Interaction of 1,1′-Bi(4-anilino)naphthalene-5,5′-Disulfonic Acid with α-Crystallin*

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The hydrophobic sites in α-crystallin were evaluated using a fluorescent probe 1,1′-bi(4-anilino)naphthalene-5-sulfonic acid (bis-ANS). Approximately one binding site/subunit of α-crystallin at 25 °C was estimated by equilibrium binding and Scatchard analysis (K_d = 1.1 μM). Based on fluorescence titration, the dissociation constant was 0.95 μM. The number of bis-ANS binding sites nearly doubled upon heat treatment of the protein at 60 °C. Likewise, the exposure of α-crystallin to 2–3 M urea resulted in increased binding of bis-ANS. Above 3 M urea there was a rapid loss in the fluorescence indicating the loss of interaction between bis-ANS and protein. The α-crystallin refolded from 6 M urea showed tryptophan fluorescence emission similar to the native α-crystallin. However, the refolded α-crystallin showed a 60% increase in bis-ANS binding, suggesting distinct changes on the protein surface resulting from exposure to urea similar to the changes occurring due to heat treatment. The fluorescence of tryptophan in native α-crystallin was quenched by the addition of bis-ANS. The quenching was inversely related to the amount of bis-ANS bound to α-crystallin. Additionally, the binding of bis-ANS reduced the chaperone-like activity of the protein. Photolysis of bis-ANS-α-crystallin complex resulted in incorporation of the probe to both A- and B-subunits, indicating that both subunits in native α-crystallin contribute to the surface hydrophobicity of the protein.

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 the average molecular mass of 800 kDa (2). α-Crystallin is made up of two types of subunits, designated αA and αB with molecular masses 19,832 and 20,079 kDa, respectively (2). 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), and increased expression of αB-crystallin has been demonstrated 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), and chemically denatured (28) proteins and enzymes has been demonstrated. Complex formation between α-crystallin and denatured proteins and enzymes or β- and γ-crystallins has been demonstrated (14, 18). On the basis of these 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 has been observed for the α-crystallin present in high molecular mass aggregates from aged bovine and human lens (29, 30).

It has been proposed that surface hydrophobic sites in the native α-crystallin aggregate are involved in binding of target proteins to α-crystallin during chaperone-like activity display (17). A direct correlation between the extent of α-crystallin hydrophobicity and chaperone-like activity has been demonstrated (31–34). Liang and co-workers (35) in their recent study used recombinant αA- and αB-homopolymers and reported that the relative fluorescence enhancement of ANS1 is greater with αB compared with αA and concluded that αB has higher hydrophobicity. However, so far the amino acid sequences that contribute to the hydrophobic site(s) have not been identified. In a recent report, Smulders and de Jong (36) described that the N-terminal domain of recombinant murine αB-crystallin binds hydrophobic probe bis-ANS. We have recently reported that amino acid residues 57–69 and 93–107 of αB-crystallins interact with heat-denaturating alcohol dehydrogenase (37). Liang and Li (38) reported that there are about 40 ANS binding sites/native α-crystallin. Stevens and Augusteyn (39) have disputed the study of Liang and Li and reported that there is one ANS binding site/24 subunits of α-crystallins. It is rather difficult to explain the stoichiometry of ANS binding to α-crystallin in view of the proposed complex but ordered structure for α-crystallin (2).

In the present study we have determined the binding of bis-ANS to α-crystallin by equilibrium dialysis. The data presented here show the binding of bis-ANS to both A- and B-subunits of α-crystallin and transfer of the energy from protein tryptophan to the bound fluorophore. Furthermore, we show that prior binding of bis-ANS to α-crystallin can affect the chaperone-like activity.

EXPERIMENTAL PROCEDURES

Materials—bis-ANS was 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, ε_385 = 16,790 cm⁻¹ M⁻¹ (40). Ultra pure urea was purchased from U. S. Biochemical Corp. Yeast alcohol dehydrogenase (ADH) was obtained from Sigma. 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-Separa-

tions) as described earlier (21). The α-crystallin thus obtained was

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‡ The abbreviations used are: ANS, 1-anilinonaphthalene-8-sulfonic acid; bis-ANS, 1,1′-bi(4-anilino)naphthalene-5,5′-disulfonic acid; ADH, alcohol dehydrogenase; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography.
Hydroporphic Sites in α-Crystallin

Equilibrium Binding of bis-ANS to α-Crystallin—Bovine α-crystallin (0.3 μg) was incubated with 0–40 μM bis-ANS in 50 mM sodium phosphate buffer, pH 7.0, containing 100 mM NaCl (buffer A) for 1 h at 25 °C. The entire sample was then subjected to equilibrium dialysis in micro-dialysis tubing (Technilab Instruments Inc.) using 1.2-ml volumes of α-crystallin and bis-ANS. Following equilibration for 24 h with stirring, the concentration of bis-ANS in each chamber was measured by its absorbance at 385 nm. The number of bis-ANS binding sites/α-crystallin was determined by Scatchard analysis (41).

