Temperature dependent chaperone-like activity of alpha-crystallin

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Received 21 March 1995

Abstract Alpha-crystallin, a multimeric protein present in the eye lens, is known to have chaperone-like activity in preventing the aggregation of enzymes and other crystallins. We have studied the chaperone-like activity of this protein towards the aggregation of insulin B chain, induced by reducing the interchain disulphide bond with dithiothreitol. At room temperature, there is no detectable protection (at a 1:1 (w/w) ratio of insulin: α-crystallin) against the aggregation of insulin B chain by α-crystallin, whereas it completely prevents this aggregation at 40°C. We have monitored the temperature dependence of the protection of aggregation by α-crystallin; the protection increases sharply above 30°C and reaches almost 100% by 41°C. Probing the hydrophobic surfaces of α-crystallin with the hydrophobic fluorophore 8-anilino-1 naphthalene sulfonate suggests that the hydrophobic surfaces of α-crystallin are exposed to a greater extent above 30°C. A complete prevention of the aggregation is achieved at 27.6°C by increasing the concentration of α-crystallin by more than 8 fold. Similar temperature dependent chaperone-like activity of α-crystallin is observed towards the aggregation of zeta-crystallin, an enzyme crystallin from guinea pig. We have earlier shown that α-crystallin exposes hydrophobic surface(s) at temperatures above 30°C. These results support our earlier hypothesis [Raman, B. and Rao, Ch.M. (1994) J. Biol. Chem. 269, 27264–27268] that the chaperone-like activity of α-crystallin is more pronounced in its structurally perturbed state.

Key words: Chaperone; Aggregation; Insulin; Alpha-crystallin; Zeta-crystallin

1. Introduction

Alpha-crystallin of the eye lens is a multimeric protein, made up of the two homologous gene products of α_A and α_B. It had been thought to be a lens specific, structural protein until a few years ago. Identification of this protein in several non-lenticular tissues such as heart, muscle and kidney [1–4] suggests that it might have a role to play in other functions. It is structurally related to small heat-shock proteins (Hsps), behaves in several ways like Hsps [5–8] and its expression can be induced by thermal [5] and osmotic stress [9]. Like some Hsps [10–12] it is known to interact with membranes [13,14], cytoskeletal elements [15,16], and to modulate the intermediate filament assembly [17]. Mixed assemblies of α_B and some Hsps have been observed both in vivo [18,19] and in vitro [8].

α-Crystallin is heat stable up to 100°C [20] and recent studies have shown that it exhibits chaperone-like activity in preventing the aggregation of other proteins [21–26]. It binds ATP [27] and possesses autokinase activity [28]. Whether the chaperone-like activity of α-crystallin has functional significance in the eye lens is not clear yet. However, the chaperone-like activity of α-crystallin has been shown to decrease on treatment with calpain II in vitro and in selenite induced cataract in vivo [29]. It is possible that α-crystallin might chaperone the formation of transparent and refractive eye lens and also may help keep it that way by interacting with aged/damaged proteins [25]. Thus, one can speculate that failure of α-crystallin may lead to loss of transparency as in cataract.

We hypothesised that α-crystallin prevents aggregation of non-native structures by providing appropriately placed hydrophobic surfaces; a structural transition above 30°C enhances the protective ability, perhaps by increasing or reorganizing the hydrophobic surfaces [25]. In order to test our hypothesis and to have a better understanding of the chaperone-like activity of α-crystallin, we have investigated the effect of α-crystallin on the aggregation of B chain of insulin on treatment with dithiothreitol (DTT) and on the aggregation of zeta-crystallin, an enzyme crystallin from the eye lens of guinea pig. Results clearly support our hypothesis. Thus, structural perturbation appears to be important in the chaperone-like activity of α-crystallin.

