

Phosphorylation of α B-crystallin alters chaperone function through loss of dimeric substructure

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Running title: Effect of phosphorylation on α B-crystallin

Summary

Phosphorylation is the most common post-translational modification of the α -crystallins in the human lens. These phosphorylated forms are not only important because of their abundance in ageing lenses and the implications for cataract, but also since 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 α B-crystallin. Here we show that phosphorylation of α B-crystallin at S45 results in uncontrolled aggregation. By using an innovative tandem mass spectrometry approach, we demonstrate how this alteration in behaviour stems from disruption of dimeric sub-structure within the polydisperse α B-crystallin assembly. This structural perturbation appears to disturb the housekeeping role of α B-crystallin, and consequently has important implications for the disease states caused by protein aggregation in the lens and deposition in non-lenticular tissue.

Introduction

The mammalian small heat shock protein (sHSP)¹ α B-crystallin, though systemically expressed (1), is found primarily in the eye lens where it associates with the closely related α A-crystallin into large hetero-oligomers. While its lenticular function is also structural, both α B-, α A-, 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 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 post-translational modifications which are thought to disrupt their structure (7-9). For α B-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 α B-crystallin both inside and outside the lens (11), the *in vivo* significance of these modifications and their effect on chaperone-like activity remains unclear (12).

Phosphorylation of α -crystallin has been reported variously to have no effect on chaperone activity (13,14), to reduce chaperone activity (15), and cause a decrease in oligomeric size (16). Furthermore, it has been shown that *in vitro* phosphorylation of α B-crystallin compromises its inhibitory activity toward actin polymerization (17). The use of site-directed mutagenic substitution of aspartate for serine residues, to mimic phosphorylated

α B-crystallin, led to the report of smaller oligomers with significantly reduced chaperone efficacy (18).

α B-crystallin, like many mammalian sHSPs, is inherently polydisperse, and this has presented many difficulties for structural biologists (12). Nanoelectrospray ionization mass spectrometry (nanoESI-MS) (19) is capable of providing unique insight into the subunit composition and dynamics of large noncovalent complexes such as sHSPs (20,21). While 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 α B-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 α B-crystallin (22).

Here we apply this approach to investigate the effect of phosphorylation upon the quaternary structure of α B-crystallin. We used aspartate mutations of serine residues known to be phosphorylated *in vivo* (10) [S19D (1P- α B) and S19/45D (2P- α B)] and compared their properties with wild-type α B-crystallin (WT- α B). 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 sub-structure, as determined by nanoESI-MS. Since sHSPs, including α B-crystallin, are upregulated 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 behaviour of sHSPs, but also in elucidating the reasons for their accumulation associated with such diseases.

Experimental Procedures

Preparation of α B-crystallins

PCR technology was used for generation of human α B-crystallin with serine to aspartate mutations at S19 and S45. For generation of the point mutation S19D, DNA of wild type α B-crystallin was used as a template for the PCR amplification and the following oligonucleotide primers were used:

forward 5'-TTTCCACGACCCCAGCCGCCTCT-3',

reverse 5'-CGGCTGGGGTCGTGGAAAGAAAAGAA-3'.

Two restriction sites were added to the PCR product: at the 5' Nde1 site, and at the 3' Xho1 site. The expression vector, PET 21b(+) was linearized with Nde1 and Xho1, and subsequently ligated with the gel purified PCR product using the same cohesive ends. The ligation product was then transformed into *E. 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 Nde1 And Xho1 and analyzed by gel electrophoresis. Clones which contained the DNA insert of mutated α B-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'-TTCCCTGGATCCCTTCTACCTTCGGCCA-3',

reverse 5'-AGAAGGGATCCAGGGAAGTAGACGTC-3'.

The proteins were expressed and purified as described previously (24).

Chaperone Assay

The chaperone efficiency of α B-crystallin and the phosphorylation mimics towards reduced α -lactalbumin was assessed by using a modified assay (25). Briefly, apo- α -lactalbumin

(0.5 mg/ml final) was mixed with α B-crystallin and each of the mimics (0.235 mg/ml final) in the presence of 20 mM DTT. The final sample volumes were 100 μ l, in 200 mM ammonium acetate. The mixtures, as well as a control of apo- α -lactalbumin were placed in identical cuvettes in a heated (37 °C) multi-cell block. Their apparent absorbance at 360 nm was monitored in parallel over a 30 min period in a Cary 400 Scan spectrophotometer.

