

# Interaction of human recombinant $\alpha$ A- and $\alpha$ B-crystallins with early and late unfolding intermediates of citrate synthase on its thermal denaturation

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**Abstract** We have investigated the role of recombinant human  $\alpha$ A- and  $\alpha$ B-crystallins in the heat-induced inactivation and aggregation of citrate synthase. Homo-multimers of both  $\alpha$ A- and  $\alpha$ B-crystallins confer protection against heat-induced inactivation in a concentration-dependent manner and also prevent aggregation. Interaction of crystallins with early unfolding intermediates of citrate synthase reduces their partitioning into aggregation-prone intermediates. This appears to result in enhanced population of early unfolding intermediates that can be reactivated by its substrate, oxaloacetate. Both these homo-multimers do not form a stable complex with the early unfolding intermediates. However, they can form a soluble, stable complex with aggregation-prone late unfolding intermediates. This soluble complex formation prevents aggregation. Thus, it appears that the chaperone activity of  $\alpha$ -crystallin involves both transient and stable interactions depending on the nature of intermediates on the unfolding pathway; one leads to reactivation of the enzyme activity while the other prevents aggregation. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:**  $\alpha$ -Crystallin; Molecular chaperone; Citrate synthase; Unfolding intermediate; Interaction

## 1. Introduction

The mammalian eye lens  $\alpha$ -crystallin exists as a hetero-multimer composed of two homologous gene products,  $\alpha$ A- and  $\alpha$ B-crystallin, in ratios varying from species to species [1].  $\alpha$ B-Crystallin belongs to the family of small heat-shock proteins [2].  $\alpha$ -Crystallin exhibits chaperone-like activity by preventing thermal and non-thermal aggregation of proteins [3–5]. Both  $\alpha$ A- and  $\alpha$ B-crystallins are capable of forming stable homo-multimers [6,7].  $\alpha$ B-Crystallin is more efficient in preventing the aggregation of proteins than  $\alpha$ A-crystallin [8,9].  $\alpha$ B-Crystallin is more vulnerable to heat-induced structural perturbations than  $\alpha$ A-crystallin [9]. Non-lenticular tissues like the heart muscles, kidney and the brain express  $\alpha$ B-crystallin under conditions of stress [10].  $\alpha$ A-Crystallin is not stress-inducible and its presence in non-lenticular tissues is rather limited. Trace amounts of  $\alpha$ A-crystallin are found in the spleen [11].

It is now established that, like most other molecular chap-

erones,  $\alpha$ -crystallins form complexes with partially unfolded proteins and prevent their aggregation. Earlier studies from our laboratory [12,13] as well as from other laboratories [14] showed that the bound protein possesses significant structural elements:  $\alpha$ -crystallin forms stable complexes with the aggregation-prone partially unfolded state(s) having properties similar to those of molten globules [12,13]. Though molecular chaperones exhibit certain common properties such as binding to the partially unfolded state(s) of the target proteins and preventing their aggregation, they differ in their ability to confer protection against the stress-induced inactivation of enzyme. For example, Hsp90 stabilises citrate synthase in heat-induced inactivation, while GroEL accelerates the inactivation process at high concentrations [15,16]. The eye lens  $\alpha$ -crystallin (hetero-oligomer composed of  $\alpha$ A- and  $\alpha$ B-crystallin) has been shown to stabilise catalase from heat-induced inactivation [17]. The molecular mechanisms involved in preventing the thermal inactivation, however, are not well understood.

Considering the distinct differences between  $\alpha$ A- and  $\alpha$ B-crystallins in terms of their expression patterns, chaperone-like activities and thermal stabilities, it is important to investigate the differences, if any, in the abilities of  $\alpha$ A- and  $\alpha$ B-crystallins to confer protection against heat-induced inactivation of enzyme. We have investigated the chaperone-like activity of the recombinant human  $\alpha$ A- and  $\alpha$ B-crystallin towards heat-induced aggregation as well as inactivation of citrate synthase. Citrate synthase is ideally suited for this study because (i) it gets inactivated at 43°C, a temperature relevant to physiological heat-shock conditions and (ii) the unfolding intermediates formed during its thermal denaturation have been well characterised. The enzyme, on thermal denaturation, initially forms reactivatable intermediates that do not aggregate to an appreciable extent. These intermediates subsequently form aggregation-prone intermediates [15]. The presence of these distinct intermediates allows us to investigate the nature of interaction with each of these intermediates. Our study provides insight into the molecular mechanisms involved in the chaperone-like activity of  $\alpha$ -crystallin in heat-induced inactivation as well as aggregation of the enzyme.

