Temperature-induced exposure of hydrophobic surfaces and its effect on the chaperone activity of α-crystallin

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Abstract **α-Crystallin, the major protein of the ocular lens, is known to have extensive similarities to small heat shock proteins and to act as a molecular chaperone. The exposure of hydrophobic surfaces on α-crystallin was studied by fluorescence spectroscopy using the hydrophobic probe bis-ANS. Upon heating the protein undergoes a conformational transition which is associated with a marked increase in surface hydrophobicity. This transition, which occurs between approximately 38 and 50°C, lacks reversibility. The increase in surface hydrophobicity correlates with the increased chaperone activity of the protein. These results indicate that hydrophobic interactions play a major role in the chaperone action of α-crystallin.**

Key words: α-Crystallin; Surface hydrophobicity; Molecular chaperone; Thermal transition

1. Introduction

α-Crystallin, the major protein of eye lens, is believed to play a prominent role in the maintenance of the transparency and refractive properties of the eye [1,2]. The protein consists of two highly homologous 20 kDa subunits, αA and αB [1–4]. The A and B chains self-associate to form a large macromolecular complex of a molecular mass of about 800 kDa. The models proposed for the architecture of α-crystallin oligomer include a three layered structure [5], a micellar structure [6], a combination of a micellar and layer model [7], a rhombic dodecahedron [8] and a pore-like structure [9]. However, none of these models is consistent with all experimental findings. In the absence of high resolution structural data, the molecular structure of α-crystallin complex is speculative and remains a matter of controversy.

A number of important developments have occurred in recent years which shed a new light on the potential physiological role of α-crystallin. Believed to be strictly lens-specific protein until recently, α-crystallin has now been found to be expressed in many non-lenticular tissues [10–12]. Furthermore, the protein was found to share extensive structural and functional similarities with the family of ubiquitous small heat shock proteins [12–16]. Finally, it has been shown that α-crystallin, as well as other small HSPs, can act in vitro as molecular chaperone by preventing the aggregation of denaturated (or partially) denatured) proteins [1,17–20]. While this chaperone activity has been postulated to play an important role in vivo [17], the molecular mechanisms of the chaperone action of α-crystallin remain unknown. An important step towards unraveling these mechanisms is to understand the conformational requirements of the interaction between α-crystallin and the substrate proteins.

In a recent study [21] it was shown that the ability of α-crystallin to protect irradiation-induced aggregation of γ-crystallin is strongly temperature dependent. This suggested the potential involvement of conformational transitions in modulating the chaperone activity of α-crystallin. In this communication we have used the hydrophobic fluorescent probe, bis-ANS, to characterize the conformational state of α-crystallin as a function of temperature. Our results indicate that at temperatures above approximately 38°C the protein undergoes an irreversible transition that results in an increased exposure of hydrophobic surfaces. The increase in surface hydrophobicity correlates with the enhancement of the chaperone activity of α-crystallin.

2. Materials and methods

2.1. Materials

α-Crystallin was isolated from young bovine lenses. The protein was fractionated by gel filtration on a Sephadex G-200 column (5 × 75 cm) using 0.01 M Tris, pH 7.8, as an elution buffer. The pooled α-crystallin fractions were further purified by Agarose A-5m chromatography in the same buffer to remove the minor high molecular weight component. The above procedure yields an oligomeric protein of a molecular mass of approximately 800,000. The purified α-crystallin was concentrated and stored at −70°C. Insulin and DTT were obtained from Sigma Chemical Co. (St. Louis, MO) and the fluorescence probe bis-ANS was purchased from Molecular Probes (Eugene, OR). Protein concentration was determined by absorbance measurements using extinction coefficients of 0.8 $(mg/ml)^{-1}$ cm^{-1} (at 280 nm) and 0.95 $(mg/ml)^{-1}$ cm^{-1} (at 275 nm) for α-crystallin and insulin, respectively.

2.2. Fluorescence measurements

Fluorescence of bis-ANS was measured using the SLM 8000 or Perkin Elmer 650-40 fluorometer equipped with a thermostated cell holder. Samples contained 13 μM bis-ANS and 0.06 mg/ml α-crystallin in 50 mM phosphate buffer, pH 7.2. An excitation wavelength of 390 nm was used, and the excitation and emission slits were set at 4 nm. Thermal transition curves were obtained by gradually increasing (or decreasing) the temperature and incubating the sample for 15 min at each temperature prior to obtaining a fluorescence reading. The temperature was monitored by a thermocouple inserted into the cuvette and is accurate within 0.2°C.

