Recognition Sequence 2 (Residues 60−71) Plays a Role in Oligomerization and Exchange Dynamics of αB-Crystallin†

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ABSTRACT: Previously, using the peptide scan method, we have determined that residues 42−57 and 60−71 in αB-crystallin (TSLSPFYLRPPSFLRA, named recognition sequence 1 or RS-1, and WFDTGLSEMRLE, named recognition sequence 2 or RS-2) are involved in interaction with αA-crystallin. To understand the significance of the RS-2 region in interactions between αA- and αB-crystallins, W60R, F61N, and S66G mutants of αB-crystallin were made and tested for their ability to interact with αA-crystallin. W60R and S66G mutations increased the oligomeric size of αB-crystallin by 1.6- and 2.7-fold respectively, whereas the F61N mutation had no effect. The tryptophan fluorescence intensity of αBS66G was 1.5-fold higher than that for the wild type. The intrinsic fluorescence of αBF61N was marginally lower than that of αB, whereas the fluorescence intensity of αBW60R decreased by 40% compared with that of αB. The relative availability of hydrophobic sites in the mutants was in the following order: αBS66G ≫ αB = αBF61N = αBW60R. The far-UV CD profiles for the wild type and αB-crystallin mutants indicated no significant changes in their secondary structures, except for αBS66G, which showed an increase in α-helical content. The near-UV CD profiles of αBW60R and αBF61N were nearly similar to that of wild type αB. On the other hand, αBS66G beyond 270 nm exhibited a signature completely different from that of wild type αB. Mutations did not alter the chaperone-like activity of these proteins. The W60R mutation did not affect the rate of subunit exchange between αB- and αA-crystallins. On the other hand, the S66G mutation increased the subunit exchange rate by 100%, whereas the F61N mutation decreased the rate of subunit exchange between αB- and αA-crystallins by 36%. Our results establish the importance of residues 60−71 in oligomerization of αB-crystallin and subunit interaction between αB- and αA-crystallins.

α-Crystallin, a major protein of the vertebrate lens, is a member of the small heat shock protein (sHSP)[1] family (*1*). It consists of two types of subunits, designated αA- and αB-crystallins, each with a molecular mass of 20 kDa. These subunits share sufficient sequence homology, and they can form homooligomers and heterooligomers (*2*). Heterooligomers with a 3:1 to 1:1 ratio of αA:αB exist in vivo depending on age (*3*), and the thermostability of the heterooligomer has been shown to be greater than that of either homooligomer alone (*4*). The sequence of both αA and αB subunits can be categorized into three domains: an N-terminal region, consisting of residues 1−66, a central α-crystallin domain or C-terminal domain, comprising residues 67−161, and a C-terminal extension, ranging from residue 162 to 173 in αA-crystallin and from residue 162 to 175 in αB-crystallin (*1*). Several studies have established the critical role played by the N-terminal region in oligomerization of sHSPs (*5*−*7*). Spin labeling studies have suggested a role for the α-crystallin domain in subunit interactions (*8*), whereas studies employing yeast two-hybrid (*9*) and mammalian two-hybrid systems (*10*) have indicated the involvement of the entire C-terminal domain of αB-crystallin in the interaction with αA-crystallin. Study of C-terminally truncated αA-crystallin has established a role for the C-terminal extension in its oligomerization (*11*).

All sHSPs tend to oligomerize into high-molecular mass forms, and oligomerization has been shown to be a prerequisite for chaperone-like function and subunit exchange (*4*). However, another study suggests that oligomerization may not be necessary for chaperone-like function as a truncated form of human αB-crystallin comprising residues 57−157 has been shown to exist as a dimer but still retain its chaperone activity (*12*). Also, citraconylation of all the Lys residues of αA- and αB-crystallins has been shown to disrupt their oligomerization, leading to the formation of tetramers with fully preserved chaperone-like function (*13*). In an earlier report, Sharma and Ortwerth (*14*) have shown that α-crystallin cross-linked by dimethyl suberimidate displays significant chaperone-like activity. This observation was corroborated in a recent study by Augusteyn (*15*), which also suggested that chaperone function of α-crystallin is a surface phenomenon and dissociation into smaller species was not required for the display of full activity.

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[1] Abbreviations: sHSP, small heat shock proteins; CS, citrate synthase; ADH, alcohol dehydrogenase; bis-ANS, 1,1′-bis(4-anilino)-naphthalene-5,5′-disulfonic acid; PBS, phosphate-buffered saline; FRET, fluorescence resonance energy transfer; MALS, multiangle light scattering; r_g, root-mean-square radius; r_h, hydrodynamic radius; QELS, quasi-elastic light scattering; SEC, size exclusion chromatography.

