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Functional Elements in Molecular Chaperone α-Crystallin: Identification of Binding Sites in αB-Crystallin

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α-Crystallin, the predominant eye lens protein with sequence homology to small heat shock proteins, acts like a molecular chaperone by suppressing the aggregation of damaged crystallins and proteins. To gain an insight into the amino acid sequences in α-crystallin involved in chaperone-like function, we used a cleavable, fluorescent, photoactive, crosslinking agent, sulfosuccinimidyl-2(7-azido-4-methylcoumarin-3-acetamido)-ethyl-1,3′ dithiopropionate (SAED), to derivatize yeast alcohol dehydrogenase (ADH) and allowed it to complex with bovine α-crystallin at 48°C. The complex was photolyzed and reduced with DTT and the subunits of α-crystallin, αA- and αB-, were separated. Fluorescence analysis showed that both αA- and αB-crystallins interacted with ADH during chaperone-like function. Tryptic digestion, amino acid sequencing, and mass spectral analysis of αB-crystallin revealed that APSWIDTGLSEMR (57-69) and VLGDVIEVHGKHEER (93-107) sequences were involved in binding with ADH.

α, β- and γ-Crystallins constitute the major portion of the eye lens fiber cells (1). Among the crystallins, α-crystallin is the most abundant protein, existing as a polydisperse aggregate with average molecular weight 800 kDa (2). α-Crystallin is made-up of two types of subunits, designated αA- and αB- with molecular weights 19,832 Da and 20,079 Da respectively. The sequences of the subunits of α-crystallin have high homology to small heat shock proteins (3, 4). α-Crystallin subunits, once thought to be lens specific, are now widely known to be present in other tissues as well (5-8). In addition, increased expression of αB-crystallin has been documented in some neurological disorders (6, 9, 10).

Recently, the ability of native α-crystallin to suppress the aggregation of heat denatured (11-26), UV-irradiated (26, 27) as well as chemically denatured (28) proteins and enzymes has been demonstrated. Complex formation between α-crystallin and denatured proteins, enzymes or β- and γ-crystallins have been demonstrated (14, 18). On the basis of those in vitro data, it has been proposed that α-crystallin acts as a chaperone in vivo to maintain the lens clarity and that α-crystallin loses this ability during aging. Consistent with this hypothesis, a decreased chaperone-like activity for α-crystallin present in high molecular weight aggregates from aged bovine and human lens has been demonstrated (23, 29). Data from the studies with lens homogenates and lenses incubated at elevated temperatures have provided evidence for an in vivo role for chaperone-like function of α-crystallin (30, 31).

There has been an intense study on the ability of α-crystallin to suppress aggregation of denatured proteins (12-31), since this activity was first reported by Horwitz (11). While some of the studies were directed towards understanding the importance of specific amino acid residues in αA- and αB-crystallins in chaperone-like activity (20-22) others were directed towards understanding the effect of chemical and post-translational modifications on α-crystallin chaperone-like activity (19, 27, 32-34). Low molecular weight thiol compounds such as pantethine and oxidized glutathione have been shown to modulate α-crystallin chaperone-like activity (25). By limited trypsin digestion (34), as well as recombinant methods (26), it has been shown that truncated αA-crystallin has lower chaperone-like activity. Analysis of α-crystallin from selenium induced cataractous rat lens has shown a decrease in chaperone-like activity and a concurrent truncation of C-terminus (35). Furthermore, de Jong et. al. (24) have shown that immobilization of the flexible C-terminal extension of bovine αA-crystallin results in loss of chaperone-like activity of the protein. However, the observation that mutation of Asp69 to Ser affects the chaperone activity of αA-crystallin (20) and the presence of residual activity in C-terminally truncated αA-crystallin (26) suggest that regions other than the proposed C-terminus are also involved in the chaperone-like

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function of αA-crystallin. By site directed mutagenesis Crabbe and associates (22) have shown that mutation within the phenylalnine rich N-terminal region abolishes the chaperone-like activity of αB-crystallin whereas substitutions at the C-terminus had no effect on chaperone-like activity. Based on deuterium exchange study results Smith et. al. (36) suggested that the hydrophobic regions of αA- 32-37, 72-75 and αB-28-34 may be involved in chaperone-like activity display by α-crystallin.