Size-exclusion chromatography of α B-crystallin:substrate complexes

Samples were prepared as for the chaperone assay, except that; (1) after 15 minutes at 37°C, the reaction was quenched by placing the mixtures on ice; (2) the final concentration of α B-crystallin was 0.6 mg/ml. The entire volume of each sample was loaded onto a Superdex 200HR 10/30 size-exclusion column (Amersham Pharmacia) and eluted at 0.4 ml/min with 200 mM ammonium acetate, at 8 °C. The column was calibrated with Sigma gel filtration markers.

Circular Dichroism

Far-UV circular dichroism spectra of the proteins were obtained by using a Jasco J-810 spectropolarimeter. Measurements were performed at 25 °C in a cylindrical 0.1 mm path length cell, and represent the sum of 15 accumulations per sample. Protein concentrations were carefully standardized to 1.2 mg/ml in 200 mM ammonium acetate buffer.

Size Exclusion Chromatography-Light Scattering

The native proteins were analyzed by using size-exclusion chromatography with online light scattering, absorbance, and refractive index detectors (26). A Superose 6HR 10/30 column (Amersham Pharmacia) was connected in line to a UV detector (Pharmacia UV-900), a Dawn-EOS (Wyatt-Technology) multi-angle laser light scattering detector, and an Optilab-

DSP (Wyatt-Technology) refractive index detector. Samples were loaded onto the column at a concentration of 1.5 ml/ml and eluted with 200 mM ammonium acetate, 1 mM DTT, pH 7.0.

Mass Spectrometry Analysis

Approximately 2 mg of the of the purified protein was buffer exchanged by loading onto a Superdex 200HR 10/30 size-exclusion column (Amersham Pharmacia) and eluting at 0.4 ml/min with 200 mM ammonium acetate, at 8 °C. Fractions corresponding to the peak elution volume were pooled and concentrated to 1 mg/ml using a Millipore Biomax centrifugal filtration device.

Nanoelectrospray mass spectrometry experiments were performed on a Q-ToF 2 instrument (Micromass UK Ltd) which has been modified for high mass operation (27). Conditions were carefully chosen to allow the ionization and detection of the α B-crystallins without disrupting the non-covalent interactions which maintain the quaternary structure. To dissociate the oligomers, ions of the selected m/z range were collided with argon atoms in the collision cell of the mass spectrometer. The asymmetric distribution of charge between the products allowed the unambiguous identification of the products. The experimental conditions employed for the recording of these MS and MS/MS spectra are the same as described previously (22).

Results

Effect of phosphorylation on α B-crystallin chaperone activity

To investigate chaperone function we incubated aliquots of the three α B-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- α B and 1P- α B delayed the onset of aggregation until ~15 mins, after which an exponential increase in apparent absorbance occurred. At 30 mins both reached a similar value to that of the control experiment of α -lactalbumin in the absence of α B-crystallin. By contrast, the 2P- α B caused a dramatic increase in apparent absorbance from ~8 mins, exceeded that of the control at ~11 mins, 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- α B and 1P- α B produced similar elution profiles, with major and minor components having retention times of 25 and 21 mins (labelled 1 and 2), corresponding to approximate molecular masses of 600 kDa and 1200 kDa respectively. Peak 1 is α B-crystallin, which elutes as a single symmetrical peak in the absence of substrate (Fig. 2B). Peak 2 we attribute to species containing both chaperone and substrate. These are more prevalent in the elution profile of 2P- α B than in WT- α B and 1P- α B. The elution profile for 2P- α B exhibited the same peaks observed for WT- α B and 1P- α B as well as an additional peak of molecular mass >1200 kDa (3). A third component eluting after ~45 mins (not shown) was assigned to free α -lactalbumin. These results demonstrate very similar chaperone-like properties for wild-type and 1P- α B, but imply the co-aggregation of substrate and chaperone to form higher molecular weight species in the case of 2P- α B.

