Identification of 1,1'-Bi(4-anilino)naphthalene-5,5'-disulfonic Acid Binding Sequences in α-Crystallin*

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The hydrophobic binding sites in α-crystallin were evaluated using fluorescent probes 1,1'-bi(4-anilino)naphthalenesulfonic acid (bis-ANS), 8-anilino-1-naphthalene sulfonate (ANS), and 1-azidonaphthalene 5-sulfonate (1,5-AZNS). The photolysis of bis-ANS-α-crystallin complex resulted in incorporation of the probe to both αA- and αB-subunits. Prior binding of denatured alcohol dehydrogenase to α-crystallin significantly decreased the photoincorporation of bis-ANS to α-crystallin. Localization of bis-ANS incorporated into αA-crystallin resulted in the identification of residues QSLFR and HFSPEDLTVK as the fluorophore binding regions. In αB-crystallin, sequences DRFSVNLNVK and VLGDVIEVHGK were found to be the bis-ANS binding regions. Of the bis-ANS binding sequences, HFSPEDLTVK of α-crystallin and DRFSVNLNVK and VLGDVIEVHGK of αB-crystallin were earlier identified as part of the sequences involved in their interaction with target proteins during the molecular chaperone-like action. The hydrophobic probe, 1,5-AZNS, also interacted with both subunits of α-crystallin. Localization of 1,5-AZNS binding site in αB-crystallin lead to the identification of HFSPEDLTVK as the interacting site in this subunit of α-crystallin. Glycated α-crystallin displayed decreased ANS fluorescence and loss of chaperone-like function, suggesting the involvement of glycation site as well as ANS binding site in chaperone-like activity display.

α-Crystallin is one of the most predominant eye lens proteins. Its concentration in lens fiber cells is about 40% of the total protein in lens (1). α-Crystallin exists as a polydisperse aggregate with an average molecular mass of 800 kDa (2). The two types of subunits, designated αA and αB, each of which has a molecular mass of 20 kDa, arrange themselves in yet undefined ways to form the aggregate (2). During aging, α-crystallin undergoes extensive modifications culminating in the formation of super aggregates and highly cross-linked light-scattering molecules (2). The sequences of the subunits of α-crystallin have high homology to small heat shock proteins (3, 4) and are highly conserved between species. α-Crystallin subunits, once thought to be lens-specific, are now widely known to be present in other tissues as well (5–8). Despite extensive studies carried out in the past, the quaternary structure or the structure-function of α-crystallin or its subunits has remained an enigma and challenge for researchers.

Recently, the ability of native α-crystallin to suppress the aggregation of heat-denatured (9–20), UV-irradiated (20, 21), as well as chemically denatured (22) proteins and enzymes has been demonstrated. It has been proposed that surface hydrophobic sites in the α-crystallin aggregate are involved in the binding of α-crystallin to target proteins during the display of chaperone-like activity (13, 23). A direct correlation between the extent of α-crystallin hydrophobicity and chaperone-like activity has been demonstrated by several studies (13, 23–27). However, the amino acid sequence that contributes to the site responsible for the binding of denatured proteins and hydrophobic site specific probes is not fully understood.

We have shown earlier that both A- and B-subunits in α-crystallin interact with bis-ANS1 in 1:1 stoichiometry at 37 °C (26). The number of bis-ANS molecules binding to α-crystallin increases if the protein is exposed to higher temperatures or denaturing agents prior to the addition of the fluorophore (26, 27). Furthermore, it has been shown that binding of bis-ANS to α-crystallin (25) or α-crystallin (26) diminishes the chaperone-like activity of the protein. In the present study we have determined the bis-ANS binding sequences in α-crystallin by photocross-linking, peptide mapping and sequencing. The data presented here also show that the bis-ANS binding sequences are also the chaperone sites in α-crystallin.

EXPERIMENTAL PROCEDURES

Materials—bis-ANS, ANS, and 1,5-AZNS were obtained from Molecular Probes, Inc. (Junction City, OR). The stock solutions of bis-ANS were prepared in 95% alcohol, and the concentration was determined by absorbance at 385 nm using an extinction coefficient, ε₂₈₅ = 16,790 cm⁻¹ M⁻¹ (28). Lysyl endopeptidase was purchased from Wako Bioproducts. Sequence grade trypsin was obtained from Sigma. β-crystallin (29) was isolated from bovine lenses (15). 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 trimethylaminoethyl-fractogel column (EM-Separations) as described earlier (26, 30). The α-crystallin thus obtained was >99% pure as judged by SDS-PAGE, and this preparation was used in this study.

