Mimicking phosphorylation of α B-crystallin affects its chaperone activity

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α B-crystallin is a member of the sHsp (small heat-shock protein) family that prevents misfolded target proteins from aggregating and precipitating. Phosphorylation at three serine residues (Ser¹⁹, Ser⁴⁵ and Ser⁵⁹) is a major post-translational modification that occurs to α B-crystallin. In the present study, we produced recombinant proteins designed to mimic phosphorylation of α B-crystallin by incorporating a negative charge at these sites. We employed these mimics to undertake a mechanistic and structural investigation of the effect of phosphorylation on the chaperone activity of α B-crystallin to protect against two types of protein misfolding, i.e. amorphous aggregation and amyloid fibril assembly. We show that mimicking phosphorylation of α B-crystallin results in more efficient chaperone activity against both heat-induced and reduction-induced amorphous aggregation of target proteins. Mimick-

ing phosphorylation increased the chaperone activity of α B-crystallin against one amyloid-forming target protein (κ -casein), but decreased it against another (cc β -Trp peptide). We observed that both target protein identity and solution (buffer) conditions are critical factors in determining the relative chaperone ability of wild-type and phosphorylated α B-crystallins. The present study provides evidence for the regulation of the chaperone activity of α B-crystallin by phosphorylation and indicates that this may play an important role in alleviating the pathogenic effects associated with protein conformational diseases.

Key words: amyloid, α B-crystallin, chaperone, phosphorylation, protein aggregation, small heat-shock protein.

INTRODUCTION

During a protein's life cycle, exposure to conditions of physiological stress (e.g. heat, changes in pH, oxidation) can lead to destabilization and formation of partially folded intermediates that expose hydrophobic regions to solution, which may interact resulting in large-scale aggregation and precipitation. sHsps [small Hsps (heat-shock proteins)] act in a chaperone manner to confer protection against cellular stress by recognizing, interacting with and stabilizing partially unfolded intermediates that have entered this 'protein off-folding pathway' [1]. However, unlike the classical bacterial chaperonin GroEL, sHsps including α B-crystallin do not directly participate in refolding of the partially unfolded proteins, except in the presence of another chaperone protein, e.g. Hsp70 [2,3]. In many cases, protein aggregation and precipitation are highly deleterious to cell viability and are the hallmark of diseases generally classified as protein misfolding or conformational diseases, e.g. Parkinson's, Alzheimer's and Creutzfeldt–Jakob diseases and cataract. As such, sHsps are thought to play a key role in preventing or alleviating diseases characterized by protein misfolding and aggregation [4–6].

α B-crystallin is an sHsp primarily found in the eye lens, where it associates with the closely related α A-crystallin to form large hetero-oligomeric species. However, α B-crystallin is also constitutively expressed in many non-lenticular tissues, including the brain, lung and cardiac and skeletal muscles [7]. As with other members of the sHsp family, the expression of α B-crystallin is dramatically up-regulated in response to stress and pathological conditions [4,8–10]. Both inside and outside the lens, a major post-translational modification described for α B-crystallin is

phosphorylation at three serine residues (Ser¹⁹, Ser⁴⁵ and Ser⁵⁹) [11,12], which is mediated by at least two distinct mitogen-activated protein kinase enzymes [11–14].

Various types of cellular stress, such as heat, oxidation and increased intracellular calcium levels, stimulate the phosphorylation of α B-crystallin [11,15]. In the lens, phosphorylation also increases with age; however, even in the young lens, α B-crystallin may be extensively phosphorylated [16–18]. In the brain, some of the α B-crystallin isolated from the proteinaceous aggregates of patients with degenerative diseases [19], amyloid plaques and Lewy bodies is phosphorylated [20]. However, it is not known whether there is a preference for the phosphorylated forms of α B-crystallin to interact with the proteins associated with these deposits.

A limited number of studies have investigated the role of phosphorylation on the chaperone action of α B-crystallin and only against amorphously aggregating proteins. Studies performed *in vitro* have used either purified forms of the phosphorylated protein from lenses, or recombinant proteins that have been designed to mimic serine phosphorylation by replacing it with a negatively charged aspartic or glutamic acid residue at the same position. One study, using bovine lenses and isoelectric focusing to obtain non-phosphorylated and phosphorylated α B-crystallin, showed a 60 % reduction in the ability of the phosphorylated forms to prevent the cytochalasin-D-induced depolymerization of actin from filaments compared with the non-phosphorylated form [21]. Another, employing ion-exchange chromatography to purify monophosphorylated α B-crystallin from bovine lenses, reported a 30 % decrease in the ability of this form to prevent the heat-induced aggregation of β _L-crystallin [22]. Studies using phosphorylation mimics have

Abbreviations used: ANS, 8-anilino-1-naphthalene-sulphonic acid; α B-1P, S19D α B-crystallin; α B-2P, S19D/S45D α B-crystallin; α B-3P, S19D/S45D/S59D α B-crystallin; α B-WT, wild-type α B-crystallin; DTT, dithiothreitol; FRET, fluorescence resonance energy transfer; Hsp, heat-shock protein; sHsp, small Hsp; MALS, multi-angle light scattering; RCM κ -casein, reduced and carboxymethylated κ -casein; SEC, size-exclusion chromatography; TEM, transmission electron microscopy; ThT, thioflavin T.

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indicated that an increase in phosphorylation leads to a decrease in the oligomerization of the protein [23] and a disruption in dimeric substructure within the oligomer [24]. One of these studies showed a slight decrease in the ability of a triple phosphorylation mutant (S19D/S45D/S59D) to prevent the thermal aggregation of lactate dehydrogenase or to refold denatured firefly luciferase, compared with the wild-type recombinant protein [23]. The other reported that, when used in a reduction assay of α -lactalbumin, the double phosphorylation mimic (S19D/S45D) promoted rather than inhibited protein precipitation [24], which was ascribed to co-aggregation and precipitation of reduced α -lactalbumin and the chaperone to form a higher-molecular-mass species. This may be due to the phosphorylation mimics having an increased binding affinity to destabilized target proteins compared with the wild-type protein [25]; however, it would be expected that this would lead to an increase in chaperone function rather than a decrease. Thus the overall effect of phosphorylation on the function of α B-crystallin remains controversial, and it is for this reason that we sought to re-address this issue.

