Suppression of DTT-induced aggregation of abrin by αA- and αB-crystallins: a model aggregation assay for α-crystallin chaperone activity in vitro[1]

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Abstract **The eye lens small heat shock proteins (sHSP), αA- and αB-crystallins, have been shown to function like molecular chaperones, both in vitro and in vivo. It is essential to assess the protective effect of αA- and αB-crystallins under native conditions to extrapolate the results to in vivo conditions. Insulin and α-lactalbumin have widely been used to investigate the chaperone mechanism of α-crystallin under native conditions. Due to its smaller size, insulin B-chain may not represent the binding of putative physiological substrate proteins. As it stands, the aggregation of α-lactalbumin and binding of α-crystallin to it varies under different experimental conditions. Abrin, a ribosome inactivating protein isolated from the seeds of *Abrus precatorius*, consists of a 30 kDa A-chain and a lectin-like B-chain of 33 kDa joined by a single disulfide bond. Reduction of the disulfide link between the two chains of abrin leads to the aggregation of the B-chain. In this study, we demonstrate that dithiothreitol (DTT)-induced aggregation of abrin B-chain could be monitored by light scattering similar to that of insulin. Moreso, this process could be suppressed by recombinant human αA- and αB-crystallins in a concentration dependent manner, notably by binding to aggregation prone abrin B-chain. SDS–PAGE and HPLC gel filtration analysis indicate that there is a soluble complex formation between α-crystallin and abrin B-chain. Interestingly, in contrast to insulin, there is no significant difference between αA- and αB-crystallin in suppressing the aggregation of abrin B-chain at two different temperatures (25 and 37°C). HSP26, an another small heat shock/α-crystallin family protein, was also able to prevent the DTT-induced aggregation of abrin. These results suggest that due to relatively larger size of its B-chain (33 kDa), compared to insulin B-chain (about 3 kDa), abrin may serve as a better model substrate for in vitro chaperone studies of α-crystallin and as well as other sHSP.** © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

1. Introduction

α-Crystallin, the major eye lens protein, belongs to small heat shock protein (sHSP) family, due to its structural and functional similarities [1–3]. The lenticular α-crystallin consists of αA and αB subunits of 20 kDa each and exists as a polydisperse oligomer with an average molecular mass of 600–800 kDa [2]. More recently α-crystallin has been shown to be present in a number of non-lenticular tissues [2–5], suggesting that it may have a more general cellular function. Like all other sHSP, α-crystallin acts as a molecular chaperone by preventing the aggregation of other proteins denatured by heat or other stress conditions [2,6–10]. Although, the mechanism of chaperone function of α-crystallin is not fully understood, it was shown that α-crystallin specifically recognizes aggregation-prone, non-native structures that occur early on the denaturation pathway of protein [11–13]. Numerous studies have shown that the chaperone activity of the proteins is dependent on the presence of surface-exposed hydrophobic patches [7,14–16]. α-Crystallin forms stable complexes with a wide variety of chemically or thermally denatured proteins [9,11,13,17,18]. Both αA and αB subunits participate in chaperone activity [10,13,16,19,20]. Many of the above mentioned in vitro studies and some studies with lens epithelial cell lines [21,22] and α-crystallin knockout mice [23,24] suggest that the chaperone function of α-crystallin is of considerable importance in vivo and in particular in the prevention of cataract formation.

In view of the suggested physiological roles of α-crystallin, a variety of substrates and strategies have been used in studying the mechanism of chaperone function of α-crystallin. Except insulin assay, in many aggregation assays, α-crystallin is shown to undergo some structural perturbation due to heat, chemical (like guanidine hydrochloride) or physical (UV light) factors [15,19,25,26]. Therefore insulin has been widely used to study the chaperone function of α-crystallin under native conditions. Though, not as widely as insulin, α-lactalbumin is also used to study the chaperone activity of α-crystallin under native conditions. However, α-lactalbumin requires specific experimental conditions for aggregation and the extent of aggregation as well as the unfolding intermediates vary accord-

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ing to the experimental conditions [17,18,25,27]. α-Crystallins have also been shown to prevent the aggregation/inactivation of many enzymes caused by heat, UV radiation, sugars and other chemicals [19,28,29,30]. Though heat-induced aggregation of citrate synthase [19,29] and alcohol dehydrogenase [10,19] have been commonly used to assay chaperone activity, the lowest temperature required to induce considerable aggregation is 40–45°C. Although, insulin aggregation has advantages, extrapolating the mechanism(s) based on binding of insulin B-chain to α-crystallin may not be appropriate due to its small size (3 kDa). Moreso, many, if not all, putative physiological substrate proteins of α-crystallin may be relatively larger in size at least to the order of about 7–10 times the insulin B-chain. In this context, we investigated whether dithiothreitol (DTT)-induced aggregation of abrin can be used to understand the chaperone function of α-crystallin under native conditions.