2.3. Chaperone activity assay

Chaperone activity of α-crystallin was probed as described by Farahbakhsh et al. [20]. This assay measures the ability of α-crystallin to protect the aggregation of reduced insulin B-chain. Briefly, 0.2 mg of insulin in 0.6 ml of 50 mM phosphate buffer, pH 7.2 (in the absence or presence of α-crystallin), was reduced with 20 mM freshly prepared DTT and the kinetics of B-chain aggregation was followed by measuring the apparent absorption at 400 nm due to light scattering. The measurements were performed at 22°C using a Cary 1 spectrophoto-

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Abbreviations: bis-ANS, 1,1′-bi(4-anilino)naphtalene-5,5′-disulfonic acid; HSP, heat-shock protein; DTT, dithiothreitol.

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meter and a 1 cm pathlength cuvette. α-Crystallin used in these measurements was preincubated at various temperatures for 15 min, and then allowed to equilibrate at room temperature for approximately 12 h prior to the experiment. The concentration of α-crystallin in the aggregation assay was 0.13 mg/ml.

3. Results

3.1. Binding of bis-ANS

The fluorescence quantum yield of a dye 8-anilino-1-naphthalene sulfonic acid (ANS), or its dimeric conjugate bis-ANS, depends strongly on the polarity of the environment, increasing upon binding to hydrophobic sites of proteins [22]. This class of compounds has been widely used as a probe to monitor conformational changes in proteins and to asses surface exposure of hydrophobic sites [23–26]. The fluorescence of bis-ANS in an aqueous buffer is very weak, with a maximum at around 540 nm. In the presence of α-crystallin the maximum of the emission spectrum shifts to 492 nm and there is a strong increase in the fluorescence quantum yield (Fig. 1). The above spectral characteristics indicate that α-crystallin has exposed hydrophobic surfaces which are capable of binding bis-ANS. The presence of such surfaces on α-crystallin is fully consistent with the previous results of Liang and Li [27].

Fig. 2 shows temperature dependence of the fluorescence intensity of bis-ANS in the presence of α-crystallin. In the temperature range up to approximately 36°C there is a linear decrease in the emission intensity of the probe. Since a slope of this decrease is very similar to that observed for bis-ANS in an organic solvent dimethyl formamide (data not shown), we conclude that the initial drop in fluorescence of α-crystallin-bound bis-ANS is unrelated to the conformational state of the protein, but merely represents thermal inactivation of the excited state of the probe. Further increase in temperature results in a relatively rapid increase in fluorescence intensity, until it reaches a maximum at approximately 50°C. Finally, upon further heating of the sample, the upward portion of the fluorescence vs. temperature curve is followed by a linear decline phase which continues up to at least 80°C (data up to only 60°C are shown in Fig. 2). The rise in fluorescence intensity of bis-ANS between approximately 38 and 50°C reflects increased binding of the probe, most likely as a result of the exposure of additional hydrophobic sites on the surface of the protein molecule. The apparent midpoint temperature of this conformational transition is about 45°C.

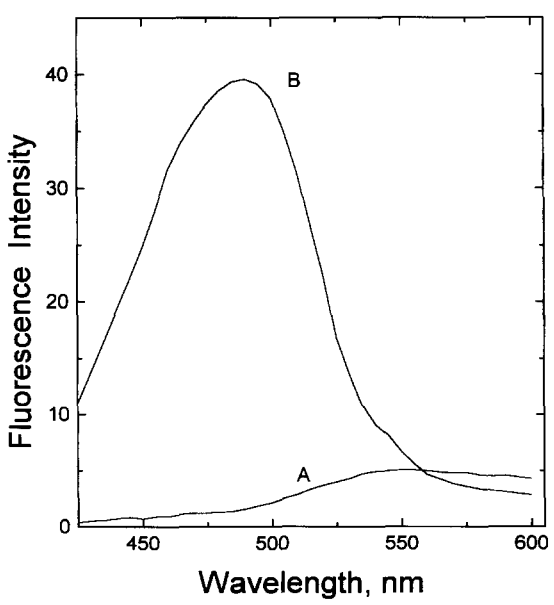

Fig. 1. Fluorescence emission spectra of bis-ANS in 50 mM phosphate buffer, pH 7.2 (A), and in the presence of 0.06 mg/ml of α-crystallin (B); concentration of bis-ANS was 13 μM.

