Effect of the chaperone-like alpha-crystallin on the refolding of lysozyme and ribonuclease A

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Abstract Alpha-crystallin exhibits chaperone-like properties in preventing aggregation of proteins. We have studied the effect of alpha-crystallin on the refolding of denatured-disulfide intact and denatured-reduced lysozyme and RNase A. Alpha-crystallin does not have any effect on the refolding of both the denatured-disulfide intact enzymes. However, it inhibits the aggregation and oxidative renaturation of denatured-reduced lysozyme. Interestingly, it has no effect on the refolding of denatured-reduced RNase A. In order to probe the molecular basis of this differential behavior of alpha-crystallin towards lysozyme and RNase A, we have carried out circular dichroism and fluorescence studies on the refolding of denatured-reduced RNase A. It exhibits an extended conformation with little difference in the exposed hydrophobicity during the refolding process. We have earlier shown the presence of an aggregation-prone, refolding-competent, molten-globule-like intermediate on the refolding pathway of lysozyme. Alpha-crystallin binds to this intermediate, prevents its aggregation and inhibits its oxidative refolding. It was earlier believed that alpha-crystallin, unlike other chaperones, does not recognize intermediates on the refolding pathway but only recognizes intermediates on the unfolding pathway of proteins. Our present study clearly shows that it recognizes the refolding intermediates as well.

Key words: Alpha-crystallin; Chaperone; Substrate conformation; Lysozyme; RNase A

1. Introduction

Alpha-crystallin, a multimeric protein present in the eye lens in large amount, has been shown to exhibit chaperone-like activity in preventing the aggregation of other proteins [1]. It is made up of two homologous gene products, αA and αB; bovine α-crystallin consists of both αA and αB in the ratio of 3:1 respectively [2]. Both the polypeptide chains exist in non-lenticular tissues [3,4] and exhibit chaperone-like activity, however, to different extents [5]. α-Crystallin shares both sequence and secondary structural homology with small heat shock proteins (sHsps) and behaves in several ways like sHsps [6–9].

We have earlier shown that α-crystallin prevents the UV-induced aggregation of γ-crystallin under physiologically relevant temperatures [10] and the aggregation of β- and γ-crystallin upon co-refolding [11]. Post-translational modifications such as glycation, oxidation, crosslinking etc., [12,13] and photolysis with UV radiation appear to affect the function of α-crystallin [14,15]. α-Crystallin from cataractous human lenses [12] and from selenite-induced cataract in animal model [16] has been shown to exhibit reduced chaperone-like activity. It has been shown to reduce the inactivation of enzymes such as the glycation-induced inactivation of malate dehydrogenase [17]. Brady et al. [18] showed that targeted disruption of the mouse αA-crystallin gene induces lens opacity. These studies suggest that the chaperone-like activity of α-crystallin may be important in the formation and maintenance of eye lens transparency.

To understand the molecular mechanism of chaperone function, it is important to study the conformational aspects of target proteins which are recognized by α-crystallin. An earlier study from our laboratory showed that α-crystallin binds to the molten-globule state of carbonic anhydrase [19]. Das et al. [20] have shown that the conformational state of γ-crystallin bound to α-crystallin is compact in nature. Das and Surewicz [21] reported that α-crystallin only recognizes intermediates on the unfolding pathway but not on the refolding pathway of proteins. In the present study, we have investigated the effect of α-crystallin on the refolding of denatured-disulfide intact and denatured-reduced hen egg white lysozyme and bovine pancreatic RNase A. We show that α-crystallin can interact with refolding intermediates and also provides an insight into the conformational states of refolding intermediates which can interact with α-crystallin.

2. Materials and methods

2.1. Materials

Hen egg white lysozyme, RNase A, cytidine 3′:5′ cyclic monophosphate (cyclic CMP) and DL-cystine hydrochloride were purchased from Sigma Chemical Company, USA. 8-anilinonaphthalene-1-sulfonic acid (ANS) was obtained from Aldrich Chemical Company, USA. Dithiothreitol (DTT) was purchased from SISCO Research Laboratories, India. Guanidinium chloride (GdmCl) was purchased from Serva, Heidelberg. Lysozyme was further purified as described by Saxena and Wetlaufer [22]. α-Crystallin from calf eye lens was isolated and purified as described in our earlier study [10].