Fluorescence Titration of bis-ANS Binding to α-Crystallin—Fluorescence measurements were made on a Perkin-Elmer Spectrofluorimeter model 650–40 with a 3600 data station. The concentration of protein used was approximately 0.125 μM. The excitation and emission slit width were set to 4 nm. Samples with bis-ANS were excited at 390 nm, and the emission was measured at 490 nm in a cuvette with a 1-cm path length or recorded between 400 and 600 nm. Briefly, bis-ANS at several fixed concentrations was mixed with α-crystallin, and the fluorescence was measured. All the measurements were made at 25 °C. The fluorescence intensities of the samples were corrected for the absorption of the dye by the relation (42), 

\[ F_m = F_{obs} \times (OD_{620} + OD_{650}) / 2, \]

where \( OD_{620} \) and \( OD_{650} \) are the optical densities at excitation and emission wavelengths, respectively. To see the effect of urea on bis-ANS binding to α-crystallin, a known amount of protein was mixed with 0–6 M urea in buffer A. After 14 h at 5 °C, bis-ANS was added, and the fluorescence spectra was recorded as above. Blanks without protein were prepared in same buffer.

For energy transfer experiments, an excitation wavelength of 295 nm was used to reduce the involvement of tyrosine fluorescence from the protein. The efficiency of energy transfer (EET) (43) from tryptophan to bis-ANS was calculated from the formula,

\[ EET = 1 - (Q_d/Q_o), \]

where \( Q_d \) and \( Q_o \) are the relative fluorescence intensities of energy donor (tryptophan) in the presence and the absence of energy acceptor (bis-ANS), respectively. The quenching of tryptophan fluorescence upon the addition of bis-ANS was analyzed according to the equation

\[ F_o - F = (F_o - F_{corr}) - K_{d} \times [\text{bis-ANS}]. \]

A plot of \( F_o - F \) versus \( [\text{bis-ANS}] \) yields a straight line whose slope equals \( K_{d} \) of bis-ANS.

Photoincorporation of bis-ANS into α-Crystallin—α-Crystallin (1 mg) was mixed with 100 μg of bis-ANS in 1 ml of buffer A, and the excess bis-ANS was removed by dialysis using buffer A. After dialysis the amount of bis-ANS was obtained (excitation, 390 nm). Bis-ANS-bis-ANS was treated with 5 mM dithiothreitol for 2 h and then dialyzed against buffer A. The identities of α-A- and α-B-subunit peaks were confirmed by SDS-PAGE and pooled separately. The fluorescence spectrum of both the α-A- and α-B-crystallin fractions was obtained (excitation, 390 nm).

Thermal Denaturation and Light Scattering Assay—The capacity of the bis-ANS treated α-crystallin to protect against heat-induced aggregation of ADH was determined according to the procedure described earlier (19). Briefly, 400 μg of ADH was incubated in 50 mM PO₄, pH 7.0, and 0.1 M NaCl (buffer A) in the presence or the absence of different amounts of the bis-ANS-α-crystallin in a final volume of 1.0 ml. bis-ANS-α-crystallin was prepared by saturating bis-ANS binding sites in α-crystallin at 48 °C and removing excess bis-ANS by dialysis. The aggregation of proteins at the specified temperature was followed by recording the increase in light scattering as a function of time in a Perkin-Elmer Lambda 3 spectrophotometer equipped with multichannel detector attached to a circulating water bath.

RESULTS

Interaction of bis-ANS with α-Crystallin—bis-ANS is an environment-sensitive probe that shows an emission maximum at 533 nm in aqueous medium (46). When bis-ANS binds to a hydrophobic protein such as α-crystallin, its fluorescence intensity increases severalfold, and the emission maxima is blue shifted (32). Titration of α-crystallin (0.125 μM) with bis-ANS (0–10 μM) gave a hyperbolic plot as shown in Fig. 1, suggesting the saturation of bis-ANS binding sites in α-crystallin. The titration data, when analyzed by a double reciprocal plot (47), gave a \( K_d \) value of 0.95 μM for the binding of bis-ANS to α-crystallin.