While the above studies implicated several regions of αA- and αB-crystallin in chaperone-like function, the full extent of the binding site in the α-crystallin-target protein interaction has not been investigated. We have begun studies using a novel heterobifunctional crosslinker sulfosuccinimidyl-2(7-azido-4-methylcoumarin-3-acetamido)-ethyl-1,3′ dithiopropionate (SAED) to identify the regions/amino acid sequences in αB-crystallin that are involved in chaperoning of yeast alcohol dehydrogenase (ADH). We report below successful identification of binding sites in B subunit of molecular chaperone α-crystallin.

EXPERIMENTAL PROCEDURES

α-Crystallin was isolated from the water-soluble extract of young bovine lens cortex. ADH and sequence grade trypsin were obtained from Sigma Chemical Co. (St. Louis, MO). SAED was from Pierce Chemical Co. All other chemicals were of the highest grade commercially available.

Preparation of α-crystallin. α-crystallin was isolated from young bovine lens cortex by gel filtration on Sephadex G-200 and ion-exchange chromatography on TMAE-fractogel column (EM-Separations) as described earlier (21). In brief, the cortex from the decapsulated lens was homogenized in 50 mM Tris-HCl buffer, pH 7.5 and centrifuged at 30 000g for 30 min. The supernatant was fractionated on a Sephadex G-200 column (5 × 27 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.5. The α-, β- and γ-crystallin fractions were pooled and concentrated. The α-crystallin was rechromatogrphed on the same Sephadex G-200 column and the protein from the central portion of the alpha peak was further purified by TMAE-fractogel chromatography (2.5 × 10 cm). The bound α-crystallin was eluted by a linear NaCl gradient (0-0.5 M). The α-crystallin thus obtained was >99% pure as judged by SDS-PAGE and used in current study.

Thermal denaturation and light scattering assay. The capacity of α-crystallin and its isolated subunits to protect against heat induced aggregation of ADH was measured according to the procedure described earlier (12, 21). Briefly, a known amount of target protein (300-500 μg) was heated in 50 mM PO_4, pH 7.0 and 0.1 M NaCl (buffer A) in the presence or absence of different amounts of α-crystallin in a final volume of 1.0 ml. The aggregation of proteins at the specified temperature was followed by recording the increase in absorbance at 360 nm as a function of time in a Perkin Elmer Lambda 3 spectrophotometer equipped with multicell transporter attached to a circulating water bath.

Derivatization of ADH with SAED. ADH was derivatized with SAED using the procedure described earlier for soybean trypsin inhibitor (37). In brief, ADH (5 mg) in 0.5 ml H_3BO_3 pH 8.5 was reacted in the dark with 0.5 mg of SAED in dimethylformamide. After 15 min the mixture was quenched by the addition of Tris (50 mM final). The SAED labeled ADH was separated from the free reagent by gel filtration on Sephadex G-25 (1.5 × 30 cm) equilibrated in 50 mM sodium phosphate buffer, pH 7 containing 100 mM NaCl.

Complexing of SAED-derivatized ADH with α-crystallin and crosslinking. The ADH-SAED (2 mg) was mixed with an equal amount of α-crystallin and incubated at 48° C in dark for 1 h. The photoactivation of the azido group of the crosslinker and the covalent crosslinking of ADH to α-crystallin was accomplished by exposing the reaction mixture to 150W tungsten lamp for 10 min.

Separation of labeled αA- and αB-crystallin from ADH. The α-crystallin ADH complex was treated with 100 mM 2-mercaptoethanol and 4 M GnHCl for 2 h and filtered. The αA- and αB- subunits and the ADH were separated from one another by HPLC using a C18 column (Vydac C18, 218TP1010, The Separation Group, Hesparia, CA) and linear gradient formed between 0.1% TFA in water and 0.1% TFA in acetonitrile. The elution was monitored by absorbance at 280 nm. All the fractions were tested for fluorescence (350 nm EX; 450 nm EM) in a Perkin Elmer Spectrofluorimeter. The identities of various subunit peaks were confirmed by SDS-PAGE and pooled separately. The fluorescence spectrum of both the αA- and αB-crystallin fractions was obtained (Ex 350 nm).

