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R120G **α**B-crystallin promotes the unfolding of reduced **α**-lactalbumin and is inherently unstable.

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Abstract

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Keywords

?B-crystallin, lens protein, non-lenticular tissue, H NMR spectroscopy

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R120G αB-crystallin promotes the unfolding of reduced α-lactalbumin and is inherently unstable.

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Abstract

α-Crystallin is the principal lens protein which, in addition to its structural role, also acts as a molecular chaperone, to prevent aggregation and precipitation of other lens proteins. One of its two subunits, αB-crystallin, is also expressed in many non-lenticular tissues, and a natural missense mutation, R120G, has been associated with cataract and desmin-related myopathy, a disorder of skeletal muscles (Vicart et al., 1998, Nature Genet. 20:92-95). In the present study, real-time ^{1}H NMR spectroscopy showed that the ability of R120G αB-crystallin to stabilize the partially folded, molten globule state of α-lactalbumin was significantly reduced in comparison with wild type αB-crystallin. The mutant showed enhanced interaction with, and promoted unfolding of reduced α-lactalbumin, while it showed limited chaperone activity for other target proteins. Using NMR spectroscopy, gel electrophoresis and mass spectrometry (MS) it was observed that, unlike the wild type protein, R120G αB-crystallin is intrinsically unstable in solution with unfolding of the protein over time leading to aggregation and progressive truncation from the C-terminus. Light scattering, MS and size-exclusion chromatography data indicated that R120G αB-crystallin exists as a larger oligomer than wild type αB-crystallin, and its size increases with time. It is likely that removal of the positive charge from R120 of αB-crystallin causes partial unfolding, increased exposure of hydrophobic regions and enhances its susceptibility to proteolysis, thus reducing its solubility and promoting its aggregation and complexation with other proteins. These characteristics may explain the involvement of R120G αB-crystallin with human disease states.

Abbreviations

1D – one dimensional; DRM - desmin-related myopathy; DTT - dithiothreitol; MS – mass spectrometry; ppm – parts per million; SEC - size exclusion chromatography; sHsp - small heat-shock protein; TOCSY – Total COrrelation SpectroscopY

Introduction

The vertebrate lens is composed of a very high concentration of proteins, the main group of which is the crystallins, whose higher order structural arrangement enables the refraction of light to ensure proper vision. The principal lens protein is α-crystallin which, in addition to its structural role, also functions as a molecular chaperone to interact and complex with the β- and γ-crystallins to prevent their aggregation and precipitation [1]. The crystallins are very stable proteins and the lack of protein turnover in all but the outer (epithelial) layer of the lens means that they have to be very long lived. With age, however, many post-translational changes occur to the crystallin proteins leading to localized unfolding and the potential for aggregation and precipitation, characteristic of cataract formation. The chaperone action of α-crystallin helps to minimize these events [2].

α-Crystallin is a member of the small heat-shock protein (sHsp) family of molecular chaperones [3-5]. sHsps are found in all organisms and, in humans, are comprised of ten proteins [6]. In addition to being found in the lens, sHsps are present in many tissues. Lens α-crystallin is comprised of two related subunits, A and B, but only αB-crystallin is expressed extralenticularly to any significant extent. For example, high levels of αB-crystallin are found in cardiac and skeletal muscle and are also present in the brain, lung and retina. Intracellularly, it has been proposed that the A subunit stabilizes the B subunit and knockout of the αA-crystallin gene in mice causes aggregation of αB-crystallin [7, 8]. sHsps have subunit masses in the range of ~ 12-43 kDa but, in the main, exist as large oligomeric species [3-5]. The mammalian sHsps, including α-crystallin, are found as heterogeneous oligomers, e.g. αB-crystallin has a size distribution from 200 to 800 kDa [9] with an average mass of around 560 kDa [10].

The occurrence of a natural missense mutation of αB-crystallin, R120G, was first reported by Vicart et al. [11], and closely followed the discovery that a naturally occurring mutation at the equivalent position in αA-crystallin, R116C, caused congenital

cataract in humans [12]. In αA- and αB-crystallin, R116 and R120, respectively, are located within the conserved α-crystallin domain. The R120G αB-crystallin mutation has been linked to a number of diseases including desmin-related myopathy (DRM), an inherited muscle disorder in humans characterised by intrasarcoplasmic accumulation of desmin, and cataract [11]. Desmin filaments play an important role in cardiomyocytes where they maintain the structural integrity of the cell by linking adjacent myofibrils to each other, to the cell membrane and to the nuclear envelope [13]. R116C αA-crystallin causes cataract in the lens (where αA-crystallin is mainly located), but because αB-crystallin also has considerable extra-lenticular distribution, it is perhaps not surprising that the R120G αB-crystallin mutant is responsible for the occurrence of DRM in addition to cataract.