Taken together these experiments show that there is a marked difference in the chaperone behaviour of 2P- α B compared with that of WT- α B and 1P- α B. We found that WT- α B and 1P- α B suppress aggregation equally (Fig. 1A), by the formation of large complexes with the substrate (Fig. 1B). 2P- α B, 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 α B-crystallin

As protein function is intrinsically related to structure, we have examined these α B-crystallins for any structural differences associated with this difference in chaperone activity. The effect of phosphorylation on secondary structure was examined by using far-UV circular dichroism (Fig. 2A). All three proteins displayed minima at $\sim$ 213 nm. Relative to WT- α B the intensity of this minimum was increased slightly toward negative ellipticity for 1P- α B, and more so for 2P- α B. Thus, phosphorylation at two serine residues in the N-terminus of α B-crystallin results in an increase in, or stabilization of, secondary structure.

The effects of these local structural perturbations on the global organization of α B-crystallin were investigated by using SEC coupled to a multi-angle laser light scattering detector (SEC-MALS) to measure the oligomeric distribution of the α B-crystallins (Fig. 2B). This technique enables molecular mass determination which is independent of the interactions between the proteins and SEC column. Each protein eluted as a single symmetrical peak at $\sim$ 13 ml, and this peak broadened with increasing phosphorylation. The MALS data show that WT- α B species exist within the range 400 to 800 kDa, with an average molecular mass of $\sim$ 540 kDa. This mass range was 450 to 830 kDa, and 320 to 940 kDa, for 1P- α B and 2P- α B

respectively, with both having an average molecular mass similar to WT- α B at ~540 kDa.

These results reveal that phosphorylation does not affect the average molecular mass of α B-crystallin, but rather causes an increase in polydispersity.

While 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 (MS/MS) can detect such distinctions, permitting relative quantification of the constituent oligomers in a polydisperse protein (22). We obtained mass spectra of the intact α B-crystallin assemblies (Fig. 3). All three proteins exhibited peaks between 7000 and 13000 m/z, with the peak at ~10000 m/z constituting the most intense peak in each spectrum. These spectra are, however, not interpretable directly due to 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 (schematic in Fig. 4). The peak corresponding to all the oligomeric species, each with two charges per subunit (10080, 10120, 10160 m/z for WT- α B, 1P- α B and 2P- α B respectively), was isolated and subjected to collisions with argon atoms in the collision cell of the mass spectrometer (22). Figure 4 shows the spectrum we obtained from such an analysis of WT- α B. Peaks arising from the oligomers which have lost subunits – ‘stripped oligomers’ - were observed in the range of 12000 to 33000 m/z, whereas the corresponding highly-charged monomers gave rise to signal below 3000 m/z.

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 to 28 subunits, hence originating from oligomers of 24 to 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 α B-crystallins to be 28.1, 27.2, and 27.4 subunits for WT- α B, 1P- α B and 2P- α B respectively, in close agreement with the ~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- α B forms oligomers with an even numbers of subunits in preference to those with an odd number, that this preference is somewhat diminished for 1P- α B, and is not observed for 2P- α B. From these observations it is clear that human α B-crystallin contains some dimeric sub-structure 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 α B-crystallin, by preparing aspartate mimics at two sites commonly found *in vivo*. It has been reported previously that the oligomeric state of WT- α B 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- α B 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 α B-crystallin and other mammalian sHSPs.

We demonstrated by investigating the aspartate mutant 1P- α B crystallin that phosphorylation at S19 has little effect on the chaperone activity or distribution of oligomers in the assembly. By contrast, by examining 2P- α B, we show that additional phosphorylation at S45 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 α 2 helix in the N-terminus of the sHSPs (32). Although no crystal structure exists for α B-crystallin, the related sHSP16.9 from wheat has been shown to be composed of 12 α -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 α -crystallin domain interactions provide a general cohesion amongst 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 α B-crystallin uses similar N-terminal interactions to stabilize quaternary sub-structure, 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 α B-crystallin assemblies. Moreover, phosphorylation at S45 (Fig. 6B) would result in the disruption of inter-subunit contacts by the $\alpha 2$ helices, leading to a loss of dimeric sub-structure. 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- α B.