Photoincorporation of Bis-ANS into α-Crystallin—Photoincorporation of bis-ANS to α-crystallin was carried out as described earlier (26), with slight modification of the original procedure described by Scale et al. (31). Following photolysis, the sample was analyzed by HPLC and SDS-PAGE, and the fluorescence associated with protein bands was documented by photography using TMAX 100 film (Eastman Kodak Co.) under UV light (360 nm). The gel was later stained with Coomassie Blue. The efficiency of bis-ANS incorporation to α-crystallin during 15-min photolysis was determined by quantitative densitometry function of Image-1 system (Universal Imaging Corp.).

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1 The abbreviations used are: bis-ANS, 1,1'-bi(4-anilino)naphthalene-5,5'-disulfonic acid; HPLC, high pressure liquid chromatography; 1,5-AZNS, 1-azidonaphthalene 5-sulfonate; ANS, 8-anilino-1-naphthalenesulfonate; HSP, heat shock protein; PAGE, polyacrylamide gel electrophoresis; ADH, alcohol dehydrogenase.
To investigate whether prior binding of denatured proteins to α-crystallin prevents bis-ANS binding and photoincorporation, α-crystallin and alcohol dehydrogenase (ADH) (1:6 ratio) were incubated at 48 °C for 1 h. Following incubation, the reaction mixture was cooled to 25 °C, and bis-ANS was added. The final bis-ANS concentration was 12.5 μM. Photolysis of the sample was carried out as above for 15 min, and the aliquots were subjected to SDS-PAGE under reducing conditions. The fluorescent bands were photographed as above and the gel was stained with Coomassie Blue.

**Separation of Bis-ANS-labeled A- and B-crystallin**—The photo-lyzed α-crystallin-bis-ANS complex was treated with 5 mM dithiothreitol (DTT) and the reduced A- and B-subunits were separated from one another by HPLC using a C18 column (215TP1010 from the Separation Group, Hesparsa, CA) and linear gradient (0–60% over a period of 1 h) formed between 0.065% trifluoroacetic acid in water and 0.065% trifluoroacetic acid in acetonitrile. The flow rate was 1 ml/min. The elution was monitored by absorbance (280 nm) and fluorescence (390 nm excitation and 490 nm emission). bis-ANS-labeled A- and B-crystallin were further purified by SDS-PAGE and recovered by electrophoresis, and the SDS was removed by ether precipitation (32).

**Identification of Bis-ANS-labeled Peptides**—The bis-ANS-labeled αA- and β-crystallins were digested with lysyl endopeptidase (1:30, enzyme/protein) for 4 h at 37 °C. The peptides were separated by reverse phase HPLC on a Vydac C18 column (215TP54) equilibrated with 20 mM sodium phosphate buffer, pH 6.5, in 95% acetonitrile (solvent A). The elution of bound peptides was carried out with a linear gradient (0–60%) formed by solvent A and solvent B (20 mM phosphate buffer, pH 6.5, in 95% acetonitrile). A flow rate of 1 ml/min over 120 min was maintained, and 1-ml fractions were collected. The elution was monitored at 220 nm for absorption and fluorescence (390 nm excitation and 490 nm emission). The amino-terminal sequences of bis-ANS-labeled peptides were determined by Edman degradation on an Applied Biosystems PROCISE CLC protein sequencing system.

**Photoincorporation of 1,5-AZNS to α-crystallin and Identification of Binding Site in α-crystallin**—The photoincorporation of 1,5-AZNS to α-crystallin was accomplished using a procedure described by Dockter and Koseki (33). 1 mM 1,5-AZNS and 1.25 μM α-crystallin were used in this following photoincorporation, αA- and B- subunits of α-crystallin were separated by HPLC as above using a C18 column. Although the fluorophore was incorporated to both the subunits, only αA-subunit was further analyzed to determine the 1,5-AZNS incorporation site. Labeled βA-crystallin was digested with sequencing grade trypsin (1:50 ratio), and the resulting peptides were separated as described earlier (30). The elution profile was monitored at 220 nm. All fractions were tested for fluorescence in a Perkin-Elmer Spetrophotometer model 650-40 (excitation and emission maxima of 334 and 440 nm, respectively). The major fluorescence peptide eluting at 45 min from the HPLC column was subjected to amino acid sequencing in an Applied Biosystems 470A sequencer.

