-crystallins toward heat-induced aggregation of ζ-crystallin (0.1 mg/ml) (*A*) and citrate synthase (0.02 mg/ml) (*B*) at 43 °C.** The percentage protection is calculated with respect to the aggregation of the enzymes in buffer alone. In the case of aggregation of ζ-crystallin, the concentrations of αA-crystallin and αABc were 5 μg/ml, whereas αB-crystallin and αBAc were 2.5 μg/ml. In the case of citrate synthase aggregation, a weight ratio of 1:1 of citrate synthase to α-crystallins was used.

We have observed that αBAc precipitates in the 10–35% ammonium sulfate fraction whereas αABc precipitates in the 30–60% ammonium sulfate fraction similar to wild type αA- and αB-crystallins. These observations suggest that αBAc differs considerably from the other proteins in its columbic interactions and solubility, although the predicted pI values do not differ significantly.

To study the effect of exchange of the C-terminal extensions on chaperone function, we compared the ability of the chimeras and the wild type proteins to prevent protein aggregation. We have used various model systems wherein aggregation is initiated either by thermal or non-thermal means. Fig. 2 shows the chaperone-like activity of the crystallins on the DTT-induced aggregation of insulin at 37 °C at different chaperone to insulin ratios. αA- and αB-crystallin differ significantly in their protective ability, consistent with the observation of other studies (7, 42); at 0.3:1 (w/w) ratio of chaperone to insulin, αA- and αB-crystallins showed about 13 and 50% protection, respectively. The chimera, αABc, does not prevent insulin aggregation significantly at the above-mentioned chaperone to insulin ratio. Interestingly, αBAc offers almost complete protection at the same ratio (Fig. 2). The percentage protection offered by αA- and αB-crystallin increases progressively with increasing concentrations of the chaperone, αB being more effective than αA (Fig. 2). However, increasing concentrations of αABc do not increase the percentage protection significantly; even at 2:1 (w/w) ratio of αABc to insulin the protection is only about 40% (Fig. 2). It is important to note that αBAc, even at very low concentrations, can prevent insulin aggregation almost completely; the percentage protection reaches almost 100% even below the 0.3:1 (w/w) ratio of αBAc to insulin (Fig. 2). Thus, these results show that αBAc prevents the aggregation of insulin much better than αB-crystallin, whereas αABc is less effective than αA-crystallin.

We have also studied the chaperone-like activity of these proteins against heat-induced aggregation of ζ-crystallin (Fig. 3*A*) and citrate synthase (Fig. 3*B*) at 43 °C. In corroboration with the results on DTT-induced aggregation of insulin, we found αBAc to be the most effective, whereas αABc is the least effective in preventing the aggregation of ζ-crystallin and citrate synthase. We have also studied the effect of the wild type and the chimeric proteins on the photo-aggregation of aldolase and found the results qualitatively comparable with those obtained for the DTT-induced aggregation of insulin, with αBAc showing more protection than αB and αABc showing no protection (data not shown).

TABLE I

The difference in percent protection between the chimeric α-crystallins and their respective wild type proteins (ΔP) with various aggregation systems used. Minus sign indicates lower protection offered by the chimera than the wild type

System	Mode of aggregation	$\Delta P_{(\alpha ABc\text{-}P\alpha A)}$	$\Delta P_{(\alpha BAc\text{-}P\alpha B)}$
Insulin	DTT-induced (37 °C)	−13	49
Zeta crystallin	Thermal (43 °C)	−46	14
Citrate synthase	Thermal (43 °C)	−30	38

It is to be noted that the exact percentage protection offered by these proteins and the stoichiometry of chaperone to target protein varies considerably from system to system. As can be seen from Fig. 3*A*, aggregation of ζ-crystallin is suppressed by even one-fortieth of the chimeric protein αBAc. Horwitz has found that α-crystallin, even at one-twentieth of the concentration prevented the aggregation of β- or γ-crystallin (17). It appears that even very low concentrations of α-crystallin are sufficient to prevent the aggregation of other crystallins. Aggregation of a small percentage of molecules can lead to measurable turbidity. The extent of aggregation varies from protein to protein. Under steady state, only a fraction of the total target protein undergoes aggregation. The fraction committed to aggregation depends on many factors including concentration, extent, and complementarity of the interacting hydrophobic surfaces. Table I shows the difference in the percentage protection offered by the chimeric protein and the respective wild type protein ($\Delta P_{(P\alpha chi\text{-}P\alpha wild)}$) for the various aggregation model systems used in this study. The data show that this value varies quite drastically between the systems. This makes the quantitative estimation of the increase or decrease in the chaperone activity of the mutant or engineered proteins with respect to their wild type counterparts difficult. However, in the present case the order of the chaperoning ability of the chimeric and wild type α-crystallins remains: αBAc > αB > αA > αABc, independent of the aggregation system used.