In the present study, we have used recombinant proteins designed to mimic phosphorylation of α B-crystallin [α B-1P (S19D α B-crystallin), α B-2P (S19D/S45D α B-crystallin) and α B-3P (S19D/S45D/S59D α B-crystallin)] to undertake a comprehensive survey of their relative chaperone activity against different types of protein aggregation, i.e. disordered amorphous aggregation and ordered amyloid fibril formation. Amyloid fibril aggregation leads to highly structured cross- β -sheet fibrillar arrays of proteins that can be observed as thread-like structures, sometimes assembled further into larger aggregates or plaques [26,27]. They are characteristic of the protein deposits formed in diseases such as Alzheimer's, Parkinson's and Creutzfeldt–Jakob diseases. In general, we found that phosphorylation of α B-crystallin leads to an increase in the chaperone activity of the protein against aggregating target proteins. However, the target protein and solution conditions were found to be important factors that regulate the effect of phosphorylation on the chaperone action of α B-crystallin. Using a variety of biophysical techniques, structural characterization and comparison of the phosphorylation mimics and the wild-type protein were also undertaken.

MATERIALS AND METHODS

Proteins including κ -casein and α -lactalbumin from bovine milk, bovine pancreas insulin, bovine liver catalase and yeast alcohol dehydrogenase were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and used without further purification. Prior to use, the κ -casein was reduced and carboxymethylated as described earlier [28]. The 18-mer coiled-coil α -helical peptide described previously [29], with an additional C-terminal tryptophan residue (cc β -Trp), was synthesized by CS Bio Co. (San Carlos, CA, U.S.A.). Bovine β ₁-crystallin was purified via SEC (size-exclusion chromatography) using methods described elsewhere [17,30]. ThT (thioflavin T), ANS (8-anilino-1-naphthalene-sulphonic acid), DTT (dithiothreitol) and 2-mercaptoethanol were obtained from Sigma. Uranyl acetate was obtained from Agar Scientific (Stansted, Essex, U.K.). α B-WT (wild-type α B-crystallin) and its phosphorylation mimics (α B-1P, α B-2P and α B-3P) were expressed and purified as described previously [24,30]. SDS/PAGE analysis of the purified α B-crystallin proteins indicated that they contained less than 5% contaminating proteins. The concentrations of proteins used in these studies were determined by protein assays based on the Bradford method (Bio-Rad, Hemel Hempstead, Herts., U.K.) and/or by spectrophotometric methods using a Cary 5000 UV–visible spectrophotometer

(Varian, Melbourne, VIC, Australia) and calculated molar absorption coefficients based on amino acid sequences.

Intrinsic and extrinsic fluorescence

Intrinsic tryptophan fluorescence spectra were recorded using a Cary Eclipse fluorescence spectrophotometer (Varian) equipped with temperature control. The excitation wavelength was set at 295 nm and emission was monitored between 300 and 400 nm. The excitation and emission slit widths were set at 5 nm. The α B-WT and phosphorylation mimics were measured at 100 μ g/ml in 50 mM phosphate buffer (pH 7.2) or 100 mM ammonium acetate buffer (pH 6.8). Samples were maintained at the designated temperatures for 30 min before being assayed.

For the ANS binding studies, a stock solution of methanolic ANS (100 mM) was diluted 1000-fold into a 100 μ g/ml protein solution in 50 mM phosphate buffer (pH 7.2). Emission fluorescence spectra were monitored (400–600 nm) following excitation at 350 nm. FRET (fluorescence resonance energy transfer) analysis was performed on the same samples by excitation at 295 nm and monitoring the emission fluorescence spectra (320–560 nm). The excitation and emission slit widths for these studies were set at 5 nm. Samples were maintained at the designated temperatures for 30 min before being assayed.

Chaperone activity assays

The aggregation and precipitation of the target proteins, incubated in the presence of increasing amounts of the α B-crystallins and monitored by either ThT fluorescence or turbidity assay (see below), were performed in duplicate using a 96-microwell plate. At the end of the assay the samples were collected and centrifuged (15 000 g, 15 min, 4°C) to separate the soluble and insoluble (pellet) fractions. The pellet was washed with an equal volume of the same buffer used in the aggregation assay.

ThT assays

The formation of amyloid fibrils by target proteins was monitored using an *in situ* ThT binding assay method adapted from [31]. We used two model target proteins that form amyloid fibrils under physiological conditions: RCM κ -casein (reduced and carboxymethylated κ -casein) and cc β -Trp. RCM κ -Casein (500 μ g/ml) and cc β -Trp (150 μ g/ml) were incubated at 37°C for 15 h in 50 mM phosphate buffer (pH 7.2) and 50 mM phosphate buffer (pH 7.8) respectively. In order to assess the effect of pH, in some experiments RCM κ -casein was incubated at 37°C for 15 h in 50 mM phosphate buffer (pH 7.8; the same pH as that used with cc β -Trp). Samples were prepared in duplicate and incubated with 10 μ M ThT in a 96-microwell plate. The plates were sealed to prevent evaporation, and the fluorescence levels were measured with a Fluostar Optima plate reader (BMG Labtechnologies, Melbourne, VIC, Australia) with a 440 nm/490 nm excitation/emission filter set. The change in ThT fluorescence measured after its initial decrease [due to an increase in temperature from room temperature (25°C) to 37°C] is presented. The change in ThT fluorescence in the absence of the target protein was negligible for each assay.

Turbidity assays

Light scattering at 340 nm was measured and recorded using a Fluostar Optima plate reader (BMG Labtechnologies). The change in light scattering at 340 nm for each sample is presented in the graphs. The change in light scattering in the absence of the target protein was negligible for each assay.