The bis-ANS binding to α-crystallin was also determined by equilibrium dialysis method. α-Crystallin was dialyzed against bis-ANS in buffer A at 4 °C for 24 h following a initial incubation for 1 h at 25 °C. After the dialysis the amount of bis-ANS bound to α-crystallin was estimated on the basis of 385 nm absorption of bis-ANS-α-crystallin complex. Scatchard analysis (41) of bis-ANS bound to different concentrations of α-crystallin is shown in Fig. 2. The \( K_d \) and \( n \) values calculated from the graph were 1.15 μM and 40, respectively. An average molecular mass of 800 kDa for α-crystallin (2) was used in these calculations.

Effect of Heat Treatment on bis-ANS Binding to α-Crystallin—Earlier reports have shown that interaction of ANS or bis-ANS with α-crystallin is temperature-dependent (31–34). When α-crystallin was incubated with excess of bis-ANS at 48 or 70 °C for 1 h and dialyzed to remove the unbound dye, considerably higher amounts of bis-ANS remained bound to α-crystallin compared with bis-ANS-α-crystallin incubated at 25 °C. α-Crystallin incubated at 48 °C was able to bind 80 ± 4 mol of bis-ANS, whereas prior heat treatment of α-crystallin at 70 °C resulted in binding of 110 ± 6 mol of bis-ANS. This translated to 2 and 2.7 binding sites/subunit of α-crystallin at 48 and 70 °C, respectively.

Effect of Urea on bis-ANS Binding to α-Crystallin—The effect of varying concentration of urea on bis-ANS binding to α-crystallin is shown in Fig. 3. Maximum dye binding to α-crystallin was observed in the presence of 3.0 M urea. The increase in bis-ANS fluorescence at low urea concentrations and the sharp decrease in the fluorescence above 3 M urea suggest an initial exposure of the buried binding sites at low urea concentration and the destruction of protein quaternary, tertiary, and secondary structures at higher urea concentration. The bis-ANS emission maximum also red shifted in samples with urea (data not shown). Fig. 3 also shows the change in tryptophan emission maximum at various concentrations of urea used. The
Energy Transfer from Tryptophan to Bound bis-ANS—Energy transfer was detected from the overlap of the tryptophan fluorescence emission spectrum of \( \alpha \)-crystallin and excitation spectrum of bis-ANS. The emission spectra of \( \alpha \)-crystallin and the \( \alpha \)-crystallin-bis-ANS complex when excited at 295 nm are shown in Figs. 5 and 6. In the absence of bis-ANS, the fluorescence was emitted maximally at 336 nm due to the excitation of tryptophan residues in \( \alpha \)-crystallin. Upon addition of bis-ANS to \( \alpha \)-crystallin there was a decrease in the tryptophan fluorescence at 336 nm concurrent with an increase in the fluorescence at 488 nm. Because free bis-ANS does not fluoresce when excited at 295 nm, the 488 nm band represents emission due to tryptophan-excited bis-ANS bound to \( \alpha \)-crystallin. The isoemmissive point was 420 nm. A \( K_d \) value of 1.4 \( \mu \)M was obtained for the binding of bis-ANS to \( \alpha \)-crystallin by the analysis of tryptophan quenching data (44). This value is not significantly different from the \( K_d \) value obtained by equilibrium dialysis method. A similar value was obtained when the tryptophan quenching data were analyzed by a

FIG. 4. \( \alpha \)-Crystallin interaction with native and urea-denatured and refolded \( \alpha \)-crystallin. Experimental details are under “Experimental Procedures.” Broken line, native \( \alpha \)-crystallin; solid line, refolded \( \alpha \)-crystallin.

FIG. 5. Energy transfer between the tryptophan residues and bound bis-ANS in \( \alpha \)-crystallin. Excitation wavelength was 295 nm. Protein concentration was 1 mg/ml, and the added bis-ANS concentrations were 0 (trace 1), 2.2 (trace 2), 4.4 (trace 3), 6.6 (trace 4), 8.8 (trace 5), 11 (trace 6), and 13.2 \( \mu \)M (trace 7).