Trypsin digestion of labeled αB-crystallin and identification of the coumarin-labeled peptide. The methylcoumarin labeled αB-crystallin obtained after HPLC separation was dried, redissolved in Tris-HCl, pH 8.0 and digested for 24 h at 37° C with trypsin using a 1:50 ratio of enzyme to substrate. After 24 h digestion the reaction mixture was fractionated by reversed phase HPLC using a 4.6 × 250 mm C18 column (Vydac 218TP), and a flow rate of 1ml/min. A 0-60% gradient formed by mixing solvent A and B over 60 minutes was used to elute the bound peptides. Solvent A was 0.1% TFA in H_2O; solvent B was 0.1% TFA in CH_3CN. UV absorbance was monitored at 220 nm. One ml fractions were collected. All the fractions were tested for the fluorescence (350 nm EX; 450 nm EM) in a Perkin Elmer Spectrofluorimeter. The fluorescent peptides were repurified by HPLC and concentrated by vacum centrifugation. The peptides having bound coumarin were sequenced at the Protein Core Facility (University of Missouri, Columbia, MO). The apparent molecular weight of fluorescent peptides was determined by Matrix Assisted Laser Desorption Mass Spectrometry (MALDI) at the Biomedical Research Core Facilities of the University of Michigan, Ann Arbor, MI.

RESULTS AND DISCUSSION

Yeast alcohol dehydrogenase (ADH) is a tetramer of 39 kDa subunits. It denatures, aggregates and precipitates upon heating at 48° C (21) a temperature at which α-crystallin undergoes minimal structural changes (38). In the presence of α-crystallin, however, ADH does not precipitate, but rather binds to α-crystallin to form binary complexes allowing the reaction mixture to remain clear (21). Sulfosuccinimidyl 2-(7-azido-4-methylcoumarin-3-acetamido) ethyl-1,3′ dithio propionate (SAED), is a cleavable, heterobifunctional, aminoreactive, water-soluble, photoreactive crosslinker (Fig. 1A). SAED molecule has two reactive groups, a sulfo-NHS ester for coupling with amino group of a donor molecule, a terminal photoactivable azido coumarin moiety to react with a proximal amino acids on the interacting target protein, and a central disulfide linker to release the labeled target after cleavage. SAED can be used as a means of transferring a fluorophore from one protein to another protein to which it binds (37).