2.2. Preparation of denatured and denatured-reduced enzymes

Denatured enzymes were prepared by dissolving the enzymes (11.5 mg lysozyme and 12 mg RNase A) in 1 ml of 50 mM Tris-acetate buffer (pH 8.1) containing 6.5 M GdmCl. The sample was incubated at 25°C for about 16 h. Denatured-reduced enzymes were prepared similarly except the buffer also contained 80 mM DTT.

2.3. Refolding experiments

Refolding of the denatured enzymes was performed by a 100-fold dilution of the denatured enzyme into 100 mM Tris-acetate buffer pH 8.1 (refolding buffer) either in the absence or in the presence of 1 mg/ml α-crystallin at 25°C or at 45°C. Small aliquots were withdrawn at different time intervals and the enzyme activity was measured. Refolding of denatured-reduced enzymes was performed similarly in the absence or in the presence of 0.5 mg/ml α-crystallin, by a 100-fold dilution of the denatured-reduced stock into the refolding buffer that also contained 1 mM cystine hydrochloride at 37°C.

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2.4. Circular dichroism and ANS binding experiments

Refolding of denatured-reduced RNase A was performed as mentioned above in the refolding buffer alone (without α-crystallin and cystine hydrochloride) and the far UV circular dichroism (CD) spectrum of this sample was recorded between 3 and 6 min after initiation of the refolding. CD spectra were recorded using a JASCO J-715 spectro-polarimeter. CD spectra of native and denatured RNase A (in 6.0 M GdmCl) were also recorded.

Fluorescence spectra of native RNase A and lysozyme were recorded in the presence of 50 μM ANS using a Hitachi F-4000 fluorescence spectrophotometer. The fluorescence of ANS bound to the refolding lysozyme or RNase A was performed by adding ANS 3 min after the initiation of refolding of the denatured-reduced enzymes into the buffer lacking cystine hydrochloride. The excitation wavelength was set at 365 nm. The excitation and emission band passes were set at 5 nm. All spectra were recorded in correct spectrum mode.

2.5. Enzyme assay

Lysozyme activity was determined at 25°C essentially as described by Fischer et al. [23]. The rate of enzymatic lysis of *M. lysodeikticus* cells, suspended in 0.1 M phosphate buffer (pH 6.3), was obtained by measuring the decrease in turbidity of the cell suspension at 450 nm as a function of time using a Hitachi U-2000 UV-visible spectrophotometer.

The activity of RNase A was measured using the method described by Crook et al. [24] using cyclic CMP as substrate. Ten μg of RNase A sample was added to 0.1 mg/ml of cyclic CMP in 100 mM Tris-HCl buffer (pH 7.13) (the ionic strength adjusted to 0.2 with NaCl) and the increase in optical density at 284 nm was monitored as a function of time. The rate of increase in the optical density is the measure of the activity of the enzyme. The percentage renaturation yields in the refolding studies of the enzymes were calculated with respect to the activity of the native enzymes.

3. Results and discussion

The formation of chaperone-target protein complex which prevents the aggregation of partially folded states of the target proteins is an important step in the chaperone function. α-Crystallin is known to prevent the heat-induced aggregation of other proteins like a molecular chaperone by forming a stable complex. We have investigated the effect of α-crystallin on the refolding process of denatured-disulfide intact and denatured-reduced hen egg white lysozyme and bovine pancreatic RNase A to understand the effect of the chaperone-like α-crystallin on the refolding process of the enzymes.