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Using peptide scans, we have identified two amino acid sequences of αB-crystallin involved in interaction with αA-crystallin (*16*). These two regions are residues 42−57 and residues 60−71, designated as recognition sequence 1 (RS-1; TSLSPFYLRPPSFLRA) and recognition sequence-2 (RS-2; WFDTGLSEMRLE), respectively (*16*). Site-directed mutagenesis of the RS-1 sequence showed the importance of this region in subunit interaction with αA-crystallin (*15*). A similar strategy was used to identify the oligomerization and substrate interaction sites in *Bradyrhizobium japonicum* sHSPB, a protein homologous to αB-crystallin (*17*). With protein pin array technology, five sequences in αB-crystallin were shown to be involved in interaction with both αA- and αB-crystallins (*18*). One of the sequences, from residue 37 to 54 (*17*), overlaps with the RS-1 sequence (residues 42−57 of αB-crystallin) we have identified in our previous study (*16*).

The study presented here investigates the role of RS-2 in subunit interactions between αA- and αB-crystallins. We mutated RS-2 at three positions: W60 with R (αBW60R), F61 with N (αBF61N), and S66 with G (αBS66G). We studied the effect of such mutations on structure−function properties of αB-crystallin. A Trp at position 60 was replaced with a bulky, basic amino acid, Arg, to determine whether charge replacement has an effect on subunit interaction with αA-crystallin. Phe at position 61 is a hydrophobic residue, and it was replaced with Asn, a polar amino acid, to investigate whether a hydrophobic residue is important for interaction with αA. Ser at positions 19, 45, and 59 constitutes the phosphorylation sites in αB-crystallin (*19*). To determine the role of Ser at position 66 in αA−αB oligomer formation, we replaced it with neutral Gly. Of the three mutants, W60R and S66G increased the oligomeric size of the proteins. The F61N mutation slowed the subunit exchange rate with αA-crystallin by 36%, whereas the S66G mutation accelerated the subunit exchange with αA-crystallin by 100%. These results demonstrate that the RS-2 region of αB-crystallin has an important role in oligomerization and subunit interaction between αB- and αA-crystallins.

EXPERIMENTAL PROCEDURES

Construction, Expression, and Purification of Wild Type αA-Crystallin, Wild Type αB-Crystallin, and Mutant αB-Crystallins. Human αA- and αB-crystallin cDNA (obtained from J. M. Petrash, Washington University, St. Louis, MO) were cloned into the pET23d vector (Novagen) at the NcoI−HindIII site. αBW60R, αBF61N, and αBS66G mutants were constructed using a Quik-Change site-directed mutagenesis kit (Stratagene). Mutations were confirmed by automated DNA sequencing. The proteins were expressed in *Escherichia coli* BL21(DE3)pLysS cells (Invitrogen), as described by Horwitz et al. (*20*), and were purified according to the method previously described (*21*). The purity of the recombinant proteins was checked by SDS−PAGE, and the mass was confirmed by ESI mass spectrometry.

Molecular Size Determination by Dynamic Light Scattering. To obtain the native molecular mass of recombinant αB-crystallins, protein samples were passed through the TSK4000 gel filtration column connected to a HPLC system, which is on-line with a Dawn-EOS multiangle laser light scattering detector (Wyatt Technology Corp., Santa Barbara, CA) and a refractive index detector (Shimadzu). Multiangle light scattering (MALS) permits the determination of the molar mass and size from the measurement of the angular dependence of scattered light. Coupled with size exclusion chromatography (SEC), it permits the determination of polydispersity and provides estimates of the absolute molar mass and size, which cannot be obtained by conventional chromatographic detection methods (*22*). In a SEC−MALS separation, it is generally assumed that each slice contains molecules of a single or at least very narrow molecular weight, M_i. Therefore, once a separation has been achieved and the collected data have been processed, the effective mass and size moments can be calculated (*22*) over each peak selected using the following equations:

$$\text{number average mass, } \overline{M_n} = \frac{\sum n_i M_i}{\sum n_i}$$

$$\text{weight average molar mass, } \overline{M_w} = \frac{\sum n_i M_i^2}{\sum n_i M_i}$$

where n_i is the number of molecules with molar mass M_i and n_i is proportional to C_i/M_i, where C_i is the weight concentration of species i.

Polydispersity is a ratio of the weight average molar mass ($\overline{M_w}$) to the number average molar mass ($\overline{M_n}$) (*22*).

Tryptophan Fluorescence Measurements. The intrinsic fluorescence spectra of the wild type and mutant αB-crystallins were recorded using a Jasco FP-750 spectrofluorimeter. Protein samples (0.2 mg/mL) in 0.05 M PO_4 buffer containing 0.15 M NaCl (pH 7.4) were excited at 295 nm, and emission spectra were recorded between 300 and 400 nm.

Bis-ANS Fluorescence Measurements. To 0.2 mg/mL wild type and mutant proteins taken in 0.05 M PO_4 buffer containing 0.15 M NaCl was added 10 μM bis-ANS. The samples were excited at 385 nm, and the emission spectra were recorded from 400 to 600 nm using a Jasco spectrofluorimeter.