Heat-stress assay

Bovine liver catalase (500 μ g/ml) or bovine β _L-crystallin (500 μ g/ml) was incubated at 60 °C for 80 min in 50 mM phosphate buffer (pH 7.2). Alcohol dehydrogenase from yeast (500 μ g/ml) was incubated at 42 °C for 100 min in 50 mM phosphate buffer (pH 7.2) with 100 mM NaCl and 2 mM EDTA.

Reduction assay

Bovine pancreas insulin (250 μ g/ml) was incubated at 37 °C for 80 min in 50 mM phosphate buffer (pH 7.2), and α -lactalbumin (500 μ g/ml) was incubated at 37 °C for 80 min in 50 mM phosphate buffer (pH 7.2) containing 100 mM NaCl or in 100 mM ammonium acetate (pH 6.8). Aggregation and precipitation were initiated by addition of DTT to a final concentration of 10 mM (insulin) or 20 mM (α -lactalbumin).

SDS/PAGE

SDS/PAGE was conducted on 15 or 20 % (v/v) acrylamide gels using standard techniques. Samples were mixed with an equal volume of reducing gel sample buffer such that the final concentration of 2-mercaptoethanol was 2.5 % (v/v), and then heated (95 °C, 5 min) before loading on to gels.

Size-exclusion FPLC and light scattering

SEC of samples (100 μ l) was performed on a Superdex 200HR 10/30 column (Amersham Biosciences) and eluted at 0.4 ml/min with the corresponding buffer used in the aggregation assay, i.e. 50 mM phosphate buffer (pH 7.2). The column was calibrated with gel-filtration markers (Bio-Rad).

SEC-MALS [SEC coupled with MALS (multi-angle light scattering)] was performed using a Superose 6HR 10/30 column (Amersham Biosciences) and a DAWN EOS multi-angle laser light-scattering detector (Wyatt Technology, Santa Barbara, CA, U.S.A.). Samples were loaded on to the column at a concentration of 1.5 mg/ml and eluted with 100 mM ammonium acetate (pH 6.8) or 50 mM phosphate buffer (pH 7.2) containing 100 mM NaCl at a flow rate of 0.5 ml/min.

TEM (transmission electron microscopy)

Formvar and carbon-coated nickel electron-microscopy grids were prepared by the addition of 2 μ l of protein sample, washed with 3 $\times$ 10 μ l of Milli-Q and negatively stained with 10 μ l of uranyl acetate (2 %, w/v). Samples were viewed using a Philips CM100 transmission electron microscope (Philips, Eindhoven, the Netherlands) at a magnification range of 25 000–64 000 using an 80 kV excitation voltage.

Temperature-dependent denaturation

The thermal stability of the α B-crystallin proteins (250 μ g/ml) in 50 mM phosphate buffer (pH 7.2) containing 100 mM NaCl or in 100 mM ammonium acetate buffer (pH 6.8) was monitored by measuring the light scattering of the sample at 360 nm using a Cary 5000 UV–visible spectrophotometer equipped with a Peltier temperature controller. The temperature of the samples was increased at a rate of 1 °C $\cdot$ min⁻¹. The change in absorbance at 360 nm is presented in the graphs.

MS analysis

Nanoelectrospray MS experiments were performed on a Q-ToF II spectrometer (Micromass UK) that has been modified for high-

mass operation [32]. Conditions were the same as those described previously [24,33].

NMR spectroscopy

¹H-NMR spectra were acquired at 600 MHz on a Varian Inova-600 NMR spectrometer with the parameters outlined in [34], except that the sweep width was 6000 Hz. The mixing time in the NOESY spectrum was 100 ms, and the spin lock period in the TOCSY spectrum was 60 ms. The concentration of α B-WT and α B-3P was 10 mg/ml dissolved in 10 mM phosphate buffer (pH 7.0) and 10 % ²H₂O/90 % H₂O (v/v).

RESULTS

Structural analyses of α B-WT and its phosphorylation mimics

We examined, using a variety of biophysical techniques, the structure of the recombinant phosphorylation mimics compared with that of the recombinant wild-type protein. Intrinsic tryptophan fluorescence of the proteins measured at 37 °C showed that both α B-WT and α B-3P had similar emission intensities, although the λ_{max} of α B-3P was slightly longer (α B-WT, 340 nm; α B-3P, 342 nm) and the fluorescence emission intensity was slightly lower than α B-WT (Figure 1A), indicating that the tryptophan residues in α B-3P are slightly more solvent-exposed under these conditions. The α B-2P mimic (λ_{max} = 343 nm) had the highest and α B-1P (λ_{max} = 339 nm) the lowest fluorescence emission intensities at 37 °C. After heating to 60 °C, the fluorescence emission intensity decreased and the λ_{max} shifted to longer wavelengths for all the proteins (Figure 1B). The biggest relative decrease in emission intensity and largest shift in λ_{max} was for α B-3P (λ_{max} = 346 nm), indicating that the environment of its tryptophan residues changed the most upon heating (i.e. they become more solvent-exposed).

The ANS fluorescence emission spectra measured at 37 °C showed little difference in exposed clustered hydrophobicity between α B-WT, α B-1P and α B-3P (Figure 1C). In contrast, α B-2P had a lower fluorescence emission intensity, indicating that it exposes fewer clustered regions of hydrophobicity. At 60 °C, the fluorescence emission intensity decreased and λ_{max} moved to longer wavelengths for all the proteins, reflecting a loss of exposed hydrophobicity as the proteins unfold (Figure 1D). Again, α B-3P showed the biggest decrease in fluorescence intensity upon heating, suggesting that it unfolds the most readily.

We next conducted FRET analysis in order to probe dynamic structural changes in the proteins. FRET is a technique to study conformational changes in proteins in which the emission of tryptophan residues (at $\sim$ 350 nm) is used to excite nearby ANS fluorophores. FRET was shown for all proteins by a decrease in the tryptophan fluorescence and a concomitant increase in their normal ANS fluorescence (compare Figures 1A–1D with Figures 1E and 1F). At 37 °C, α B-WT, α B-1P and α B-3P all had comparable levels of FRET, indicating that the tryptophan residues are in close proximity to the clustered regions of exposed hydrophobicity in each of these proteins. The α B-2P had lower levels of FRET, indicating an increase in the distance between its tryptophan residues and hydrophobic regions compared with the other proteins. The FRET for each protein decreased after heating to 60 °C, which is indicative of an increase in the distance between these tryptophan residues and the surrounding hydrophobic sites due to unfolding of the protein.