Lysozyme is one of the most extensively studied enzymes for its refolding properties [22,23,25]. As the concentration of the enzyme to be refolded increases the renaturation yield drastically decreases due to aggregation [25]. We have earlier investigated the refolding of denatured and denatured-reduced lysozyme [26]. Refolding of denatured lysozyme even at 1 mg/ml concentration did not result in any aggregation and almost 100% activity of the enzyme was recovered. However denatured-reduced lysozyme aggregated even at the lowest concentration studied (50 μg/ml) [26]. We found that under appropriate concentrations of thiol/disulfide reagents it can be refolded to its active state with high renaturation yields [26].

Table 1
Refolding of denatured lysozyme (at 0.115 mg/ml) and RNase A (at 0.12 mg/ml) in the absence and in the presence of 1 mg/ml α-crystallin

Enzymes	Time (min)	% Recovery of activity[a]			
		25°C		45°C	
		−α	+α	−α	+α
Lysozyme	1	80	86	78	80
	8	90	92	83	86
RNase A	1	83	90	–	–
	7	83	89	–	–

[a]The % recovery of activity is with respect to the activity of the native enzymes.

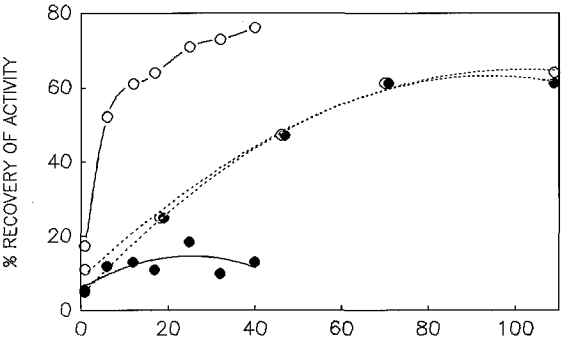

Fig. 1. Oxidative refolding of denatured-reduced lysozyme (——) and RNase A (- - -) at 0.115 mg/ml and 0.12 mg/ml respectively in the absence (○) and in the presence (●) of 0.5 mg/ml α-crystallin at 37°C. The refolding buffer, 100 mM Tris-acetate (pH 8.1), also contained 1 mM cystine hydrochloride. The percentage recovery of activity is with respect to the activity of the native enzymes.

Table 1 shows the percentage recovery of activity of lysozyme upon refolding from its denatured state in the absence and in the presence of α-crystallin. The denatured-disulfide intact lysozyme refolds to its active state within a short time period (e.g. 1 min). α-Crystallin neither has a significant effect on the renaturation yield, nor does it slow down the renaturation process as evident from the table. Since α-crystallin exhibits temperature-induced structural alterations which are important in its chaperone-like activity [10,27,28], we have performed refolding of denatured lysozyme at 45°C. Even at this temperature, α-crystallin neither affects the renaturation yield nor slows down the renaturation process.

Refolding of denatured-reduced lysozyme in the absence of disulfide exchange reagents at 0.15 mg/ml by a 100-fold dilution into the refolding buffer alone, resulted in aggregation and yielded a turbidity value of 0.83 (measured as optical density at 500 nm). On the other hand, refolding of it similarly in the buffer that also contains 0.5 mg/ml α-crystallin results in much reduced aggregation and yields a turbidity value of only 0.3. Thus, α-crystallin prevents the aggregation of the enzyme significantly upon refolding it from its denatured-reduced state.

We have earlier shown that the refolding pathway of denatured-reduced lysozyme involves an intermediate state which possesses significant amount of secondary structure while lacking tertiary structure [26]. This intermediate binds the hydrophobic dye, ANS with greater avidity compared to the native or fully unfolded enzyme. This property of the intermediate is similar to that of the molten-globule state of proteins. This aggregation-prone intermediate can be oxidatively refolded to its active form in the presence of disulfide reagents such as cystine or GSSG [26]. Hence, we have investigated the effect of α-crystallin under this condition which results in productive folding of the enzyme to its native state. Fig. 1 shows that the denatured-reduced lysozyme refolds with about 80% renaturation yield in the absence of α-crystallin. The presence of α-crystallin inhibits this oxidative renaturation of the denatured-reduced lysozyme. This suggests that α-crystallin interacts with the refolding-competent intermediate of lysozyme, preventing the aggregation of this intermediate