Abrin is a type II ribosome inactivating protein isolated from the seeds of *Abrus precatorius*. It consists of two subunits, a 30 kDa A-chain and a lectin-like B-chain of 33 kDa joined by a single disulfide bond [31]. The A-chain is the toxic one having the RNA *N*-glycosidase activity and brings about inhibition of protein synthesis [32]. The B-chain is essential for the entry of the toxin into the cell as it binds to the cell surface glycoproteins or receptors [31,32]. In vitro, reduction of a disulfide link between the two chains of abrin by DTT or 2-mercaptoethanol leads to aggregation of the B-chain. This property of abrin B-chain could be utilized for studying the activities of many chaperones including α-crystallin by quantifying their capacity in preventing this aggregation. However, so far, abrin aggregation has not been employed to study the chaperone activity of any molecular chaperone.

In the present communication, we demonstrate that abrin B-chain aggregates when reduced with DTT with similar kinetics to that of insulin B-chain aggregation and αA- and αB-crystallins prevent this aggregation in a concentration-dependent manner, suggesting that abrin could be used as an in vitro substrate to understand chaperone mechanism of α-crystallin.

2. Materials and methods

2.1. Purification of abrin

The protein was purified from the seeds of *A. precatorius* as described previously [33]. Briefly, the seed kernels were soaked overnight in 5% acetic acid and homogenized. The crude extract was subjected to 30 and 90% ammonium sulfate precipitation followed by extensive dialysis in 10 mM phosphate-buffered saline (PBS), pH 7.4. The dialyzed protein was centrifuged and the supernatant was loaded onto the Lactamyl–Sepharose affinity column equilibrated with PBS. The unbound proteins were removed by washing the column with PBS. The bound proteins were then eluted with 0.4 M lactose. The lactose fractions were pooled and loaded onto the gel filtration column. The proteins were eluted with 20 mM PBS. The fractions corresponding to peak II were pooled, dialyzed extensively and lyophilized. Protein concentration was determined using its molar extinction coefficient $100\,170\ \text{M}^{-1}\ \text{cm}^{-1}$.

2.2. Overexpression and purification of human recombinant αA- and αB-crystallins

Bacterial (BL21) cells containing expression vectors of human αA- and αB-crystallin were kindly provided by Dr. J. Mark Petrash (Washington University, St. Louis, MO, USA). Proteins from 1 l cultures were extracted and purified to apparent homogenity according to the procedures described previously [19]. The purified recombinant α-crystallins showed on SDS–polyacrylamide gel a single band

corresponding to protein with a molecular weight of approximately 20 kDa. Protein concentration was determined spectrophotometrically using the molar extinction coefficient, ε_{280}, of 16 500 and 19 000 M^{-1} cm^{-1} for αA- and αB-crystallin, respectively [10].

2.3. Chaperone activity

Chaperone activity of purified recombinant human αA- and αB-crystallins and HSP26 (a gift from Prof. J. Buchner, Technical University Munich) was assessed by measuring their ability to prevent DTT-induced aggregation of insulin B-chain [8,14].

2.4. Abrin aggregation

The aggregation of abrin (675 μg/ml) in 50 mM sodium phosphate buffer, pH 7.4 containing 100 mM NaCl, was initiated by the addition of 30 mM DTT in 1 ml reaction volume at required temperatures (mentioned in the legends to figures). The extent of B-chain aggregation was measured as a function of time by monitoring light scattering at 400 nm in a Cary Bio 100 spectrophotometer.

2.5. Suppression of abrin aggregation by αA- and αB-crystallins and HSP26

The suppression of aggregation of abrin B-chain by αA- and αB-crystallins and HSP26 was studied by incubating abrin with the required concentrations of either αA- or αB-crystallin or HSP26 for 10 min. Aggregation was initiated by the addition of 30 mM DTT and the extent of aggregation was measured as mentioned above. The relative chaperone activity of αA- and αB-crystallin and HSP26 was calculated considering abrin aggregation in the absence of crystallins and HSP26 as 100% after 70 min of incubation with DTT.

