The Selective Inhibition of Serpin Aggregation by the Molecular Chaperone, α-Crystallin, Indicates a Nucleation-dependent Specificity*

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Small heat shock proteins (sHsps) are a ubiquitous family of molecular chaperones that prevent the misfolding and aggregation of proteins. However, specific details about their substrate specificity and mechanism of chaperone action are lacking. α1-Antichymotrypsin (ACT) and α1-antitrypsin (α1-AT) are two closely related members of the serpin superfamily that aggregate through nucleation-dependent and nucleation-independent pathways, respectively. The sHsp α-crystallin was unable to prevent the nucleation-independent aggregation of α1-AT, whereas α-crystallin inhibited ACT aggregation in a dose-dependent manner. This selective inhibition of ACT aggregation coincided with the formation of a stable high molecular weight α-crystallin-ACT complex with a stoichiometry of 1 on a molar subunit basis. The kinetics of this interaction occur at the same rate as the loss of ACT monomer, suggesting that the monomeric species is bound by the chaperone. 4,4′-Dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (Bis-ANS) binding and far-UV circular dichroism data suggest that α-crystallin interacts specifically with a non-native conformation of ACT. The finding that α-crystallin does not interact with α1-AT under these conditions suggests that α-crystallin displays a specificity for proteins that aggregate through a nucleation-dependent pathway, implying that the dynamic nature of both the chaperone and its substrate protein is a crucial factor in the chaperone action of α-crystallin and other sHsps.

A large number of in vitro studies have shown that many proteins undergo inappropriate conformational changes, due to changes in solution conditions or mutation, that result in self-association and aggregate formation (1). This chain of events forms the molecular basis for a range of disorders such as Alzheimer’s disease, Huntington’s disease, prion encephalopathies, and the serpinopathies, which have collectively been associated and aggregate formation (1–3). This scaffold presents a highly mobile reactive center that can be used to mediate specific interactions with non-native proteins as they unfold (6–8). α-Crystallin is the principal lens protein. It exists as a heteromultimer with an average mass of 800 kDa and is composed of two closely related subunits, A and B, each of ~20 kDa in mass. Until recently, α-crystallin was believed to be a lens-specific protein, protecting the other lens proteins from the precipitation associated with cataract blindness as well as providing transparency and refractive qualities (6–8). However, both αA- and αB-crystallin have been found in many extralenticular tissues including the liver and brain, although only αB-crystallin is induced under stress conditions.

Unlike members of the heat shock family of proteins such as GroEL/Hsp60, for which the chaperone mechanisms have been extensively characterized, less is understood about the precise mechanism and specificity of the sHsps. α-Crystallin typically sequesters aggregation-prone proteins into a high molecular weight complex and protects them from irreversible aggregation. However, under certain permissive folding conditions, bound substrates can be released, and in cooperation with other chaperones, in particular Hsp70 (6–8), regain their native structures. α-Crystallin undergoes a temperature-dependent conformational change that increases its chaperone activity. This structural change results in the exposure of an increased number of hydrophobic residues, suggesting that hydrophobic interactions play a key role in the chaperone activity of α-crystallin. Interestingly, these heat-induced changes are also accompanied by increased subunit exchange within the α-crystallin oligomer, indicating that, as with other sHsps, much smaller subunit oligomers may be the active chaperone species (6–8).

Here, we investigate the substrate specificity of α-crystallin using two members of the serine proteinase inhibitor (serpin) superfamily, α1-antitrypsin (α1-AT) and α1-antichymotrypsin (ACT) (9). Both serpins aggregate under mildly denaturing conditions, such as elevated temperature, via a loop-sheet mechanism that has been shown to occur for a number of pathological variants (10–16). The native serpin conformation consists of three β-sheets (A–C) surrounded by nine α-helices (A–I) (17). This scaffold presents a highly mobile reactive cen...
ter loop, extending from the fifth strand of the A β-sheet. Under conditions of partial denaturation, caused by environmental changes or mutation, this reactive center loop can insert into the A β-sheet of another serpin, thereby promoting loop-sheet polymerization (13). Recent investigations into the mechanisms of serpin polymerization have suggested that individual serpins aggregate via different pathways. For example, α1-AT polymerizes through the formation of an intermediate ensemble that self-associates to form dimers which coalesce to form multimers (18, 19). However, the highly homologous plasma serpin, ACT, polymerizes through a nucleation-dependent process (20).

In this study, the substrate specificity of α-crystallin is investigated. We observe inhibition of the nucleation-dependent aggregation of ACT due to a specific interaction between ACT and α-crystallin. By contrast, α-crystallin does not prevent the nucleation-independent aggregation of α1-AT, suggesting that α-crystallin has the ability to specifically inhibit nucleation-dependent aggregation.

