Insights into Hydrophobicity and the Chaperone-like Function of αA- and αB-crystallins

AN ISOTHERMAL TITRATION CALORIMETRIC STUDY*

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α-Crystallin, composed of two subunits, αA and αB, has been shown to function as a molecular chaperone that prevents aggregation of other proteins under stress conditions. The exposed hydrophobic surfaces of α-crystallins have been implicated in this process, but their exact role has not been elucidated. In this study, we quantify the hydrophobic surfaces of αA- and αB-crystallins by isothermal titration calorimetry using 8-anilino-1-naphthalenesulfonic acid (ANS) as a hydrophobic probe and analyze its correlation to the chaperone potential of αA- and αB-crystallins under various conditions. Two ANS binding sites, one with low and another with high affinity, were clearly detected, with αB showing a higher number of sites than αA at 30 °C. In agreement with the higher number of hydrophobic sites, αB-crystallin demonstrated higher chaperone activity than αA at this temperature. Thermodynamic analysis of ANS binding to αA- and αB-crystallins indicates that high affinity binding is driven by both enthalpy and entropy changes, with entropy dominating the low affinity binding. Interestingly, although the number of ANS binding sites was similar for αA and αB at 15 °C, αA was more potent than αB in preventing aggregation of the insulin B-chain. Although there was no change in the number of high affinity binding sites of αA and αB for ANS upon preheating, there was an increase in the number of low affinity sites of αA and αB. Preheated αA, in contrast to αB, exhibited remarkably enhanced chaperone activity. Our results indicate that although hydrophobicity appears to be a factor in determining the chaperone-like activity of α-crystallins, it does not quantitatively correlate with the chaperone function of α-crystallins.

Small heat shock proteins (sHSPs)1 form a large family of proteins of 12–43 kDa found ubiquitously in cells as large (200–800 kDa) oligomeric complexes (1, 2). α-Crystallin, a member of the sHSP group, constitutes a major portion of the eye lens cytoplasm. Its concentration in the lens fiber cells can make up to 50% of the total protein that exists as a polydisperse aggregate (2–4). Although the average size of α-crystallin is ∼700 kDa, its size can range from 360 to >1000 kDa depending on the source, the purification protocols employed, and the presence of posttranslational modifications (2–4). Lenticular α-crystallin is a hetero-oligomer with two subunits, αA and αB, mostly present in a stoichiometry of 3:1. αA and αB are 20 kDa each and share ∼60% sequence identity (3–5). Apart from their presence in the lens, α-crystallins are also found in many non-lenticular tissues. For example, αB-crystallin is expressed in the heart, skeletal muscle, kidney, and brain (5). Increased levels of αB-crystallin have been observed in many neurodegenerative disorders and tumors (5, 6). Unlike αB-crystallin, αA-crystallin appears to be largely lens-specific. Both homopolymers and heteropolymers of α-crystallin exhibit chaperone-like activity similar to that of other sHSPs (4–7). Several studies have demonstrated that α-crystallin suppresses protein aggregation, protecting them from heat, chemicals, and UV light irradiation (4–7). Hence, in addition to providing refractive properties to the eye lens, α-crystallin is instrumental in maintaining transparency of the eye lens with its chaperone-like activity (4–8).

Despite high sequence homology, the relative importance of αA- and αB-crystallins is not completely understood (4, 5). Whereas αA-crystallin knock-out mice develop cataracts, αB knock-out mice show degeneration of specific skeletal muscles with no symptoms of cataracts (8, 9). Likewise, αA-crystallin is more effective than αB-crystallin in conferring protection against UVA light stress-induced apoptosis in lens epithelial cells (10). Primary cultures of αA/−/− lens epithelial cells showed genome instability and hyperproliferation, suggesting that αB-crystallin regulates cell division through the stabilization of cytoskeleton filaments (10–12). Regarding the ability of αA- and αB-crystallins to act as chaperones, different studies have reported conflicting results (14–18). At this point, it is not clear whether the apparent differences in tissue specificity and distinct physiological demands/necessities of αA- and αB-crystallins have differentially influenced their chaperone ability and other structural properties.