## 2. Materials and methods

### 2.1. Materials

Pig heart citrate synthase, cytosolic malate dehydrogenase (MDH), acetyl CoA,  $\beta$ -NADH and the Ellman's reagent, 5,5'-dithio-bis(2-ni-

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trobenzoic acid) or DTNB, were purchased from Sigma, St. Louis, MO, USA. Catalase was purchased from Worthington Biochemical Corporation, NJ, USA. H<sub>2</sub>O<sub>2</sub> and oxaloacetate was purchased from Sisco Research Laboratories, Mumbai, India.

## 2.2. Over-expression and purification of $\alpha$ A- and $\alpha$ B-crystallins

Recombinant human  $\alpha$ A- and  $\alpha$ B-crystallins were prepared as described by Kumar et al. [18]. *Escherichia coli* BL21 ( $\lambda$ DE3) cells containing the pET21a- $\alpha$ A and  $\alpha$ B plasmids were induced by adding 1 mM isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG; final concentration) after they reached the mid-log phase (OD<sub>600 nm</sub> was 0.6). The cells were allowed to grow for another 3 h in the presence of IPTG, harvested and stored at  $-70^{\circ}\text{C}$  until further use. The cell pellets were suspended in 50 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 7.4 (TNE buffer), containing 10  $\mu$ l of 50 mM phenylmethylsulfonyl fluoride and 80  $\mu$ l of lysozyme (10 mg/ml) at a ratio of 3 ml buffer/g wet weight of cells, and kept on ice for 30 min. Sodium deoxycholate (4 mg/g of cell pellet) was added and the cells vortexed. DNase I (20  $\mu$ g/g of pellet) was added to the suspension and kept at  $37^{\circ}\text{C}$  until it was no longer viscous. The suspension was then centrifuged at 12000 rpm for 30 min.  $\alpha$ A- and  $\alpha$ B-crystallins were found to be in the soluble fraction. The supernatant containing  $\alpha$ A- or  $\alpha$ B-crystallin was used for further purification. The supernatant was then subjected to ammonium sulphate fractionation.  $\alpha$ A- and  $\alpha$ B-crystallins precipitate out in the 30–60% saturated ammonium sulphate fraction. This precipitate was dissolved and loaded onto a Sephacryl S-300 HR column (130  $\times$  1 cm). The fractions corresponding to  $\alpha$ A- or  $\alpha$ B-crystallin were pooled, concentrated and dialysed against 50 mM Tris-HCl buffer pH 7.4 containing 100 mM NaCl. The concentrated samples of  $\alpha$ A- or  $\alpha$ B-crystallin were then passed through a Mono Q FPLC column to separate minor contaminants. A linear gradient of 0.1–1 M NaCl was used to elute the proteins.  $\alpha$ A-Crystallin elutes at 300 mM NaCl while  $\alpha$ B-crystallin elutes out at 100 mM NaCl. The concentrations of  $\alpha$ A- and  $\alpha$ B-crystallin were calculated using the subunit molar extinction coefficients at 280 nm ( $\epsilon_{280}$ ) of 16500 and 19000 M<sup>-1</sup> cm<sup>-1</sup>, respectively [19].

## 2.3. Aggregation of citrate synthase

Citrate synthase (0.025 mg/ml) was incubated at various temperatures (40–47 $^{\circ}\text{C}$ ) in 40 mM HEPES-KOH buffer, pH 7.5, in the presence or in the absence of 0.025 mg/ml  $\alpha$ A- or  $\alpha$ B-crystallin. The aggregation was monitored on a Hitachi F-4000 spectrofluorimeter. The excitation and emission band passes were set at 3.0 nm. The temperature of the sample was maintained using a Julabo circulating water-bath. The internal temperature of the cuvette was monitored using a Physitemp type T microcouple.

## 2.4. Gel filtration chromatography

The formation of a complex between  $\alpha$ B-crystallin and the unfolding intermediates of citrate synthase was studied using gel filtration chromatography on a Superose-6 HR 10/30 column using a Pharmacia FPLC system. The samples were analysed for their molecular masses using a Superose-6 column. A mixture of 0.75 mg/ml  $\alpha$ B-crystallin and 0.06 mg/ml citrate synthase was incubated at 43 $^{\circ}\text{C}$ , either for 5 or 60 min in 40 mM HEPES buffer, pH 7.5. The samples were then centrifuged at 10000 rpm for 15 min to remove insoluble protein, if any, present in the sample and loaded on to the column. The samples were equilibrated in 50 mM Tris-HCl buffer, pH 7.5, containing 50 mM NaCl.

## 2.5. Thermal inactivation of enzymes

Citrate synthase (15  $\mu$ M) was diluted 100-fold into 40 mM HEPES-KOH buffer, pH 7.5, and pre-incubated at 43 $^{\circ}\text{C}$ . Small aliquots (10  $\mu$ l) of the denaturing enzyme were withdrawn at various time points and assayed for residual activity. To test the chaperoning ability, different concentrations of  $\alpha$ A- or  $\alpha$ B-crystallin were pre-incubated at 43 $^{\circ}\text{C}$  for 5 min in 40 mM HEPES-KOH buffer, pH 7.5, prior to adding the enzyme.