It is now well established that the interaction between chaperone molecule and target protein largely involves hydrophobic forces; in some cases electrostatic forces also play a role. Fig. 4*A* shows that the fluorescence intensity of the probe, bis-ANS, bound to αBAc is higher compared with that of the probe bound to αB-crystallin, suggesting more accessible hydrophobic sur-

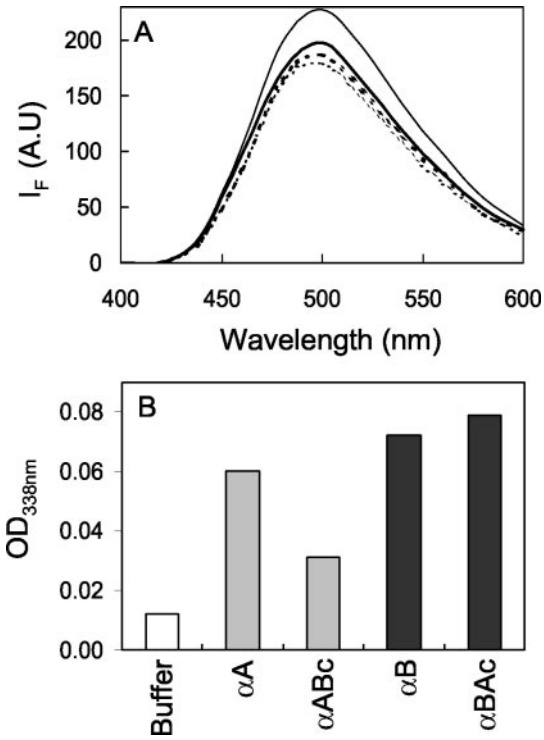

FIG. 4. *A*, fluorescence spectra of bis-ANS bound to αA-crystallin (*thick dashed line*), αABc (*thin dashed line*), αB-crystallin (*thick solid line*), and αBAc (*thin solid line*). The concentrations of α-crystallins were 0.15 mg/ml. I_F (*A.U.*) represents fluorescence intensity in arbitrary units. *B*, solubilization of pyrene in buffer alone and in the presence of the wild type and the chimeric α-crystallins. The concentration of α-crystallins was 0.3 mg/ml. The solubility of pyrene was measured as optical density at 338 nm ($OD_{338\ nm}$).

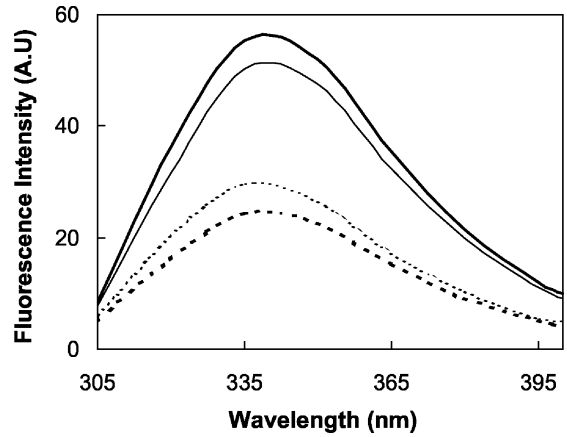

FIG. 5. **Intrinsic tryptophan fluorescence spectra of αA-crystallin (*thick dashed line*), αABc (*thin dashed line*), αB- crystallin (*thick solid line*), and αBAc (*thin solid line*).** The concentration of α-crystallins was 0.15 mg/ml. Excitation wavelength was 295 nm.