**Glycation of α-crystallin, Chaperone Assay, and ANS Binding**—Glycation of α-crystallin was carried out in 0.1 M phosphate buffer, pH 7.0, using 10 mg/ml protein and 20 mM l-ascorbic acid (34). After incubation at 37 °C for 4 weeks, the reaction mixture was dialyzed, and the glycated protein was tested with Edman degradation on an Applied Biosystems 470A sequencer. These peptides probably have the sequence HFSPEDLTVK and HFSPEDLTVKVQED. The ADH was heat-denatured and allowed to bind to α-crystallin prior to the addition of the fluorophore (compare lanes 1 and 2 in Fig. 1). The result shows a significant decrease in photoincorporation of bis-ANS to α-crystallin when ADH was heat-denatured and allowed to bind to α-crystallin prior to the addition of the fluorophore (compare lanes 1 and 2 in Fig. 1).

**Effect of Denaturing Protein Bound to α-crystallin on the Photoincorporation of Bis-ANS**—Earlier we showed that prior binding of bis-ANS to α-crystallin diminishes the chaperone-like activity of the protein (26). To determine whether the binding of denaturing proteins to α-crystallin at the chaperone site can affect subsequent bis-ANS photoincorporation, α-crystallin and ADH (1:6 ratio) were incubated at 48 °C for 1 h prior to the addition of bis-ANS and photolysis. SDS-PAGE of such an experiment is shown in Fig. 1. The result shows a significant decrease in photoincorporation of bis-ANS to α-crystallin when ADH was heat-denatured and allowed to bind to α-crystallin prior to the addition of the fluorophore (compare lanes 1 and 2 in Fig. 1).

**Photoincorporation of Bis-ANS to α-crystallin Subunits and Localization of Incorporated Bis-ANS**—To determine the bis-ANS binding sites in α-crystallin, the fluorophore was initially allowed to bind to purified α-crystallin by the addition of saturating amounts of the probe and removal of the excess by dialysis to minimize nonspecific photoincorporation of free bis-ANS activated during photolysis. Photolysis of the α-crystallin-bis-ANS complex by UV-A light (366 nm) resulted in covalent incorporation of the fluorophore to both αA- and β-subunits as we reported earlier (26).

In order to identify the sites of bis-ANS incorporation into αA- and β-crystallins, α-crystallin was modified with bis-ANS, and the subunits were separated by HPLC and purified by SDS-PAGE. The purified bis-ANS-modified proteins were later digested with lysyl endopeptidase. The resulting fluorescent peptides were separated from other peptides by reverse phase HPLC. The HPLC profile of αA-crystallin peptides and the fluorescence is shown in Fig. 2. Edman degradation of fluorescent peptides eluting at 37 and 40 min revealed the same NH4-terminal sequence HFSPE. These peptides probably have the sequence pHEDTFTIK and HPEDEDTKYQED-FVEIHKG, corresponding to residues 79–88 and 79–99 of α-crystallin (Fig. 3), since we used lysyl endopeptidase to digest αA-crystallin. Incomplete digestion at Lys-88 due to the bis-ANS incorporation at/or near Lys-88 may have generated the latter peptide. Furthermore, the peptide eluting at 40 min yielded low levels of Phe (10 pmol) in the second cycle compared with 20 pmol of Ser in the third sequencing cycle, indicating that Phe-80 was modified by bis-ANS during photoincorpora-
FIG. 2. HPLC separation of bis-ANS-labeled α-crystallin peptides. α-crystallin was isolated from the bis-ANS-labeled α-crystallin and digested with lysyl endopeptidase as described under "Experimental Procedures." The peptides were separated by reverse phase HPLC, and the relative fluorescence is shown by a graph. Solid line, fluorescence (390 excitation/490 emission); broken line, A220. Inset, HPLC profile of α-peptide eluting at 89 min in Fig. 2 digested with trypsin.