When α-crystallin solution with bis-ANS is heated to 60°C and then gradually cooled, the emission intensity vs. temperature curve does not coincide with that obtained during the heating cycle. Instead, as the temperature is decreased, there is a relatively steep increase in the fluorescence intensity of the heat-pretreated sample (Fig. 2). At temperatures below approximately 50°C, the fluorescence measured during the cooling cycle reaches a markedly higher level than that recorded in the first heating run. For example, at 20°C fluorescence of the sample that was preheated to 60°C is approximately 1.7 as high as that of the sample that was never exposed to high temperatures. The relatively high fluorescence level of heat-pretreated sample remains esentially unchanged upon the incubation at room temperature for at least 24 h. When the sample cooled from 60°C was subjected to a second heating cycle, the fluorescence intensity versus temperature curve was very similar to that obtained during the cooling cycle (Fig. 2).

In order to gain further insight into the nature of thermally induced conformational transition in α-crystallin we have performed an additional experiment in which aliquots of α-crystallin were preincubated for 15 min at different temperatures ranging from 20 to 60°C. The samples were then brought to room temperature and allowed to equilibrate for 5 h before bis-ANS was added and fluorescence intensity was measured. As shown in Fig. 3, when α-crystallin was preincubated at temperatures up to approximately 40°C, upon cooling the protein assumed the conformation in which the binding of bis-ANS was essentially unchanged compared to that of the non-treated protein. However, when higher preincubation temperatures were used, the conformation adopted by the protein after cooling was characterized by significantly increased affinity for bis-ANS. Regardless of the preincubation temperature, the maximum of the emission spectrum remained unchanged at 492 nm. Taken together, the results of the fluorescence measurements indicate that α-crystallin undergoes a conformational transition in the temperature range between 38 and 50°C. Once exposed to elevated temperature (within or above the transition region), upon cooling the protein does not return to its original conformational state but adopts a conformation which is characterized by a markedly enhanced affinity for bis-ANS.

3.2. Chaperone activity of α-crystallin

In order to assess the effect of temperature-induced conformational changes on the chaperone activity of α-crystallin we have used the assay which monitors the ability of α-crystallin to prevent the aggregation of insulin B chains. Reduction of the insulin interchain disulfide bond leads to aggregation and precipitation of the B chain. As shown previously [20], this process can be suppressed by α-crystallin which binds the aggregation-prone protein. The extent of protection of B chain from aggregation depends on the relative concentration of insulin and the chaperone, with complete protection occurring at a stoichio-

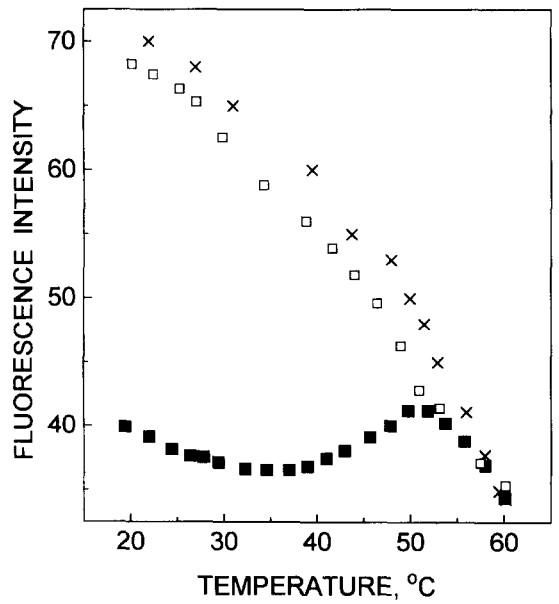

Fig. 2. Temperature dependence of fluorescence emission intensity (490 nm) of bis-ANS (13 μM) in the presence of α-crystallin (0.06 mg/ml) in 50 mM phosphate buffer, pH 7.2. (■) First heating cycle; (×) first cooling cycle; (□) second heating cycle. See text for details.