2. Materials and methods

2.1. Isolation and purification of α-crystallin

Fresh calf lenses were homogenized in Tris-HCl buffer, pH 7.2, containing 100 mM NaCl, 1 mM EDTA, and 0.02% sodium azide and centrifuged at 5000 × *g* at 4°C for 20 min. The soluble proteins in the supernatant were fractionated by gel filtration on a column of Bio-Gel A-1.5 m (1.8 × 180 cm) at 4°C. The fractions corresponding to α_L-crystallin (second peak) were pooled and concentrated at the above mentioned temperature by ultrafiltration using an Amicon ultrafiltration unit. The α_L concentrate was further purified by gel filtration on Bio-Gel A-5m column (1.8 × 180 cm), and the fractions were pooled and concentrated by ultrafiltration as described above. The concentrate of purified α-crystallin was stored at −20°C until required. It is important to note that the sample was kept at or below 4°C during the entire isolation procedure in order to ensure that it remained in its higher molecular weight form.

2.2. Preparation of zeta-crystallin

Zeta-crystallin from guinea pig lens was purified using a Blue Sepharose CL-6B affinity column as described by Rao and Zigler [30]. Fresh guinea pig lenses were homogenized in Tris-HCl buffer, pH 7.4, containing 0.5 mM EDTA and 5 mM β-mercaptoethanol and centrifuged at 5000 × *g* for 15 min. The supernatant was loaded on a Blue Sepharose CL-6B column equilibrated with the homogenizing buffer. The bound zeta-crystallin was eluted with the homogenizing buffer containing 1 M NaCl and desalted by dialysis against buffer. The zeta-crystallin isolated by this procedure was free from NADPH as tested by absorption spectroscopy. The purity of the zeta-crystallin was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the protein concentration was determined by the modified Lowry method [31].

2.3. Assay for protein aggregation

Insulin at a concentration of 0.2 mg/ml (10 mM phosphate buffer,

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SSDI 0014-5793(95)00440-8

pH 7.4, containing 100 mM NaCl) in the presence or absence of the required amount of α-crystallin was equilibrated at the required temperature for 5 min with constant stirring in the sample holder using a Julabo thermostated water bath. The actual temperature of the sample in the cuvette was monitored by a Physitemp microthermocouple thermometer system. The reduction of insulin was initiated by adding 30 μl of 1 M DTT to 1.5 ml of the sample and the extent of aggregation of the insulin B chain was measured as a function of time by monitoring the scattering by setting the excitation and emission monochromator of the Hitachi F4000 fluorescence spectrophotometer at 465 nm with excitation and emission band passes of 1.5 nm.

Thermal aggregation of zeta-crystallin was monitored by the following procedure. The buffer (10 mM phosphate buffer, pH 7.4, containing 100 mM NaCl) was equilibrated at the required temperature for 5 min with constant stirring in the sample holder as mentioned above. Then, 60 μl of 5 mg/ml zeta-crystallin solution was added to a final volume of 1.5 ml and the extent of aggregation was measured as a function of time by the scattering of 465 nm light as mentioned above. In another experiment the buffer containing 0.2 mg/ml α-crystallin was equilibrated at the required temperature. Zeta-crystallin was then added and aggregation measured as described above.

2.4. Fluorescence measurements

α-Crystallin (0.3 mg/ml) in 10 mM phosphate buffer, pH 7.4, containing 100 mM NaCl was incubated with 20 μM of sodium 8-anilino-1-naphthalene sulfonate (ANS) at 20°C for 2 h. This sample was equilibrated at the required temperature in the sample holder of Hitachi F4000 fluorescence spectrophotometer using a Julabo thermostated water bath for 5 min. The actual temperature of the sample was monitored as mentioned above. Fluorescence spectra were recorded with an excitation wavelength of 365 nm. The excitation and emission band passes were 3 nm and 1.5 nm, respectively.

