Phosphorylation of αB-Crystallin Alters Chaperone Function through Loss of Dimeric Substructure

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Phosphorylation is the most common posttranslational modification of the α-crystallins in the human lens. These phosphorylated forms are not only important because of their abundance in aging lenses and the implications for cataract but also because they have been identified in patients with degenerative brain disease. By using mimics corresponding to the reported in vivo phosphorylation sites in the human lens, we have examined the effects of phosphorylation upon the chaperone-like properties and structure of αB-crystallin. Here we show that phosphorylation of αB-crystallin at Ser-45 results in uncontrolled aggregation. By using an innovative tandem mass spectrometry approach, we demonstrate how this alteration in behavior stems from disruption of dimeric substructure within the polydisperse αB-crystallin assembly. This structural perturbation appears to disturb the housekeeping role of αB-crystallin and consequently has important implications for the disease states caused by protein aggregation in the lens and deposition in non-lenticular tissue.

The mammalian small heat shock protein (sHSP) αB-crystallin, although systemically expressed (1), is found primarily in the eye lens where it associates with the closely related αA-crystallin into large hetero-oligomers. While its lenticular function is also structural, both αB- and αA-crystallin and the hetero-oligomer α-crystallin (2) have been shown to display molecular chaperone activity in vitro and to arrest the aggregation of the β- and γ-crystallins in the lens (3). Unlike ATP-dependent chaperones, the α-crystallins and other sHSPs are not thought to actively refold non-native proteins but rather to incorporate them into large complexes, thereby preventing their non-specific aggregation (4–6). This property of the α-crystallins is regarded as crucial in the maintenance of lens transparency.

As there is no protein turnover in the central part of the lens, the exceptionally long lifetime of lenticular proteins means that the α-crystallins are susceptible to the accumulation of a variety of posttranslational modifications that are thought to disrupt their structure (7–9). For αB-crystallin in the lens, the major modifications have been identified as phosphorylation at serine residues 19, 45, and 59 (10). Despite phosphorylation being a common feature of αB-crystallin both inside and outside the lens (11), the in vivo significance of these modifications and their effect on chaperone-like activity remain unclear (12). Phosphorylation of α-crystallin has been reported primarily to have no effect on chaperone activity (13, 14), to reduce chaperone activity (15), and to cause a decrease in oligomeric size (16). Furthermore, it has been shown that in vitro phosphorylation of αB-crystallin compromises its inhibitory activity toward actin polymerization (17). The use of site-directed mutagenic substitution of aspartate for serine residues to mimic phosphorylated αB-crystallin led to the report of smaller oligomers with significantly reduced chaperone efficacy (18).

αB-Crystallin, like many mammalian sHSPs, is inherently polydisperse, and this has presented many difficulties for structural biologists (12). Nano electrospray ionization mass spectrometry (19) is capable of providing unique insight into the subunit composition and dynamics of large noncovalent complexes such as sHSPs (20, 21). Although the homogeneity of many of the non-mammalian sHSPs permits a relatively simple interpretation of subunit stoichiometry by means of this technique, it has not been possible, however, to interpret data from polydisperse proteins such as αB-crystallin and other mammalian sHSPs. The large number of oligomeric species involved leads to a substantial overlap of peaks, and therefore the spectra obtained cannot be interpreted unambiguously. We have recently shown, however, that tandem mass spectrometry (MS/MS), in which discrete packets of ions are isolated and subjected to collisions, is capable of defining the relative populations of different oligomers within the polydisperse assembly of αB-crystallin (22).

Here we apply this approach to investigate the effect of phosphorylation upon the quaternary structure of αB-crystallin. We used aspartate mutations of serine residues known to be phosphorylated in vitro (10) (S19D (1P-αB) and S19D/S45D (2P-αB)) and compared their properties with wild-type αB-crystallin (WT-αB). We show here that these proteins differ substantially in their ability to reduce protein aggregation and that these differences are directly linked to alterations in their quaternary substructure, as determined by nanoelectrospray ionization mass spectrometry. Because sHSPs, including αB-crystallin, are up-regulated in a number of neurodegenerative diseases, cancers, and desmin-related myopathy (23), this structure-function relationship has implications not only for...
the mechanism of the chaperone-like behavior of sHSPs but also for elucidating the reasons for their accumulation associated with such diseases.