Inactivation of the cytosolic MDH was performed at 55 $^{\circ}\text{C}$ . 15  $\mu$ M MDH was diluted 100-fold into 40 mM HEPES-KOH buffer, pH 7.5, in the presence or in the absence of  $\alpha$ A- or  $\alpha$ B-crystallin pre-incubated at 55 $^{\circ}\text{C}$  for 5 min. The samples were denatured for 5 min at 55 $^{\circ}\text{C}$  and the residual activity measured. The percentage activity retained was calculated with respect to the activity of the native enzyme.

Catalase was diluted to a final concentration of 0.15  $\mu$ M and inactivated at 60 $^{\circ}\text{C}$  for 5 min in the presence or in the absence of  $\alpha$ A- or

$\alpha$ B-crystallin pre-incubated at 60 $^{\circ}\text{C}$  for 5 min. The enzyme samples were thermally denatured for 5 min at 60 $^{\circ}\text{C}$ .

## 2.6. Assay for enzymes

Citrate synthase activity was measured essentially as described by Srere et al. [20]. The activity assay is based on the first step of the citric acid cycle, in which citrate synthase catalyses the condensation of oxaloacetic acid (OAA) and acetyl-CoA to citrate and coenzyme A. Coenzyme A reacts with stoichiometric amounts of DTNB. This reaction can be monitored as an increase in absorption at 412 nm. The reaction mixture contained 940  $\mu$ l of TE (50 mM Tris-HCl, pH 7.5, containing 2 mM EDTA), 10  $\mu$ l of 10 mM OAA, 10  $\mu$ l of 10 mM DTNB and 30  $\mu$ l of 5 mM acetyl-CoA. The reaction was started by adding 10  $\mu$ l of 0.15  $\mu$ M citrate synthase. The activity assay was performed at 25 $^{\circ}\text{C}$  and the change in absorbance recorded using a Hitachi U-2000 spectrophotometer.

MDH activity was measured in 50 mM phosphate buffer, pH 7.5, containing 0.5 mM oxaloacetate and 0.3 mM NADH. The reaction was initiated by the addition of 25  $\mu$ l of the diluted enzyme. The reaction velocity was determined by measuring the decrease in absorbance at 340 nm resulting from the oxidation of NADH over a period of 2 min. The reaction buffer was prepared just before the assay and used within 1 h. The cytoplasmic form of the enzyme used in these studies is much more hydrophilic and stable compared to the mitochondrial MDH [21].

Catalase activity was assayed essentially as described by Beers and Sizer [22]. H<sub>2</sub>O<sub>2</sub> (30%) was diluted in 0.05 M potassium phosphate buffer so that the OD of the peroxide solution at 240 nm was approximately 1.0. The assay was initiated by the addition of catalase at the required concentration. The activity was measured as the disappearance of peroxide which was followed by a decrease in OD<sub>240 nm</sub> with time. The percent residual activity of the heat-inactivated enzyme was calculated with respect to the activity of the native enzyme at the same concentration.

## 2.7. Oxaloacetate-induced reactivation of citrate synthase

Citrate synthase was heat-denatured as described above for 5 min in the presence or absence of  $\alpha$ A- or  $\alpha$ B-crystallin. The samples were incubated with 0.1 mM oxaloacetate at 25 $^{\circ}\text{C}$  for 5 min. The residual activity of the samples was then measured.

## 3. Results and discussion

We have investigated the chaperone-like activity of the recombinant  $\alpha$ A- and  $\alpha$ B-crystallin towards heat-induced aggregation as well as inactivation of citrate synthase. The enzyme exists as a homo-dimer of a polypeptide chain of 48 kDa. Since it aggregates at around physiological heat-shock temperature and its enzyme activity can be assayed easily, it serves as a good substrate protein for studying the activity of molecular chaperones [15]. It is, therefore, ideally suited to our investigation on the effect of  $\alpha$ -crystallin on heat-induced inactivation of the enzyme as well as its aggregation kinetics.