Although the mechanism of chaperone function is not completely understood, the ability of α-crystallin to specifically recognize aggregation-prone non-native proteins and prevent their subsequent insolubilization is well established (3, 4, 7). Numerous studies implicate surface-exposed hydrophobic sites on α-crystallin and other sHSPs in binding to partially unfolded proteins (14–23). The finding that increased exposure of hydrophobic surfaces on structurally perturbed α-crystallin is associated with increased chaperone-like function substantiates the role of hydrophobicity in the chaperone function of sHSP (14–16, 21). However, enhanced chaperone-like activity with an increase in temperature was not similarly associated.

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1 The abbreviations used are: sHSP, small heat shock protein; ANS, 8-anilino-1-naphthalenesulfonic acid; DTT, dithiothreitol; ITC, isothermal titration calorimetry.
with increased hydrophobicity with recombinant αA- and αB-crystallins (14, 16). αA- and αB-crystallins differ not only in their hydrophobic character with temperature but also in their secondary and tertiary structure, molecular size, and other physicochemical properties (14–16). This makes an exact correlation between hydrophobicity and chaperone activity difficult to establish.

Historically, hydrophobicity has been measured qualitatively and quantitatively by spectroscopic methods like fluorescence and equilibrium dialysis, mostly by using two common fluorescent dyes, 8-anilino-1-naphthalenesulfonic acid (ANS) and bis-ANS (19, 20, 22, 24). In such cases, indirect methods such as van’t Hoff analysis for obtaining thermodynamic parameters to elucidate ANS binding are often limited. For instance, one study reported a stoichiometry of one ANS binding site per 24 subunits (22), whereas others have reported one ANS or bis-ANS binding site per subunit of α-crystallin (14, 19, 24). More sensitive and accurate methods are needed to resolve such ambiguities. High sensitivity isothermal titration calorimetry (ITC) is one such method for quantifying the hydrophobic sites and measuring the thermodynamic parameters directly and accurately. In the present study, we have employed ITC to determine the number of binding sites and the thermodynamics of ANS binding to αA- and αB-crystallins. We correlate hydrophobicity and chaperone activity of αA- and αB-crystallins at different temperatures to get greater insight into the role of hydrophobicity in the chaperone-like function of α-crystallin.

**EXPERIMENTAL PROCEDURES**

**Materials**—ANS, β-mercaptoethanol, acrylamide, bis-acrylamide, bovine serum albumin, isopropyl 1-thio-β-D-galactopyranoside, DTT, and insulin were obtained from Sigma. Sephacryl 300 HR and MonoQ were from Amersham Biosciences. All other chemicals were procured from local companies.

**Purification of Recombinant αA- and αB-crystallins**—Bacterial (BL21) cells containing expression vectors (pET23d) of human αA- and αB-crystallins were a generous gift from Dr. J. Mark Petrasch (Washington University, St. Louis, MO). Proteins from 1-liter cultures were extracted and purified according to the procedures described previously (14). Briefly, αA- and αB-crystallins were overexpressed in *Escherichia coli* BL21 cells containing the respective vectors by isopropyl 1-thio-β-D-galactopyranoside induction and purified using MonoQ anion exchange and Sphacyl S-300 gel filtration columns. The purity/homogeneity of αA and αB was determined by using SDS-PAGE. Concentrations of αA and αB were calculated using molar extinction coefficients, (ε)_280, of 16,500 and 19,000 μmol cm⁻¹, respectively.

**Chaperone Activity Assay**—The chaperone activities of αA-crystallin and αB-crystallin were assessed by measuring each one’s ability to prevent the aggregation of insulin denatured by the reduction of disulfide bonds. The aggregation assay was performed essentially as described previously (14). Apparent absorption at 400 nm due to DTT-induced aggregation of insulin was monitored as a function of time in the absence and presence of αA- and αB-crystallins using a Cary100 UV-visible spectrophotometer. For a chaperone assay performed in the presence of ANS, αA- and αB-crystallins were preincubated with saturating amounts of ANS under specific conditions as shown in Table III, and excess ANS was removed by dialysis.