Circular Dichroism Studies. Changes in protein secondary structure were measured by far- and near-UV CD spectra in an AVIV circular dichroism spectrometer. Proteins were used at a concentration of 0.2 mg/mL for far-UV and at 3 mg/mL for near-UV measurements. The reported CD spectra are the average of eight scans.

Chaperone-like Activity. The ability of the wild type and mutant proteins to prevent protein aggregation was determined using citrate synthase (CS) and alcohol dehydrogenase (ADH) as substrates.

CS Aggregation Assay. CS, 75 μg (Roche Molecular Biochemicals) in 1 mL of 40 mM HEPES-KOH buffer (pH 7.4), was heated at 43 °C for 1 h in the presence of 20 and 40 μg of the wild type and each mutant protein, respectively. The extent of aggregation was measured by monitoring the light scattering at 360 nm in a Shimadzu spectrophotometer.

ADH Aggregation Assay. Aggregation of ADH, 800 μg (Sigma), was carried out at 37 °C in 0.05 M PO_4 buffer containing 0.15 M NaCl and 100 mM EDTA in the presence of 20 or 40 μg of either wild type or mutant αB-crystallins, respectively. Light scattering was measured up to 100 min.

Table 1: Determination of the Molar Mass and Polydispersity of Wild Type αB-Crystallin and αB-Crystallin Mutants Using Dynamic Light Scattering

protein	homooligomeric molar mass (g/mol)	molar mass range (g/mol)	heterooligomeric molar mass (3αA:1αB, g/mol)	polydispersity	r_g/r_h
αB-crystallin	572000	476500−699000	698000	1.011	0.48
αBW60R	934000	772400−1198000	941000	1.031	0.93
αBF61N	607000	481000−946000	684000	1.055	3.00
αBS66G	1548000	641700−3954000	769000	1.340	0.81

Determination of Protein Stability. Stability of wild type αB and the αB mutants was determined at 37 and 43 °C, the temperatures at which the chaperone assays were conducted. Wild type αB and αB-crystallin mutants, 100 μg each, were incubated separately in 0.05 M PO_4 buffer containing 0.15 M NaCl (pH 7.4) in the absence of the target protein. The absorbance at 360 nm was monitored for 60 min.

Labeling of Recombinant αA-, αB-, and αB-Crystallin Mutants. Purified αA-crystallin was labeled with Alexa fluor 488, and wild type and mutant αB-crystallins were labeled with Alexa fluor 350 according to the recommendations of the manufacturer (Molecular Probes). Briefly, α-crystallin subunits were mixed with Alexa fluor dye in PBS supplemented with 100 mM sodium bicarbonate. The reaction was allowed to proceed for 1 h at room temperature. Labeled proteins were then separated from excess Alexa fluor dye by passing the mixture through a gel (Biogel P-30) supplied with the kit. The first fluorescent peak, representing the labeled protein, was collected, and the slower running free dye band was discarded.

Fluorescence Resonance Energy Transfer Measurements. The rate of subunit exchange between αB-crystallin (both the wild type and mutants) and αA-crystallin was measured using the fluorescence resonance energy transfer (FRET) technique. Alexa fluor 350-conjugated wild type αB-crystallin and its mutants were used as the energy donors, and Alexa fluor 488-conjugated αA-crystallin was the energy acceptor. αB-350, αBW60R-350, αBF61N, and αBS66G-350 [25 μg of each (separately)] and 75 μg of αA labeled with Alexa fluor 488 were taken in PBS (a 3:1 αA:αB ratio was used to mimic the in vivo situation), and the sample was incubated at 37 °C. Subunit exchange was monitored by exciting the sample at 346 nm (excitation wavelength for Alexa fluor 350) and measuring the emission spectra from 400 to 600 nm (emission for Alexa fluor 488 is at 520 nm) for 2−3 h. As the exchange progresses, there is a decrease in the fluorescence intensity of the donor with a concomitant increase in acceptor fluorescence. The rate of subunit exchange was calculated as described by Bova et al. (*5*).

RESULTS AND DISCUSSION

Previous studies have shown that both the N-terminal region and the α-crystallin domain are involved in subunit interactions of α-crystallin subunits (*6*, *8*). Removal of the first 56 or 63 residues dramatically reduces the size of the subunit, resulting in dimers or trimers with no subunit exchange, whereas deletion of the first 19 residues does not alter the size of αA-crystallin or the rate of subunit exchange (*5*). Peptide scan studies from our laboratory also suggest that residues 42−57 and 60−71 in αB-crystallin are involved in the interaction with αA-crystallin (*16*). Thus, a sequence between residues 19 and 71 appears to be important for oligomerization and subunit interactions. To determine the role of residues 60−71 in αB-crystallin (recognition sequence 2 or RS-2) in subunit interactions with αA-crystallin, we mutated W60 to R, F61 to N, and S66 to G by site-directed mutagenesis and studied the effect of the mutations on structural and functional properties of the recombinant proteins and on their interaction with αA-crystallin.