Fig. 1A shows the aggregation of citrate synthase as a function of time at different incubation temperatures as monitored by the light scattering of the sample. The enzyme does not aggregate significantly at temperatures up to 41 $^{\circ}\text{C}$ . An appreciable extent of aggregation is observed at 43 $^{\circ}\text{C}$ , and it is considerably enhanced at 45 and 47 $^{\circ}\text{C}$ . The onset of aggregation is preponed and the extent of aggregation is increased as a function of temperature. This shows that the aggregation-prone partially unfolded state(s) of the enzyme is significantly populated at 43 $^{\circ}\text{C}$  and higher temperatures. The decrease in the light scattering at 47 $^{\circ}\text{C}$  after an initial sharp increase is due to the formation of large aggregates. Fig. 1B compares the chaperone-like activity of  $\alpha$ A- and  $\alpha$ B-crystallins in preventing the aggregation of citrate synthase at 43 $^{\circ}\text{C}$ . It is evident from Fig. 1B that, when identical concentrations of

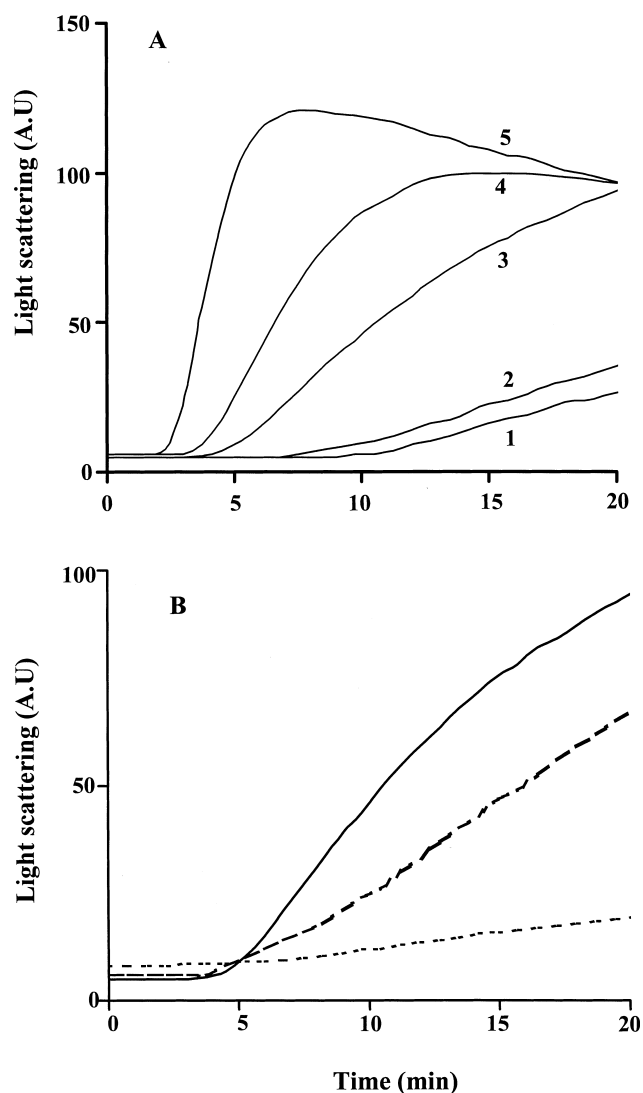


Fig. 1. A: Curves 1–5 represent aggregation of citrate synthase (25 µg/ml) in 40 mM HEPES–KOH buffer pH 7.5 at 39, 41, 43, 45 and 47°C, respectively. B: Aggregation of citrate synthase (25 µg/ml) at 43°C in the absence (solid line) and in the presence of 25 µg/ml αA-crystallin (long-dash line) or αB-crystallin (short-dash line).

αA- and αB-crystallin are used, αB-crystallin is more potent in preventing the aggregation than αA-crystallin. This result is consistent with the earlier reports on the chaperone-like activity of the homo-multimers of αA- and αB-crystallin towards the aggregation of insulin upon reducing the interchain disulphide bonds [8,9].

We have investigated the effect of αA- and αB-crystallin on the inactivation kinetics of the enzyme at 43°C. Fig. 2 shows that the enzyme loses almost 90% of its activity within 1200 s (20 min). The enzyme loses about 70% activity in 300 s (5 min), while it does not show significant aggregation within this time-period (see Fig. 1). This result shows that the inactivation precedes the aggregation process. Under our experimental conditions, the inactivation process follows the exponential first order kinetics with a rate constant of inactivation ( $k_{\text{inact}}$ ) of  $4.1 \times 10^{-3} \text{ s}^{-1}$ . In the presence of 11 µM (subunit concentration using the molecular mass of 20 kDa) αA- or αB-crystallin, the rate constants  $k_{\text{inact}}$  were determined to be  $2.9 \times 10^{-3}$  and  $2.0 \times 10^{-3} \text{ s}^{-1}$ , respectively. These results show

that, both αA- and αB-crystallins decrease the rate of inactivation process and hence confer thermal protection to the enzyme. However, αB-crystallin decreases the rate of inactivation process more effectively than αA-crystallin. The inset in Fig. 2 shows that αA- and αB-crystallins confer thermal protection to the enzyme in a concentration-dependent manner. Bovine serum albumin is not found to significantly affect the inactivation kinetics (data not shown). This shows that the effect observed with α-crystallin is due to its chaperone-like activity and not a non-specific protein effect.