Nanoelectrospray MS was used to compare the oligomeric distributions of α B-WT and α B-3P. As has been previously shown for this polydisperse protein, the mass spectra exhibited broad peaks

Figure 1 Mimicking phosphorylation of α B-crystallin affects its structure as monitored by fluorescence spectroscopy

Intrinsic tryptophan fluorescence at 37 °C (A) and 60 °C (B), ANS fluorescence at 37 °C (C) and 60 °C (D) and FRET analysis at 37 °C (E) and 60 °C (F) of α B-WT ($\blacklozenge$), α B-1P ($\blacktriangle$), α B-2P ($\blacktriangleright$) and α B-3P ($\square$). The proteins (100 μ g/ml) were incubated in 50 mM phosphate buffer (pH 7.2) at each temperature for 30 min before the spectra were measured.

and an elevated baseline, consistent with the overlap of peaks arising from a multitude of different sized constituent oligomers [24]. In this case, the α B-WT spectrum contained a mixture of broad and sharp peaks between 7000 and 13 000 m/z . In contrast, peaks in the spectrum of α B-3P were less well defined over the same m/z range and were superimposed upon a significantly elevated baseline (Figure 2A). The fact that this baseline elevation continues beyond 14 000 m/z suggests that the range of oligomers in α B-3P extends to a much higher mass range than the wild-type protein. The major peaks in the spectra for α B-WT and α B-3P were 10 080 and 10 200 m/z respectively, corresponding to the species of which all oligomers carry two charges per subunit. These peaks were isolated using a tandem MS approach that enables the relative quantification of the constituent oligomers within assemblies [24], and it was found that the highest relative

proportion of subunits in an oligomer for both α B-WT and α B-3P was 28 (Figure 2B). Both proteins form major oligomers of between 24 and 32 subunits; however, there is a clear bias towards oligomers with an even number of subunits in α B-WT that is not apparent in α B-3P. Furthermore, in the case of α B-3P, the distribution of minor oligomers around the median was skewed towards those containing larger numbers of subunits (results not shown).

1 H-NMR spectroscopy was undertaken on α B-WT and α B-3P to investigate structural differences between the proteins. Cross-peaks from the highly mobile flexible C-terminal extension of 12 amino acids [35] were observed in 1 H-NMR TOCSY and NOESY spectra of both α B-WT and α B-3P (results not shown). No extra cross-peaks were observed in the α B-3P spectra compared with those of α B-WT, indicating that no additional regions of marked

Figure 2 Oligomeric distribution of α B-WT and α B-3P

(A) Nanospray mass spectra of α B-WT and α B-3P. The peaks at 10080 and 10200 m/z , corresponding to species with two charges per subunit for α B-WT and α B-3P respectively, were isolated and subjected to collision-induced dissociation. The doubly stripped oligomers were used to calculate the oligomeric distribution of the intact proteins as described previously [24]. (B) Histograms quantifying the relative abundance of the various oligomeric species for α B-WT and α B-3P.

flexibility are present in α B-3P compared with α B-WT. However, the intensity of the cross-peaks in the spectra of α B-3P was reduced, implying a slightly reduced flexibility of its C-terminal extension compared with α B-WT. This may reflect structural alterations in the domain core of α B-3P compared with α B-WT, which lead to α B-3P having a much larger mass range than α B-WT (see below).

The effect of mimicking phosphorylation on the chaperone activity of α B-crystallin against aggregating target proteins

Amyloid fibril assembly

We employed two different models to examine the effect of phosphorylation on the ability of α B-crystallin to prevent ordered protein aggregation in the form of amyloid fibril formation: (i) RCM κ -casein, an unstructured protein in its native state; and (ii) cc β Trp, a modified form of the cc β peptide that exists in a triple-helical coiled-coil configuration in its native state (results not shown). Importantly, both form amyloid fibrils under physiological conditions [29,36] and so are of relevance to amyloid formation by aggregating proteins *in vivo*. Fibril formation by RCM κ -casein (500 μ g/ml), as monitored by an increase in ThT binding, showed a gradual increase over the time course of the assay (Figure 3A). Analysis of RCM κ -casein by SEC before fibril formation showed that it predominately exists as a high-molecular-mass oligomer of more than 1 MDa and a species of apparent mass approx. 40 kDa, corresponding to the κ -casein

Figure 3 Phosphorylation mimics of α B-crystallin are more effective at preventing amyloid fibril formation by RCM κ -casein

(A) ThT binding curves of RCM κ -casein (500 μ g/ml) incubated at 37 °C in 50 mM phosphate buffer (pH 7.2) in the absence (■) or presence of α B-WT (◆), α B-1P (▲), α B-2P (◇) or α B-3P (□). The chaperone was at 500 μ g/ml and the ThT fluorescence was monitored by an *in situ* assay for 15 h. The change in ThT fluorescence of each sample is shown. This experiment was performed four times and the results shown are representative. (B) SEC of RCM κ -casein before and after fibril formation and after incubation in the presence of α B-WT and α B-3P. The proteins were loaded on to a Superdex 200HR 10/30 column and eluted in 50 mM phosphate buffer (pH 7.2) at a flow rate of 0.4 ml/min. Calibration of the column was performed using (1) Blue Dextran, void; (2) thyroglobulin, 670 kDa; (3) γ -globulin, 158 kDa; (4) ovalbumin, 44 kDa; and (5) myoglobin, 17 kDa.

dimer (Figure 3B). After fibril assembly, only a single peak that was eluted in the void volume of the column was evident, indicating that most of the protein exists in high-molecular-mass species. The addition of α B-WT (500 μ g/ml) (1.0:1.0 molar ratio of RCM κ -casein/ α B-crystallin) to the sample resulted in a decrease in ThT binding (Figure 3A). SEC indicated the formation of a high-molecular-mass complex between these two proteins, since α B-WT alone had a calculated average molecular mass of approx. 600 kDa and this peak shifted to an earlier elution