In the study presented here, both wild type and mutant αB-crystallins were expressed in *E. coli* BL21(DE3)pLysS cells and were purified by gel filtration and ion exchange chromatography. Molecular masses were determined by electrospray mass spectrometry and were as follows: 20158.9 Da for wild type αB, 20128.9 Da for αBW60R, 20125.9 Da for αBF61N, and 20128.9 Da for αBS66G. To investigate the effect of mutation on oligomerization of αB-crystallin, we analyzed the oligomeric sizes of wild type αB and αB mutants using dynamic light scattering. The observed oligomeric sizes of various proteins are listed in Table 1. From Table 1, it is clear that the F61N mutation had no effect on the oligomeric size, whereas W60R and S66G mutations enhanced the oligomeric size by 1.60- and 2.70-fold, respectively. In addition, the polydispersity of mutant αB-crystallins was also higher than that observed for wild type αB-crystallin, with αBS66G exhibiting the highest polydispersity. Also, by comparing the hydrodynamic radius r_h, measured by QELS, to the root-mean-square radius or radius of gyration, r_g, measured by static multiangle light scattering, we can gain information about the molecular conformation (*22*). For compact objects, r_g is smaller than r_h. The ratio of r_g to r_h increases as the molecule become less compact (*22*). From Table 1, we can deduce that of all the recombinant proteins, wild type αB-crystallin is more compact, followed by αBS66G and αBW60R, and αBF61N is less compact. Also, αA- and αB-crystallin (wild type to mutant) were mixed in 3:1 ratio and incubated at 37 °C for 3 h to allow the subunit exchange to reach equilibrium, and then their heterooligomeric mass was determined using dynamic light scattering. Table 1 reveals that wild type αB and αBF61N exhibit similar heterooligomeric molar masses, whereas αBW60R, which forms 1.6-fold larger homooligomers, also results in heterooligomers which are 1.35-fold larger than the wild type heterooligomers. On the other hand, αBS66G which forms 2.7-fold larger homooligomers leads to the formation of only 1.1-fold larger heterooligomers than the wild type counterparts. These findings suggest that residues 60−71 play an important role in the quaternary structural organization of αB-crystallin. This seems to be in agreement with a recent report from Koretz's group (*21*) which suggests that if residues in the N-terminal region can form specific interactions with residues in the conserved core shell, they can affect the oligomeric size as well as shape of the protein.

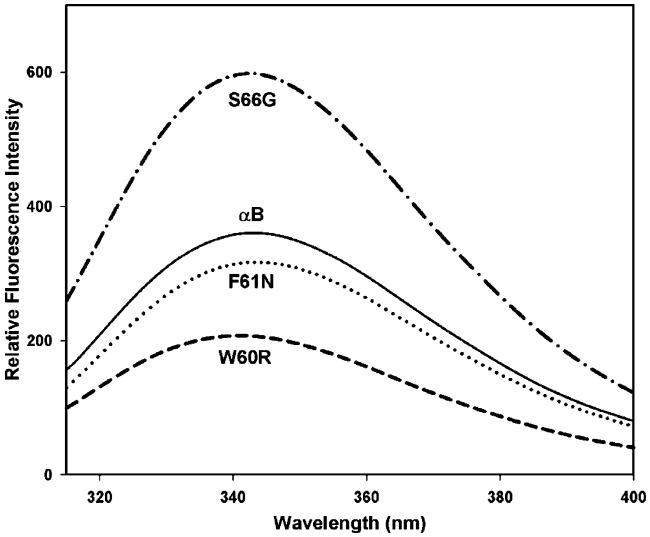

FIGURE 1: Intrinsic tryptophan fluorescence spectra for the wild type and αB-crystallin mutants. Protein samples (200 μg) in phosphate buffer were excited at 295 nm.

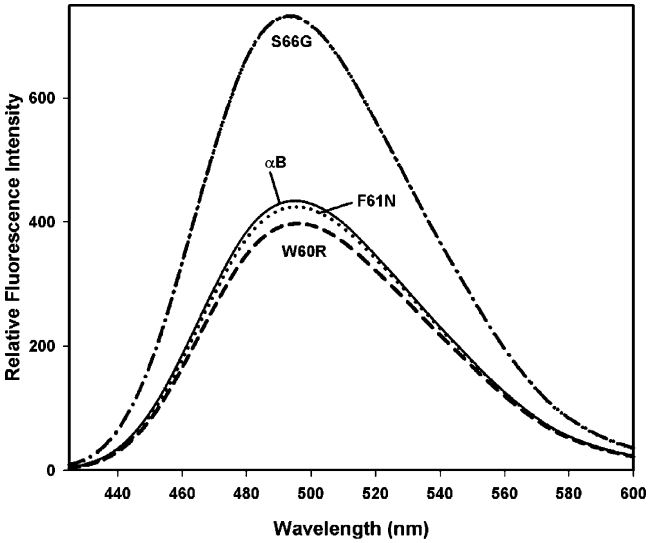

FIGURE 2: Fluorescence spectra of bis-ANS-bound wild type and mutant αB-crystallins.