EXPERIMENTAL PROCEDURES

Preparation of aB-Crystallin—PCR technology was used for generation of human aB-crystallin with serine-to-aspartate mutations at Ser-19 and Ser-45. For generation of the point mutation S19D, DNA of wild-type aB-crystallin was used as a template for the PCR amplification, and the following oligonucleotide primers were used: forward, 5’TTCCTGGAGCCGCCGCTCT-3’, and reverse, 5’-CGGCTGGGGTCTGAAAGAAGAA-3’. Two restriction sites, at the 5’ NdeI site and at the 3’ XhoI site, were added to the PCR product. The expression vector PET 21b(+) was linearized with NdeI and XhoI and subsequently ligated with the gel-purified PCR product using the same cohesive ends. The ligation product was then transformed into *Escherichia coli* One Shot cells by standard methods. After amplification of the construct plasmid, DNA was extracted by using the QiAprep Spin system. The DNA was digested with NdeI and XhoI and analyzed by gel electrophoresis. Clones that contained the DNA insert of mutated aB-crystallin were sequenced to confirm the sequence of the insert. A clone that contained the point mutation S19D was used as a template for the PCR amplification to generate the point mutation S45D. The following oligonucleotide primers were used: forward, 5’TTCCTGGATCCTTCTACCTGAGCCA-3’, and reverse, 5’-AGAAGGGATCAGGGAAGTATGACCTGAC-3’. The proteins were expressed and purified as described previously (24).

Chaperone Assay—The chaperone efficiency of aB-crystallin and the phosphorylation mimics toward reduced a-lactalbumin were assessed by size-exclusion chromatography and multi-angle light scattering. All of the proteins elute at ~13 ml, and the width of the peaks increases with successive phosphorylation (lines). The molecular mass decreases across the peaks, illustrating the polydispersity of these proteins (circles). Increasing phosphorylation results in a steeper negative gradient, indicative of an increase in polydispersity.

Size-Exclusion Chromatography of aB-Crystallin/Substrate Complexes—Samples were prepared as for the chaperone assay with the following exceptions. 1) After 15 min at 37 °C, the reaction was quenched by placing the mixtures on ice, and 2) the final concentration of apo-a-lactalbumin, were placed in identical cuvettes in a heated (37 °C) multicell block. Their apparent absorbance at 360 nm was monitored in parallel over a 30-min period in a Cary 400 Scan spectrophotometer.

**Effect of Phosphorylation on aB-Crystallin Activity.** *A*, the rate of a-lactalbumin (aLac) aggregation is significantly reduced in the presence of WT-aB and 1P-aB relative to the control in the absence of aB-crystallin. By contrast 2P-aB rapidly co-aggregates with the substrate, effectively promoting precipitation. 

**Effect of phosphorylation on the structure of aB-crystallin.** *A*, far UV circular dichroism of WT-aB (green), 1P-aB (orange), and 2P-aB (red). The increase toward negative ellipticity observed for 2P-aB demonstrates that phosphorylation at Ser-45 causes an increase in, or stabilization of, secondary structure. 

**Effect of phosphorylation on the structure of aB-crystallin.** *B*, CD spectra of WT-aB (green), 1P-aB (orange), and 2P-aB (red) examined by SEC coupled to multiangle light scattering. All of the proteins elute at ~13 ml, and the width of the peaks increases with successive phosphorylation (lines). The molecular mass decreases across the peaks, illustrating the polydispersity of these proteins (circles). Increasing phosphorylation results in a steeper negative gradient, indicative of an increase in polydispersity.
Effect of Phosphorylation on αB-Crystallin Chaperone Activity

To investigate chaperone function we incubated aliquots of the three αB-crystallins with an aggregation-prone substrate protein, reduced α-lactalbumin, and monitored their apparent absorbance due to light scattering (Fig. 1A). The results demonstrate that WT-αB and 1P-αB delayed the onset of aggregation until ~15 min, after which an exponential increase in apparent absorbance occurred. At 30 min both reached a similar value to that of the control experiment of α-lactalbumin in the absence of αB-crystallin. By contrast, the 2P-αB caused a dramatic increase in apparent absorbance from ~8 min, exceeded that of the control at ~11 min, and increased exponentially.

To investigate the species formed during these reactions, we used size-exclusion chromatography (SEC) to examine the solutions after 15 min of incubation (Fig. 1B). Both WT-αB and 1P-αB produced similar elution profiles, with major and minor components having retention times of 25 and 21 min (Fig. 1B, labeled 1 and 2), corresponding to approximate molecular masses of 600 and 1200 kDa, respectively. Peak 1 is αB-crystallin, which elutes as a single symmetrical peak in the absence of substrate (Fig. 2B). We attribute Peak 2 to species containing both chaperone and substrate. These are more prevalent in the elution profile of 2P-αB than in the elution profiles of WT-αB and 1P-αB. The elution profile for 2P-αB exhibited the same peaks observed for WT-αB and 1P-αB as well as an additional peak of molecular mass >1200 kDa (Peak 3). A third component eluting after ~45 min (not shown) was assigned to free α-lactalbumin. These results demonstrate very similar chaperone-like properties for wild type and 1P-αB but imply the co-aggregation of substrate and chaperone to form higher molecular weight species in the case of 2P-αB.