Figure 4 Fibril formation by RCM κ -casein in the presence of α B-crystallin

Electron micrographs of RCM κ -casein (500 μ g/ml) after incubation at 37 °C in 50 mM phosphate buffer (pH 7.2) for 15 h in the absence (A) or presence of α B-WT (B) or α B-3P (C). The chaperone was present at 500 μ g/ml. Scale bar, 500 nm.

time in the presence of RCM κ -casein. The RCM κ -casein high-molecular-mass peak that was eluted in the void volume of the column was reduced significantly in the presence of α B-WT (Figure 3B). The phosphorylation mimics were more effective in preventing the increase in ThT binding associated with fibril formation by RCM κ -casein, and α B-3P completely abolished it (Figure 3A). The SEC data showed that α B-3P was eluted later ($\sim$ 430 kDa average molecular mass) and as a broader peak than α B-WT. A high-molecular-mass complex between α B-3P and RCM κ -casein was evident by the shift in the elution peaks of the individual components to earlier elution times (Figure 3B). Altering the pH of the buffer in the *in situ* ThT assay to that used for cc β -Trp (i.e. pH 7.8, see below) had no effect on the overall trends in these data (results not shown).

Electron micrographs of negatively stained RCM κ -casein fibrils showed them to be thread-like structures, approx. 100–900 nm in length (Figure 4A), similar to those reported previously [36]. In the presence of the α B-crystallins the number and length of these fibrils appeared to be reduced. In the presence of α B-WT, short prefibrillar species approx. 50–100 nm in length were observed (Figure 4B), although the predominant species were large amorphous aggregates similar to those seen when the α B-crystallins were incubated alone (results not shown). No fibrillar or prefibrillar species were observed by TEM in samples incubated in the presence of α B-3P (Figure 4C), or the other phosphorylation mimics (results not shown). These samples only contained amorphous aggregates, similar to those seen for RCM κ -casein before incubation [36], or when the α B-crystallins were incubated alone (results not shown).

We next tested the chaperone activity of the phosphorylation mimics against an extended form of cc β . At 37 °C, the increase in ThT fluorescence associated with fibril formation by cc β -Trp (150 μ g/ml) was sigmoidal and included a lag phase of 150 min followed by a growth phase, which reached a plateau after

600 min (Figure 5A). The presence of the α B-crystallins at 130 μ g/ml (a 1.0:0.1 molar ratio of cc β -Trp/ α B-crystallin) decreased ThT fluorescence, and therefore, by inference, peptide selfassembly, such that it was almost completely abolished in the presence of α B-WT (Figure 5A). Similarly, SDS/PAGE of pelleted material demonstrated that cc β -Trp precipitates from solution when incubated in the absence of the chaperone protein (Figure 5B), whereas in the presence of α B-WT the peptide remains soluble. Increasing the number of negative charges used to mimic phosphorylation of α B-crystallin decreased the protective action of the chaperone in terms of ThT binding (Figure 5A) and increased the proportion of cc β -Trp that precipitated from solution (Figure 5B).

Electron micrographs of cc β -Trp after incubation demonstrated the formation of unbranched amyloid fibrils varying in length between 100 and 700 nm (Figure 6A). In the presence of α B-WT, only a small number of fibrils were observed by TEM (Figure 6B), and those that were present appeared to have a different morphology from those typical of cc β -Trp alone, i.e. thinner, more cylindrical fibrils were observed as well as thread-like tangles (Figures 6B and 6C). When cc β -Trp was incubated with α B-3P, both types of fibrils were apparent, i.e. both long straight fibrils typical of those adopted by the cc β -Trp peptide alone (Figure 6D), and thin thread-like tangles of fibrils seen in the presence of α B-WT (Figure 6E).

Heat-induced amorphous aggregation

The effect of phosphorylation on the ability of α B-crystallin to prevent amorphous aggregation was examined by employing the heat-induced aggregation of the target proteins, β_L -crystallin (a natural target of α B-crystallin in the lens), catalase and alcohol dehydrogenase (Figure 7). Turbidity was monitored by measuring the change in absorbance due to light scattering at 340 nm. When

Figure 5 Mimicking phosphorylation of α B-crystallin decreases its ability to prevent amyloid fibril formation by cc β -Trp

(A) ThT binding curves of cc β -Trp (150 μ g/ml) incubated at 37°C in 50 mM phosphate buffer (pH 7.8) in the absence (■) or presence of α B-WT (◆), α B-1P (▲), α B-2P (◇) or α B-3P (□). The chaperone was at 130 μ g/ml and the ThT fluorescence was monitored by an *in situ* assay for 15 h. The change in ThT fluorescence of each sample is shown. (B) After the ThT assay, the insoluble (pellet) fractions were separated and the proteins were resolved by SDS/PAGE. This experiment was performed a minimum of three times and the results shown are representative.

Figure 6 Fibril formation by cc β -Trp in the presence of α B-crystallin

Electron micrographs of cc β -Trp (150 μ g/ml) after incubation at 37°C in 50 mM phosphate buffer (pH 7.8) for 15 h in the absence (A) or presence of α B-WT (B, C) or α B-3P (D, E). The chaperone was present at 130 μ g/ml. Scale bar, 500 nm.

Figure 7 Phosphorylation mimics of αB -crystallin are more effective at preventing the heat-induced amorphous aggregation of target proteins

Aggregation curves of (A) β_L -crystallin (500 $\mu\text{g/ml}$) and (C) catalase (cat) incubated at 60°C in 50 mM phosphate buffer (pH 7.2) and of (E) alcohol dehydrogenase (ADH) incubated at 42°C in 50 mM phosphate buffer (pH 7.2) containing 100 mM NaCl and 2 mM EDTA. The target proteins were incubated in the absence (■) or presence of αB -WT (◆), αB -1P (▲), αB -2P (◇) or αB -3P (○) and the change in light scattering was measured at 340 nm. In (A, C), the chaperones were added at a final concentration of 100 $\mu\text{g/ml}$; in (E), they were added at a final concentration of 50 $\mu\text{g/ml}$. (B, D, F) After the aggregation assay, the soluble and insoluble fractions were separated and the proteins were resolved by SDS/PAGE. Each experiment was performed a minimum of three times and results shown are representative.

