Experiments with mini-α-crystallin (KLVIFLD-VKHFSPEDELTVK) showed that Phe\(^{71}\) in α-crystallin could be essential for the chaperone-like action of the protein (Sharma, K. K., Kumar, R. S., Kumar, G. S., and Quinn, P. T. (2000) J. Biol. Chem. 275, 3767–3771). In the present study we replaced Phe\(^{71}\) in rat α-crystallin with Gly by site-directed mutagenesis and then compared the structural and functional properties of the mutant protein with the wild-type protein. There were no differences in molecular size or intrinsic tryptophan fluorescence between the proteins. However, 1,1′-bi(4-anilino)naphthalene-5,5′-disulfonic acid interaction indicated a higher hydrophobicity for the mutant protein. Both wild-type and mutant proteins displayed similar secondary structure during far UV CD experiments. Near UV CD signal showed a slight difference in the tertiary structure around the 285–295 region for the two proteins. The mutant protein was totally inactive in suppressing the aggregation of reduced insulin, heat-denatured citrate synthase, and alcohol dehydrogenase. However, a marginal suppression of β\(_h\)-crystallin aggregation was observed when mutant α-crystallin was included. These results suggest that Phe\(^{71}\) contributes to the chaperone-like action of α-crystallin. Therefore we conclude that the 70–88 region in α-crystallin, identified by us earlier, is the functional chaperone site in α-crystallin.

α-Crystallins are major refractive proteins in the vertebrate eye lens. When isolated from the lens they exist as polydisperse aggregates having an average molecular mass of ~800 kDa (1, 2). α-Crystallin is composed of two subunits, αA and αB, which have considerable sequence homology between them and with other heat shock proteins (3, 4). Recently α-crystallin subunits were also reported to be present in nonlenticular tissues like heart, brain, and kidney (5–8). The significance of their presence in nonlenticular tissues is not clear. However, a marginal suppression of the aggregation of reduced insulin, heat-denatured citrate synthase, and alcohol dehydrogenase.

In brief,1 go of cells was suspended in 5 ml of reagent at room temperature and 1 l of cells was suspended in 5 ml of reagent at room temperature and vortexed gently. Protease inhibitor mixture set III (Novagen) was then added. The cell suspension was treated with 1 ml (25 units) of benzoxaneml of Bugbuster reagent and incubated at room temperature on a shaking platform for 30 min. The extract was centrifuged at 10,000 × g for 20 min.

Site-directed mutations of recombinant protein provide an excellent means of studying the role of constituent amino acids in the functional properties of the protein. Site-directed mutations of recombinant protein provide an excellent means of studying the role of constituent amino acids in the functional properties of the protein. Site-directed mutations were conducted on α-crystallin either to identify the region responsible for chaperone-like function or to explain the role of α-crystallin in hereditary cataracts and certain other diseases (29–38). The majority of these studies report either no change in chaperone-like function or a partial loss of this function. We report here, for the first time, a complete loss in the functional property of a mutant α-crystallin at and slightly above physiological temperatures. The results also indicate the presence of additional sites in α-crystallin that become available at elevated temperatures. We conclude here that the region identified by us earlier as chaperone site contributes to the chaperone-like activity of αA-crystallin.

EXPERIMENTAL PROCEDURES

Preparation of the Mutant Clone—Rat αA-crystallin cDNA cloned in pET21b was kindly donated by Dr. Suraj Bhat (UCLA). αF71G mutant was constructed using a QuickChange site-directed mutagenesis kit (Stratagene). The following set of primers were used: 5′-CTGACCGG-GACAAGGTGTGTCATCTTCTTG-3′ and 5′-CCAAGAAGATGACAC-CCTGTTCGCGGTCA-3′. The mutation was confirmed by automated DNA sequencing.