FIG. 6. Concentration dependence of energy transfer between tryptophan and bound bis-ANS. \( \alpha \)-Crystallin was excited at 295 nm, and emission was measured at 338 and 490 nm. bis-ANS at 150 \( \mu \)M was added in 15-\( \mu \)l aliquots, and emission was measured after 30 s. Measured fluorescence intensities were corrected for the absorption of bis-ANS at 295, 338, and 490 nm, respectively. ■, 338 nm fluorescence; ●, 490 nm fluorescence.
modified Stern-Volmer method (48, 49). The maximum transfer efficiency of 0.9 was obtained when the quenching data were analyzed by the method of Wallach et al. (50). The transfer efficiency was also calculated by the method described by Stryer (43) and found to be 0.83.

**Effect of bis-ANS Binding on Chaperone-like Activity of α-Crystallin**—The effect of prior binding of bis-ANS to α-cristallin on its chaperone-like activity is shown in Fig. 7. Only a marginal decrease in the α-cristallin chaperone-like activity was observed when tested with ADH.

**bis-ANS Incorporation to α-Crystallin**—It has been shown that bis-ANS can be incorporated to specific hydrophobic sites in proteins by UV activation and that the dye incorporated into the proteins remains sensitive to the polarity of its general environment (45). Furthermore, it has been shown that bis-ANS binding sequences in molecular chaperones can be identified by photocross-linking of the dye to the protein, peptide mapping, and sequencing (45). During this study bis-ANS was initially allowed to bind to α-cristallin by the addition of saturating amounts of the probe. The excess probe was removed by dialysis. The α-cristallin-bis-ANS complex was photolyzed by UV-A light (366 nm). Fig. 8 shows the SDS-PAGE of the bis-ANS-α-cristallin complex subjected to a 10-min photolysis. The fluorescence seen in the 20-kDa region (Fig. 8, lane 3, left panel) was due to the covalently bound bis-ANS. Lane 2 of Fig. 8 contains the unphotolyzed α-cristallin-bis-ANS complex. The αA- and αB-subunits of photolyzed α-cristallin-bis-ANS complex were separated by HPLC. SDS-PAGE of αA and αB is shown in Fig. 8 (lanes 4 and 5). Both lanes show fluorescence in αA- and αB-cristallin protein band region. These data suggest that both αA- and αB-subunits may be contributing to the bis-ANS binding sites in α-cristallin.

**DISCUSSION**

Although the presence of surface hydrophobic sites on α-cristallin has been known for a number of years (51), only recently has considerable interest been shown in the hydrophobic sites within α-cristallins because these sites have been implicated in chaperone-like function of the protein (17, 31–36, 38, 39). There is, however, disagreement with respect to the number of hydrophobic sites available within α-cristallin as determined by the environment-sensitive probe ANS (38, 39). We used bis-ANS instead of ANS to determine the nature of hydrophobic sites in α-cristallin. Consistent with the report published earlier (32), our data show that α-cristallin has hydrophobic sites capable of binding environment-sensitive probe bis-ANS. bis-ANS binds to α-cristallin very tenaciously. Unlike ANS-α-cristallin, dialysis of bis-ANS-α-cristallin for 2 days in buffer A did not dissociate the protein bound bis-ANS. We determined the affinity of bis-ANS to α-cristallin by three methods. Although the fluorescence titration method gave a $K_d$ of 0.95 μM (Fig. 1), the equilibrium dialysis method and the tryptophan quenching studies gave $K_d$ and $K_{d_{app}}$ values of 1.15 μM (Fig. 2) and 1.4 μM (Fig. 6), respectively. The difference in dissociation constants determined by the three methods was not significant. These values are five to eight times lower than the $K_d$ value for ANS binding to α-cristallin reported recently (39).

We determined the number of bis-ANS binding sites/subunit of α-cristallin by equilibrium dialysis and measuring the absorbance of bound bis-ANS at 385 nm rather than the fluorescence titration method for the following reasons. The titration method is likely to give incorrect values if all the binding sites are not homogenous. The quantum yield of the dye bound at different sites in subunits may vary significantly. It has also been shown recently that αA- and αB-cristallins have different ANS binding characteristics (35). From earlier studies we know that there is no constant stoichiometry between the two types of subunits (2). Therefore if we do not know the contribution of each type of binding site to the total sites, a reliable estimate of the total binding sites cannot be made by fluorescence titration studies. In view of this an estimate of binding sites determined earlier by fluorescence titration is likely to include significant error. The equilibrium dialysis method we employed involves the direct estimation of the bound dye on the basis of molar absorbance. The absorbance of bis-ANS is not altered upon binding to proteins. Our estimate of one bis-ANS binding site/α-cristallin subunit at 25 °C is much higher than one ANS binding site/24 subunits estimated by titration method (39). The estimation of one binding site/24 subunits of α-cristallin cannot be explained by any of the proposed structural models (2) for the protein. The increase in the bis-ANS binding sites at elevated temperature, the earlier demonstration that α-cristallin displays higher chaperone-like activity if exposed to elevated temperatures (32–34), and the estimated stoichiometry of interactions between the target protein and α-cristallin during chaperoning (18) suggest that one to three hydrophobic sites may be involved in binding a target protein.