3. Results and discussion

Upon reducing the disulphide bond between the two chains of insulin with reducing agents such as dithiothreitol (DTT), the B chain of insulin aggregates and precipitates out leaving the A chain in solution [32]. Recently Farahbakhsh et al. have shown that α-crystallin prevents the aggregation of insulin B chain at 25°C [26]. They have observed that the aggregation of insulin B chain is completely prevented at a weight ratio of α-crystallin to insulin of more than 6:1. It is interesting to note that α-crystallin prevents the thermal aggregation of several proteins at 10 fold lesser concentrations than that of the target protein [21]. We have earlier shown that the chaperone-like activity of α-crystallin is enhanced at temperatures above 30°C.

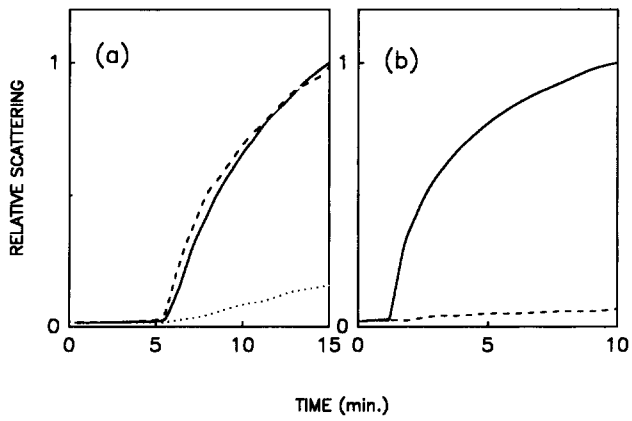

Fig. 1. Aggregation of insulin B chain at (a) 27.6°C and (b) 40.6°C. Aggregation was monitored by measuring scattering of 465 nm light. (—) 0.2 mg/ml insulin alone, and at an insulin to α-crystallin weight ratio of 1:1 (– – –) and 1:8 (···).

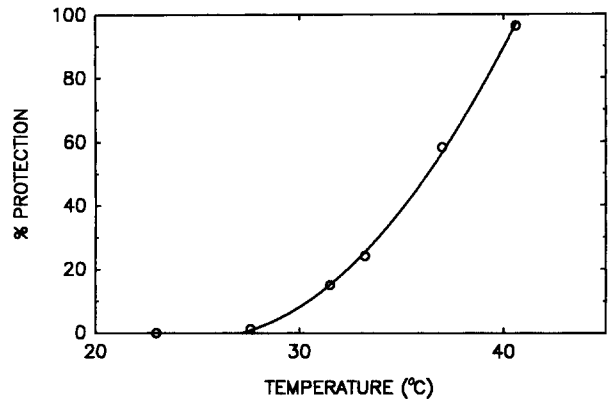

Fig. 2. Temperature dependent prevention of aggregation of insulin (0.2 mg/ml) by α-crystallin (0.2 mg/ml). Percentage protection is calculated using the equation, $((I_o-I_\alpha)/I_o) \times 100$; where I_o is the light scattering in the absence and I_α in the presence of α-crystallin at a fixed time after initiation of the reaction with DTT.

Thus, on the basis of our earlier studies, we expect a much better protection of aggregation by α-crystallin at higher temperatures. To test our hypothesis and to gain an insight into the mechanism of the chaperone-like activity of α-crystallin, we have investigated its ability to prevent the aggregation of insulin B chain as a function of temperature.