β_L -crystallin (500 $\mu\text{g/ml}$) was incubated in the absence of the αB -crystallins, a marked increase in light scattering due to protein precipitation occurred after an initial lag phase of 20 min and reached a plateau after 70 min (Figure 7A). After 80 min, most of the β_L -crystallin had precipitated from solution (Figure 7B). All the αB -crystallins (100 $\mu\text{g/ml}$) were effective at suppressing heat-induced aggregation of β_L -crystallin (a 1.0:0.2 molar ratio of β_L -crystallin/ αB -crystallin); however, the phosphorylation mimics were more effective than αB -WT, with αB -3P being the most effective. Similarly, whereas in the presence of αB -WT a small amount of β_L -crystallin was present as an insoluble species (Figure 7B), the addition of the phosphorylation mimics resulted in β_L -crystallin only being detected in the soluble fraction of the sample.

When catalase (500 $\mu\text{g/ml}$) was incubated at 60°C in the absence of the αB -crystallin proteins, after an initial lag phase, of 18 min, a large increase in light scattering was observed, which reached a plateau by 45 min (Figure 7C). After 80 min, most of the catalase had precipitated from solution, as indicated by SDS/PAGE (Figure 7D). The presence of the αB -crystallins (100 $\mu\text{g/ml}$) (a 1.0:0.6 molar ratio of catalase/ αB -crystallin) increased the lag phase (to 21 min) and reduced the magnitude of light scattering due to protein precipitation. In the presence of αB -WT, there was a decrease in the proportion of catalase that pelleted from solution (Figure 7D). Increasing the number of negative charges designed to mimic phosphorylation of αB -crystallin increased the chaperone's protective ability, such that all of the catalase remained soluble after 80 min in the presence of

Figure 8 Effect of mimicking phosphorylation of α B-crystallin on its chaperone ability to prevent DTT-induced amorphous aggregation of target proteins

Aggregation curves of (A) insulin (250 μ g/ml) and (C, E) α -lactalbumin (500 μ g/ml) incubated in the absence (■) or presence of α B-WT (◆), α B-1P (▲), α B-2P (◇) or α B-3P (□). (A) Insulin was incubated at 37 °C in 50 mM phosphate buffer (pH 7.2) with 10 mM DTT. α -Lactalbumin was incubated at 37 °C in (C) 50 mM phosphate buffer (pH 7.2) containing 100 mM NaCl with 20 mM DTT, or (E) 100 mM ammonium acetate buffer (pH 6.8) with 20 mM DTT. In each case, the chaperone (250 μ g/ml) was added to the reaction mixture and the change in light scattering was measured at 340 nm. (B, D, F) After the aggregation assay, the soluble and insoluble fractions were separated and the proteins were resolved by SDS/PAGE. Each experiment was performed a minimum of three times and the results shown are representative.

α B-1P, α B-2P and α B-3P. Interestingly, in this example and other cases described below, when insoluble precipitate was formed in the presence of the chaperone proteins (e.g. catalase incubated in the presence of α B-WT), it was made up of both the target protein and chaperone. This suggests that, in attempting to prevent aggregation, α B-crystallin may become saturated and co-aggregate with the target proteins, leading to their co-precipitation.

Alcohol dehydrogenase (500 μ g/ml), incubated at 42 °C in the absence of the α B-crystallins, after a lag phase of 15 min, showed a large increase in light scattering that reached a plateau after 80 min (Figure 7E). After 100 min, most of the alcohol dehydrogenase was still soluble in solution; however, some was insoluble as was evident by SDS/PAGE (Figure 7F). All of the α B-crystallins (50 μ g/ml) (a 1.0:0.2 molar ratio of alcohol dehydro-

genase/ α B-crystallin) inhibited the increase in light scattering due to the heat-induced aggregation of alcohol dehydrogenase; the phosphorylation mimics were more effective than α B-WT (Figure 7E). The presence of the α B-crystallins resulted in none of the alcohol dehydrogenase being detected in the insoluble fraction when it was analysed by SDS/PAGE (Figure 7F).

Reduction-induced amorphous aggregation

DTT-induced aggregation of insulin (250 μ g/ml) commenced after 10 min and reached a plateau after 45 min (Figure 8A). After 80 min, most of the B-chain of insulin in the sample existed as a precipitate, while the A-chain, which is resistant to DTT-induced aggregation [37], remained soluble (Figure 8B). In the presence

Figure 9 Effect of solution condition on the thermal stability and oligomeric distribution of α B-WT and α B-3P

Thermal denaturation curves of α B-WT ($\blacklozenge$) and α B-3P ($\square$) in 50 mM phosphate buffer (pH 7.2) containing 100 mM NaCl (**A**) and 100 mM ammonium acetate buffer (pH 6.8) (**B**). The proteins were incubated at 250 μ g/ml and the temperature was increased at the rate of $1^\circ\text{C} \cdot \text{min}^{-1}$. Precipitation of the proteins was monitored by measuring the change in light scattering at 360 nm. (**C**) SEC coupled with multi-angle laser light scattering. α B-WT and α B-3P (1.5 mg/ml) were loaded on to a Superose 6HR 10/30 column and eluted with 100 mM ammonium acetate (pH 6.8) at a flow rate of 0.5 ml/min. The solid lines represent the absorption profile of the eluted protein, and circles represent the molecular mass obtained as a function of the elution volume.

of α B-WT (125 μ g/ml) (a 1.0:0.15 molar ratio of insulin/ α B-crystallin), the degree of light scattering was reduced. At the same ratios, the phosphorylation mimics were much more effective at preventing the precipitation of the insulin B-chain; α B-2P and α B-3P inhibited the increase in light scattering the most (Figure 8A).