Expression and Purification of Wild-type and Mutant αA-crystallin—The proteins were expressed in Escherichia coli BL21(DE3) cells (Novagen) as described by Horwitz et al. (39). The proteins were isolated from the cell pellet using Bugbuster protein extract reagent (Novagen). In brief, 1 g of cells was suspended in 5 ml of reagent at room temperature and vortexed gently. Protease inhibitor mixture set III (Novagen) was then added. The cell suspension was treated with 1 ml (25 units) of benzoxaneml of Bugbuster reagent and incubated at room temperature on a shaking platform for 30 min. The extract was centrifuged at

The abbreviations used are: bis-ANS, 1,1′-bi(4-anilino)naphthalene-5,5′-disulfonic acid; CS, citrate synthase; ADH, alcohol dehydrogenase; HPLC, high pressure liquid chromatography.
aggregation was monitored up to 1 h. This is slightly higher than the earlier published values for rat (Fig. 2). The mass was calculated from the calibration curve generated by using Sigma molecular weight marker standards. The proteins exist in oligomeric form. For analysis of the molecular size determination, size determination was carried out using a Amersham Biosciences Hiload 16/60 Superdex 200 gel filtration column equilibrated with 0.05 M PO₄ buffer containing 0.15 M NaCl (pH 7.4). The purity of the proteins was checked by SDS-PAGE, and the mass was determined by mass spectrometry.

Characterization of Recombinant α-A-crystallin—In the present study, the recombinant proteins were purified by a combination of ion exchange and reverse phase HPLC. The structural differences between the wild-type and mutant αA-crystallin were analyzed by spectroscopic methods. Tryptophans in the proteins used was 1.5 and 0.35 mg/ml for near and far UV CD, respectively. The reported CD spectra are the averages of four scans. Our results show no change in the tryptophan region of the recombinant proteins, as evidenced by the similar fluorescence emission maximum (340 nm) and intensity (Fig. 3). Unfolding of proteins increases the exposure of hydrophobic surfaces that can be probed with bis-ANS fluorescence. Tryptophan fluorescence measurements—The intrinsic fluorescence spectra of the wild-type and mutant αA-crystallin were recorded using a Jasco spectrophluorometer. Insulin Aggregation Assay—The aggregation of insulin (0.4 mg/ml) was heated at 45 °C in the presence of various amounts of mutant and wild-type proteins. The aggregation was monitored at room temperature.

RESULTS

Characterization of Recombinant α-A-crystallin—In the present study, the recombinant proteins were purified by a combination of ion exchange and reverse phase HPLC. The purified proteins were dissolved in urea and refolded by extensive dialysis. The proteins thus obtained were highly pure (Fig. 1). The ES mass spectrometry analysis revealed molecular masses of 19,799 and 19,709 daltons, which would be expected for the wild-type and mutant αA-crystallin respectively. Like the α-crystallin subunits isolated from eye lens, recombinant proteins exist in oligomeric form. For analysis of the molecular mass of the homomultimers, the purified recombinant proteins were chromatographed on a Superdex-200 column. Both wild-type and mutant proteins showed similar elution profiles, corresponding to an oligomeric mass of 7.1 × 10⁵ daltons (Fig. 2). This is slightly higher than the earlier published values for rat αA-crystallin (37). The discrepancy can be attributed to the buffer conditions used in analysis, because it is known that the mass of the purified protein varies depending on the buffer condition (40, 41). During these studies we also observed a similar mass for reconstituted homopolymers of bovine lens αA-crystallin.

The structural differences between the wild-type and mutant proteins were analyzed by spectroscopic methods. Tryptophans of the protein have a fixed solvent accessibility, and any change in their environment leads to an altered fluorescence emission pattern and intensity. Our results show no change in the tryptophan region of the recombinant proteins, as evidenced by the similar fluorescence emission maximum (340 nm) and intensity (Fig. 3). Unfolding of proteins increases the exposure of hydrophobic surfaces that can be probed with bis-ANS fluorescence (26). We see an increase in bis-ANS binding to the αA71G mutant (Fig. 4), indicating an increased hydrophobicity comprised with the wild-type protein.

The secondary and tertiary structures of wild-type and mutant αA-crystallin were determined by far and near UV CD spectral analysis. The far UV profile showed a characteristic β-sheet conformation with a slight increase in the negative intensity of the mutant protein (Fig. 5). Both proteins showed similar amounts of α-helix, β-sheet, and random coil (42). Near
UV CD spectra showed a slight increase in the negative intensity of the mutant protein (Fig. 6). Although significant portions of the near UV spectra for the two proteins were similar, only minor changes were seen in the 285–295 nm region of the spectra, suggesting some differences in the tyrosine and/or tryptophan microenvironments of the mutant protein compared with the wild-type αA-crystallin. Surprisingly enough, there was no alteration in the signal caused by phenylalanine in the 250–270-nm region. In summary, the data in Fig. 6 do not suggest a significant difference in the tertiary structure between wild-type and mutant αA-crystallin.