EXPERIMENTAL PROCEDURES

Materials—4,4′-Dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bis-ANS) was purchased from Molecular Probes.

Production of Proteins—Recombinant ACT was expressed and purified as described previously with the following modifications (21). After chromatography on a Q-Sepharose matrix, fractions containing inhibitory activity against bovine chymotrypsin were purified further by SP and DEAE chromatography. Recombinant wild type α1-AT was expressed and purified as described previously (22). The purity of these proteins was assessed by SDS- and non-denaturing-PAGE. The protein concentration of each serpin was determined using previously described methods.

Aggregation of ACT due to a specific interaction between ACT and α-crystallin. We observe inhibition of the nucleation-dependent aggregation of ACT (closed circles) and in the presence of 50 μg/ml preformed polymer (open circles).

RESULTS

ACT and α1-AT Aggregate through Nucleation-dependent and -independent Pathways, Respectively—Aggregation of ACT at 50 °C occurs with a sigmoidal increase in light scatter, in agreement with a previous study (20) (Fig. 1a). At an ACT concentration of 2.5 μM, there is an initial lag period of ~20 min prior to a gradual increase in light scatter that was complete after 70 min. The rate of ACT aggregation was concentration-dependent, over a range of 1–10 μM ACT, with the reaction reaching completion at much earlier times for higher protein concentrations (Fig. 1a). The initial lag phase showed an inverse correlation with protein concentration, with a much shorter lag time at higher ACT concentrations. In accord with a recent study (20), the rate of ACT aggregation was also sensitive to nucleation. The addition of preformed ACT polymer (5 μM) promoted polymerization (Fig. 1b) and decreased the duration of the lag phase from 20 min to less than 10 min, with the full reaction reaching completion by 45 min. These effects were demonstrated to be concentration-dependent as the presence of greater concentrations of preformed polymer shortened the lag phase to less than 5 min.
The aggregation of $\alpha_1$-AT followed by light scatter measurements showed distinct dissimilarities to that of ACT (Fig. 1c).

$a_1$-AT polymerization at 50 °C did not occur with a sigmoidal increase in light scatter, as no observable lag phase was present. Under these conditions, a hyperbolic increase in light scatter occurred that was significantly slower than that observed for ACT, reaching completion after 400 min (Fig. 1c, open circles). In addition, no nucleation dependence of $\alpha_1$-AT aggregation was observed even at a seed concentration of 50 μg/ml (Fig. 1c). Cumulatively, these data demonstrate that the mechanisms of ACT and $\alpha_1$-AT aggregation are significantly different.

$\alpha_1$-Crystallin Inhibits Only Nucleation-dependent Serpin Aggregation—Light scatter changes were used to examine the influence of $\alpha_1$-crystallin on the aggregation reaction of both ACT and $\alpha_1$-AT. In these experiments, $\alpha_1$-crystallin was able to suppress the light scatter increase associated with the thermal aggregation of ACT aggregation in a dose-dependent manner (Fig. 2a). The presence of equimolar amounts of $\alpha_1$-crystallin almost completely suppressed ACT aggregation (Fig. 2a). Thus, under these conditions, the interaction between ACT and $\alpha_1$-crystallin appeared to have a stoichiometry of approximately one-to-one on a molar subunit basis. In contrast, $\alpha_1$-crystallin was unable to suppress thermal aggregation of $\alpha_1$-AT even at a 2-fold molar subunit excess of $\alpha_1$-crystallin (Fig. 2b). When $\alpha_1$-crystallin was incubated alone under the same conditions, there was no change in light scatter (data not shown). These data strongly indicate that $\alpha_1$-crystallin displays substrate specificity in its chaperone action.

Having established that $\alpha_1$-crystallin inhibits ACT aggregation, we used size exclusion chromatography to gain insight into the interaction between the two proteins. Fig. 3a shows the elution profile of ACT (2.5 μM) incubated at 50 °C over a 2-h time period in the absence of $\alpha_1$-crystallin. As expected, at 0 min, native monomeric ACT eluted as a single sharp peak at of ~45 kDa. Progressively with time, the monomeric ACT peak reduced in size as more material eluted in the void volume of the column, indicating the formation of large soluble ACT polymers. After 80 min, the size of peak corresponding to the polymerized material decreased dramatically as the polymerized protein dropped out of solution and was removed by cen-
Inhibition of Serpin Polymerization by α-Crystallin