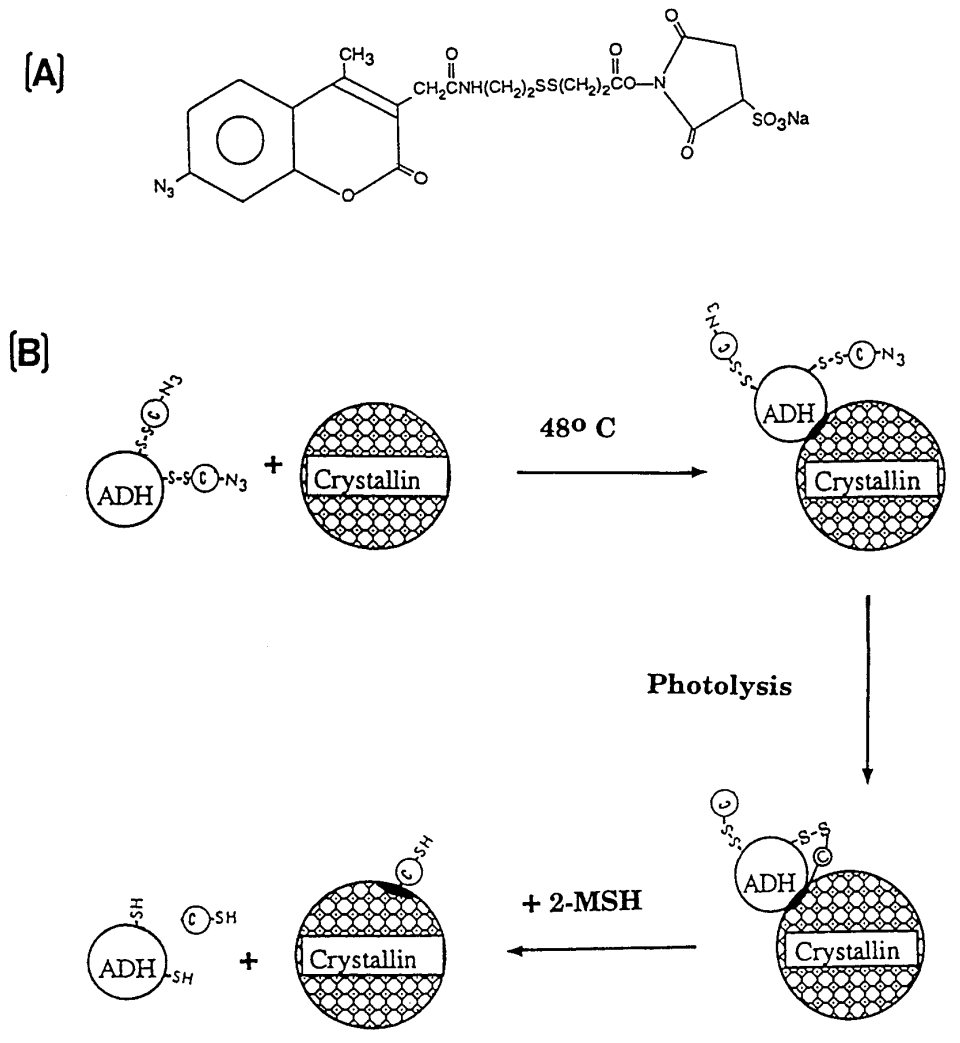

FIG. 1. SAED structure of the scheme for specific labeling of the binding region in α-crystallin. (A) SAED structure. (B) Scheme. ADH was derivatized with SAED and mixed with an equal amount of α-crystallin (Wt/Wt) and incubated in the dark at 48°C to elicit the chaperone-like activity and binding. The mixture was then exposed to a bright light to initiate photolysis of the azide bond and covalent linkage of SAED-ADH to α-crystallin. Cleavage of the disulfide bond within the crosslinker using 2-mercaptoethanol and subsequent separation of the α-crystallin subunits by HPLC yield the fluorescence labeled αA- and αB-crystallin. In the figure, N_3 represents the terminal photoactivable azide of SAED. © is the coumarin fluorophore. S-S is the cleavable portion of the crosslinker (figure is not to scale).

α-Crystallin shows chaperone-like activity towards ADH derivatized with SAED. Fig. 1B shows the scheme used for ADH derivatization and the crosslinking to α-crystallin. All the steps up to the photolysis stage were carried out under dim red light with reaction vessels covered in aluminum foil. ADH was first derivatized with SAED and separated from the free reagent by gel filtration on a Sephadex G-25 column equilibrated in buffer A. This step resulted in the covalent linkage of SAED to ADH through an acetylation step with a concurrent loss of the sulfosuccinimidyl moiety. The overall modification of the available amino groups in ADH by this step was less than 50 percent as determined by absorption of the derivatized ADH (37). SAED derivatized ADH showed heat denaturation and aggregation pattern similar to the unmodified ADH (Fig. 2). Addition of α-crystallin in 1:1 weight ratio completely suppressed the aggregation of SAED-ADH. Gel filtration analysis of SAED-ADH incubated with α-crystallin at 48° C for 1 h showed co-elution of SAED-ADH with α-crystallin, whereas the SAED-ADH and α-crystallin incubated at 25° C for 1 h eluted as separate peaks at the elution volumes expected for α-crystallin and SAED-ADH (data not shown). These results suggest that partial modification of ADH with SAED does not abolish its ability to complex with α-crystallin during heat denaturation.

Both A and B subunits of α-crystallin interact with ADH during chaperone-like activity display. SAED-ADH was mixed with α-crystallin in 1:1 weight ratio

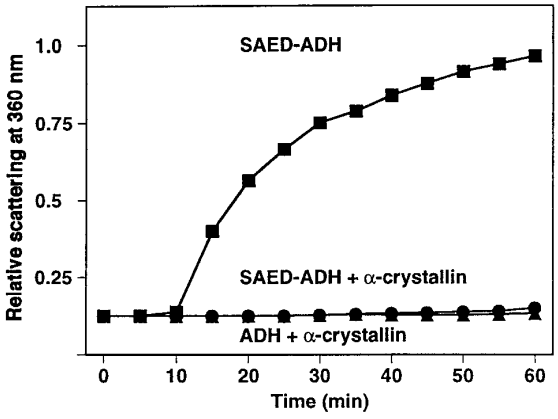

FIG. 2. Inhibition of temperature-induced aggregation of SAED-ADH by α-crystallin. ADH was derivatized as descibed under methods. The chaperone-like activity assay was done at 48°C as described under general methods. ADH and α-crystallin were used in a 1:1 weight ratio.