2.6. SDS–PAGE

Abrin aggregation in the presence or absence of either αA- or αB-crystallin was analyzed on 12% polyacrylamide gels in the presence of SDS. The aggregation of abrin in the presence or absence of αA- and αB-crystallin was initiated as described above. After 70 min, samples were centrifuged at $10\,000 \times g$ for 15 min and supernatant was collected. The precipitate was washed twice with phosphate buffer and resuspended in the same buffer. Required amount of supernatant and redissolved precipitate samples were treated with equal volumes of 2× SDS sample buffer and electrophoresis was carried out in a Bio-Rad mini gel apparatus.

2.7. Gel filtration

The formation of complex between α-crystallins or HSP26 and the B-chain of abrin was studied by gel filtration chromatography on a 600×7.5 mm TSK-G2000 SW column (Tosoh Co., Japan) using a Shimadzu HPLC system. The column was equilibrated with 0.1 M sodium phosphate buffer, pH 6.7, containing 0.1 M sodium sulfate at
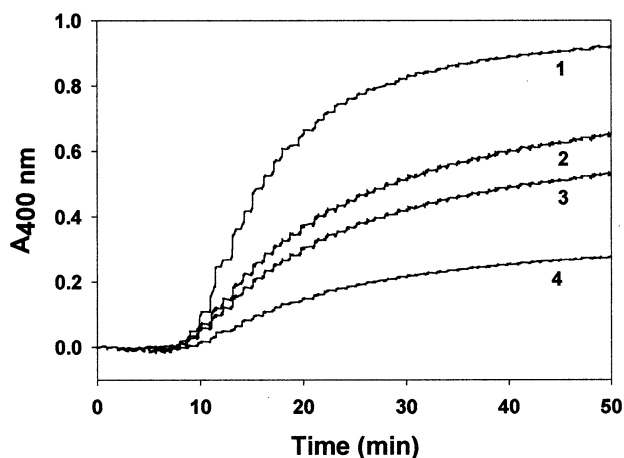