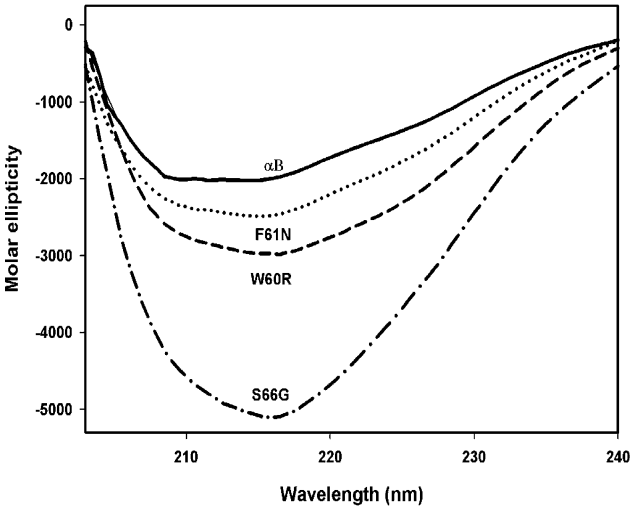

FIGURE 3: Far-UV CD spectra of recombinant αB-crystallins. Two hundred micrograms of protein was used to record spectra using a 0.05 cm path length cell.

Intrinsic fluorescence spectra indicated some differences between the wild type and αB mutant proteins (Figure 1). The tryptophan fluorescence intensity of αBS66G was 1.65-fold higher than that observed for wild type αB. The fluorescence intensity of αBF61N was marginally lower than that of αB. The fluorescence emission maximum was the same (343 nm) for wild type αB, αBS66G, and αBF61N. On the other hand, the fluorescence intensity of αBW60R was 40% lower than that of wild type αB. This difference was expected as one of the Trp residues in αB was mutated to Arg. In addition to the reduction in the fluorescence intensity of αBW60R, the fluorescence emission maximum also exhibited a 2 nm blue shift to 341 nm (from 343 nm) observed for the wild type, indicating the buried nature of the remaining Trp in αBW60R (Figure 1). This finding is consistent with an earlier report which showed that the Trp at position 9 is relatively buried (*24*). The higher fluorescence intensity for αBS66G could be due to the change in the Trp microenvironment of the protein, mainly of Trp^{60}, which is close to the mutated site.

To investigate whether the observed changes in oligomeric size and intrinsic fluorescence are accompanied by altered surface hydrophobicity, we probed wild type αB and αB mutants with bis-ANS. Bis-ANS is a hydrophobic probe, and its fluorescence intensity increases upon binding to hydrophobic regions in the proteins (*25*). Figure 2 shows that αB, αBF61N, and αBW60R have similar availability of hydrophobic surfaces, whereas αBS66G has 40% more available surface. It appears that the structural changes induced by S66G mutation led to the exposure of hydrophobic surfaces.

The far-UV CD spectra of wild type αB and αB-crystallin mutants indicated no significant change in the secondary structure of αB, αBW60R, and αBF61N, apart from the increased negative ellipticity for αBS66G (Figure 3). Negative ellipticity is generally associated with helical structures; therefore, secondary structural elements were estimated in αBS66G and wild type αB according to the self-consistent method of analysis as described by Sreerama and Woody (*26*). For αBS66G, the α-helix content increased from 3.6 to 7.65% in wild type αB at the expense of β-sheet content, which decreased from 39.8 to 34.15% in the wild type. The S66G mutation also led to a minor increase in β-turn and random coil contents (data not given). Therefore, in addition to causing an increase in oligomeric size, intrinsic fluorescence, and surface hydrophobicity, the S66G mutation also altered the secondary structure of αB-crystallin.

The near-UV CD spectra of wild type αB, αBF61N, and αBW60R were similar with some minor variations in αBF61N and αBW60R between 270 and 290 nm; transitions in this region arise mainly from Tyr and/or Trp residues. αB, αBF61N, αBW60R, and αBS66G exhibited maxima at 259 and 265 nm that are characteristic of phenylalanine fine structure (Figure 4). The negative vibronic transitions around 293 nm are contributed by Trp residues. For αBS66G, the change observed in Trp fluorescence intensity is also reflected in its tertiary structure. The protein exhibited a signature totally different from that of wild type αB and other αB mutants beyond 270 nm. It exhibited increased negative ellipticity instead of positive ellipticity, which was observed in the wild type. It also exhibited negative transitions at 290 nm instead of at 293 nm which was observed for αB and other αB mutants. The S66G mutation altered the Trp