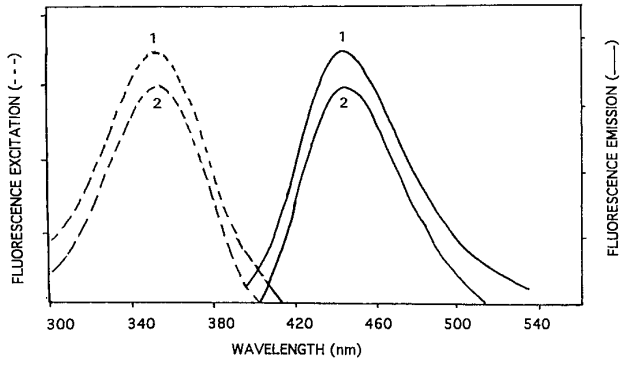

FIG. 3. Fluorescence excitation and emission spectra of the HPLC purified αA- and αB-crystallins with bound coumarin fluorophore. The samples were HPLC separated αA- and αB- from the SAED-ADH-α-crystallin complex. The experimental details are given under methods. 1, αA-crystallin; 2, αB-crystallin.

in buffer A and allowed to form complex at 48° C for 1 h. The crosslinking of SAED-ADH to α-crystallin, through the photolysis of the azide bond, was initiated by exposing the reaction mixture to long wavelength bright light at room temperature. The crosslinked complex was then treated with 100 mM 2-mercaptoethanol. The cleavage of the crosslinker with 2-mercaptoethanol resulted in the transfer of the fluorphore from SAED-ADH to α-crystallin subunits at the binding sites. The labeling of αA- and αB- by fluorescence transfer was confirmed by SDS-PAGE analysis as well as by isolation of αA- and αB- subunits by HPLC and fluorescence measurement. Both αA- and αB-crystallins migrated as single protein bands during SDS-PAGE and exhibited coumarin fluorescence when the gels were exposed to 350 nm light (data not shown). We purified the labeled αA- and αB-crystallins by HPLC using a C18 column. The emission spectrum (Ex 350) of labeled αA- and αB-crystallins was recorded in a Perkin Elmer Spectrofluorimeter. The spectra shown in Fig. 3 suggests that both αA- and αB-crystallins contain the transfered fluorophore. While previous studies have shown that isolated αA- and αB-crystallins display chaperone-like activity (11, 20-22), the data presented in Fig. 3 is the direct evidence supporting the participation of both αA- and αB- subunits in the in vitro chaperone-like activity assay. When α-crystallin-ADH-SAED was reduced prior to photolysis, to inactivate the azido group and cleave s-s bond, there was no transfer of coumarin to α-crystallin. When an excess of unlabeled ADH was included in the reaction mixture during complex formation, the fluorescent label transfer from SAED-ADH was proportionately diminished (data not shown). It is known that SAED modified proteins do not transfer the coumarin to the second protein unless the two proteins are interacting (37).

Identification of chaperone sites in αB-crystallin. We determined the amino acid sequences of αB-crystallin peptides that crosslinked with SAED-ADH. In order to do this, the methylcoumarin labeled αB-crystallin was digested with sequence grade trypsin. The fluorescent peptides from the trypsin digest were separated from the rest of the peptides by HPLC (Fig. 4). The HPLC separation of fluorescent peptides gave two peaks, eluting at 49 min and 51 min. The two peaks were collected separately, repurified and subjected to sequencing as well as molecular weight determination.

The Edman sequencing of the 49 min and 51 min peptide fractions showed N-terminus sequence of APSWIDT and VLGDVIEVHG respectively. These peptides showed a relative molecular mass of 1754.4 Da and 2009 Da when analyzed by MALDI (spectra not shown). Trypsin digestion of αB-crystallin generates several peptides of which the peptides beginning with APSWIDT and VLGDVIEVHG will have sequence APS-