In the presence of equimolar amounts of α-crystallin, the loss of monomeric ACT was observed as in the absence of α-crystallin. However, this loss of monomer was not accompanied by the formation of polymeric ACT; instead, the size of the α-crystallin peak increased. SDS-PAGE analysis of this fraction confirmed that it contained both ACT and αA- and αB-crystallin (Fig. 4b, lane 3). The elution volume of the α-crystallin (Fig. 4a) did not change over the time course; however, the peak became significantly broader, tending toward lower elution volumes. In the presence or absence of α-crystallin, the loss of monomeric ACT reached completion around 80 min (Figs. 3a and 4a), in good agreement with the light scatter data (Fig. 1a). In both cases, however, ∼12% of the initial monomeric material persisted after heating. This material was intact, monomeric, and inactive against chymotrypsin (data not shown). In addition, it could not be unfolded by 8 M urea (Fig. 3b), suggesting that it is in the latent conformation of ACT in which the reactive center loop is inserted into its own A β-sheet to give an inactive monomer (26).

The kinetics of monomeric ACT loss and ACT-α-crystallin complex formation were analyzed by integration of the peaks from the gel filtration data (Figs. 3a and 4a) to obtain information about the relative quantities of the protein species (Fig. 5). These data fitted well to a single exponential function, and the rate of ACT loss was virtually identical either in the absence or in the presence of α-crystallin (kapp = 0.028 ± 0.003 min⁻¹ and 0.029 ± 0.001 min⁻¹, respectively). Similarly, the formation of the ACT-α-crystallin complex could be fitted to a single exponential function, giving a comparable rate of 0.033 ± 0.006 min⁻¹.

Spectroscopic Analysis of ACT α-crystralalin Interaction Reveals Binding of a Non-native ACT Species—Far-UV CD was used to assess the conformation of ACT in complex with α-crystallin by collecting the fraction containing the ACT-α-crystallin peak from the SEC and measuring the far-UV CD spectra (Fig. 6, thin broken line). α-Crystallin heated alone under the same conditions (thin solid line) gave a broad spectrum with a minimum at ∼215 nm, consistent with previous data (27). This spectrum was subsequently subtracted from that of the ACT-α-crystallin complex (thick broken line). As previous studies have shown that the secondary and tertiary structures of α-crystallin are not grossly altered when in complex with a protein substrate (28), this difference spectrum is assumed to represent the conformation of ACT in the complex. When compared with the native ACT spectrum at an identical concentration (thick solid line), ACT in complex with α-crystallin appears to have lost ∼20% of its ellipticity at 222 nm. Previous work has shown that there is a dramatic increase in

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bis-ANS fluorescence when the polymyogenic precursor of ACT is formed (29). We examined the kinetics of this conformational change in the presence and absence of a-crystallin. Fig. 7 shows a dramatic increase in bis-ANS fluorescence intensity as a result of ACT undergoing a conformational change upon incubation at 50 °C; this change occurred with a rate of 1.06 ± 0.01 min⁻¹. In agreement with previous studies (6–8), we found that a-crystallin itself binds more bis-ANS when it undergoes its heat-induced activation (Fig. 7, inset, broken black line). Therefore, a-crystallin alone was incubated at 50 °C prior to the addition of ACT, such that the bis-ANS fluorescence changes associated with the a-crystallin conformational change could be separated from those of ACT. The change in bis-ANS fluorescence intensity when ACT was added to the solution containing a-crystallin occurred in a single kinetic phase with a rate of 0.95 ± 0.01 min⁻¹, which is similar to the rate obtained in the absence of a-crystallin. The amplitudes of this change were also similar, whether in the presence or absence of a-crystallin, suggesting that under both conditions, ACT adopts a similar partially unfolded state. Taken together with the far-UV CD results, these data are consistent with ACT being in a non-native conformation when complexed to a-crystallin.