Fig. 1. The chaperone activity of αA- and αB-crystallins and HSP26 at 25°C. Insulin (0.4 mg/ml in 50 mM phosphate buffer, pH 7.4) was reduced with 20 mM DTT and the aggregation of the insulin B-chain in the absence (trace 1) and presence of 0.3 mg/ml αA- (trace 2), HSP26 (trace 3) and αB-crystallin (trace 4) was monitored by measuring the apparent absorption at 400 nm.
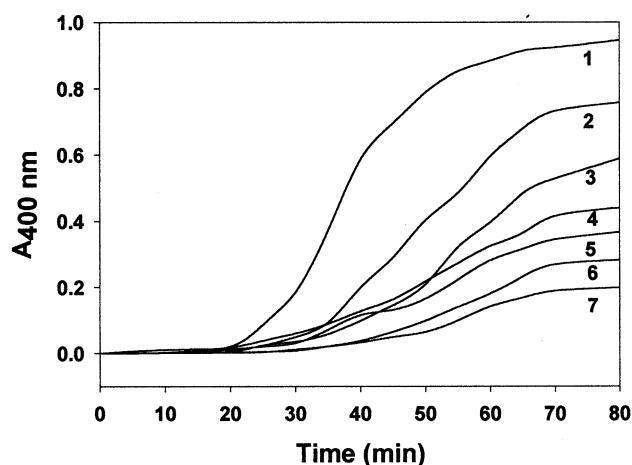
Fig. 2. DTT-induced aggregation of abrin B-chain and suppression by αB-crystallin at 25°C. Abrin (0.675 mg/ml in 50 mM phosphate buffer, pH 7.4, containing 100 mM NaCl) was reduced with 30 mM DTT in a final volume of 1 ml and the aggregation of the B-chain in the absence (1) and presence of 0.10 (2), 0.20 (3), 0.30 (4), 0.40 (5), 0.5 (6) and 0.60 mg/ml of αB-crystallin (7) was monitored by measuring the apparent absorption at 400 nm. The graph is a representative plot of the three individual experiments.
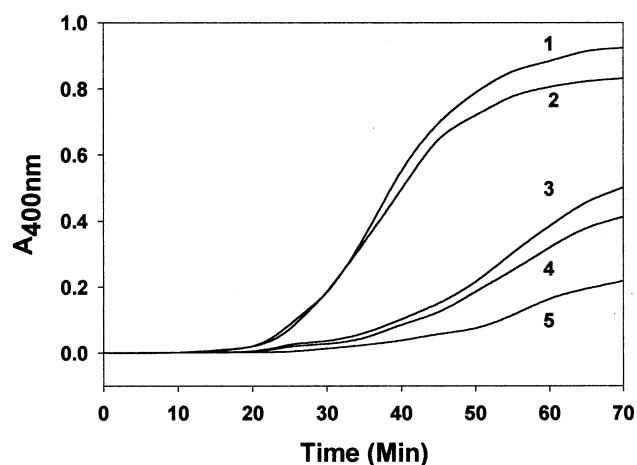
Fig. 3. Suppression of abrin B-chain aggregation by αA- and αB-crystallins and HSP26 at 25°C. Abrin (0.675 mg/ml in 50 mM phosphate buffer, pH 7.4, containing 100 mM NaCl) was reduced with 30 mM DTT and the aggregation of the abrin B-chain in the absence (1) and presence of 0.5 mg/ml αA- (3), HSP26 (4) and αB-crystallin (5) was monitored by measuring the apparent absorption at 400 nm. As a negative control we have also assessed the aggregation of abrin B-chain in the presence of 0.5 mg/ml BSA (2).
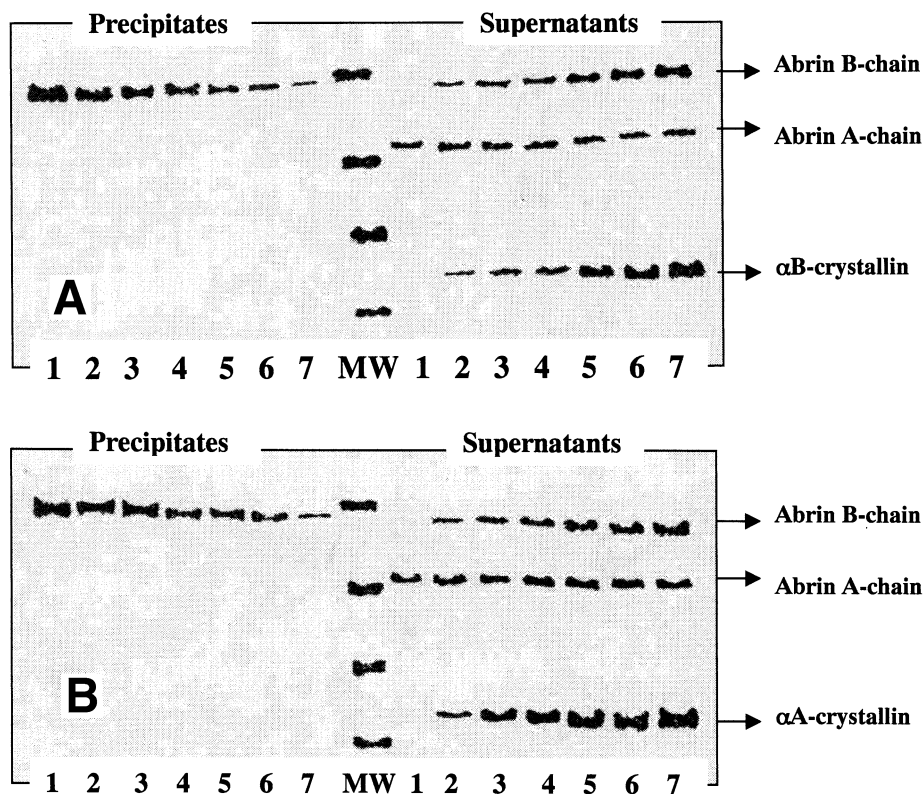