faces on αBAc. This observation parallels the result that αBAc exhibits more chaperone-like activity as compared with αB-crystallin, suggesting that the more hydrophobic surfaces that the probe reports may reflect the more accessible substrate-binding sites. The fluorescence spectra of the probe bound to αABc and αA-crystallin show that the extent of binding of bis-ANS to αABc is marginally lower than that bound to αA-crystallin (Fig. 4*A*). We have shown previously that pyrene, an aromatic hydrocarbon, can be used to study the hydrophobic surfaces of α-crystallin (18). It is almost insoluble in aqueous solution, and its solubility increases in the presence of α-crystallin. Fig. 4*B* shows that the solubility of pyrene (measured as the optical density at 338 nm) in the presence of αA-crystallin is lower than that in the presence of αB-crystallin. Solubility of pyrene in αABc is almost half that in αA-crystallin. αBAc marginally exhibits more solubilization capacity than αB-crystallin as shown in Fig. 6*B*. Thus, our bis-ANS binding and pyrene solubility data, in general, make a correlation with the chaperone-like activity of the proteins. However, we found that the chimeric proteins do not differ significantly from their respective wild type proteins in their ability to bind to another commonly used hydrophobic probe, ANS (data not shown). In other words, ANS binding studies did not show the correlation between the probe binding properties and chaperone activity as shown by bis-ANS and pyrene. Our own studies[2] and those from another laboratory (61) show that ANS binding and chaperone activity of some mutants of α-crystallin do not correlate. Our earlier study (18, 28) on temperature-dependent chaperone activity and ANS binding or pyrene solubilization of the wild type α-crystallin showed good correlation. It is possible that ANS can bind and report all hydrophobic sites, some of which may not be large enough to be able to interact with target proteins. Thus, it appears that the target protein binding/chaperone sites are hydrophobic whereas not all the hydrophobic surfaces are chaperone sites.

Because our results show that the chimeric α-crystallins differ from each other, as well as from their respective wild type counterparts, in their properties to bind hydrophobic probes, we investigated their tertiary structures by intrinsic tryptophan fluorescence and near-UV circular dichroism. The tryptophan fluorescence spectra of the chimeric proteins do not overlap with those of their respective wild type α-crystallins; the fluorescence intensity of αBAc is lower than that of αB whereas the fluorescence intensity of αABc is more than that of αA-crystallin (Fig. 5). αA- and αB-crystallins have a conserved tryptophan residue at the 9th position whereas αB-crystallin has an additional tryptophan at the 60th position; the tryptophans in both are in the N-terminal domain. Swapped C-terminal extensions do not have tryptophan residues, and thus our intrinsic tryptophan fluorescence spectra show that exchanging the C-terminal extensions between αA- and αB-crystallin could influence to some extent the microenvironment of the tryptophan residues situated in the distal N-terminal domains. Fig. 6, *A* and *B* shows that the near-UV CD spectra of αABc and αBAc also do not overlap with those of their wild type counterparts. The near-UV CD spectra of αABc exhibits increased ellipticity whereas that of αBAc exhibits decreased ellipticity as compared with that of αA- and αB-crystallin, respectively. This result shows that the chiral structure around the aromatic amino acid residues is significantly altered upon exchange of the C-terminal extensions of αA- and αB-crystallin. Because variation is also seen in the region below 280 nm where tyrosine and phenylalanine can also absorb, our results suggest that subtle changes occur in the side chain packing around these amino acid residues, as well. Thus, our hydrophobic probe binding studies, tryptophan fluorescence, and near-UV CD spectral studies indicate detectable changes in the tertiary structure of αA- and αB-crystallin upon swapping their C-terminal extensions. Far-UV CD spectra of αABc and αA-crystallin and αBAc and αB-crystallin are compared in Fig. 6, *C* and *D*, respectively. The spectra of αABc and αA-crystallin overlap with each other within experimental error indicating that there is no significant difference in the secondary structure of these proteins. However, the far-UV CD spectra of αBAc and αB-crystallin show some differences (Fig. 6*D*) indicating

[2] L. V. S. Kumar, T. Ramakrishna, B. Singh, and Ch. M. Rao, unpublished data.