FIG. 3. α- and β-crystallin sequence showing the bis-ANS binding region. The bis-ANS binding sequences are shown in bold. The alcohol dehydrogenase binding sequences in α-crystallin and melittin binding sequence in αα-crystallin are taken from Refs. 30 and 40, respectively, and underlined. The melittin binding region in α- and β-crystallin marked with a double underline is taken from Ref. 40. The 1,5-AZNS binding sequence in α- and β-crystallin is shown in italics.

duction. We could not determine the location of bis-ANS insertion site in peptide eluting at 37 min by examining the results of five sequence cycles used to determine the identity of the peptide.

The fluorescent peptide eluting at 89 min showed the NH₂-terminal sequence RTLGPF. The peptide showed a mobility equivalent to 6.5 kDa on SDS-PAGE (data not shown). Therefore, the peptide eluting at 89 min is likely to represent the residues 12–70 of αα-crystallin. Analysis of the fluorescent material eluting at 72 min failed to show any amino acid during sequencing cycles. Since the same peak also did not contain appreciable 220 nm absorption, it is likely that the fluorescence at this region may be due to the peptide-bound unstable bis-ANS or its derivative. To localize the bis-ANS-bound amino acid in peptide eluting at 89 min (Fig. 2), the peptide was subjected to trypsin digestion and HPLC analysis. Fig. 2, inset, shows the HPLC profile of the trypsin digest. The single fluorescent peptide eluting at 66 min (Fig. 2, inset) was sequenced as described earlier. The observed sequence for the 66-min peptide, QSLFR, corresponds to residues 50–54 of αα-crystallin (Fig. 3). During the sequencing of this peptide the yield of Phe was low (82 pmol) compared with other amino acids (which were in the range of 110–160 pmol), suggesting a possible modification of Phe-53 in αα-crystallin by bis-ANS.

The two fluorescent peptides of αβ-crystallin, generated by lysyl endopeptidase digestion, eluted from the HPLC column at 38 min as a doublet (Fig. 4). The same peaks also showed maximal fluorescence emission at 490 nm when excited at 390 nm and revealed NH₂-terminal sequence DRFSV and VLGDV. These two αβ-peptides can only be from the sequence DRFSVNLVDV and VLGDVIEVHK (Fig. 3), since we have used lysyl endopeptidase for digestion. We were unable to conclude which of the amino acid in αβ-crystallin formed a cross-link with bis-ANS by examining the results from five cycles of sequencing reaction used to determine the identity of the fluorescence peptide. Although the fluorescent material eluting at 72 min was the largest fluorescent peak, it gave no amino acids during sequencing cycles. Since the same peak also did not contain measurable 220 nm absorption, it is likely that the fluorescence at this region may be due to the peptide-bound unstable bis-ANS or its derivative. Since the chemistry of the reaction is not known at the present time, further studies are required to see whether the 72-in fluorescent peak was due to the bis-ANS that was originally bound to the two peptides we have identified or to a different peptide.

Interaction of 1,5-AZNS with α-Crystallin—1,5-AZNS is another, less specific, photoreactive agent used to study the hydrophobic sites in proteins (33, 35). When bovine α-crystallin was treated with 1 mM 1,5-AZNS at 25 °C and photolyzed, about 40 nmol of 1,5-AZNS was incorporated to each mole of α-crystallin (800 kDa), suggesting that on average there exists one 1,5-AZNS binding site per subunit of α-crystallin at 25 °C. Further analysis of 1,5-AZNS-α-crystallin by HPLC revealed that both αα- and αβ-crystallins were labeled with 1,5-AZNS during the experiment (data not shown), as with bis-ANS (26).

The 1,5-AZNS binding site in αβ-crystallin was determined by peptide mapping and sequencing of the fluorophore-containing peptide. A peptide eluting from C18 column at 45 min (Fig. 5) with a sequence HFSPEELK was found to have incorporated 1,5-AZNS. This sequence corresponds to residues 83–90 in bovine αβ-crystallin (Fig. 3). Examination of the chromatographic profiles obtained during the sequencing of the fluorescent peptide showed that Asp in the peptide was modified by...
1,5-AZNS during photolysis. Although αA-crystallin was also labeled with 1,5-AZNS, we did not analyze the sample further.