In the case of the DTT-induced aggregation of α -lactalbumin, the effect of phosphorylation on α B-crystallin's chaperone activity was found to be dependent on the solution conditions. When α -lactalbumin (500 μ g/ml) was reduced and precipitated in 50 mM phosphate buffer (pH 7.2) containing 100 mM NaCl, α B-2P and α B-3P (250 μ g/ml) (a 1.0:0.3 molar ratio of α -lactalbumin/ α B-crystallin) were most effective in suppressing the light scattering associated with protein precipitation (Figure 8C). SDS/PAGE indicated that a higher proportion of the target and chaperone protein remained soluble as the number of negative charges that mimic phosphorylation of α B-crystallin increased (Figure 8D). The opposite trend was observed when α -lactalbumin (500 μ g/ml) was reduced by DTT in 100 mM ammonium acetate buffer (pH 6.8) (Figure 8E). The increase in light scattering associated with precipitation of α -lactalbumin commenced 15 min after the addition of DTT and reached a plateau after 50 min. Not all of the α -lactalbumin precipitated under these conditions, as seen using SDS/PAGE (Figure 8F). In the presence of α B-WT (250 μ g/ml), the aggregation was initially suppressed, but the degree of light scattering increased after 25 min such that after 80 min it was greater than when α -lactalbumin was incubated in the absence of the chaperone. When analysed by SDS/PAGE, both

α -lactalbumin and α B-WT were found in the pellet, indicating that they interact and then co-precipitate (Figure 8F). The increase in light scattering was more rapid in the presence of the phosphorylation mimics (Figure 8E) and, as the number of negative charges that mimic phosphorylation increased, less of the chaperone protein remained in solution, such that none of α B-3P was soluble after 80 min (Figure 8F). While pH changes were found to have a significant effect on the rate of aggregation of α -lactalbumin (for example, changing the pH of the ammonium acetate buffer from pH 6.8 to 7.2 increased the lag phase in the aggregation curve by 20 min and decreased the light scattering due to protein precipitation by 40%), the overall trends in chaperone ability of the α B-crystallins to prevent its aggregation in each buffer were the same.

Mimicking phosphorylation of α B-crystallin affects its thermal stability and oligomeric distribution

In order to decipher the differences observed in the relative ability of the wild-type and phosphorylation mimics to chaperone reduced α -lactalbumin under different solution conditions (Figures 8C and 8E), we examined the stability of the chaperone proteins in the different buffers used in these assays. In phosphate buffer (50 mM, pH 7.2, containing 100 mM NaCl), α B-WT underwent two temperature-dependent structural transitions, one at 65°C and another at 78°C (Figure 9A). In contrast, α B-3P did not undergo a significant thermal transition until 82°C (Figure 9A). However, when incubated in ammonium acetate

buffer (100 mM, pH 6.8), α B-3P was significantly less stable than α B-WT; most of α B-3P precipitated from solution at 63 °C, compared with 78 °C for α B-WT (Figure 9B). The intrinsic tryptophan fluorescence of α B-WT or α B-3P was not different when incubated in either the phosphate or ammonium acetate buffers (results not shown), indicating that the difference in stability is not due to a detectable unfolding of α B-3P in the ammonium acetate buffer.

We therefore investigated differences in the oligomeric distribution of α B-WT and α B-3P in these two buffer systems. When incubated in 50 mM phosphate buffer (pH 7.2), both proteins are eluted as a symmetrical peak, although α B-3P was eluted much later than α B-WT (Figure 3). Horwitz [38] has previously shown by SEC-MALS that under these conditions α B-WT exists within the range of 480–640 kDa, with an average molecular mass of 580 kDa. Upon introduction of three negative charges that mimic phosphorylation, this range increases (320–730 kDa) and average molecular mass decreases (440 kDa). In contrast, when these proteins are incubated in ammonium acetate, α B-WT exists within the range of 400–780 kDa, with an average molecular mass of 530 kDa (Figure 9C) and, although α B-3P has the same average molecular mass as α B-WT (530 kDa), it is much more polydisperse (330–950 kDa), in a similar manner to that reported for α B-1P and α B-2P [24].

DISCUSSION

The negative charge introduced into the recombinant α B-crystallin by replacing the serine residues with aspartate is designed to mimic the natural phosphorylation state of the protein. This method has been employed previously for α B-crystallin and shown to have similar effects to those of endogenously phosphorylated α B-crystallin with regard to its oligomeric distribution [23], subcellular localization [39] and cellular trafficking [40]. Thus, using this rationale, we have employed α B-crystallin phosphomimics to investigate the chaperone action of α B-crystallin on amyloid fibril formation and amorphous aggregation. Our results show that the negative charges introduced into α B-crystallin by phosphorylation have a major effect on its chaperone ability against target proteins undergoing both types of aggregation.

In α B-crystallin, the three sites of phosphorylation are in the N-terminal domain of the protein, outside of the conserved C-terminal ' α -crystallin' domain. Intrinsic fluorescence shows that phosphorylation results in structural changes in the N-terminal domain of α B-crystallin. The α B-WT, α B-1P and α B-3P proteins all had very similar ANS and FRET fluorescence spectra at 37 °C, indicative of similar regions of clustered hydrophobicity and their proximity to accessible tryptophan residues. Since α B-3P had the biggest change in ANS and FRET fluorescence upon heating from 37 to 60 °C, these data suggest that its structure is altered the most by heating, which may facilitate its higher affinity for heat-stressed target proteins used in the present study. When incubated in phosphate buffer, α B-3P was found to be more thermostable than α B-WT (Figure 9). Its relative instability in acetate buffer probably reflects the relatively higher destabilizing effect of this ion on protein solubility compared with phosphate buffer, as indicated by the Hofmeister salt series [41]. With regard to α B-crystallin, our results suggest that phosphorylation accentuates this difference in protein solubility in the presence of different buffer ions. This is supported by the data showing that α B-WT and α B-3P exist in different oligomeric distributions in different buffer systems and have a greater mass range in ammonium acetate (Figure 9).