**DISCUSSION**

sHsps possess the ability to bind to partially folded proteins and maintain their solubility. The mechanisms by which this chaperone function is carried out are poorly characterized. Previous studies have implied that yeast Hsp26, a Hsp, undergoes dissociation to produce a species that can bind the protein substrate (30). These smaller species then reassemble to form the large complexes with bound substrate (30). a-Crystallin also undergoes a quaternary structural rearrangement upon complex formation with substrate proteins (31). It is generally believed that sHsps have little substrate specificity, i.e. they interact and complex to substrate proteins that are aggregating due to mutual associations between hydrophobic surfaces (6–8). In other words, sHsps are highly promiscuous molecular chaperones. Our finding that a-crystallin is only capable of repressing the aggregation of ACT but not a₁-AT suggests that a-crystallin may display substrate specificity based upon either structural differences between the two proteins and/or their different aggregation mechanisms. ACT and a₁-AT display high sequence homology, with 44% of their residues being absolutely conserved (32). The crystal structures of both ACT (33) and a₁-AT (17) have been solved and are almost identical. The most significant difference between the two native serpins is in the length of their reactive center loop regions, with that of ACT being four residues longer. It is unlikely that this small structural difference could play a role in the preferential binding of a-crystallin to ACT. However, what is important for the interaction of these proteins with a-crystallin is the partially unfolded conformations that they can adopt during aggregation. Previous studies with a₁-AT indicate that aggregation proceeds through a single partially unfolded species that self-associates (18, 19, 34). In contrast, a recent study demonstrated that two partially folded species were formed during the aggregation of ACT (20). It is feasible that the non-native species of a₁-AT and ACT may be sufficiently different in conformation to explain the specificity of a-crystallin interaction. However, given the high sequence homology and adoption of similar folding intermediates (29, 35), it seems unlikely that the relatively subtle differences in structure between ACT and a₁-AT would have a major role in determining the substrate specificity of a-crystallin for ACT over a₁-AT particularly because of the broad substrate specificity of a-crystallin and other sHsps as determined from many studies of Hsp chaperone action (6–8). Instead, a much more tangible contribution to specificity may be the distinct mechanism and kinetic differences between the aggregation processes of the two serpins.

The inhibitory effect of a-crystallin on ACT aggregation was dose-dependent, with full repression occurring at a ratio of one ACT molecule per subunit of a-crystallin (Fig. 2a). This stoichiometry is in agreement with that found in the chaperone interaction of sHsps with other stressed substrate proteins, e.g. α-lactalbumin (36). In this case, and as with all of the other substrate proteins shown previously to interact with a-crystallin (37–44), the aggregation profile is sigmoidal (Figs. 1a and 2a), indicative of a nucleation-dependent mechanism. a-Crystallin does not prevent the aggregation of a₁-AT, which occurs via a simple polymerization process involving the sequential addition of a₁-AT monomers to form a chain in an essentially one-way process. By contrast, nucleation-dependent aggregation is a dynamic process involving equilibria between various aggregated states. Although a-crystallin typically exists as a
large heteromultimer, previous studies have clearly demonstrated the dynamic nature of the quaternary structure that arises from extensive subunit exchange (45). Thus, similar dynamic interchange of subunits occurs in both the chaperone, α-crystallin, and its substrate protein, ACT, during their interaction.

Previously, we have proposed that dynamic interchange of both proteins facilitates their mutual incorporation during chaperone action into a complex without the requirement for an external input of energy, i.e. via ATP hydrolysis (36). It would seem from the evidence presented herein that a similar mechanism is operating during the interaction of ACT with α-crystallin. Furthermore, as is apparent from comparing Fig. 2, a and b, the aggregation of α1-AT occurs over a much longer time scale than that of ACT. α-Crystallin is a much more efficient chaperone when it interacts with slowly aggregating proteins (36). Thus, if aggregation kinetics were the only criterion in determining whether α-crystallin bound to either serpin, it would have been expected to prevent the aggregation of α1-AT much more effectively than ACT. Clearly, this is not the case. Elevated temperature increases the rate of subunit exchange in α-crystallin in addition to causing partial unfolding of the protein and exposure of greater hydrophobicity (6–8). It is to the exposed hydrophobic substrate-binding region(s) that partially folded substrate proteins are thought to bind. The loss of monomeric ACT is unaffected by the presence of partially folded substrate proteins are thought to bind. The loss is to the exposed hydrophobic substrate-binding region(s) that are in a misfolding event distinct from aggregation, and the data without this event clearly demonstrate the dynamic nature of the quaternary structure that arises from extensive subunit exchange (45). The apparent frequency of liver pathology in individuals with ACT deficiency is much lower than those with α1-AT, leading to the notion that more efficient pathways of clearance exist for misfolded ACT (49). The effective and specific suppression of the nucleation-dependent polymerization of ACT by α-crystallin (and therefore most likely other Hsps), coupled with the co-expression of both of these proteins within liver tissue, implies a potential role for Hsps in the clearance of misfolded ACT.

In conclusion, the analysis of the interaction between α1-AT and ACT with α-crystallin provides new insights into the substrate specificity of the chaperone, which is based upon the mechanism and kinetics of the substrate protein aggregation process. α-Crystallin, and by inference, all Hsps, have a preference for nucleation-dependent aggregating substrate proteins, implying that dynamic exchange of the chaperone subunits and the aggregating substrate protein is an important determinant in their mutual chaperone association.

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Inhibition of Serpin Polymerization by α-Crystallin

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