These results clearly demonstrate two aspects of the chaperone function of α-crystallin: (i) α-crystallin is able to protect the enzyme activity as well as prevent aggregation of the heat-induced denaturation of the enzyme; (ii) the differential abilities in preventing the aggregation of proteins exhibited by the homo-multimers of αA- and αB-crystallin [8,9] are also observed in the differential protection of the enzyme activity. However, the differences in the protection offered by αA- and αB-crystallins in heat-induced inactivation of citrate synthase is less compared to that observed in the prevention of heat-induced aggregation of the enzyme (see Figs. 1 and 2). The observed differences may be due to several factors such as the differences in the exposed hydrophobic surfaces and the spatial organisation of these surfaces in αA- and αB-crystallin, the differences in the nature of the interacting species in the heat-inactivation and aggregation processes and the reactivation kinetics of the interacting species of the enzyme in the presence of αA- and αB-crystallins.

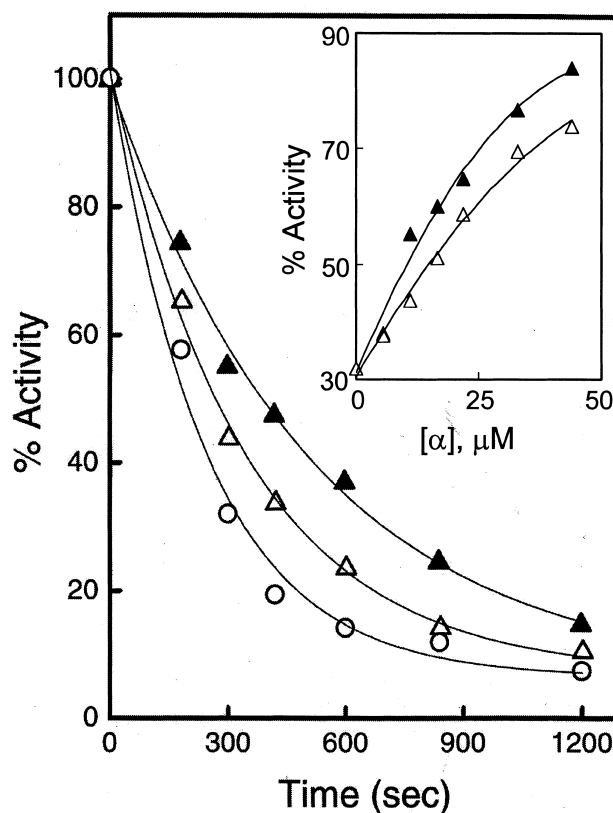


Fig. 2. Inactivation of 0.15 µM citrate synthase as a function of time in the absence (○) or in the presence of 11 µM αA-crystallin (△) or αB-crystallin (▲). Inset shows residual activity at the end of 5 min of inactivation at 43°C with increasing concentrations of αA- (△) or αB-crystallin (▲).