Taken together these experiments show that there is a marked difference in the chaperone behavior of 2P-αB compared with that of WT-αB and 1P-αB. We found that WT-αB and 1P-αB suppress aggregation equally (Fig. 1A) by the formation of large complexes with the substrate (Fig. 1B). 2P-αB, by contrast, co-aggregates with the substrate, as evidenced by reaching a higher apparent absorbance than the control in the chaperone assay (Fig. 1A). This leads to the formation of additional, even larger chaperone-substrate complexes (Fig. 1B).

Effect of Phosphorylation on the Structure of αB-Crystallin—As protein function is intrinsically related to structure, we have examined these αB-crystallins for any structural differences associated with this difference in chaperone activity. The

**Fig. 3.** Nanoelectrospray mass spectra of the αB-crystallins. WT-αB (bottom), 1P-αB (middle), and 2P-αB (top) show signal between 7000 and 13,000 m/z. These spectra are characteristic of polydisperse proteins and are not interpretable directly (22).

**Fig. 4.** Tandem mass spectrometry analysis of WT-αB. Isolation of the main peak in the spectrum of WT-αB and collision-induced dissociation result in dissociation of the oligomers into monomers at low m/z and stripped oligomers at high m/z (22). Up to three monomers can be removed from the oligomers in this way (schematic). In the region of the spectrum assigned to the doubly stripped oligomers, the peaks are sufficiently separated to allow identification of the individual oligomers that constitute the polydisperse assembly of WT-αB. This spectrum is magnified 16-fold above 12,500 m/z.
Effect of phosphorylation on αB-crystallin

The effect of phosphorylation on the population of the different oligomers in αB-crystallin. A comparison of part of the doubly stripped oligomer region († in Fig. 4) shows the number of subunits and charge state assignments (x-mer ††) for WT-αB (bottom), 1P-αB (middle), and 2P-αB (top). Histograms quantifying the relative populations of the different oligomeric species, determined from all of the doubly stripped oligomers (22), are inset. A preference for oligomers composed of an even number of subunits is observed for WT-αB and 1P-αB but is absent in 2P-αB, indicating a disruption of quaternary substructure upon phosphorylation.

Although SEC-MALS provides an average mass of the oligomers in the volume being sampled, it is not capable of distinguishing the different species therein. We have recently demonstrated, however, that a tandem mass spectrometry approach can detect such distinctions, permitting relative quantification of the constituent oligomers in a polydisperse protein (22). We obtained mass spectra of the intact αB-crystallin assemblies (Fig. 3). All three proteins exhibited peaks between 7000 and 13,000 m/z, with the peak at ~10,000 m/z constituting the most intense peak in each spectrum. These spectra are, however, not interpretable directly because of the large number of overlapping charge states. To overcome this problem we used collision-induced dissociation of the oligomers in the gas phase (Fig. 4) (22). The mechanism of such an MS/MS approach, as applied to oligomeric assemblies, can be described briefly as follows (Fig. 4, schematic). The peak corresponding to all of the oligomeric species, each with two charges/subunit (10,080, 10,120, and 10,160 m/z for WT-αB, 1P-αB, and 2P-αB, respectively), was isolated and subjected to collisions with argon atoms in the collision cell of the mass spectrometer (22). Fig. 4 shows the spectrum we obtained from such an analysis of WT-αB-crystallin by homology with sHSP16.9 from wheat. A, two of the monomers within the αHSP dodecamer are colored (blue and gold) and demonstrate how the resolved N-terminal arms of sHSP16.9 link together in a pairwise interaction of the α2 helices (28). An expansion of this area (B) reveals that Ser-26 (homologous to Ser-45 in αB-crystallin (32)) is located in the α2 helix. Phosphorylation at this residue would destabilize the interactions between the helices and therefore disrupt the quaternary substructure of αB-crystallin.

450–830 kDa and 320–940 kDa for 1P-αB and 2P-αB, respectively, with both having an average molecular mass similar to WT-αB at ~540 kDa. These results reveal that phosphorylation does not affect the average molecular mass of αB-crystallin but rather causes an increase in polydispersity.