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 (32,33). We propose that the disruption of dimeric interfaces caused by phosphorylation alters this equilibrium, thereby increasing substrate affinity such that phosphorylated α B-crystallin actively co-aggregates with unfolding substrates. Phosphorylated α B-crystallin has been found in patients with degenerative brain disease (11) and high levels of phosphorylation, particularly at S45 of α B-crystallin, in aged human lenses (10) may contribute to the onset of age-related nuclear cataract. The levels of phosphorylated α B-crystallin in non-lenticular tissues are, for the most part, unknown (34). However given that α B-crystallin is upregulated 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 in understanding the accumulation of α -crystallins associated with such diseases.

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Footnotes

¹ The abbreviations used in this work are:

(s)HSP : (small) heat shock protein

MALS : multi-angle laser light scattering

MS : mass spectrometry

MS/MS : tandem mass spectrometry

nanoESI : nanoelectrospray ionization

SEC : size-exclusion chromatography

Figure Legends

Figure 1

Effect of phosphorylation on α B-crystallin chaperone activity. **A**, The rate of α -lactalbumin aggregation is significantly reduced in the presence of WT- α B (green) and 1P- α B (orange) relative to the control in the absence of α B-crystallin (black). By contrast 2P- α B (red) rapidly co-aggregates with the substrate, effectively promoting precipitation. **B**, SEC of incubations of reduced α -lactalbumin and WT- α B (lower), 1P- α B (middle) and 2P- α B (upper). In the absence of substrate, the α B-crystallins elute as a single symmetrical peak. This peak is present in the mixtures after incubation (1), but peaks corresponding to complexes formed between the chaperone and substrate are also observed (2). An additional peak is observed in the void volume in the case of 2P- α B (3), reflecting the co-aggregation behaviour observed in **A**.

Figure 2

Effect of phosphorylation on the structure of α B-crystallin. **A**, Far-UV circular dichroism of WT- α B (green) 1P- α B (orange) and 2P- α B (red). The increase towards negative ellipticity observed for 2P- α B demonstrates that phosphorylation at S45 causes an increase in, or stabilization of secondary structure. **B**, WT- α B (green), 1P- α B (orange) and 2P- α B (red) examined by SEC coupled to multi-angle light scattering. All the proteins elute at $\sim$ 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.

Figure 3

Nanoelectrospray mass spectra of the α B-crystallins. WT- α B (lower), 1P- α B (middle) and 2P- α B (upper) show signal between 7000 and 13000 m/z. These spectra are characteristic of polydisperse proteins, and are not interpretable directly (22).

Figure 4

Tandem mass spectrometry analysis of WT- α B. Isolation of the main peak in the spectrum of WT- α B and collision-induced dissociation results 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 which constitute the polydisperse assembly of WT- α B. This spectrum is magnified 16-fold above 12500 m/z.

Figure 5

The effect of phosphorylation on the population of the different oligomers in α B-crystallin. Comparison of part of the doubly-stripped oligomer region (* in Fig. 4) showing the number of subunits and charge state assignments ($X\text{mer}^{Y+}$) for WT- α B (left), 1P- α B (middle) and 2P- α B (right). Histograms quantifying the relative populations of the different oligomeric species, determined from all the doubly stripped oligomers (22), are inset. A preference for oligomers composed of an even number of subunits is observed for WT- α B and 1P- α B, but is absent in 2P- α B, indicating a disruption of quaternary sub-structure upon phosphorylation.

Figure 6

Location of S45 in α B-crystallin by homology with sHSP16.9 from wheat. **A**, Two of the monomers within the sHSP dodecamer are coloured (blue, gold), and demonstrate how the resolved N-terminal arms of sHSP16.9 link together in a pair wise interaction of the α 2 helices (28). An expansion of this area, **B**, reveals that S26, (homologous to S45 in α B-crystallin (32)), is located in the α 2 helix. Phosphorylation at this residue would destabilise the interactions between the helices, and therefore disrupt the quaternary sub-structure of α B-crystallin.