Small Heat Shock Protein Activity Is Regulated by Variable Oligomeric Substructure*

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The α-crystallins are members of the small heat shock protein family of molecular chaperones that have evolved to minimize intracellular protein aggregation; however, they are also implicated in a number of protein deposition diseases. In this study, we employed novel mass spectrometry techniques to investigate the changes in quaternary structure associated with this switch from chaperone to adjuvant of aggregation. We replicated the oligomeric rearrangements observed for post-translationally modified α-crystallins, without altering the protein sequence, by refolding the α-crystallins in vitro. This refolding resulted in a loss of dimeric substructure concomitant with an augmentation of substrate affinity. We show that packaging of small heat shock proteins into dimeric units is used to control the level of chaperone function by regulating the exposure of hydrophobic surfaces. We propose that a bias toward monomeric substructure is responsible for the aberrant chaperone behavior associated with the α-crystallins in protein deposition diseases.

The small heat shock proteins (sHSPs) 4 αA- and αB-crystallin are most prevalent in the vertebrate eye lens, where they are found co-assembled and maintain lens transparency by preventing other proteins from forming light-scattering aggregates (1), but are also found in other tissues (2). It is clear that these proteins have the ability to sequester substrates that have become partially unfolded under conditions of stress to preserve them in a state competent for refolding (3–6). However, molecular mechanisms of their function and regulation remain incompletely understood, particularly regarding aberrant behavior in the transitions that occur to bring about disease states (7).

Structurally, the sHSPs are characterized by their low monomeric molecular mass, their assembly into oligomers, and the presence of a well conserved “α-crystallin” domain (3–6). Although crystal structures exist for some of the monodisperse members of the family (3), the extreme polydispersity of many of the mammalian sHSPs, including the α-crystallins, has hampered crystallographic analysis (4). Common to the sHSPs for which high resolution structures have been published is the existence of dimers as “building blocks” of the larger oligomer (3, 6). We have reported that recombinant αA and αB form a range of oligomers with a notable preference for an even number of subunits (9,10). This suggests that dimeric substructure is a key characteristic of the sHSPs, and indeed, dimers have been proposed as the active “chaperoning unit” of the sHSPs (6). In our previous study on αB isolated from the lens under denaturing conditions, we observed a difference in the proportion of oligomers containing an even or odd number of subunits (11). This observation, along with recent work showing structural changes in the α-crystallins upon renaturation from urea (12), suggests that in vitro refolding provides a means of modulating the quaternary structure of these proteins. Here, we exploit this phenomenon to examine the structural and functional properties of α-crystallins with identical sequence yet differing quaternary structure. Using novel mass spectrometry (MS) approaches, we show that not only do these proteins exist as different sized oligomers, but also that these oligomers have variable building blocks. Moreover, we correlate the loss of dimeric substructure with an augmentation of substrate binding and suggest that it is these changes in local oligomeric organization that provide the molecular mechanism for sHSP regulation.

EXPERIMENTAL PROCEDURES

Expression and Purification—The expression vector pET21b(+) containing the genes for either bovine αA-crystallin or human αB-crystallin was introduced into the BL21(DE3) strain of Escherichia coli by standard methods. Protein expression and purification were performed using methods described previously (13).

1-Anilino-8-naphthalenesulfonic Acid (ANS) Binding—αA and αB solutions of 13.5 mg/ml were used. To effect unfolding of the protein, 12 mg of urea was added to 20 μl of each solution, giving a final volume of 30 μl (6.67 M urea), followed by incubation at room temperature for 30 min. The proteins were refolded by dilution to 0.2 mg/ml with 1320 μl of phosphate-buffered saline (PBS; 100 mM sodium chloride and 50 mM phosphate, pH 7.2). Matching controls were prepared by adding the urea after dilution. ANS (5 mg/ml of stock solution in methanol) was added to each sample (resulting in a methanol concentration of 0.37% (v/v) in the samples), and the fluorescence emission was measured between 400 and 600 nm at an
excitation wavelength of 370 nm using a Hitachi F4500 fluorometer. The experiment was also performed, as described above, using 200 mM ammonium acetate instead of PBS as diluent.

**Kynurenine Binding**—60 mg of urea was added to 100 μl of 4 mg/ml αB in PBS and left at room temperature for 30 min. The protein was refolded by dilution to 1 ml with PBS. Matching controls were prepared by adding the urea after dilution. Urea was removed from the diluted solutions by five consecutive concentration and dilution steps using Biomax concentrators (Millipore). 25 μl of kynurenine (5 mg/ml in PBS) and 2 μl of chloroform were added before being sealed and incubated at 37 °C for 14 days. No significant proteolysis was observed to occur by MS analysis.