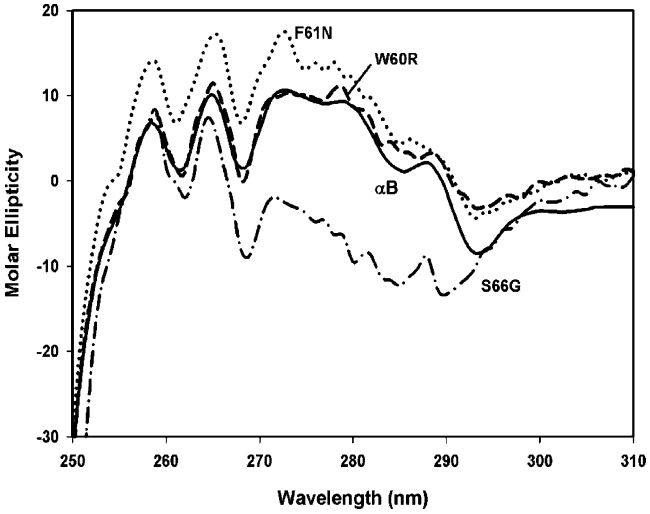

FIGURE 4: Near-UV CD spectra of wild type and mutant αB-crystallins.

microenvironment of the protein. As S66 is close in position to W60, replacement of Ser may have altered the interaction of aromatic chromophores (Tyr and Trp), thereby leading to changes in Tyr and Trp absorbing regions of αBS66G. It is well-known that unlike wild type αA-crystallin, wild type αB-crystallin does not exhibit a well-defined near-UV profile beyond 270 nm (*27*). The contribution of Tyr and/or Trp residues is not clearly discernible. This could be a possible reason for not being able to decipher any changes in the Tyr and/or Trp absorbing regions of αBW60R despite removal of a Trp residue. It is not surprising that no major changes were observed in the Phe fine structure (250−270 nm) in αBF61N as only one Phe of 14 residues was mutated.

The stability of wild type αB and αB mutants was determined by measuring light scattering of the proteins at both 37 and 43 °C, the temperatures used in the chaperone assays. At both temperatures, αB mutants behaved like wild type αB and did not exhibit any scattering or aggregation, indicating that none of the mutations altered protein stability (data not shown).

To investigate whether structural changes lead to changes in function, we measured the chaperone-like activity of αB mutants as well as their ability to interact with αA-crystallin. The chaperone-like activity of αB-crystallin mutants was determined using different substrates and different conditions. EDTA-induced aggregation of ADH at 37 °C and thermal aggregation of citrate synthase (CS) at 43 °C were monitored in the presence of wild type αB and αB mutants. With both substrates, αBW60R (Figure 5A,D), αBF61N (Figure 5B,E), and αBS66G (Figure 5C,F), at 20 μg exhibited slightly less chaperone-like activity. However, at 40 μg, they completely suppressed aggregation of ADH (Figure 5A−C) and CS (Figure 5D−F) like wild type αB. This suggests that mutations did not affect the chaperone-like property of recombinant αB-crystallins. The increased exposure of hydrophobic sites in αBS66G did not result in enhanced chaperone-like function, indicating that new hydrophobic sites do not contribute toward chaperone-like activity. This is also consistent with our previous observation that subunit interaction sites and chaperone sites are distinct as binding of the RS-1 peptide did not alter the chaperone-like activity of αA-crystallin (*16*). Also, the synthetic RS-2 peptide does not show any chaperone activity of its own (data not shown), whereas residues 70−88 in αA-crystallin and 73−92 in αB-crystallin constitute the chaperone sites (*28, 29*) and exhibit chaperone-like activity. In the study presented here, as the mutations were created in the αA interaction site in αB-crystallin, that is, RS-2, which constitutes residues 60−71, we observed changes only in the oligomeric size and structure of the proteins but not in their chaperone function. We can once again deduce that the subunit interaction region and the chaperone region are different though they may reside in the same domain. Similar results were obtained in the case of sHSPB; Narberhaus' group proposed the oligomerization sites to be different from substrate interaction sites (*17*). On the other hand, on the basis of protein pin array studies, Clark's group (*30*) showed that the same sequences in αB-crystallin are involved in subunit interaction and binding to the substrate proteins.

In another study, mutation of Met^{68} in rat αB-crystallin to Thr, Val, and Ile did not result in significant changes in molecular mass or secondary and tertiary structures (*31*). The Thr mutant showed substantial loss in chaperone activity, whereas Val and Ile mutants showed significant improvement in chaperone function (*31*). However, subunit interaction was not investigated.

Unlike our previous study that demonstrated the binding of the synthetic RS-1 peptide to αA-crystallin (*16*), this study revealed that the synthetic RS-2 peptide did not bind to αA-crystallin (data not shown). Various methods, including gel filtration to separate the complex of RS-2 and αA-crystallin and fluorescence polarization to demonstrate complex formation between RS-2 and αA-crystallin, were used in an attempt to demonstrate the binding of RS-2 with αA-crystallin. This finding suggests that RS-2 has a very low affinity for αA-crystallin. Nevertheless, the following results point out the importance of the RS-2 sequence of αB-crystallin in subunit interaction with αA-crystallin.