metry of approximately one molecule of B chain per 1.7 mol of α-crystallin monomer [20]. In the present study we have used a relatively low concentration of α-crystallin, so that only partial protection from aggregation could be observed. Fig. 4 shows the results of a representative experiment in which three different samples of α-crystallin were used: one that was never heated above room temperature, one that was preincubated at 50°C and one that was preincubated at 60°C. Clearly, the protein preincubated at high temperature is significantly more potent in suppressing aggregation of insulin B-chain than that which was never exposed to elevated temperatures. Furthermore, a correlation exists between the chaperone activity of various samples of α-crystallin and the exposure of hydrophobic surfaces as assessed by the bis-ANS binding assay (see data of Fig. 3).

4. Discussion

The mechanism by which α-crystallin recognizes and captures the aggregation-prone folding intermediates of target proteins is at present unknown. In the case of other classes of molecular chaperones, especially the chaperonin GroEL from *E. coli*, it has been widely postulated that surface hydrophobicity is a necessary characteristic of both the substrate as well as the chaperone itself [25,26,28–30]. The presence of appropriately placed hydrophobic patches has been also suggested to be a prerequisite for the effective chaperoning by α-crystallin [21]. However, the experimental assessment of conformational requirements of the latter protein is complicated by the fact that the chaperone activity of α-crystallin is typically studied by probing its ability to suppress the aggregation of thermally denatured proteins. The aggregation experiments are usually performed at elevated temperatures that, depending on the stability of a substrate protein, range between approximately 45 and 65°C [17,19,31]. Therefore, in order to elucidate the molecular interactions between α-crystallin and thermally denaturated (or partially denaturated) substrates it is essential to understand how the conformation of α-crystallin itself is modulated by elevated temperature.

It should be noted here that α-crystallin is often referred to as a protein of a very high thermal stability [7,32]. However, our recent Fourier-transform infrared spectroscopy study has challenged this notion by providing evidence that the protein undergoes a major loss of its native secondary structure in the temperature range between approximately 50 and 70°C [33,34]. Furthermore, more subtle structural changes, which represent partial dissociation of the oligomeric α-crystallin complex, have been reported at lower temperatures [7,35]. In the present study, we have focused on the surface hydrophobicity of α-crystallin and its modulation by temperature. The fluorescence of a hydrophobic probe bis-ANS clearly indicates that upon heating α-crystallin undergoes a structural/conformational transition between approximately 38 and 50°C. This transition is manifested by an increase exposure of hydrophobic surfaces. Furthermore, the thermotropic changes in α-crystallin detected by bis-ANS binding appear to lack reversibility. Once exposed to the temperatures within or above the transition region, the protein upon cooling does not return to its original conformational state but adopts a conformation characterized by significantly increased surface hydrophobicity.

It has been reported that the apparent size of α-crystallin oligomer changes, in an irreversible manner, in response to incubation at elevated temperatures, reaching a minimum at around 37°C [35]. The above temperature is very close to that corresponding to the onset of the conformational transition detected by bis-ANS binding. However, it is highly unlikely that the temperature-induced increase in surface hydrophobicity and the previously described changes in the size of α-crystallin

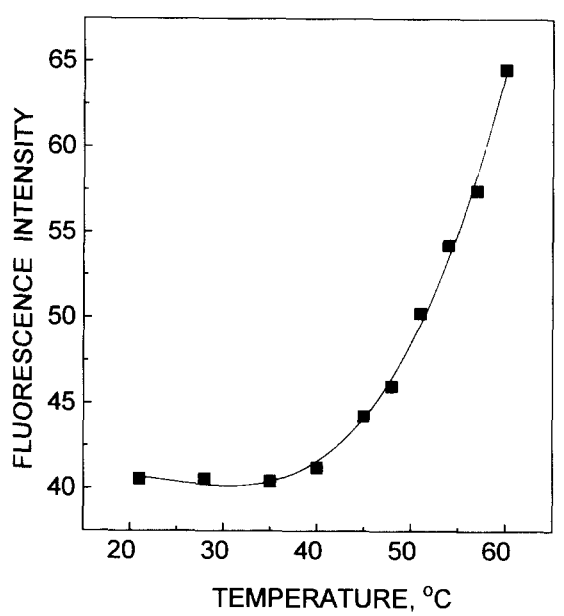

Fig. 3. Fluorescence emission intensity at 22°C of bis-ANS (13 μM) in the presence of α-crystallin as a function of preincubation temperature. Samples of α-crystallin (0.06 mg/ml in 50 mM phosphate buffer) were preincubated at different temperatures for 15 min and cooled down to room temperature. bis-ANS (13 μm final concentration) was then added and fluorescence intensity measured at 490 nm at room temperature.