**MS Analysis**—Nanoelectrospray MS of the kynurenine-modified αB was performed using a Q-ToF Ultima spectrometer (Waters). Samples were desalted and denatured using C₁₈ ZipTips (Millipore) prior to analysis.

Tandem MS (MS/MS) experiments were performed on a modified Q-ToF 2 instrument as described previously (11). Prior to analysis, ~2 mg of α-crystallin (30 mg/ml in PBS with or without 6.67 mM urea) was loaded onto a Superdex 200 gel filtration column (GE Healthcare) and eluted at 0.3 ml/min with 200 mM ammonium acetate. Chromatography resulted in refolding and/or buffer exchange.

**Chaperone Assay**—Apo-α-lactalbumin (0.5 mg/ml final concentration) was mixed with αB or recombinant αB (αBₐ, both at 0.24 mg/ml final concentration) in the presence of 20 mM dithiothreitol. The final sample volumes were 100 μl in 200 mM ammonium acetate. The mixtures and a control of apo-α-lactalbumin were transferred to identical cuvettes and placed in a heated (37 °C) multicell block, and the apparent absorption due to light scattering at 360 nm was monitored using a Cary 400 Scan spectrophotometer.

**RESULTS**

**In Vitro Refolding Enhances the Substrate Affinity of αB-crystallin via Increased Surface Exposure**—To structurally reorganize recombinant αB, we subjected the protein to *in vitro* refolding from a high concentration of urea. We have termed this protein αBᵣ. To investigate any surface changes in the oligomers associated with this refolding, we employed two molecular probes: ANS, which fluoresces in a hydrophobic environment, and the reactive small molecule kynurenine, which can covalently bind to exposed His residues in αB (14). Fig. 1A shows the ANS fluorescence for equimolar concentrations of αB and αBᵣ in both PBS and ammonium acetate. In both cases, the fluorescence intensity of the ANS probe was greater for the refolded protein relative to the original, indicating that a small but significant increase in exposed hydrophobic surface area had occurred. This is in agreement with a recent study examining the biophysical properties of α-crystallin (12). Fig. 1B shows the stoichiometries of relative binding of kynurenine to the surface-exposed His residues of αB, with αBᵣ demonstrating ~70% more total binding. Furthermore, whereas only doubly modified chains were identified in the case of αB, a distinct proportion of αBᵣ was triply modified. These two probing techniques demonstrate that the refolded proteins are sufficiently different in structure such that they are more susceptible to both ANS binding and kynurenine modification.

To assess the effect of this structural reorganization on sHSP function, αB and αBᵣ were assayed for their ability to prevent precipitation of the aggregation-prone substrate, reduced apo-α-lactalbumin (Fig. 1C). In the absence of chaperone, a rapid increase in apparent absorbance due to light scattering was observed, indicative of the aggregation of apo-α-lactalbumin. When αB was added, the onset of apo-α-lactalbumin aggregation was considerably delayed, demonstrating the protective ability of this protein. αBᵣ was found to further reduce the level of aggregation, suggesting that αBᵣ has a higher substrate affinity than αB. Therefore, taking our chaperone and surface probing experiments together, it appears that *in vitro* refolding results in an increase in substrate affinity or capacity via the exposure of new surfaces.

**MS/MS Characterizes the Polydisperse sHSP Assemblies**—Over the last decade, MS analysis of intact protein assemblies has become a valuable addition to the structural biologists’ toolkit, allowing the determination of both structural and dynamical parameters (15). To examine the quaternary arrangement of the α-crystallins, we used a MS/MS approach we previously...
which the different dissociation steps occur (Fig. 2). Monitoring the relative abundance of ions as a function of acceleration voltage revealed that this dissociation is a sequential process and allowed us to determine the voltages at which the different oligomeric species that comprise the hetero-

oligomers having lost one or two monomers, respectively. Parent oligomers carrying two or more charges less than subunit equivalents in number of subunits, Notably, however, these proteins have lost their preference for forming even-numbered species. Thus, the dimeric substructure of the wild-type proteins, evidenced by an excess of oligomers with an even number of subunits, is lost upon refolding.

Distinct Substructures within Protein Types Are Found by Gas-phase Dissociation—To further investigate the difference between the quaternary organization of these proteins, we employed a recently developed method of probing substructure by MS (16). This approach involves a detailed examination of the dissociation pathway of protein assemblies during MS/MS. Specifically, we examined the ease of the dissociation step in which an additional monomer is removed from the singly stripped oligomers, the rationale being that different local quaternary arrangements might effect differences in dissociation behavior.