The solid line in Fig. 1a shows aggregation of insulin (0.2 mg/ml) with time on treatment with DTT as monitored by the scattering of 465 nm light at 27.6°C. The presence of α-crystallin (0.2 mg/ml) does not prevent the aggregation of insulin B chain at 27.6°C (dashed line, Fig. 1a). On the other hand, if the experiment is conducted at a higher temperature (40.6°C) the result is very different. The aggregation is completely prevented by α-crystallin (0.2 mg/ml) as shown in Fig. 1b. Fig. 2 shows the percentage protection as a function of temperature. As evident from the figure, α-crystallin is functional only above 30°C in preventing the aggregation of insulin B chain. We have earlier shown a similar transition at about 30°C in our studies on the effect of α-crystallin on photo-aggregation of γ-crystallin [25]. Gamma-crystallin aggregates upon irradiation with UV light [33]. Alpha-crystallin does not prevent this aggregation at temperatures below 30°C. The percentage protection increases sharply above 30°C. Walsh et al. have reported two endothermic transitions in the differential scanning calorimetric studies of α-crystallin [34]. The sharp transition above 30°C, in all these studies, suggests a structural change in α-crystallin which is responsible for its enhanced chaperone-like activity.

Fig. 3 shows the change in the emission maximum of ANS bound to α-crystallin with temperature. The emission maximum is red shifted gradually above 30°C (and more sharply above 50°C), indicating that the ANS bound hydrophobic surfaces of α-crystallin are becoming exposed to water. Probing the hydrophobic surfaces in α-crystallin with pyrene, we showed in our earlier study that the hydrophobic surfaces of α-crystallin are increasingly exposed above 30°C [25]. Figs. 2 and 3 together suggest that a structural change(s) in α-crystallin which exposes the hydrophobic surfaces is important for its chaperone-like activity.

It is important to note that, in our present study, the aggregation of insulin B chain is prevented more than 90% at 40.6°C.

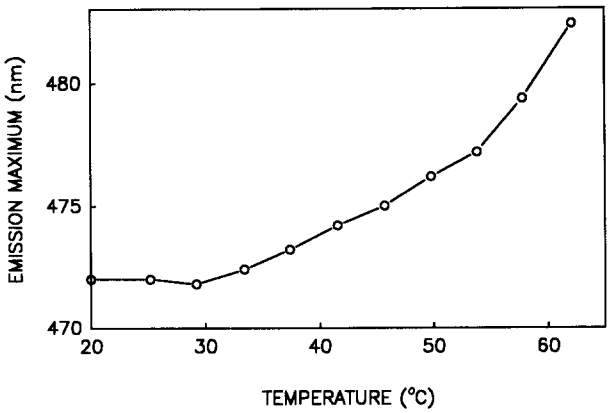

Fig. 3. Temperature dependent fluorescence of α-crystallin bound ANS (see section 2 for details).

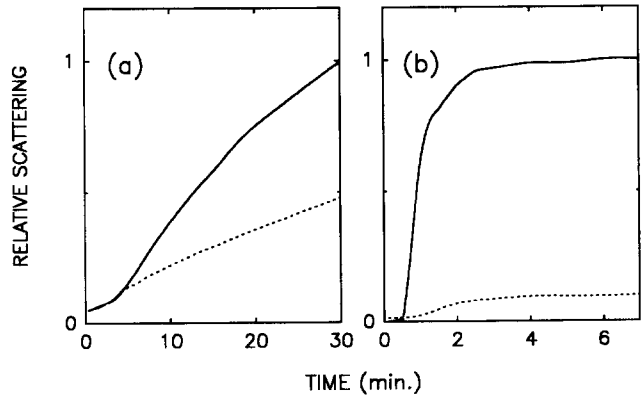

Fig. 5. Aggregation of 0.2 mg/ml zeta-crystallin in the absence (—) and in the presence (– – –) of 0.2 mg/ml α-crystallin at 41°C (a) and at 55°C (b). Aggregation was measured by monitoring the scattering of 465 nm light.

On the other hand, in our earlier study, the photo-aggregation of γ-crystallin was prevented only about 60% at this temperature. This comparison suggests that the extent of exposure of hydrophobic surfaces in the target proteins also plays a role in the chaperone-like activity.