The Chaperone-like Activity of F71G αA-crystallin—The consequence of mutation on recombinant crystallin chaperone-like activity was determined under different conditions. Reduction of insulin results in the separation of the subunits and precipitation of B chain that can be followed by measurement of light scattering. The presence of α-crystallin subunits in the assay prevents the aggregation of insulin B chain, and the solution remains clear. Fig. 7 shows the dithiothreitol-induced aggregation kinetics of insulin in the presence of both wild-type and mutant αA-crystallins. The wild-type protein showed suppression of insulin B chain aggregation that increased with the concentration of the protein in the assay tube. However, the mutant αA-crystallin completely failed to prevent the formation of light-scattering aggregates. In fact, a marginal increase in light scattering was observed in some assays. Higher concentrations of mutant protein had no effect on the aggregation of polypeptide. The chaperone-like activity of the recombinant proteins was also investigated at different temperatures. Fig. 8
shows the thermal aggregation of CS in the presence of wild-type and mutant αA-crystallin. Although the wild-type protein (50 μg) completely suppressed the aggregation of CS (75 μg), the mutant protein, as with insulin, failed to prevent the aggregation of denaturing CS. We also analyzed the ability of recombinant proteins to suppress the aggregation of ADH at 45 °C. The wild-type αA-crystallin showed increased suppression of denaturing protein aggregation with increasing concentration (Fig. 9A). Although the mutant protein appeared to suppress the aggregation of ADH at initial time points, the aggregation at 80 min was comparable with ADH by itself (Fig. 9B). Increasing the concentration of mutant protein had no effect on the aggregation of ADH. We also compared the abilities of mutant and wild-type αA-crystallin to prevent the heat-induced aggregation of βL-crystallin at 55 °C (Fig. 10). Unlike other substrates, mutant αA-crystallin showed a significant protection of βL-crystallin with increasing concentration. However, compared with the wild-type αA-crystallin, the mutant αA-crystallin was 6–10-fold less effective in suppressing βL-aggregation.

**DISCUSSION**

αA-crystallin subunit has been categorized into three domains: an N-terminal domain containing residues 1–66, a C-terminal or α-crystallin domain (central core) comprising residues 64–105, and an extended C-terminal including residues 106–173 (43–45). Most of the mutational studies on αA-crystallin were conducted either on the N-terminal domain or the C-terminal extension. Derham and Harding (46) have reviewed the mutations conducted by different laboratories on αA-crystallin and have recently reanalyzed the chaperone-like activity of several mutants (35). In the present study, we produced an αA-crystallin mutant by substituting Phe71 in the core region with a neutral amino acid Gly. This residue is highly conserved in αA-crystallin and is located in the region identified as the chaperone site of αA-crystallin (28). Biophysical characterization of the recombinant protein revealed no change in the oligomer size or tryptophan fluorescence. Because the αA-crystallin molecule has only one tryptophan at position 9, the intrinsic tryptophan fluorescence data may be of limited value.
to describe the structural changes in the central core or C-terminal domain as a consequence of the mutation. However, it will be a valuable tool for analyzing the stability of the N-terminal domain of the mutant protein. When the mutant protein was heated up to 60 °C, we did not observe any aggregation or shift in tryptophan fluorescence emission wavelength or intensity (data not shown), suggesting that the heat stability of the protein was not affected by the mutation.