Effect of Glycation of α-Crystallin on Its Chaperone-like Function and Interaction with ANS—Earlier studies have shown that glycation of α-crystallin reduces its chaperone-like activity (36). To determine whether this was due to an alteration of the hydrophobic chaperone site in α-crystallin, we measured the interaction of glycated α-crystallin with hydrophobic probe ANS. ANS, like bis-ANS is a polarity-sensitive reagent. α-Crystallin glycated with ascrobate for 4 weeks showed a 25% decrease in its ability to increase ANS fluorescence compared with the controls. When the same glycated α-crystallin was tested with βL-crystallin in a heat denaturation assay (15), a marked decrease in chaperone-like activity was observed (Fig. 6).

DISCUSSION

The presence of surface hydrophobic sites on α-crystallin has been known for a number of years (2). Since the demonstration of chaperone-like activity with α-crystallin (9), considerable interest has been shown in the hydrophobic sites within α-crystallins as these sites have been implicated in the chaperone-like function of the protein (13, 23–27). The hydrophobic sites in α-crystallin and its subunits have been studied during recent years using probes such as ANS (37), bis-ANS (26, 27), and pyrene (13). We have shown recently that UV photolysis of the α-crystallin-bound bis-ANS leads to photoincorporation of the fluorophore to the protein subunits similar to that seen with chaperone GroEL (31) and HSP18.1 (38), and pyrene (13). We have shown recently that UV photolysis of the α-crystallin-bound bis-ANS leads to photoincorporation of the fluorophore to the protein subunits similar to that seen with chaperone GroEL (31) and HSP18.1 (38), and pyrene (13). We have shown recently that UV photolysis of the α-crystallin-bound bis-ANS leads to photoincorporation of the fluorophore to the protein subunits similar to that seen with chaperone GroEL (31) and HSP18.1 (38), and pyrene (13). We have shown recently that UV photolysis of the α-crystallin-bound bis-ANS leads to photoincorporation of the fluorophore to the protein subunits similar to that seen with chaperone GroEL (31) and HSP18.1 (38), and pyrene (13). We have shown recently that UV photolysis of the α-crystallin-bound bis-ANS leads to photoincorporation of the fluorophore to the protein subunits similar to that seen with chaperone GroEL (31) and HSP18.1 (38), and pyrene (13). We have shown recently that UV photolysis of the α-crystallin-bound bis-ANS leads to photoincorporation of the fluorophore to the protein subunits similar to that seen with chaperone GroEL (31) and HSP18.1 (38), and pyrene (13). We have shown recently that UV photolysis of the α-crystallin-bound bis-ANS leads to photoincorporation of the fluorophore to the protein subunits similar to that seen with chaperone GroEL (31) and HSP18.1 (38), and pyrene (13). We have shown recently that UV photolysis of the α-crystallin-bound bis-ANS leads to photoincorporation of the fluorophore to the protein subunits similar to that seen with chaperone GroEL (31) and HSP18.1 (38), and pyrene (13). We have shown recently that UV photolysis of the α-crystallin-bound bis-ANS leads to photoincorporation of the fluorophore to the protein subunits similar to that seen with chaperone GroEL (31) and HSP18.1 (38), and pyrene (13). We have shown recently that UV photolysis of the α-crystallin-bound bis-ANS leads to photoincorporation of the fluorophore to the protein subunits similar to that seen with chaperone GroEL (31) and HSP18.1 (38), and pyrene (13). We have shown recently that UV photolysis of the α-crystallin-bound bis-ANS leads to photoincorporation of the fluorophore to the protein subunits similar to that seen with chaperone GroEL (31) and HSP18.1 (38), and pyrene (13). We have shown recently that UV photolysis of the α-crystallin-bound bis-ANS leads to photoincorporation of the fluorophore to the protein subunits similar to that seen with chaperone GroEL (31) and HSP18.1 (38), and pyrene (13).