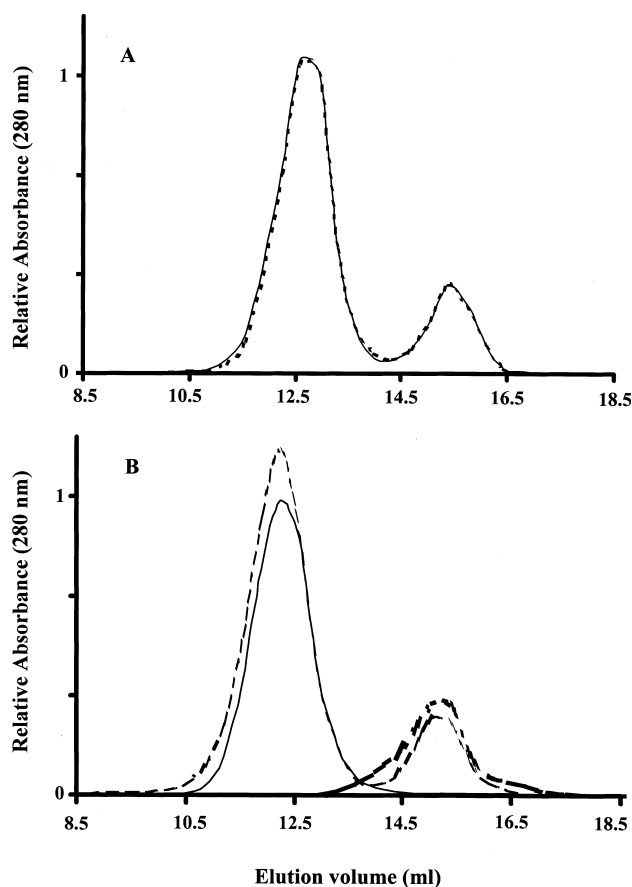


Fig. 3. Analysis of the complex formation between  $\alpha$ B-crystallin and citrate synthase by gel filtration chromatography on a Superose-6 column. A: Elution profile of a mixture of  $\alpha$ B-crystallin (0.75 mg/ml) and citrate synthase (0.06 mg/ml) (solid line) and the mixture incubated at 43°C for 5 min (dashed line). B: Elution profile of heat-treated  $\alpha$ B-crystallin (solid line), native citrate synthase (short-dash line), and the mixture of  $\alpha$ B-crystallin and citrate synthase (long-dash line). The heat-treated samples were incubated at 43°C for 90 min. The samples were then centrifuged at 10000 rpm for 15 min and loaded on the column.

It appears that prevention of aggregation as well as protection against inactivation are important in the molecular chaperone-like activity of  $\alpha$ -crystallin. It is, therefore, important to dissect out the molecular mechanisms involved in these two aspects. One possibility is that  $\alpha$ -crystallin forms a complex with the partially unfolded state of the enzyme and the bound enzyme exhibits some activity. In this context, it is important to note that the complex between  $\alpha$ -crystallin and glyceraldehyde-3-phosphate dehydrogenase [23] as well as that between  $\alpha$ -crystallin and sorbitol dehydrogenase [24] exhibits some enzyme activity. In the case of  $\alpha$ -crystallin and citrate synthase, this possibility is ruled out by our gel filtration experiments. Fig. 3A shows the elution profile of the mixture of native  $\alpha$ B-crystallin and citrate synthase before and after heat-treatment at 43°C for 5 min. At this time point, the enzyme loses almost 70% of its activity in the absence of  $\alpha$ B-crystallin, while in the presence of  $\alpha$ B-crystallin (11  $\mu$ M) it loses only 44.8% activity (Fig. 2, inset). As seen in Fig. 3A, incubation of  $\alpha$ B-crystallin with citrate synthase at 43°C for 5 min neither alters the elution profiles nor the size of the two protein peaks suggesting the absence of a stable complex between them at this time

point. In addition, the fractions from the peak where  $\alpha$ B-crystallin elutes do not exhibit any enzyme activity. However, prolonged incubation of the sample (90 min) at 43°C, results in a decrease in the citrate synthase peak and a concomitant increase and shift in the  $\alpha$ B-crystallin peak to lower elution volumes, suggesting the formation of a stable complex between  $\alpha$ B-crystallin and citrate synthase (Fig. 3B); the fractions corresponding to the complex do not exhibit enzyme activity. These results clearly rule out the above-mentioned possibility.

The second possibility for the observed protection is that  $\alpha$ -crystallin interacts transiently with the unfolding intermediate(s) of citrate synthase that are in equilibrium with its native state, thus stabilising them so that they can fold back to the active state. The temperature-induced unfolding pathway of citrate synthase has been shown to involve two early unfolding intermediates, which are characterised as being dimeric, less aggregation-prone and able to bind the substrate, oxaloacetate, which leads to refolding of the intermediates to native state [15,16]. Conversion of these intermediates to late unfolding intermediate states, which are aggregation-prone, leads to irreversible aggregation and inactivation [15,16]. We have investigated whether  $\alpha$ -crystallin can interact with these early unfolding intermediates and stabilise them. If  $\alpha$ B-crystallin interacts with and stabilises the early unfolding intermediates, their population in the equilibrium is expected to be more in the presence of  $\alpha$ B-crystallin than in its absence. We have incubated citrate synthase in the presence of increasing concentrations of  $\alpha$ B-crystallin at 43°C for 5 min. To one set of samples, 0.1 mM oxaloacetate was added and kept at room temperature for 5 min before measuring the enzyme activity. Shifting the sample to room temperature after the heat-treatment allows intermediate species in reversible equilibrium with