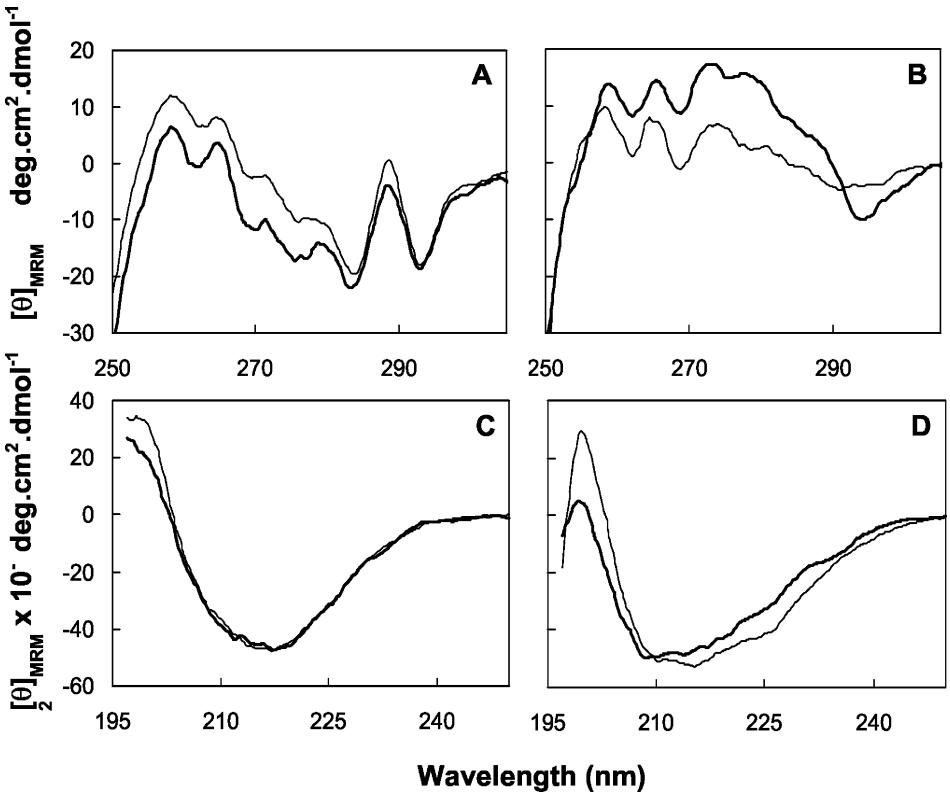

FIG. 6. **Near- (*A* and *B*) and far- (*C* and *D*) UV circular dichroism spectra of the wild type and the chimeric α-crystallins.** Spectra were recorded at a protein concentration of 1.0 mg/ml using a 1-cm path length cell for near-UV region and 0.01-cm path length cell for far-UV region. *A* and *C*, αA-crystallin (*thick solid line*) and αABc (*thin solid line*). *B* and *D*, αB-crystallin (*thick solid line*) and αBAc (*thin solid line*). $[\theta]_{MRM}$ is mean residue mass ellipticity.

that there could be marginal differences in the secondary structural elements at least in some regions of these proteins.

To compare the quaternary structure of the chimeric and wild type α-crystallins, we performed gel filtration chromatography on a Superose-6 FPLC column. Fig. 7 shows that the chimeric proteins also differ significantly from their wild type counterparts in their molecular size. The chimeric protein, αABc, elutes earlier than αA-crystallin suggesting that αABc forms larger oligomers than αA-crystallin (Fig. 7*A*). The average molecular masses of wild type αA- and αABc-crystallin were found to be ~607 and ~907 kDa, respectively, using the elution volumes of standard molecular mass proteins. Fig. 7*B* shows that the elution volume of αB-crystallin and αBAc do not vary significantly. The estimated molecular masses of αBAc and αB-crystallin are ~542 and ~574 kDa, respectively. Taken together our results show that swapping the C-terminal extensions of αA- and αB-crystallin leads to detectable structural alterations and modulating the chaperone-like activity.

It is not clear how the chaperone activity of αA- and αB-crystallins is modulated upon swapping their C-terminal extensions. Proper disposition of substrate binding sites is one of the important determinant factors in the chaperone function. Keeping the substrate-bound complex in solution is another such factor. In fact, earlier studies from our laboratory (18) showed that temperature-induced structural change accompanying increased exposure of appropriately placed hydrophobic surfaces. Further studies from our laboratory (28, 30), as well as others (21, 29, 31), confirmed and elaborated this observation and suggested exposure of specific regions of the sequence. In other words, buried or inaccessible substrate binding sites could become accessible upon certain structural perturbations brought about by either temperature or certain mutations. One of the reasons for the decreased chaperone activity of αABc in our study, which is carried out at physiologically relevant temperatures (37–43 °C), could be that the substrate binding sites are masked at these temperatures. To test this, we have carried out the chaperone assay using model systems where the aggre-