Fig. 4. SDS–PAGE proof of formation of complex between abrin B-chain and αA- or αB-crystallin. Abrin (0.675 mg/ml in 50 mM phosphate buffer, pH 7.4, containing 100 mM NaCl) was reduced with 30 mM DTT in a final volume of 1 ml and the aggregation of the B-chain in the absence (1) or presence of 0.10 (2), 0.20 (3), 0.30 (4), 0.40 (5), 0.5 (6) and 0.60 (7) mg/ml of αB-crystallin (A) and αA-crystallin (B). At the end of 70 min, incubation samples were centrifuged at $10\,000 \times g$ and supernatant was collected. Pellet was washed twice with the incubation buffer and reconstituted in 200 μl of the same buffer. Supernatant and reconstituted precipitate samples were treated with equal volumes of 2× SDS sample buffer and loaded onto a 12% polyacrylamide gel. MW, molecular weight standards (34, 29, 24 and 18 kDa in descending order).

a flow rate of 1 ml/min. Abrin (0.675 mg/ml) in the absence or presence 0.5 mg/ml of αA- and αB-crystallin and HSP26 was reduced with 30 mM DTT as described above. After 70 min the samples were centrifuged at $10\,000 \times g$ for 15 min and the supernatants (20 µl) were loaded on to the column.

3. Results and discussion

The ability of recombinant human αA- and αB-crystallins and HSP26 in suppressing the aggregation of insulin B-chain is shown in Fig. 1. While both αA- and αB-crystallins are effective in preventing the aggregation of insulin B-chain, αB is approximately three times more effective than αA at 25°C for the same amount of the protein. HSP26 is slightly better than αA-crystallin in suppressing the aggregation of insulin (Fig. 1).

Reduction of the disulfide bond bridging the abrin A- and B-chain leads to the unfolding and aggregation of the B-chain. The aggregation reaction can be monitored by simple light scattering similar to that of insulin. Fig. 2 shows the abrin B-chain aggregation kinetics due to light scattering at 400 nm. Aggregation starts after 25 min of DTT addition and keeps increasing before it reaches a saturation level by 60–70 min. Interestingly this aggregation can be suppressed by αB-crystallin in a concentration-dependent manner (Fig. 2). Similarly, αA-crystallin and HSP26 are also effective in suppressing DTT-induced aggregation of abrin B-chain (Fig. 3). However, an unrelated protein, bovine serum albumin (BSA), did not prevent the aggregation of abrin (Fig. 3). About 90% protection against abrin aggregation is seen at an approximate stoichiometry 1:1.2–1.5 of abrin B-chain and αA-, αB-crystallin and HSP26 monomers.
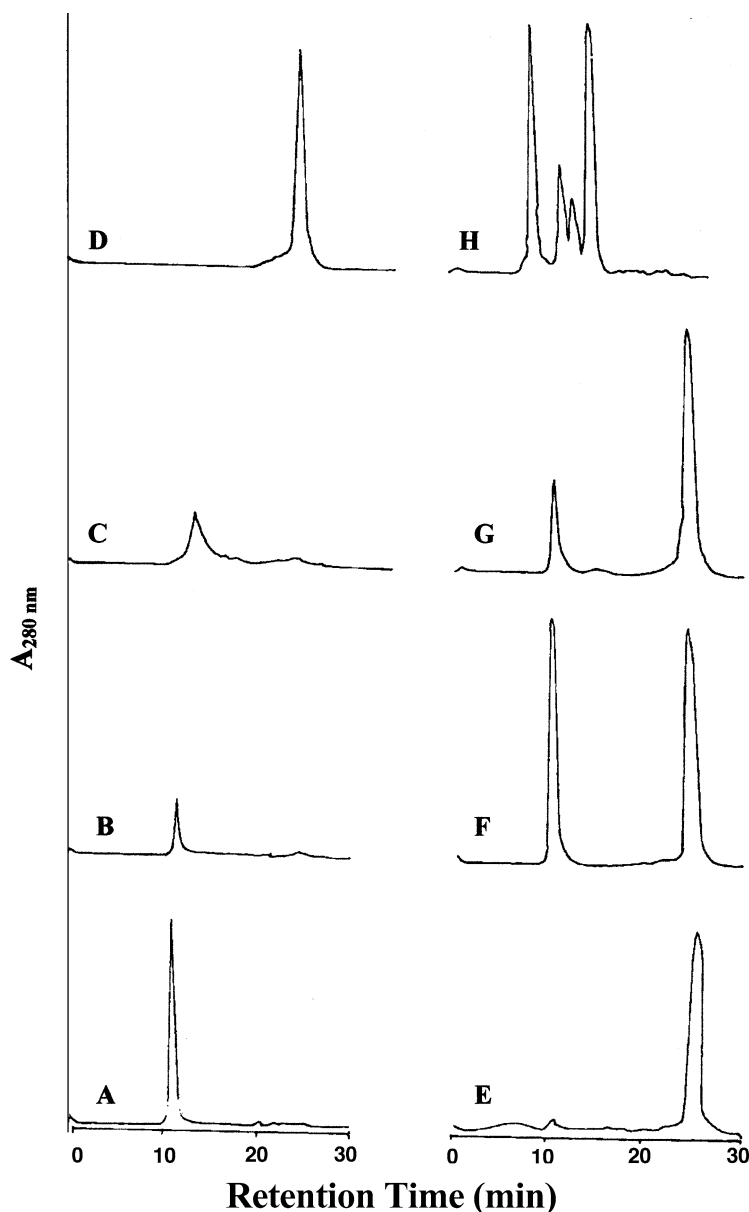