We have also studied the effect of varying weight ratio of α-crystallin to insulin at 27.6°C and at 40.6°C on the aggregation of insulin B chain. Fig. 4 shows the variation of the percentage protection with weight ratio. It is clear from the figure that protection increases dramatically at 40.6°C. Almost 100% protection is achieved with a 1:1 weight ratio, whereas at room temperature (27.6°C) even as high as 8 times more α-crystallin leads to only about 85% protection. Clearly a structural transition (as we hypothesized) is bringing about at least an 8 fold increase in the effectiveness of α-crystallin as a chaperone.

Zeta-crystallin from the eye lens of guinea pig binds NADPH and has quinone-reductase activity [35]. Rao et al. [24] have earlier shown that zeta-crystallin readily aggregates at 41°C in the absence of the nucleotide NADPH and the aggregation temperature is postponed to about 55°C in the presence of the nucleotide. Binding of NADPH, thus, stabilizes zeta-crystallin. They have also found that α-crystallin is less functional in

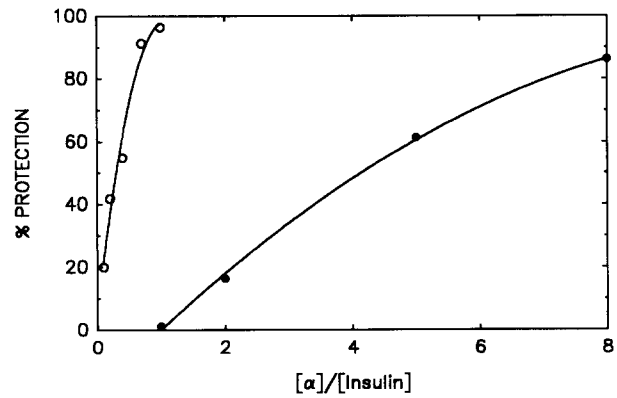

Fig. 4. Effect of the relative concentration of α-crystallin on its prevention of the aggregation of insulin B chain at (○) 27.6°C and at (●) 40.6°C. The percentage protection was calculated using the equation mentioned in the legend to Fig. 2. 0.2 mg/ml of insulin was used and the α-crystallin concentration was varied.

preventing the aggregation of zeta-crystallin at 41°C in the absence of NADPH and it completely prevents the aggregation of zeta-crystallin at 55°C in the presence of the nucleotide. They have suggested that subtle changes in the conformation of zeta-crystallin upon its binding to NADPH is responsible for the observed differential protection of thermal-aggregation of zeta-crystallin by α-crystallin. Our results, on the other hand, suggest that a structural change in α-crystallin might be responsible for its differential chaperone-like activity. In order to verify this possibility, we have modified the experiment as mentioned in section 2. We believe that α-crystallin needs to be in its temperature-perturbed state for it to be effective. Hence we kept the α-crystallin solution at 41 or 55°C and then added zeta-crystallin. We find that α-crystallin partially prevented the aggregation of zeta- crystallin at 41°C in the absence of NADPH (Fig. 5a). It almost completely prevents the aggregation of zeta-crystallin at 55°C, again in the absence of NADPH, as evident from Fig. 5b. These results reinforce the importance of structural perturbation of α-crystallin in its chaperone-like activity.

We, therefore, conclude that α-crystallin prevents aggregation of non-native structures by providing appropriately placed hydrophobic surfaces. A structural transition above 30°C enhances the protective ability of α-crystallin, perhaps by increasing or reorganizing the hydrophobic surfaces. Since α-crystallin is known to undergo quaternary structural changes with temperature [36,37], such structural perturbation might be involved in the chaperone-like activity of α-crystallin. Brunschier et al. [38] have shown a similar temperature-dependent interaction between GroEL and phage p22 tailspike protein. Above 30°C, folding intermediates bind to GroEL, whereas below 25°C GroEL has no apparent interaction with the nascent protein. Hansen and Gafni have suggested that a sharp structural transition is induced in GroEL by temperature [39]. It is, therefore, interesting to know whether all heat shock proteins (and chaperones) have similar temperature dependent function because they are required to function more efficiently at non-permissible temperatures.

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