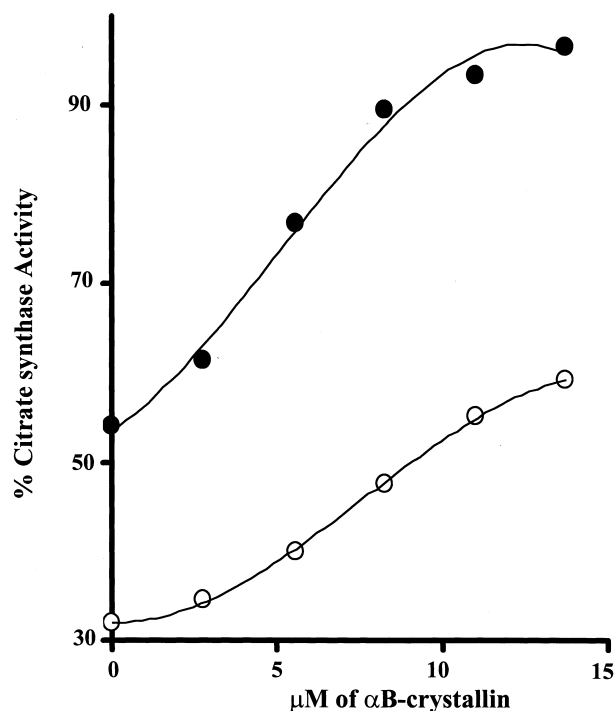


Fig. 4. Reactivation of heat-inactivated citrate synthase in the presence of increasing concentrations of  $\alpha$ B-crystallin in the presence (●) and the absence (○) of 100  $\mu$ M oxaloacetate.

the native state to fold back to the native state. Since these intermediates bind oxaloacetate and this binding is known to stabilise the enzyme, the presence of oxaloacetate facilitates the refolding process. Fig. 4 shows the percent residual activity of the enzyme in the presence of increasing concentrations of  $\alpha$ B-crystallin in the absence and in the presence of oxaloacetate. In general, as the  $\alpha$ B-crystallin concentration increases, the percent residual activity of the enzyme upon heat-inactivation increases either in the absence or in the presence of oxaloacetate. However, in the presence of oxaloacetate, this increase in residual activity is more pronounced than in its absence. For example, in the absence of  $\alpha$ B-crystallin, the population of intermediates that could fold back to native state (difference in the percent activity of the enzyme in the absence and in the presence of oxaloacetate) was 22%. In the presence of 11  $\mu$ M  $\alpha$ B-crystallin, this population increased to 38%. We have observed qualitatively similar results with  $\alpha$ A-crystallin (data not shown). These results clearly show that  $\alpha$ B-crystallin increases the populations of the early unfolding intermediates of citrate synthase. As mentioned earlier, our gel filtration experiments (Fig. 3A) do not show a stable complex formation between  $\alpha$ B-crystallin and citrate synthase at this time point (5 min). This suggests transient and reversible interactions between  $\alpha$ -crystallin and the early unfolding intermediates of the enzyme.

Based on these results, we propose a mechanism for the chaperone-like activity of  $\alpha$ -crystallin towards the heat-induced denaturation of citrate synthase (as shown in a schematic diagram in Fig. 5). Transient and reversible interactions of  $\alpha$ B-crystallin with the early unfolding intermediates ( $I_1$  and  $I_2$ ) of citrate synthase inhibit their partitioning to late aggregation-prone intermediates ( $I_n$ ). This leads to the observed increase in the equilibrium population of the early unfolding intermediate at any given time.  $\alpha$ B-Crystallin binds stably to the late aggregation-prone intermediates and prevents their aggregation (shown by formation of complex on prolonged incubation, see Fig. 3B). It is, therefore, evident that the chaperone-like activity of  $\alpha$ -crystallin towards heat-induced denaturation of citrate synthase involves two distinct mechanisms. (i) The chaperone molecule interacts transiently with the early unfolding intermediates, which are in reversible equilibrium with the native state. This limits the conversion of these early unfolding intermediates to the aggregation-prone intermediates. These intermediates refold to their native state on removing the denaturing conditions, which is observed as prevention of heat inactivation by  $\alpha$ -crystallin. (ii) The chaperone molecule interacts with the late unfolding, aggregation-prone intermediates to form a stable complex, thereby preventing these

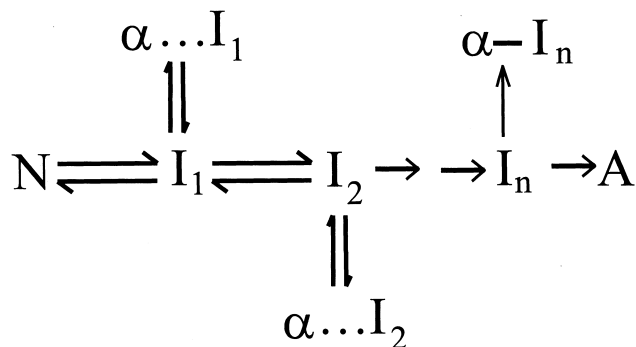


Fig. 5. Schematic representation of the possible mechanism by which  $\alpha$ A- and  $\alpha$ B-crystallin protect citrate synthase from thermal denaturation. N represents native enzyme,  $I_1$  and  $I_2$  represent the early unfolding intermediates which can be reactivated by the addition of oxaloacetate,  $I_n$  represents the late aggregation-prone intermediate while A represents the aggregated protein.  $\alpha \dots I_1$  and  $\alpha \dots I_2$  represent transient or reversible interaction.  $\alpha - I_n$  represents a stable complex.

intermediates from aggregating out of solution. These mechanisms might be involved in the chaperone-like activity of  $\alpha$ -crystallin towards heat-induced denaturation of proteins, in general.