Fig. 5. HPLC analysis of the complex formation between αB-crystallin and abrin B-chain or between HSP26 and abrin B-chain by gel filtration chromatography on TSK-G-2000SW column. Elution profile of αB-crystallin (A), HSP26 (B), abrin (C), DTT (D), abrin reduced with DTT (E) and abrin reduced with DTT in the presence of either αB-crystallin (F) or HSP26 (G). Details were given in Section 2. H: Profile of molecular weight markers with retention time in parentheses: thyroglobulin (11), BSA (14.3), ovalbumin (15.7) and carbonic anhydrase (17.5).

In the case of insulin, α-crystallin binds to the non-native conformer of insulin B-chain and prevents further aggregation [8,14]. To understand whether α-crystallin also binds to the B-chain of abrin and forms a soluble and stable complex, soluble and precipitate fractions of aggregation reaction mixtures of abrin with and without αA- and αB-crystallin were analyzed by SDS–PAGE. In the absence of α-crystallin, abrin B-chain is precipitated completely leaving A-chain in solution (Fig. 4). But when α-crystallin was present during aggregation it prevented the precipitation of B-chain from solution in a concentration-dependent manner (Fig. 4), which paralleled the decrease in light scattering (Fig. 2). These results suggest that probably α-crystallin is binding to aggregation-prone abrin B-chain mostly in a non-native conformer and forming stable soluble complexes thereby preventing the precipitation of B-chain.

Further, the complex formation between α-crystallins or HSP26 and abrin B-chain was shown by gel filtration. αB-Crystallin and HSP26 eluted in the void volume (11.1 min), abrin eluted as a 65 kDa protein (14.4 min) and DTT in exclusion volume (24. 9 min) as expected (Fig. 5). Upon reduction with DTT and after centrifugation, the native abrin peak had disappeared and the unfolded abrin A-chain (in the supernatant) eluted together with DTT in exclusion volume as abrin B-chain was precipitated out. While the area under peak for only DTT was 35 867, it was 54 788 for DTT and abrin A-chain peak together, indicating DTT and abrin A-chain coelution. This is not surprising as the subunits of many unfolded proteins have been shown to exist in expanded state or in the compact form and thereby eluting in either void volume [34] or in exclusion limits (P. Raghu, G.B. Reddy, B. Sivakumar unpublished data; [35]), respectively than the usually expected molecular weight volumes on gel filtration. Interestingly, the area under peak for αB-crystallin (41 861) and HSP26 (15 261) was higher when incubated with abrin and DTT for 70 min compared to the native αB-crystallin (16 524) and HSP26 (5929) alone, suggesting the formation of αB-crystallin–abrin B-chain or HSP26–abrin B-chain complex (Fig. 5). Interpretation of HPLC data based on area under the peak may not be direct proof to demonstrate the complex formation between chaperone and abrin B-chain. Therefore, the peaks corresponding to αB-crystallin–abrin B-chain (peak 1 of Fig. 5F) or HSP26–abrin B-chain (peak 1 of Fig. 5G) complex were further analyzed by SDS–PAGE which showed bands corresponding to αB-crystallin and abrin B-chain or HSP26 and abrin B-chain, respectively (data not shown).