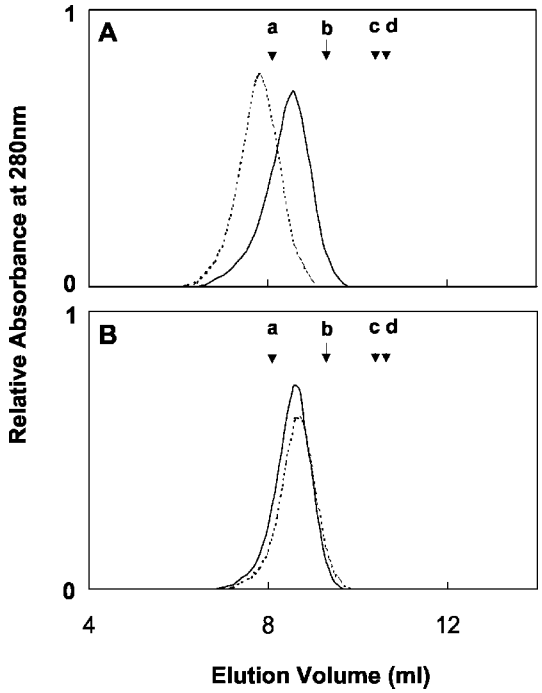

FIG. 7. **Gel filtration chromatography of the wild type and chimeric α-crystallins on a Superose-6 FPLC column.** *A*, αA-crystallin (*solid line*) and αABc (*dotted line*). *B*, αB-crystallin (*solid line*) and αBAc (*dotted line*). The elution positions of molecular mass standards are indicated by *arrows*. *a*, thyroglobulin (669 kDa); *b*, ferritin (440 kDa); *c*, catalase (232 kDa); and *d*, aldolase (158 kDa).

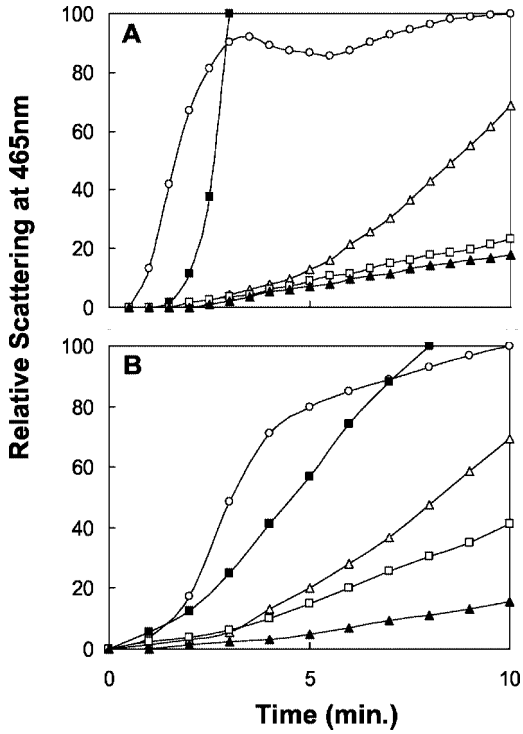

FIG. 8. **Effect of the wild type and the chimeric α-crystallin on the thermal aggregation of aldolase (0.1 mg/ml) at 60 °C (*A*) and βL-crystallin (0.1 mg/ml) at 63 °C (*B*).** Aldolase (○) and βL-crystallin (●) in buffer alone and in the presence of αA-crystallin (△), αABc (▲), αB-crystallin (□), and αBAc (■). The concentrations of α-crystallins in the cases of aldolase and βL-crystallin were 0.075 and 0.01 mg/ml, respectively.

gation is induced at very high temperatures (>60 °C). The chimera, αABc, could prevent completely the aggregation of aldolase at 60 °C and that of βL-crystallin at 63 °C (Fig. 8, *A* and *B*), suggesting that the chaperone sites of the protein could be masked at the lower, physiologically relevant temperatures, whereas they are getting exposed at the elevated temperatures. In addition, the stability of these proteins at elevated temperatures may also affect the observed activity. It is also important to note that αBAc, which exhibits high activity at lower temperatures, does not prevent the aggregation of aldolase and βL-crystallin at the elevated temperatures. We found that αBAc is not stable at these elevated temperatures as it itself undergoes aggregation to some extent under these conditions. These results also suggest that it is possible to engineer α-crystallin to obtain required chaperone activity with different temperature optima.