**Isothermal Titration Calorimetric Studies**—Titration calorimetric measurements were performed using a VP-ITC calorimeter (Microcal Inc., Northampton, MA) as described previously (25). In brief, 4–8-μl aliquots of ANS solution (10 mM) were added via a 300-μl syringe with constant stirring at 310 rpm to the sample cell containing 1.4 ml of αA- or αB-crystallin (50 μM), and heat changes accompanying these additions were recorded. An interval of 5 min between injections was chosen for the baseline to stabilize. The protein samples were extensively dialyzed against 20 mM sodium phosphate buffer, pH 7.5 (containing 100 mM NaCl, 1 mM EDTA, and 0.01% NaN₃), before titration. ANS was solubilized in the final dialysate buffer. The titration of ANS solution with buffer alone gave negligible values for the heat of dilution, which were subtracted from the actual titration data. The data so obtained were fitted using a non-linear least squares minimization method for determining the binding stoichiometry (N), binding constant (Kᵢ), and

**RESULTS AND DISCUSSION**

Despite the presence of a wealth of literature on the dependence of the chaperone function of α-crystallin on its surface hydrophobicity, a deterministic role of hydrophobicity in quantitative terms remains unresolved. There are numerous instances where hydrophobicity could not be directly correlated to the chaperone-like activity of αA- and αB-crystallins. For instance, replacement of Phe-71 with Gly in αA-crystallin resulted in loss of chaperone activity despite an increase in surface hydrophobicity with no significant alterations in structure (26). In another study, calf lens αA-crystallin was found to be more hydrophobic but showed lower chaperone activity than αB-crystallin at room temperature (18). *In vitro* modification of bovine α-crystallin with methylglyoxal enhanced the chaperone-like activity, partially in aggregation assays, although hydrophobicity showed a decrease (27). Moreover, apparent differences in the temperature-dependent behavior of αA- and αB-crystallins with respect to chaperone activity, hydrophobicity, and oligomeric size necessitate a critical evaluation of the role of hydrophobicity in αA-crystallin and αB-crystallin function.

**Isothermal Titration Calorimetry Studies**—The polydisperse nature of α-crystallin may complicate the quantification of hydrophobicity by spectroscopic methods. ITC is sensitive and appropriate because it measures the heat change directly, independent of the state of the protein. ANS binding to αA- and αB-crystallins was characterized by using ITC at 15 and 30 °C. Figs. 1 and 2 show the typical titration calorimetry profiles that resulted from the addition of 8-μl aliquots of 10 mM ANS to αA- and αB-crystallins along with the normalized integrated titration data. The results exhibit a monotonic decrease in the exothermic heat of binding with successive injections until saturation is achieved. The data best fitted to nonlinear least squares were provided by means of the two-site binding model. Stoichiometry (N) and thermodynamic parameters (changes in enthalpy (ΔH), free energy (ΔG), and entropy (ΔS)) for the binding of ANS to αA- and αB-crystallins at 15 and 30 °C are listed in Table I. Both αA-crystallin and αB-crystallin showed two sets of binding sites for ANS, low affinity and high affinity, based on the binding constants. Thermodynamic parameters

![Fig. 1. Calorimetric titration profile for the binding of ANS to native αA-crystallin at 30 °C. Panel A, exothermic heat associated with the injection of ANS into the sample cell containing αA-crystallin. Panel B, binding isotherm corresponding to the data in panel A.](http://www.jbc.org/)
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for the binding of ANS to αA- and αB-crystallins indicate that high affinity binding is driven by both enthalpy and entropy changes, whereas the entropy change is dominant for low affinity binding. These data suggest that hydrophobic forces are dominant in the ANS binding to α-crystallin. The fact that αA- and αB-crystallin have two sets of binding sites in agreement with the studies of Stevens and Augustine, who suggested two kinds of interactions for the binding of ANS to α-crystallin (22). Moreover, binding of destabilized mutants of T4 lysozyme to α-crystallin was demonstrated to be biphasic (28).