The effects of these local structural perturbations on the global organization of αB-crystallin were investigated by using SEC coupled to a multilangle laser light-scattering detector (SEC-MALS detector) to measure the oligomeric distribution of the αB-crystallins (Fig. 2B). This technique enables molecular mass determination that is independent of the interactions between the proteins and SEC column. Each protein eluted as a single symmetrical peak at ~13 ml, and this peak broadened with increasing phosphorylation. The MALS data show that WT-αB species exist within the range of 400–800 kDa, with an average molecular mass of ~540 kDa. This mass range was
To compare the spectra recorded for the three proteins, we have expanded a region assigned to oligomers from which two subunits have been removed (Fig. 5). These MS/MS spectra demonstrate that the peaks are sufficiently resolved to allow us to assign the individual species comprising the polydisperse assembly. Moreover, we can also detect differences between the relative populations of the different oligomers. Peaks corresponding to doubly stripped oligomers of 22–28 subunits, hence originating from oligomers of 24–30 subunits, are common to the spectra of all three proteins. From the intensities of the peaks in the entire region of the spectrum assigned to doubly stripped oligomers (Fig. 4), we quantified the relative abundances of the various oligomeric species (Fig. 5, insets) (22). This allowed us to calculate the mean oligomeric size of the aB-crystallins to be 28.1, 27.2, and 27.4 subunits for WT-aB, 1P-aB, and 2P-aB, respectively, in close agreement with the \( \sim 540\)-kDa (27 subunits) average molecular mass obtained by SEC-MALS for all three proteins. The peaks in the spectra and the histograms reveal the interesting distinction that WT-aB forms oligomers with an even number of subunits in preference to those with an odd number; moreover, this preference is somewhat diminished for 1P-aB and is not observed for 2P-aB. From these observations it is clear that human aB-crystallin contains some dimeric substructure and that this is compromised by successive phosphorylation.

**DISCUSSION**

We have examined the effects of serine phosphorylation on the oligomerization and chaperone-like function of aB-crystallin by preparing aspartate mimics at two sites commonly found in vivo. It has been reported previously that the oligomeric state of WT-aB is significantly reduced upon multiple phosphorylation (18); however, when SEC-MALS analyses were performed, very little difference between the average molecular masses of the proteins was observed. Rather, this analysis revealed that the oligomeric distribution became markedly broader upon phosphorylation. More significantly, a further consequence of the phosphorylation was observed in the MS/MS spectra obtained. WT-aB displayed a clear preference for assemblies containing an even number of subunits, a characteristic also observed for sHSPs from plants (28), yeast (29, 30), and bacteria (31), which exclusively form even numbered oligomers. However, the observation of significant amounts of oligomeric species containing an odd number of subunits also indicates the presence of additional non-dimeric quaternary interactions. These findings suggest that the purported “dimeric building block” (32) of the sHSPs may be partially conserved within aB-crystallin and other mammalian sHSPs.

We demonstrated by investigating the aspartate mutant 1P-aB that phosphorylation at Ser-19 has little effect on the chaperone activity or distribution of oligomers in the assembly. By contrast, by examining 2P-aB, we show that additional phosphorylation at Ser-45 resulted in significant differences in the distribution of oligomers formed by this protein and a concomitant alteration in chaperone-like function. This residue can be aligned with the sequence region attributed to the \( \alpha \)2 helix in the N terminus of the sHSPs (32). Although no crystal structure exists for aB-crystallin, the related sHSP16.9 from wheat has been shown to be composed of 12 \( \alpha \)-crystallin domains arranged into two six-membered rings. Half of the monomers are fully resolved, whereas the other half have disordered N-terminal arms (28). The \( \alpha \)-crystallin domain interactions provide a general cohesion among subunits for overall oligomeric assembly. Hydrogen bonds and hydrophobic contacts formed by the C-terminal extensions and the six ordered N-terminal \( \alpha \)2 helices confer stability to the dodecamer (Fig. 6A) (28, 32). Assuming aB-crystallin uses similar N-terminal interactions to stabilize quaternary substructure, the fact that only half of the subunits make these contacts may explain the presence of a significant proportion of odd-numbered oligomers in all of the aB-crystallin assemblies. Moreover, phosphorylation at Ser-45 (Fig. 6B) would result in the disruption of intersubunit contacts by the \( \alpha \)2 helices, leading to a loss of dimeric substructure. Comparison with the related sHSP structure therefore provides plausible explanations for the observation of assemblies with odd numbers of subunits in all three proteins as well as the significant reduction in those containing even numbers in 2P-aB.

The current model for sHSP chaperone activity involves an equilibrium between a “dormant” form and one in which exposed hydrophobic sites can bind partially unfolded substrates (4, 32). We propose that the disruption of dimeric interfaces caused by phosphorylation alters this equilibrium, thereby increasing substrate affinity such that phosphorylated aB-crystallin actively co-aggregates with unfolding substrates. Phosphorylated aB-crystallin has been found in patients with degenerative brain disease (11), and high levels of phosphorylation, particularly at Ser-45 of aB-crystallin, in aged human lenses (10) may contribute to the onset of age-related nuclear cataract. The levels of phosphorylated aB-crystallin in nontissue are, for the most part, unknown (34). However, given that aB-crystallin is up-regulated in a number of neurodegenerative diseases, cancers, and desmin-related myopathy (23), this structure-function relationship has implications not only for the mechanism of their chaperone-like activity but also for understanding the accumulation of a-crystallins associated with such diseases.

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