Previously it was reported that, despite high sequence homology, αA- and αB-crystallins behave differently with increasing temperature (even within the physiological range) with respect to their chaperone potential, secondary and tertiary structure and other physicochemical properties [19,20,36,37]. Therefore, abrin aggregation was carried out at two different physiological temperatures, 25 and 37°C, in the presence of αA or αB-crystallin or HSP26. The relative protection by αA-, αB-crystallin and HSP26 as a function of temperature is shown in Fig. 6. Though αB-crystallin is more effective than αA in suppressing the aggregation of abrin at 25°C, the difference is only marginal. Unlike with insulin [19], this difference between αA- and αB-crystallins in suppressing abrin aggregation is maintained at 37°C also. With insulin it was observed that αB-crystallin shows about three times more

chaperone activity than αA-crystallin at and around 25°C (Fig. 1) [19], but as the temperature is increased the difference between the chaperone potential of αA and αB-crystallins decreases [19].

The mode of interaction of unfolded proteins or the so-called non-native proteins with α-crystallin is of considerable interest in understanding its chaperone activity. It is believed that binding of non-native proteins to α-crystallin is driven by hydrophobic interactions [12–16]. In addition, studies have clearly shown that α-crystallin undergoes some structural/conformational changes under thermal and other conditions [14,15,19,20,25,26]. Furthermore, in glycation and steroid-induced enzyme inactivation studies, α-crystallins may also undergo modifications [30]. Therefore, it is important to choose the conditions, which do not affect α-crystallin structure and function. Insulin which can be unfolded by reduction with DTT seems to be suitable, since α-crystallin has no disulfide bonds and therefore is unaffected by the reduction. Even under physiologically relevant temperatures (37–40°C) αB is more potent as a chaperone with respect to different substrates tested including insulin [19]. The relative chaperone activity of αA and αB varies with different substrates and at different temperatures [19,20,37]. In general, the greater chaperone activity of αB (compared to αA) has been attributed to its higher surface hydrophobicity.

In addition, recently we found no substantial difference between αA and αB in protecting the glucose-6-phosphate dehydrogenase from in vitro UVB-induced inactivation [28]. Furthermore, irrespective of the similarity between abrin and insulin with respect to aggregation upon disulfide reduction, unlike with insulin, αA and αB are invariably effective in suppressing the aggregation of abrin B-chain at both the temperatures tested (Fig. 6). The assessment of 'intrinsic' chaperone activity of α-crystallin is therefore complicated by the nature of substrate proteins, effect of temperature and hydrophobicity. The present data thus provide a compelling evidence that in addition to hydrophobicity the nature of the substrate (i.e. length and topology?) may equally influence the chaperone function of α-crystallin.
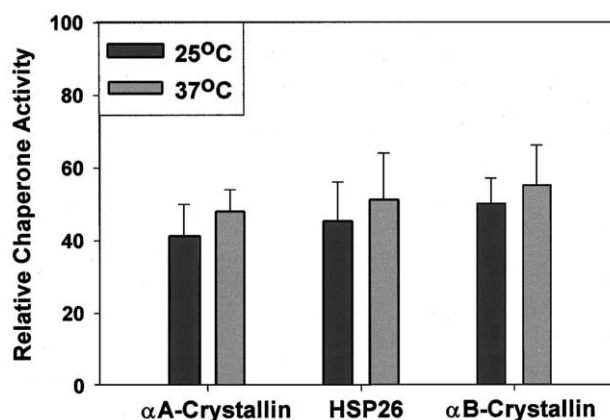

Fig. 6. The relative chaperone activity of αA-, αB-crystallin and HSP26 against the aggregation of abrin B-chain at 25 and 37°C. Abrin (0.675 mg/ml in 50 mM phosphate buffer, pH 7.4, containing 100 mM NaCl) was reduced with 30 mM DTT in a final volume of 1 ml in the presence of 0.50 mg/ml of αA-, αB-crystallin or HSP26 and the aggregation was monitored by measuring the apparent absorption at 400 nm. The relative protection was calculated as described in Section 2. Data represents mean ± S.D. of three independent experiments.

The results of the present study thus reveal that due to its relatively larger size, abrin may serve as a better substrate for characterizing the mechanism of chaperone function of α-crystallin and other sHSPs in vitro. Studies are underway to investigate the structural elements of α-crystallin that are involved in chaperoning abrin B-chain using synthetic peptides that correspond to different regions of αA- or αB-crystallins and site-directed mutagenesis approach.

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