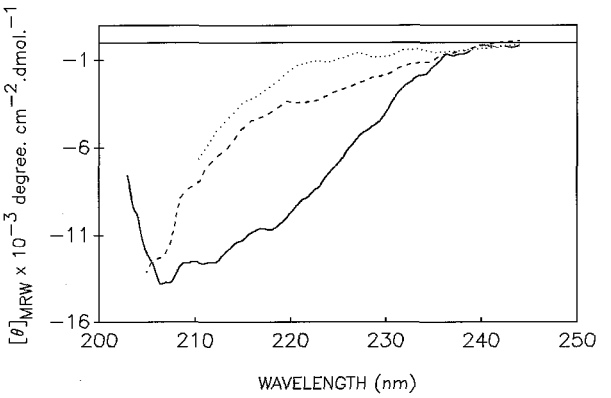

Fig. 2. Far UV-CD spectra of (——) native, (· · ·) denatured or denatured-reduced (in 6.0 M GdmCl) states of RNase A and the (- - -) refolding RNase A (obtained between 3 and 6 min of initiation of refolding of denatured-reduced enzyme by 100-fold dilution into the refolding buffer lacking cystine hydrochloride).

as well as the oxidative refolding of denatured-reduced lysozyme. Such an inhibition of regeneration of activity of an enzyme by α-crystallin under conditions which favor renaturation of the enzyme has not been shown earlier. GroEL is known to inhibit the reactivation of chicken dihydrofolate reductase, which can otherwise refold to its active state, by forming a complex [29]. The fact that α-crystallin does not inhibit the regeneration of lysozyme upon refolding from its denatured-disulfide intact state suggests that a molten-globule-like intermediate on its refolding pathway is either absent present or not sufficiently long-lived to interact with α-crystallin.

We have also studied the effect of α-crystallin on the refolding of RNase A, an enzyme which has been shown to refold to its native state [30]. It is evident from Table 1 and Fig. 1 that α-crystallin does not inhibit the reactivation of RNase A upon refolding of the enzyme either from its denatured-disulfide intact or denatured-reduced states. In order to understand the molecular basis of the differential behavior of α-crystallin towards the oxidative refolding of lysozyme and RNase A, we have investigated circular dichroism and ANS binding of a sample of denatured-reduced RNase A between 3 and 6 min of initiation of refolding by diluting into the refolding buffer containing no cystine. Fig. 2 shows the far UV-CD spectrum of this sample along with the spectra of the native and denatured enzyme (in 6.0 M GdmCl). Unlike in the case of lysozyme [26], denatured-reduced RNase A within 3–6 min of its refolding shows an extended conformation. Fig. 3A shows that this state of RNase A binds the hydrophobic dye ANS only marginally higher compared to its native state. On the other hand, the intermediate state of lysozyme exhibits 7–8-fold more solvent exposed hydrophobicity compared to its native state (Fig. 3B). These results suggest that α-crystallin does not interact with the state of RNase A having a more extended conformation and less solvent exposed hydrophobic surfaces. These results also suggest the absence of a compact, hydrophobic intermediate on the refolding pathway of RNase A which could interact with α-crystallin. This explains the lack of any effect of α-crystallin on the refolding of RNase A. Thus, it appears that α-crystallin does not interact with extended conformational states of target proteins. This conclusion is in agreement with the observation that α-crystallin does not form a stable complex with reduced carboxymethylated α-lactalbumin which assumes extended conformation [31].