As described earlier, most small heat shock proteins contain a highly conserved sequence of 80–100 residues called the α-crystallin domain, and these shsps are referred to as α-hsps (1). A few regions of this domain have been shown to bind substrate protein such as alcohol dehydrogenase and the hydrophobic probe bis-ANS (50, 51). This domain is also known to be involved in intersubunit contacts. The N-terminal domain is variable with respect to its sequence length and amino acid composition. Studies also show that the N-terminal domain aids in the assembly of functional, high molecular mass complexes. N-terminal-tagged proteins are seen to bind nickel-chelate affinity resins only in the presence of urea, suggesting the N terminus to be buried within the complex. Substrate binding regions are also mapped in the N-terminal domain of αA- and αB-crystallin (50–52). An earlier study from our laboratory (62) showed that swapping the N-terminal domains of αA- and αB-crystallins results in both structural and functional alterations. The chimeric protein having N-terminal domain of αB- and the rest of αA-crystallin (αBNAC) exhibited chaperone activity more than both the wild type α-crystallins whereas the other chimeric protein having N-terminal domain of αA- and the rest of αB-crystallin (αANBC) exhibited little or no chaperone activity (62).

The structural and functional role of the C-terminal extension is debatable (45). It is polar, but like the N-terminal domain, its sequence is of variable length and composition. It is said to function as a solubilizer of the α-crystallin complex, as its deletion from *Caenorhabditis elegans* hsp16.2 causes precipitation of the complex upon freeze-thawing (63). Deletion of 17 residues from the C terminus of αA-crystallin (R157STOP), despite having spectroscopic properties similar to wild type protein, formed large insoluble aggregates with a marked reduction in chaperone-like activity (64). Similar results were seen in the case of *Xenopus* hsp30C (65). Also, the introduction of hydrophobic residues like tryptophan in the C-terminal extension affects structural and functional integrity, reducing chaperone activity, thermostability, and flexibility (55). Many bacterial and plant hsps show altered oligomer size upon C-terminal shortening. A deletion of the conserved LXI motif in the C-terminal region of *Bradyrhizobium japonicum* shsp caused formation of smaller oligomeric species, probably dimers, whereas pea hsp17.7 variants lacking 3 to 10 amino acids from the C terminus were unable to form full-size oligomers (45). The crystal structures of *M. jannaschii* hsp16.5 and wheat hsp16.9B show the C-terminal extension to be involved in subunit interactions (46, 47). The isoleucines in the LXI motif are actually found to bind to the hydrophobic groove between two β-strands of other interacting subunits (46, 47). A recent study on shsps (hspH, hspF) from *B. japonicum* showed that truncation of the region encompassing the LXI motif results in drastic reduction in oligomeric size (from 24-mer to probably a dimer) and loss of chaperone activity (66). Mutations of the isoleucine residues in the LXI motif of hspH to alanine resulted in loss of chaperone activity and reduction in oligomeric size, indicating that the LXI motif in these bacterial shsps, with regular oligomeric structure, is crucial for their quaternary structure and chaperone function (66). Studies on α-crystallins have so far suggested that the C-terminal extensions are highly unordered, flexible, and solvent-exposed. Our present study shows clearly that exchange of the C-terminal extensions between αA- and αB-crystallin results in both structural and functional alterations; C-terminal extension influences the tertiary structure of other domains, where aromatic amino acids are present, as revealed by fluorescence and near-UV CD. It is important to note that the C-terminal extension of αA-crystallin contains the sequence of the LX(I/V) motif as IPV, whereas that of αB-crystallin contains IPI. It is possible that, as observed in the case of hsp16.9, the IP(I/V) motif present in the αB- and αA-crystallin may have some tertiary interactions either between subunits or within the subunits. Probably the Ile to Val change in the LXI motif between αB- and αA-crystallins may have different consequences on such interactions. Because the C-terminal extensions of αA- and αB-crystallin differ in their length (34 and 32 residues, respectively), the exchange results in change in length of the chimeras. Such a change may also be one of the reasons for the observed structural and functional differences of the chimeric proteins.

We conclude that upon exchanging the C-terminal extensions of αA- and αB-crystallin, the chimeric proteins differ not only in their structure and chaperone function but also with respect to their wild type counterparts. αBAc exhibits the highest chaperone activity in preventing the aggregation of proteins whereas αABc exhibits much decreased chaperone activity at

temperatures up to 43 °C. Thus, our present study shows clearly that the C-terminal extension can influence the structure and function of α-crystallin apart from the solubilizing function that is generally attributed to this region. It appears that this region can have either inter- or intramolecular tertiary interactions that contribute significantly to the function.

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