The role of hydrophobic sites and the amino acid residues that contribute to their makeup within the multimeric chaperone GroEL has been confirmed (31). The available data show that the bis-ANS binding sequences are part of the chaperone sites in GroEL (31, 39). The bis-ANS binding sites in small heat shock proteins have also been identified (38), but the chaperone sites in those proteins are yet to be determined. The two bis-ANS binding sequences in αA-crystallin, HFSPEDLTVK and HFSPEDLTVKVQEDFVEIHGK (Fig. 3), in part represent the chaperone site we identified earlier (40). On the basis of deuterium exchange studies Smith et al. (41) have also proposed that residues 72–75, in αA-crystallin, are a potential chaperone site. It should be noted that under the experimental conditions described here to determine bis-ANS binding sequences, other hydrophobic sequences in αA-crystallin, namely the residues 3–10, 27–37, or 130–145 did not label with bis-ANS. It is possible that those sequences may be buried inside the protein molecule. The only other sequence that was labeled with bis-ANS was QSLFR peptide. Although it is not a hydrophobic sequence by itself, the sequence can be interpreted as part of an extended hydrophobic region between residues 44 and 57 (Fig. 3). It should also be noted that none of the peptides arising from the COOH terminus of αA-crystallin were labeled with bis-ANS. Since a loss in chaperone activity of αA-crystallin has been correlated with COOH-terminal truncation (20, 42), and no bis-ANS binding site has been identified in that region, further studies are needed to determine the role of this region in chaperoning.

The two regions in αB-crystallin identified as bis-ANS binding sequences are at the COOH-terminal domain (Fig. 3). This is in contrast with the recent report published by Smulders and de Jong (25) on the photoincorporation of bis-ANS to recombinant rat αB-crystallin, where the authors observed incorporation of bis-ANS to the NH2-terminal domain of the protein. Since prior exposure of αA-crystallin to urea can affect the bis-ANS binding (26), it is yet to be determined whether the bis-ANS binding to rat αB-crystallin was influenced by urea used during the isolation of the recombinant protein. Of the two bis-ANS binding sequences identified in αB-crystallin during the present study (Fig. 3), the FSFSNVLQDVK portion of the DRFSVNLQDVK sequence is the same as the mellitin binding sequence determined by cross-linking studies, and the VLVDVIEVHGK sequence is one of the alcohol dehydrogenase binding sites determined earlier (30). The DRFSVNLQDVK sequence follows the alcohol dehydrogenase interacting site in αB-crystallin reported by us earlier (30). In a separate experiment we have determined that another hydrophobic site-specific probe, 1,5-AZNS, binds to αB-crystallin sequence 83–90. The 1,5-AZNS-labeled peptide is the sequence between the two bis-ANS binding sequences in αB-crystallin. The structural differences between bis-ANS and 1,5-AZNS may have contrib-

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te to the difference in the site of incorporation of these two probes to αB-crystallin. Nevertheless, both bind at the highly conserved region of the protein (3, 4).

The two bis-ANS binding sequences in αB-crystallin are separated by 10 amino acids, which have a major in vitro glycation site (43). Glycation of α-crystallin decreases ANS binding. Glycation also reduces chaperone-like activity of α-crystallin (Fig. 6). The data from this study suggest that the glycation-induced loss of chaperone-like activity reported earlier (36) may be due to the modification of Lys residues 90 and 92 of αB-crystallin that may be part of the hydrophobic/chaperone site.

The most hydrophobic region of αB-crystallin, residues 28–34, proposed as a potential chaperone site by deuterium exchange studies (39) was not labeled by bis-ANS during our study. The two bis-ANS binding sequences in αB-crystallin identified during the present study belong to a region of high homology between HSP18.1 and αB-crystallin (38). Earlier studies have shown that this region in HSP18.1 is the primary bis-ANS binding region (38). On the basis of these data it can be stated that the entire third exon sequence of αB-crystallin, which has a high degree of homology to other heat shock proteins (3), is responsible for its chaperone-like function.

Although we estimated the binding of one bis-ANS molecule per subunit of α-crystallin (26), in this study we see two sequences in each subunit as bis-ANS binding sites. The occurrence of two UV-sensitive anilinonaphthalene centers in bis-ANS and the activation of either one of the centers and insertion to the adjacent amino acid in the binding pocket may be the cause for the observation of two peptides as binding sites. Alternately, multiple conformation of α-crystallin subunits and their interaction with bis-ANS may result in labeling of more than one peptide sequence as a binding site.

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