The percentage of species that are doubly stripped (relative to singly stripped) for the four different proteins at a range of accelerating voltages, representing the ease of gas-phase removal of the second subunit, is shown in Fig. 3B. Additionally, they have been separated according to whether they contain an even or odd number of subunits. In all cases, the percentage of doubly stripped oligomers follows a sigmoidal profile. From the first derivative, we obtained the turning points shown in supplemental Table S1. For αA and αB, we did not observe a significant difference between the dissociation pathway for odd- and even-numbered oligomers, suggesting there is no difference in substructure between them. This agrees with the evidence from the histograms, which suggests that their suboligomeric organization is exclusively monomeric (Fig. 3A). In the case of αA and βB, however, there is a significant difference between the odd- and even-numbered oligomers, viz. the transition from singly to doubly stripped oligomers occurs at higher initial kinetic energies in the case of the even-numbered oligomers. These results suggest that there is a fundamental difference in the substructure of even- and odd-numbered oligomers. Furthermore, the turning points of the curves are similar for the odd-numbered oligomers of both αB and αB, and for αA and βB, whereas they are significantly different in the case of the even-numbered pairs (supplemental Table S1). This suggests that the substructure of the different oligomers of αB, as well as αB with an odd number of subunits, is the same, but that a different substructure is present for αB with an even number of subunits. The same properties were observed for βA and αA. Combining this with the evidence in Fig. 3A, that dimeric substructure is lost upon in vitro refolding, we suggest...
that wild-type \(\alpha A\) and \(\alpha B\) exist as a combination of forms, the extremes of which are oligomers with monomeric substructure and even-numbered oligomers with dimeric substructure.

**DISCUSSION**

Previously, we have shown that the substructure of \(\alpha A\) and \(\alpha B\) can be altered by post-translational modification; specifically, phosphorylation of \(\alpha B\) (9, 17) and truncation of \(\alpha A\) (10) cause a decrease and an increase in the amount of dimeric substructure, respectively. Here, we have extended this work to show that the native proteins themselves exist in an apparent equilibrium between different substructural states, viz. with dimeric or monomeric building blocks (Fig. 4A). This is in addition to both these proteins populating a polydisperse ensemble, existing in an adaptable range of oligomeric sizes (4). As such, we propose a new view of these proteins, viz. that they are heterogeneous in terms of both local and global quaternary organization and that it is likely that this heterogeneity is crucial to their function.

By causing shifts in this equilibrium without sequence modification, we have observed that disruption of the dimeric sHSP substructure to form oligomers composed of monomeric building blocks results in an augmentation of chaperone activity. Moreover, the chaperone function of these two states is different: the monomeric substructural state has greater exposed hydrophobic surface area and is consequently more active in...
protecting against protein precipitation. Similarly, small changes in hydrophobicity causing considerable differences in chaperone activity have been observed upon deamidation of the α-crystallins (18, 19). Here, we have shown that it is not the changes in primary structure caused by post-translational modification but rather the alterations in substructure that they engender that determine chaperone activity.

A recent model of sHSP chaperone function suggests that sHSP oligomers exist in two forms with differential activity: a low affinity state and a high affinity state (3, 20). We propose here, in the case of the α-crystallins, these two forms correspond to oligomers with dimeric and monomeric substructure, respectively (Fig. 4A). We suggest that it is the ratio of these two forms that determines the overall efficacy of the chaperone ensemble. Stressors, or irreversible protein modification, affect this ratio by causing sufficient destabilization such that dimeric substructure is perturbed. This leads to an increase in substrate affinity by exposing the former interfacial regions to unfolded substrate proteins. This ability to package subunits into dimers for subsequent activation represents an elegant mechanism of regulating the chaperone activity of these sHSPs.

There is a growing body of literature in which αβ has been reported to be involved in protein deposition diseases (7), potentially due to co-precipitation of the chaperone with substrate proteins in vivo (21). An explanation for this that arises from the work presented here is that mutation, post-translational modification, or some other type of alteration may disrupt the dimeric substructure of αβ such that a critical amount of binding surfaces is exceeded. Indeed, we have shown previously that phosphorylation of αβ can result in a loss of dimeric preference and consequent uncontrolled co-aggregation behavior (9). Similarly, an inheritable R120G mutation in αB has been shown to result in protein deposition disorders, including cataracts and cardiomyopathy (8, 22). In vitro measurements of R120G versus wild-type αB indicated that a dramatic increase in substrate affinity is responsible for R120G forming insoluble co-aggregates with the client proteins (21).

We hypothesize that this chaperone hyperactivity associated with some mutations and post-translational modifications is the result of a shift in the ratio of dimeric to monomeric substructure of αB. If we consider the level of αB function to be determined by position on a continuum of this ratio, then aberrant chaperone behavior occurs when the ideal substructural balance is exceeded (Fig. 4B). Thus, the chaperone function of sHSPs might be viewed as an exquisite balancing act that is, in the case of some protein-misfolding disorders, tipped such that the chaperone itself contributes to the disease.

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