We have also studied the heat inactivation of two other enzymes, MDH and catalase. A comparison of the protection offered by  $\alpha$ A- and  $\alpha$ B-crystallins towards the heat inactivation of these enzymes with that for citrate synthase is shown in Table 1. Both  $\alpha$ A- and  $\alpha$ B-crystallins confer protection on all the three enzymes during thermal inactivation,  $\alpha$ B-crystallin being more effective than  $\alpha$ A-crystallin. However, the extent of protection offered (i.e. the difference between the percentage activities of the enzyme in the absence and in the presence of the chaperone molecule) by these homo-multimers, differs significantly between the enzyme systems. It is to be noted that  $\alpha$ -crystallins at 40  $\mu$ M offer little protection to catalase (Table 1), however at higher concentrations significant protection of the enzyme activity is seen (data not shown, also see [17]). The reasons for these observed differences in protection between different enzyme systems are not clear. However, factors that dictate the extent to which these enzymes are inactivated in the presence of these homo-multimers may include the following: (i) whether or not the heat-induced unfolding pathway of the enzyme is significantly populated by early unfolding intermediate states in equilibrium with the native state that are capable of interacting with  $\alpha$ -crystallin.  $\alpha$ -Crystallin may not affect the inactivation kinetics in those cases where the unfolding pathway involves a

Table 1  
Effect of  $\alpha$ A- and  $\alpha$ B-crystallins on thermal inactivation of some enzymes

Enzyme	Inactivation temperature (°C)	% Activity			Protection offered <sup>a</sup>	
		in buffer alone	+ $\alpha$ A	+ $\alpha$ B	$\alpha$ A	$\alpha$ B
Citrate synthase	43	31.2	72.8	83.9	41.6	52.7
Citrate synthase	45	25.5	49.7	64.1	24.2	38.6
Malate dehydrogenase	55	43	53.7	54.2	10.7	11.2
Catalase	60	28.5	30.2	33.8	1.7	5.3

Enzymes (0.15  $\mu$ M) were added to 40 mM HEPES–KOH buffer (pH 7.5) either in the absence or in the presence of 40  $\mu$ M (subunit concentration)  $\alpha$ A- or  $\alpha$ B-crystallins pre-incubated at the indicated temperature for 5 min. The incubation was continued for another 5 min to allow enzyme inactivation and the enzyme activities of the samples were then measured.

<sup>a</sup>Protection offered by  $\alpha$ -crystallins is the difference between the % activities of the enzyme in the presence and in the absence of  $\alpha$ A- and  $\alpha$ B-crystallins.

highly co-operative transition from the native state to the aggregation-prone intermediates states, as it would form stable complexes with those intermediates. (ii) The rate at which the early unfolding intermediate(s) partition to the aggregation-prone intermediate states which leads to irreversible aggregation. This possibility can be verified in the case of citrate synthase. Increasing the temperature leads to an increased extent of aggregation as well as an earlier onset of aggregation as shown in Fig. 1A. This implies that the rate of partitioning of the early unfolding intermediates of citrate synthase to the aggregation-prone intermediate is increased upon increasing the temperature. As seen from Table 1, upon increasing the inactivation temperature from 43 to 45°C the protection offered by both  $\alpha$ A- and  $\alpha$ B-crystallins decreases significantly. In addition to the above factors, the temperature-induced structural changes of  $\alpha$ -crystallin [25–29] may also influence the process as the structural states of  $\alpha$ -crystallin at different inactivation temperatures of the enzymes might interact with different avidity with the early and late unfolding intermediates of the enzyme.

We conclude that the homo-multimers of both  $\alpha$ A- and  $\alpha$ B-crystallins confer protection on enzymes, particularly citrate synthase, against their heat-induced inactivation. However,  $\alpha$ B-crystallin is found to be more effective than  $\alpha$ A-crystallin. They increase the population of early unfolding intermediates of citrate synthase by transient interactions that stabilise them or decrease their partitioning to the late aggregation-prone intermediates.  $\alpha$ B-Crystallin forms a stable complex with the aggregation-prone late unfolding intermediate(s) of citrate synthase and prevents their aggregation. Thus, it appears that the chaperone activity of  $\alpha$ -crystallin involves both transient and stable interactions depending on the nature of the partially folded/unfolded intermediates states of the target proteins. These interactions may be particularly relevant in the eye lens, where  $\alpha$ -crystallin is not only required to prevent the aggregation of other partially unfolded or damaged crystallins, but also to decrease the partitioning of the early unfolding, less aggregation-prone intermediate state(s) to the aggregation-prone state by its transient interactions.

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