In an elegant study using destabilized T4 lysozyme mutants as models of partially folded intermediate states of the protein, Koteiche and Mchaourab [25] showed that serine to aspartate phosphorylation mimics of α B-crystallin have a higher binding affinity (and by inference chaperone ability) to these partially folded states of T4 lysozyme compared with the wild-type protein. It was concluded that, through phosphorylation, α B-crystallin binding is activated to levels that exceed the affinity and binding capacity of α A-crystallin for the T4L mutants [25] (α B-WT having a lower affinity for these mutants than α A-crystallin). Our results are consistent with this and other findings showing that phosphorylation increases the polydispersity of α B-crystallin, reduces its oligomeric size and diminishes its preference for assemblies with an even number of subunits [24,38]. It has been proposed that α B-crystallin acts as a chaperone via a dimeric form that dissociates from the oligomer and interacts with the target protein [1,42]. This bicomponent target protein– α B-crystallin complex is then incorporated back into the α B-crystallin oligomer. Aquilina et al. [24] suggested that, by analogy with the known structure of Hsp16.9, a related sHsp from wheat [42], the quaternary substructure of α B-crystallin is destabilized by phosphorylation of Ser⁴⁵ because introduction of a negative charge at this site results in the disruption of intersubunit contacts by the α 2-helix in the N-terminal domain. The increased prevalence of this dissociated state upon introduction of a negative charge at this site may be responsible for the enhanced chaperone activity of α B-2P and α B-3P seen against a number of target proteins used in the present study, rather than a direct interaction of the phosphorylated site with the target protein [25]. This may occur in a similar manner to other treatments (i.e. elevated temperature, presence of denaturant), which lead to structural perturbation of the α B-crystallin oligomer, an increase in the exposure of hydrophobic surfaces and/or greater subunit exchange, and an enhancement of the overall chaperone activity of the protein.

Our results indicate that α B-crystallin inhibits pathways of protein aggregation that lead to the formation of amyloid fibrils. In the two systems studied, the presence of the chaperone inhibited the increase in ThT fluorescence associated with amyloid fibril formation [43]. From our results, α B-crystallin appears to inhibit the formation of fibrils in the early stages of aggregation, since significantly fewer mature fibrils were observed in the presence of the chaperone, as judged by TEM. It possibly does so by interacting with intermediately folded states of the target protein to prevent the nucleation event that precedes fibril formation, or by interacting with prefibrillar aggregates, which would be consistent with the findings that it forms a high-molecular-mass complex with the target protein (Figure 3B) and that the complex remains in solution (Figure 5B). α B-crystallin effectively suppressed fibril formation by α -synuclein, the protein associated with Lewy bodies and plaque formation in Parkinson's disease, by interacting with α -synuclein early along its aggregation pathway [44]. Furthermore, it redirects α -synuclein from a fibril-forming pathway towards an amorphous aggregation pathway [44]. α B-crystallin also interacts with apolipoprotein C-II at its early stage of aggregation to prevent large-scale fibril formation [45].

The effectiveness of α B-crystallin to act as a chaperone against a particular target protein is due, at least in part, to the different conformational states of the target protein's intermediately folded forms. This is because α -crystallin has a differential mode of binding to target proteins that reflects the latter's free energy of unfolding [46]. The more destabilized and unfolded substrates have higher binding affinities to α B-crystallin [25]. This may account for the differences seen in the chaperone action of the phosphorylation mimics compared with α B-WT against the two fibril-forming proteins, RCM κ -casein and cc β -Trp, used in

the present study. The cc β -Trp peptide exists in a highly structured coiled-coil configuration in the native state compared with RCM κ -casein, which is essentially unstructured. Each protein's intermediate states may differ in their structure, hydrophobicity and binding affinity for α B-crystallin. Such factors may be responsible for the variation observed in the effect of phosphorylation of α B-crystallin on its ability to prevent fibril formation in these two different systems. Moreover, as a result of differences in intermediate states, variations in the chaperone ability of α B-crystallin against different target proteins are likely to arise.

Furthermore, the conditions under which aggregation occurs were also found to play an important role in the chaperone action of α B-crystallin. In the case of amorously aggregating target proteins, in general we found that increasing the number of negative charges used to mimic phosphorylation of α B-crystallin increased its chaperone ability. However, our studies involving α -lactalbumin indicated that the effect of phosphorylation on α B-crystallin is also dependent on the solution conditions under which aggregation occurred. This was shown to be due, at least in part, to the overall stability of the chaperone in solution (Figure 9). By comparing the oligomeric distribution of the proteins in these buffer systems using SEC-MALS, it was observed that local structural perturbations between these proteins are further influenced by the surrounding medium. Co-aggregation and co-precipitation of α B-crystallin with the target protein probably result from the saturation of binding sites available for substrate interaction, those with higher affinities being saturated first. This phenomenon has also been observed with the R120G mutant of α B-crystallin, a destabilized form of the protein, which probably exposes more of its binding sites(s) to solution, enhancing the rate of reaction and leading to its co-precipitation when incubated with target proteins such as reduced α -lactalbumin [34,47,48]. Thus, although the protein is a more effective chaperone in terms of its binding affinities, under certain conditions this may lead to an apparent increase in light scattering, due to its saturation and co-precipitation. Such factors are important when assessing comparative affinities and function of sHsps, and differences in assay conditions may explain the apparent disparate findings of previous studies looking at the effect of phosphorylation on the chaperone activity of α B-crystallin.

It is not known how phosphorylation and dephosphorylation of α B-crystallin are regulated in the cell. Phosphorylation of α B-crystallin occurs under normal physiological conditions *in vivo* and is stimulated by stress [11,15]. Thus it would be predicted that the activity of α B-crystallin, a protein that is required to have high activity during stress conditions in the cell, would be enhanced by phosphorylation rather than lessened. It follows that phosphorylation may provide a molecular switch to the cell that serves to regulate the binding affinity of α B-crystallin, by disrupting its oligomeric state and increasing the amount of its 'activated' form. In summary, our results show that the introduction of negative charges brought about by phosphorylation of α B-crystallin has a significant effect on its structure and chaperone ability against target proteins undergoing either amorphous or amyloid fibril-type aggregation. Our results suggest that this highly prevalent post-translational modification of sHsps may play an important role in alleviating the pathogenic effects associated with protein conformational diseases.

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