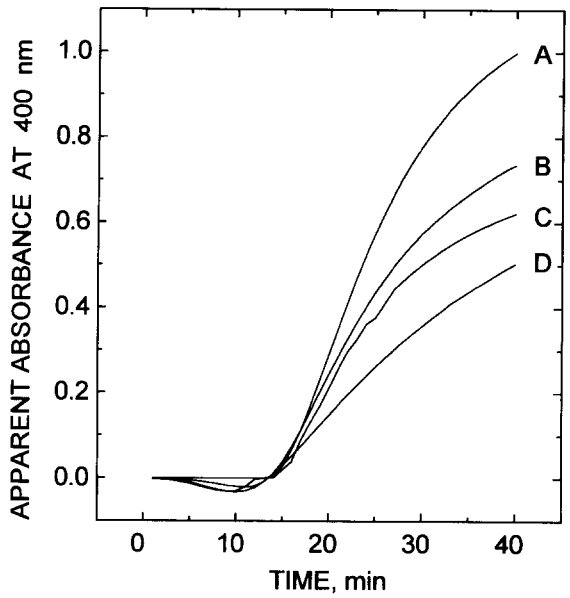

Fig. 4. DTT-induced aggregation of insulin B chain at 22°C in the absence and presence of α-crystallin (0.125 mg/ml) preincubated at different temperatures. (A) No α-crystallin; (B) α-crystallin preincubated at 22°C; (C) α-crystallin preincubated at 50°C; (D) α-crystallin preincubated at 60°C.

complex are different manifestations of the same molecular event. The response of α-crystallin size to temperature is biphasic: partial dissociation of the complex between 20 and 37°C is followed by reassociation as the temperature is increased above 37°C [35]. In contrast, no changes in surface hydrophobicity could be detected by bis-ANS binding below approximately 38°C. The molecular events responsible for the transition between 38 and 50°C likely involve subtle alterations in the tertiary structure of individual subunits and/or the reorientation of these subunits within the oligomer in a manner that results in an increased exposure of hydrophobic patches. On the other hand, the changes in bis-ANS binding at higher temperatures may represent more complex events, as a major transition in the protein backbone conformation has been detected by infrared spectroscopy between approximately 50 and 70°C [33,34].

In addition to detecting and characterizing a new thermotropic transition in α-crystallin, this study also demonstrates that there is a strong correlation between the surface hydrophobicity and the chaperone activity of the protein: as the exposure of hydrophobic surfaces is increased, α-crystallin becomes more effective in binding and preventing aggregation of a model protein on its denaturation pathway. The latter observation provides experimental evidence to support the view that binding of substrate proteins by molecular chaperones in general, and α-crystallin in particular, is largely driven by hydrophobic interactions.

While α-crystallin was believed for many years to be strictly lens-specific protein, recently this protein has been found to be present in many non-lenticular tissues [10–12]. Moreover, there is a growing evidence that α-crystallin, or at least the B-subunit, is a functional small heat shock protein. Indeed, αB has been shown to be inducible by heat and other stress conditions [15,36]. In this context, it is worthwhile noting that the conformational transition described in this study occurs within the heat shock temperature range. It has been speculated in the past that thermotropic changes in the conformation of other classes of HSPs may play a role in the heat shock response [37]. Our data support this general hypothesis and extend it to the family of small heat shock proteins. Furthermore, while in the present work the increase in surface hydrophobicity of α-crystallin has been induced by elevated temperature, it is conceivable that the exposure of hydrophobic surfaces can also be modulated by other factors. Thus, conformational transitions similar to that described in this study may constitute an integral part of the mechanisms employed to control the functional state of α-crystallin in vivo, both in the lens as well as in nonlenticular tissues.

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