Although we could not quantify the extent of A- and B-subunit contribution to the total bis-ANS binding sites on α-cristallin, the photocross-linking experiments (Fig. 8) show that both αA- and αB-subunits in α-cristallin bind bis-ANS. By cross-linking studies we have recently shown that both A- and B-subunits of α-cristallin participate in chaperone-like activity display (37). As has been reported for rat αB-cristallin (36), bovine α-cristallin also showed a decrease in chaperone-like activity after complexing with bis-ANS. Although only a partial loss in chaperone-like activity of α-cristallin was observed when it was complexed with bis-ANS (Fig. 7), the data support the hypothesis that hydrophobic sites in αA and αB are involved in chaperone activity. The high residual chaperone-like activity...
Hydrophobic Sites in α-Crystallin

activity observed for bis-ANS-α-crystallin may be due to the retention of the hydrophobic nature of the site subsequent to the binding of the probe. We have also observed that glycated α-crystallin binds less ANS and displays lower chaperone-like activity. The residual chaperone-like activity of the glycated α-crystallin was directly proportional to the residual ANS binding.

These data suggest the involvement of same site/aminino acid residues in α-crystallin during ANS binding, glycation, and chaperone-like activity.

The binding of ANS or bis-ANS to α-crystallin also increases after heat treatment (31–34), partial unfolding by urea (Fig. 3), or urea denaturation and refolding (Fig. 4). Although earlier studies have demonstrated that increased bis-ANS or ANS fluorescence is seen with α-crystallin at 25 °C that has been exposed once to higher temperature (32–34), there are no reports on the number of newly formed hydrophobic sites. Our data show that approximately 40 new bis-ANS binding sites are formed per α-crystallin molecule when it is heated to 48 °C. Retention of the additional bis-ANS sites by α-crystallin exposed to higher temperatures (48 and 72 °C) points to the inability of α-crystallin to regain its native structure after cooling. It is unlikely that in our experiments bis-ANS was trapped in α-crystallin at higher temperature because experiments where bis-ANS was added after cooling α-crystallin also showed increased bis-ANS binding. The increased binding of bis-ANS to urea-denatured and renatured α-crystallin suggests that although the refolded α-crystallin displays intrinsic fluorescence similar to the native protein as reported earlier (52), there is a measurable difference with respect to hydrophobic sites between the native protein and renatured protein (Fig. 4).

Therefore caution should be exercised in interpreting the recombinant α-crystallin chaperone-like activity data if the protein isolation step involves urea.

The increased binding of bis-ANS to structurally perturbed α-crystallin reported here is similar to that observed with molecular chaperone GroEL (49, 53, 54), but the number of bis-ANS that can bind to native or partially unfolded α-crystallin is greater. Although the oligomeric GroEL (800 kDa), binds one or two bis-ANS molecules in its native form and about 14 bis-ANS molecules in the presence of ~2.5 M urea (54), we estimated that 40 and 65 bis-ANS molecules bind to α-crystallin in its native form and in the presence of 2.5 M urea, respectively. The high number of available surface hydrophobic sites are probably responsible for the increased capacity of α-crystallin to bind denaturing proteins compared with GroEL, which is believed to bind one protein at a time (55).

The α-crystallin A-subunit has one Trp (Trp-9), whereas the B-subunit has two tryptophans (Trp-9 and 60) (2). The fluorescence emission studies have shown that two tryptophans near the N terminus are relatively buried and the other Trp is near the surface (56). The fluorescence quenching studies reported here suggest that the bis-ANS binding sites are relatively closer to the Trp. During our studies to identify the α-crystallin amino acid sequences involved in chaperone-like function, we observed that one of the binding sequences, APSWIDT-\(\cdot\)-crystallin reported here is similar to that observed with mo-

Hydrophobic Sites in α-Crystallin

2 K. K. Sharma, unpublished data.
Hydrophobic Sites in α-Crystallin

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