Oligomerization is a prerequisite for subunit exchange in sHSPs. Bova et al. (*5*) have shown that the αA-crystallin mutant in which first 19 amino acids have been deleted (αA20−173) retains its oligomeric structure (560 kDa) and exchanges subunits normally, whereas truncated αA-crystallin (with residues 56−173 or 63−173) mutants form dimers and trimers, respectively, and fail to exchange subunits. They suggested that the fundamental exchange unit is a tetramer or smaller multimers. We investigated whether the mutation in the RS-2 region which caused changes in the oligomeric size of the proteins will have any effect on their ability to exchange subunits with αA-crystallin. The subunit exchange was monitored by the FRET assay, and the initial slopes of the curves that were obtained reveal the rate of subunit exchange (Figure 6). For αB−αA interaction, the rate of subunit exchange was calculated as $(11.06 \pm 0.16) \times 10^{-4}$ s^{-1}. For αBW60R−αA interaction, the rate of subunit exchange was $(12.58 \pm 0.08) \times 10^{-4}$ s^{-1}. For αBF61N−αA interaction, the rate was $(7.0 \pm 0.15) \times 10^{-4}$ s^{-1}. For αBS66G−αA interaction, the exchange rate was $(21.9 \pm 0.32) \times 10^{-4}$ s^{-1}. The exchange rate for the wild type αA- and αB-crystallins is in a similar range as reported by Aquilina et al. (*32*). Therefore, the F61N mutation reduced the subunit exchange rate by 36%, whereas the S66G mutation increased the rate of subunit exchange by 100%.