Das and Surewicz [21] studied the chaperone-like activity of α-crystallin towards the thermal- and refolding-induced aggregation of rhodanese and found that it prevents the thermal-induced aggregation of rhodanese at 47°C but does not prevent the refolding-induced aggregation of the protein at 25°C. They concluded that unlike other molecular chaperones, α-crystallin recognizes only the non-native intermediates of proteins on their denaturing (unfolding) pathway but not on their refolding pathway. Our results on the refolding of denatured-reduced lysozyme, on the other hand, show that α-crystallin does recognize the refolding intermediates. We have earlier shown that the chaperone-like activity of α-crystallin is enhanced several fold above 30°C and the subtle changes in tertiary and/or quaternary structure of α-crystallin which lead to enhanced exposure of its hydrophobic surfaces are important in its chaperone-like activity [10,27,28]. Smith et al. [32] used hydrogen-deuterium exchange of amide proton to study α-crystallin as a function of temperature. Their observation supported our hypothesis that α-crystallin prevents the aggregation of non-native structures of target proteins by providing appropriately placed hydrophobic surfaces [10] and extended it further by suggesting the regions of α-crystallin that may become exposed with temperature [32]. Thus, the apparent discrepancy between our results and those of Das and Surewicz [21] can be explained on the basis of the temperature at which the experiments were conducted. Our recent study shows that the aggregation of the β_L-crystallin upon its

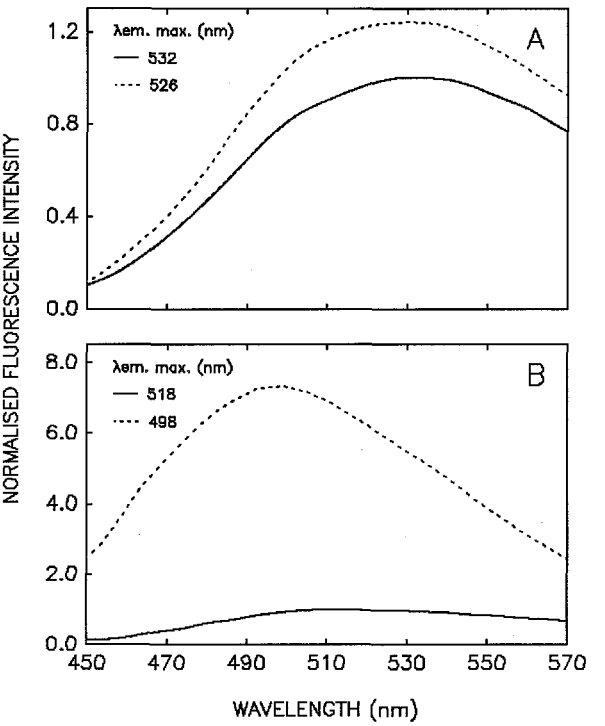

Fig. 3. Fluorescence spectra of ANS bound to RNase A (A) and lysozyme (B). (——) native and (- - -) the refolding enzymes (obtained between 3 and 6 min of initiation of refolding of denatured-reduced enzymes by 100-fold dilution into the refolding buffer lacking cystine hydrochloride). 50 μM ANS was added 3 min after initiation of refolding and spectra recorded within 6 min of the initiation.

refolding is not prevented significantly at temperatures below 30°C and is prevented above this temperature [28] suggesting that α-crystallin interacts with refolding intermediates of β_L-crystallin and prevents their aggregation. It appears that some specificity is involved in the interaction of chaperone and substrate proteins. GroEL is known to bind to flexible molten-globule state of rhodanese and chicken dihydrofolate reductase [29] but not to interact strongly with α-lactalbumin either in its more compact molten-globule state or in its reduced state which assumes extended conformation [33]. The chaperones from Hsp70 family bind to extended conformations [34]. Our studies show that α-crystallin inhibits the aggregation as well as the oxidative renaturation of lysozyme by binding to the refolding intermediate of denatured-reduced lysozyme which has substantial amount of secondary structure with no tertiary structure but with more solvent exposed hydrophobic surfaces compared to the native enzyme. On the other hand, it does not inhibit the refolding of RNase A. This may be because α-crystallin does not interact strongly with the refolding enzyme which is in an extended conformation with negligible solvent exposed hydrophobic surfaces. These results should prove useful in understanding of chaperone-substrate interactions.

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