It has been hypothesized that hydrophobic sites in α-crystallin are responsible for chaperone-like activity (22, 23). However, this is not free of controversy (29, 47). In the present study we see a complete loss in the chaperone-like function of mutant αA-crystallin at and slightly above physiological temperatures despite an increase in hydrophobicity. Smulders et al. (29) observed an increase in the chaperone-like activity of an αF74N mutant with a slight decrease in ANS binding. They concluded that there is no correlation between surface hydrophobicity and chaperone-like activity. Experiments with super-αA-crystallin have indicated that the disappearance of chaperone-like activity may be independent of hydrophobicity (47). Further, Reddy et al. (18) have shown recently that hydrophobicity is not the sole determinant of chaperone-like activity in α-crystallin. Recently, the studies with mini-αA-crystallin showed that both hydrophobicity and β-sheet conformation of the functional element are essential for chaperone-like activity (48). Although we see increased exposure of hydrophobic surfaces in the mutant, it is quite unlikely that all exposed hydrophobic patches would be involved in suppressing the substrate protein aggregation. We, as well as others, have observed bis-ANS binding to residues other than those necessary for chaperone activity (27, 49). Taking these observations together, one can conclude that although hydrophobicity is important, the extent of hydrophobicity does not reflect the chaperone-like activity of the protein.

The αF71G mutant has similar secondary structure to that of the wild type. However, the tertiary structure shows some minor changes around the 285–295-nm region. The signal in this region is produced by tyrosine or tryptophan residues. Because we did not observe any change in the tryptophan fluorescence intensity, it is possible that the alteration is in the tyrosine region. Interestingly, two tyrosine residues in αA-crystallin are found near the bis-ANS-binding region 50–54 (27). This may explain the increased bis-ANS binding of the mutant protein. However, it is unlikely that such a minor difference in the near UV CD signal would completely abolish the chaperone-like activity of the molecule. The αAR116C mutant, with structural alterations at many levels, showed only a 25% decrease in chaperone-like activity (37, 38). Further, it has been shown that α-crystallin could preserve its chaperone function despite some irreversible structural changes (50).

We have measured the chaperone-like function of the αAF71G mutant under different conditions and observed a complete loss in the activity of the mutant up to 45 °C. However, at elevated temperatures the mutant showed some suppression of β1-crystallin aggregation. It has been shown that α-crystallin undergoes a structural transition around 55 °C, resulting in the exposure of more hydrophobic patches (23, 24, 26). Our study on the stabilization of restriction enzyme (51) as well as studies conducted by others (35, 52) indicates the presence of multiple sites in α-crystallin for chaperone function. Based on the experiments with mini-αA-crystallin (28) and the complete loss of chaperone-like function of the mutant protein at physiological temperatures in this study, we conclude that the region identified by us earlier (residues 71–88) contributes to the chaperone-like function.

Plater et al. (30) reported that the F27R mutation in the N-terminal domain of αB-crystallin completely abolishes its chaperone-like activity at higher temperatures, which led them to conclude that this conserved residue is vital for chaperone function. Their report is controversial because later studies have shown that the mutant F27R is fully active (34, 35). Earlier, work showed that proteins resulting from mutation of V72N and F74N in the core region of αA-crystallin had normal activity (29). However, their conclusion was based on a single assay conducted at 58 °C. We also found some activity of the mutant with β1-crystallin around this temperature. Also, unlike Phe74, the Val72 and Phe74 residues show variations in different vertebrate lens species. The conserved Phe74 residue appears to be important for suppressing the aggregation of proteins. Other factors like charge, hydrophobicity, and structural integrity may influence the functional property to different extents. Recently, Kumar and Rao (53) produced a chimeric α-crystallin by swapping the domains of αA- and αB-crystallin and tested its effect on the chaperone-like activity. Interestingly, the αBNCαB chimeric protein which contained residues 1–79 of αA-crystallin, including a part of the functional site in αA-crystallin, was completely inactive in suppressing the aggregation of insulin. However, the αBNCαB chimeric protein containing the complete ADH binding sequence (25) and a part of the functional site in αA-crystallin had enhanced chaperone-like activity. This suggests that other residues in the chaperone site of αA-crystallin are also important in suppressing the aggregation of proteins. Therefore it would be interesting to study the role of other conserved residues on chaperone-like action.

Acknowledgments—We are grateful to Dr. P. D. Prasad for the help in site-directed mutagenesis and Jelena Kocergin in the expression and purification of recombinant proteins.

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Fig. 10. Aggregation of β1-crystallin (250 μg) in the presence of recombinant proteins. A, β1-crystallin; B, β1-crystallin + mutant αA (100 μg); C, β1-crystallin + mutant αA (200 μg); D, β1-crystallin + mutant αA (500 μg); E, β1-crystallin + wild type αA (50 μg); F, β1-crystallin + wild type αA (100 μg).