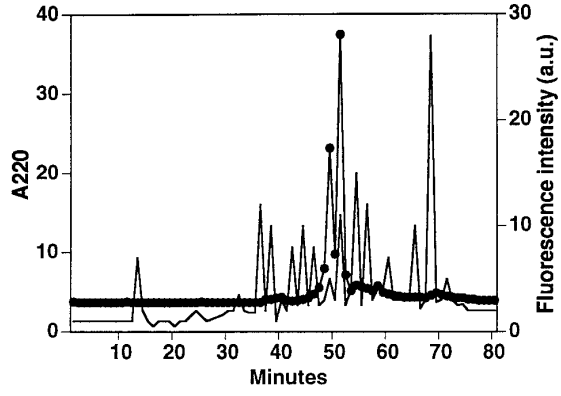

FIG. 4. HPLC separation of the tryptic fragments of coumarin fluorophore containing αB-crystallin. αB-Crystallin from previous step (Fig. 3) was digested with trypsin and the resulting peptides were separated by HPLC as described earlier. —, A_{220}; ●, fluorescence. Peptides eluting at 49 and 51 min were analyzed further.

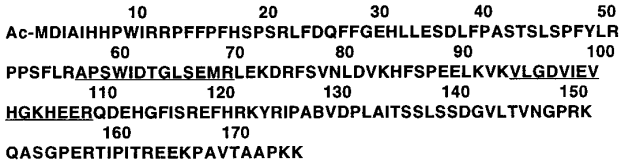

FIG. 5. Localization of ADH binding region in bovine αB-crystallin sequence. The ADH binding sequence is underlined.

WIDTGLSEMR (residues 57-69) and VLGDVIEHGKHEER (residues 93-107 if there was no cleavage at Lys 103 (39)). The calculated mol. wt. of these two peptides is 1462.7 Da and 1717.2 Da (39). Addition of one molecule of coumarin from the crosslinker to each of these two peptides will result in an increase in molecular weight by 291 Da. The molecular mass 1754.4 Da and 2009 Da determined by MALDI for the two peptides (APSWIDTGLSEMR and VLGDVIEHGKHEER) are very close to the theoretical value for a peptide with bound crosslinker. Taken together, these results indicate that APSWIDTGLSEMR (residues 57-69) and VLGDVIEHGKHEER (residues 93-107) region of αB-crystallin interact with SAED labeled ADH during chaperone-like action. The location of the binding sites in αB-crystallin sequence is shown in Fig. 5. The fact that only two peptides from αB-crystallin showed fluorescence suggests that there was specific interaction between α-crystallin subunit and SAED-ADH. A random binding of SAED-ADH to αB- present in α-crystallin tested would have given many binding sequences. It is however plausible that our experiments did not identify some other ADH binding site(s) because of the lack of reactive azido group at the interaction site on ADH or masking of a binding site(s) due to the binding of SAED-ADH to αA subunits and the resulting steric hindrance.

The ADH binding sequences in αB-crystallin determined during the present studies differ from the binding regions proposed by site directed mutagenesis of a recombinant αB-crystallin. In recombinant αB-crystallin, mutation at the phenylalnine region was shown to display decreased chaperone-like activity (22). The mutants, F24R, F27R and F27A showed complete loss of chaperone-like activity whereas the other mutant αB-crystallins, D2G and K174L and K175L displayed only partial loss of activity. Further studies are required to see if mutations at the ADH binding sequences also affect chaperone-like activity of αB-crystallin.

On the basis of hydrogen-deuterium exchange studies Smith et. al. (36) have proposed that of the three hydrophobic regions of αB-crystallin, 28-34, 61-65 and 80-89, and the 28-34 region in αB-crystallin may be involved in chaperone-like activity. One of the hydrophobic region of αB-crystallin, 61-65 is within the amino acid sequence 57-69 identified as ADH binding site in this study. Another hydrophobic region of αB-crystallin (80-89) is the preceding sequence of the second ADH binding site (residues 93-107) discovered during this study (Fig. 5). Glycation of α-crystallin leads to a loss of chaperone-like activity (33). The ADH binding site in αB-crystallin, region 93-107 is the sequence following the major glycation site, Lys 90 in αB-crystallin (41). Furthermore, Lys 90 lies between the hydrophobic region 80-89 and the putative chaperone site 93-107. Therefore it is conceivable that modification of glycation of Lys 90 perhaps leads to an alteration of charge as well as hydrophobicity at this site. Therefore, it is possible that glycation of αB- Lys 90 may have contributed to the loss of chaperone-like activity observed earlier.

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