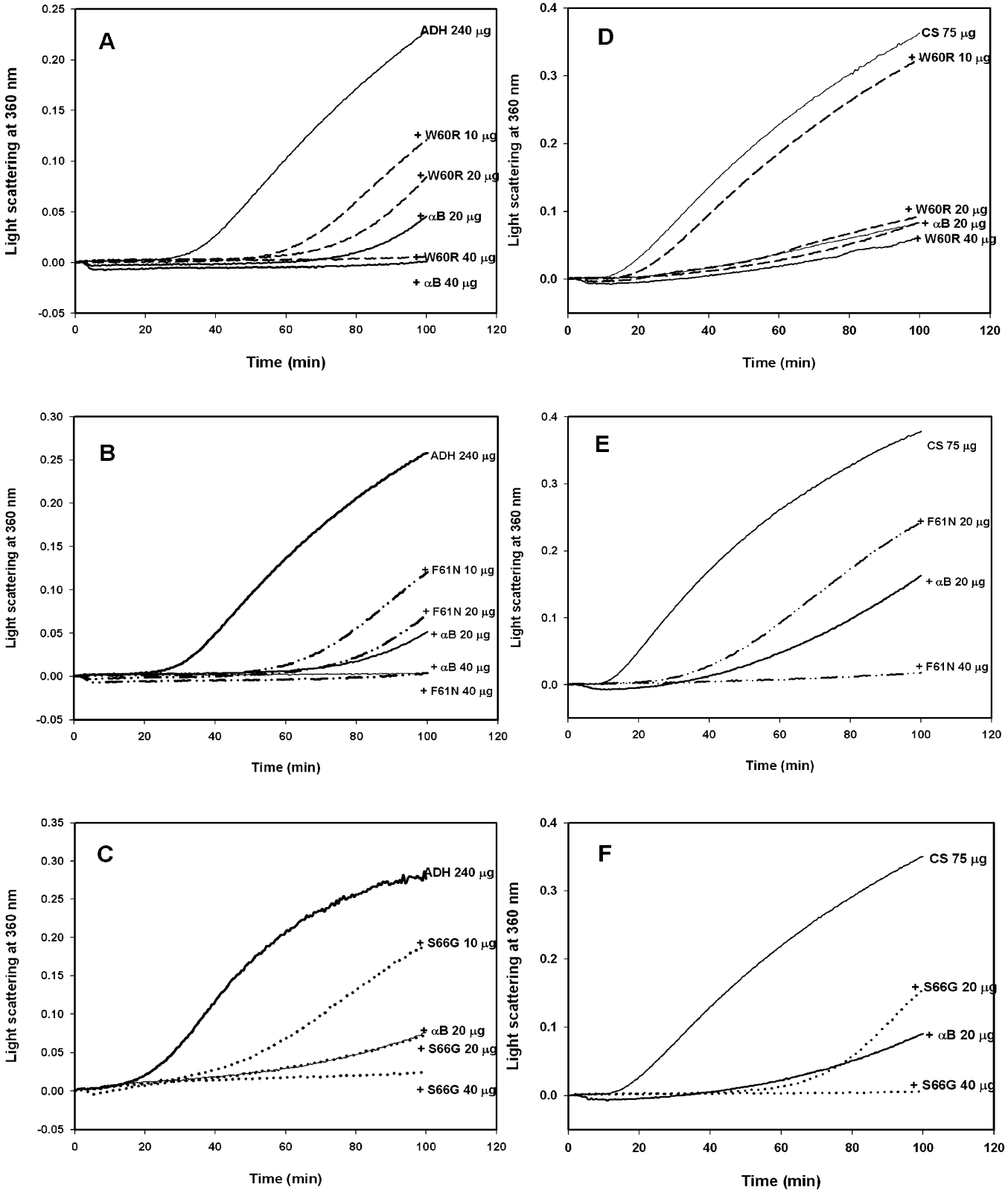

FIGURE 5: EDTA-induced aggregation of ADH [(A) αBW60R, (B) αBF61N, and (C) αBS66G] and thermal aggregation of CS [(D) αBW60R, (E) αBF61N, and (F) αBS66G] in the presence of various concentrations of recombinant αB-crystallins.

Interestingly, the W60R mutation did not have any effect on subunit exchange.

From Table 1, we know that αBS66G forms a large oligomer of 1548000 Da followed by αBW60R at 934000 Da, αBF61N at 607000 Da, and αB at 572000 Da. Although αBF61N has an oligomeric size similar to that of the wild type, it exhibits a decreased exchange rate, indicating that F61 may be involved in subunit contacts with αA. The increase in the oligomeric size of αBS66G could indicate that the removal of reactive Ser enhances the subunit interactions which are also reflected in the accelerated dynamics of the protein. A similar type of enhancement in the subunit exchange rate was observed for the αAR116C mutant. It exchanged subunits with αB-crystallin 3 times

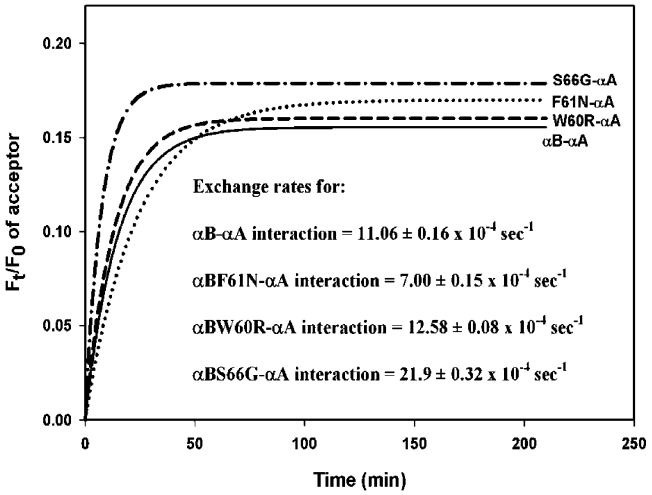

FIGURE 6: Subunit exchange studies of wild type and mutant αB-crystallins with αA-crystallin. Twenty-five micrograms of αB-crystallin-350 (wild type/mutant) was used as the energy donor, and αA-488 was the energy acceptor. F_t/F_0 represents the ratio of acceptor fluorescence intensity at 520 nm at given time intervals to the fluorescence at the beginning of the assay (time zero).

faster than wild type αA-crystallin (*28*). In addition, it also formed large oligomers of ~2000 kDa (*28*). It is well-known that charge interactions are involved in subunit−subunit interactions followed by other noncovalent interactions for the stabilization of the oligomer aggregates. The increase in the oligomeric size of αBW60R, which could be a result of introduction of Arg, did not affect the exchange dynamics of the protein.

An increase in oligomeric size may not be necessarily correlated with an increase in chaperone function or subunit exchange. Deletion of the SRLFDQFFG residues in the N-terminal region of αA- and αB-crystallins leads to smaller oligomers with enhanced subunit exchange and chaperone function (*29*). The R116C point mutation in αA-crystallin results in increased oligomeric size and subunit exchange but causes a significant loss of chaperone-like activity (*28*). In addition, the chimeric protein, αBNAC, consisting of the N-terminus of αB-crystallin and the C-terminus of αA-crystallin forms larger aggregates which display enhanced chaperone function (*30*). In this context, our study reveals interesting results. The W60R mutation leads to larger oligomers with no effect on either chaperone-like activity or subunit exchange. The F61N mutation leads to normal sized oligomers (like wild type αB), with no change in chaperone behavior, but the subunit exchange rate is reduced. On the other hand, the S66G mutation results in larger oligomers which display accelerated dynamics with normal chaperone function. Irrespective of their size, it appears that the accessibility of chaperone sites is unaltered upon mutation in the αB mutants used in the current study. Nevertheless, our results unequivocally show that residues 60−71 in αB-crystallin represent a critical motif which contributes to higher-order oligomeric assembly and subunit interactions with αA-crystallin.

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