αA-Crystallin, at 30 °C, showed one high affinity binding site per subunit for ANS and large number of low affinity binding sites. Stoichiometries of higher and lower affinity sites were greater in αB as compared with those in αA at 30 °C (Table I). This supports the view that αB is more hydrophobic than αA. At a low temperature (15 °C), αA crystallin showed an increase in the number of high affinity ANS binding sites but a decrease in the number of low affinity binding sites. Although, both αA and αB displayed similar binding stoichiometry for ANS at 15 °C, their binding constants differed particularly for low affinity binding sites. These data suggest that hydrophobic forces are greater in αB than for αA (19, 32). In the present study, we have used this approach to ascertain the enhanced chaperone function to that of increased hydrophobicity. It is believed that the enhanced chaperone activity may be due to the increased and reorganized hydrophobic sites. If this notion were true, we would expect a greater loss in chaperone activity with prior ANS binding to αA-crystallin over unheated protein has been mainly attributed to its increased aggregate size (30). Furthermore, the increase in size was observed with preheated αA-crystallin but not with αB-crystallin (31). Thus, the aggregate size of α-crystallin may play an important role in the chaperone activity of αA- and αB-crystallins.

Effect of ANS Binding on the Chaperone Activity of αA- and αB-crystallins—Some studies demonstrated that the blocking of hydrophobic sites with the incorporation of ANS or bis-ANS into α-crystallin results in a partial but not complete loss of chaperone activity (19, 32). In the present study, we have used this approach to ascertain the enhanced chaperone function to that of increased hydrophobicity. It is believed that the enhanced chaperone activity may be due to the increased and reorganized hydrophobic sites. If this notion were true, we would expect a greater loss in chaperone activity with prior ANS binding under the conditions, which enhance chaperone activity. However, the results of the present study are not in agreement with the above assumption. For instance, the percent decrease in chaperone activity due to ANS binding was more for αB than for αA (Table III), but αB displayed lower chaperone activity than αA at this temperature (Fig. 3A). However, at 30 °C the loss in activity due to ANS binding appeared to go hand in hand with chaperone activity of αA and αB (Table III and Fig. 3B). The hydrophobicity and chaperone activity paradox appears to be more striking with the preheated crystallins. The percentage loss of chaperone activity due to ANS binding was similar with preheated αA and αB irrespective of the assay temperatures, 15 and 30 °C (Table III). However, the enhanced chaperone potential was remarkable with preheated αA as compared with αB (Fig. 5).
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TABLE I

Calorimetric data of ANS binding to αA- and αB-crystallins at 15 and 30 °C

| Parameters | 15 °C | 30 °C |
|------------|-------|-------|
|            | αA    | αB    | αA    | αB    |
| N1         | 3.5 ± 0.47 | 3.1 ± 0.32 | 0.98 ± 0.06 | 5.9 ± 0.26 |
| K1 (M⁻¹) × 10⁻⁵ | 2.4 ± 0.16 | 4.6 ± 0.42 | 7.8 ± 0.66 | 2.1 ± 0.42 |
| ΔG1 (kcal/mol) | -7.0 ± 0.67 | -7.4 ± 0.61 | -6.4 ± 0.56 | -7.0 ± 0.89 |
| ΔH1 (kcal/mol) | -368 ± 22.4 | -612 ± 29.1 | -1075 ± 93.3 | -1262 ± 196 |
| S1 (cal/mol/K) | 23.4 ± 2.61 | 23.8 ± 1.62 | 18.8 ± 2.31 | 20.2 ± 3.21 |
| N2         | 12.2 ± 1.40 | 11.9 ± 1.57 | 18.0 ± 2.32 | 27.1 ± 3.05 |
| K2 (M⁻¹) × 10⁻⁵ | 1.9 ± 0.09 | 0.42 ± 0.037 | 0.73 ± 0.079 | 4.1 ± 0.60 |
| ΔG2 (kcal/mol) | -5.6 ± 0.63 | -4.7 ± 0.62 | -5.0 ± 0.65 | -10.6 ± 1.20 |
| ΔH2 (kcal/mol) | -127 ± 10.6 | -506 ± 39.2 | -243 ± 16.1 | -56.9 ± 57.0 |
| ΔS2 (cal/mol/K) | 19.2 ± 2.35 | 14.8 ± 1.25 | 16.8 ± 1.05 | 29.0 ± 2.5 |

TABLE II

Calorimetric data of ANS binding to preheated αA- and αB-crystallins at 15 °C

| Parameters | αA    | αB    |
|------------|-------|-------|
|            |       |       |
| N1         | 3.4 ± 0.26 | 3.9 ± 0.18 |
| K1 (M⁻¹) × 10⁻⁵ | 1.4 ± 0.54 | 3.6 ± 1.1 |
| ΔG1 (kcal/mol) | -6.8 ± 0.95 | -7.3 ± 0.56 |
| ΔH1 (kcal/mol) | -731 ± 85.4 | -713 ± 34.1 |
| S1 (cal/mol/K) | 21.1 ± 5.35 | 23 ± 3.65 |
| N2         | 16.2 ± 1.63 | 14.9 ± 0.71 |
| K2 (M⁻¹) | 8238 ± 2645 | 1000 ± 23 |
| ΔG2 (kcal/mol) | -5.1 ± 1.61 | -5.3 ± 1.12 |
| ΔH2 (kcal/mol) | -223 ± 45.1 | -289 ± 30.2 |
| ΔS2 (cal/mol/K) | 17.1 ± 2.53 | 17.4 ± 3.02 |

FIG. 3. Chaperone-like activity of αA- and αB-crystallins against DTT-induced aggregation of insulin at 15 (panel A) and 30 °C (panel B). Trace 1, insulin alone; trace 2, insulin with native αA-crystallin; and trace 3, insulin with native αB-crystallin. Data are average of three assays.

FIG. 4. Calorimetric titration of preheated αA- and αB-crystallins with ANS. Integrated data for net heat exchanged upon ANS binding to preheated αA [●] and αB-crystallin [□].

These findings, taken together, indicate that the correlation between hydrophobicity as assessed by the binding of hydrophobic dyes (both quantitatively and qualitatively) and chaperone activity may often be coincidental rather than a true indication of their function. Furthermore, these results suggest that factors other than hydrophobicity could play a role in the chaperone-like activity of α-crystallin. Liao et al. reported that in addition to surface hydrophobicity, structural stability plays a major role in the chaperone-like activity of α-crystallins (33). Nonetheless, the oligomeric size of sHSP (30, 31, 34) and electrostatic forces between sHSP and their denaturing substrates (18, 35) have also been proposed to play an important role. Further studies would be required to delineate the contribution of these factors in α-crystallin chaperone activity.
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Percentage loss of chaperone activity of αA- and αB-crystallin (native and preheated) in the presence of ANS at 15 and 30 °C

Data are average of three chaperone assays. The chaperone activity in the absence of ANS was considered as 100%.

|       | 15 °C  | 30 °C | 15 °C  | 30 °C |
|-------|-------|-------|-------|-------|
| Native | 62    | 62    | 65    | 63    |
| Preheated | 60    | 54    | 55    | 63    |

In summary, the data presented in this study provide the stoichiometry and the affinity of ANS binding sites of native and preheated recombinant αA-crystallin and αB-crystallin in quantitative terms vis-à-vis their contribution to chaperone activity. Together, the ITC data and the data on chaperone activity (both in the absence and presence of ANS) suggest that there is no direct quantitative correlation between hydrophobicity and chaperone-like activity. Furthermore, this approach may be employed to establish the role of hydrophobicity in the chaperone activity of other sHSPs.

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