Intermediate filament proteins such as desmin play an important structural role in skeletal muscle [14] where αB-crystallin has also been found to be present to a significant extent [15, 16]. A number of studies have shown that αB-crystallin binds to desmin and desmin filaments, particularly under conditions of cellular stress [17, 18]. The interaction of αB-crystallin with intermediate filament proteins has also been reported with the intracellular localization of the chaperone correlating to the reconstruction of the intermediate filament network of the cells following heat stress [19]. Initial studies [11] found that the R120G mutation in αB-crystallin led to the formation of aggregates involving R120G αB-crystallin and desmin and these were proposed to be a result of either the decreased ability of R120G αB-crystallin to chaperone desmin or the aggregation of R120G αB-crystallin itself, which is then followed by enmeshing of the aggregates with desmin [11]. More recent studies have expanded these observations, with mice expressing high levels of R120G αB-crystallin exhibiting a phenotype in which cardiomyocytes were affected to such an extent that hypertrophy and eventual death resulted [20]. These data also reinforced the hypothesis that desmin aggregate formation is due to a loss of function in αB-crystallin as a result of the mutation [20]. It was recently suggested that misfolding of R120G αB-crystallin causes the formation of aggregates consisting of R120G αB-

crystallin and desmin *in vivo*, which can be prevented by expression of wild type αB-crystallin or other molecular chaperones [21].

The discovery that R116C αA-crystallin and R120G αB-crystallin were related to disease states [11] led to a flurry of *in vitro* studies into the effects of these mutations on the structural and functional aspects of αA- and αB-crystallin [22-27]. The general conclusions from these studies were that both mutants have altered secondary, tertiary and quaternary structures compared to the wild type protein, which presumably combine to diminish their chaperone ability [22, 24, 28]. The positive charge of R116 in αA-crystallin has been implicated as being critical in maintaining structural integrity [28]. In this present investigation, we have extended these studies by examining the effect, in real time, of R120G αB-crystallin on the structure of one of its target proteins, reduced α-lactalbumin, and by monitoring the significant structural changes that occur to the chaperone with time.

Results

1. ^{1}H NMR spectroscopy of R120G αB-crystallin.

Real-time NMR spectroscopy.

Previously, using real-time ^{1}H NMR spectroscopy, we examined the interaction between reduced α-lactalbumin and α-crystallin isolated from bovine lenses [29-31]. From these spectra, coupled with the use of complementary spectroscopic techniques (i.e. SEC, visible and UV absorption spectroscopy and MS), it was concluded that α-crystallin stabilized and interacted with a partially folded intermediate of reduced α-lactalbumin that bore strong similarities to the well-characterised molten globule state of α-lactalbumin observed at pH 2. This species has little tertiary structure in place but retains elements of its secondary structure and is highly dynamic. The implication was that α-crystallin preferentially complexed to these types of conformational states of target proteins to prevent their aggregation and precipitation.

Wild type αB-crystallin readily suppresses the aggregation of reduced α-lactalbumin. By contrast, when R120G αB-crystallin interacts with reduced α-lactalbumin, as monitored by visible absorption spectroscopy, both proteins aggregate and precipitate. Overall, this process occurs at a faster rate than for reduced α-lactalbumin in the absence of αB-crystallin [22]. Thus, the destabilized structure of R120G αB-crystallin readily binds the partially folded α-lactalbumin species but the resultant complex is not soluble. Accordingly, R120G αB-crystallin is a very poor chaperone in preventing the precipitation of reduced α-lactalbumin. In fact, aggregation was accelerated in the presence of R120G αB-crystallin. In the experiments herein, real-time ^{1}H NMR spectroscopy was utilized to explore the detailed nature of this phenomenon, in particular the conformational state of α-lactalbumin that interacts with R120G αB-crystallin.

Figure 1 shows the aromatic region of the time course 1D ^{1}H NMR spectra of apo-α-lactalbumin following its reduction in the absence and presence of 1:1 subunit molar ratios of wild type and R120G human αB-crystallin. Since the ^{1}H NMR spectrum of αB-

crystallin does not contain any aromatic resonances [32], the changes with time in the NMR spectrum arise from effects on α-lactalbumin only. For reduced α-lactalbumin in the absence or presence of wild type αB-crystallin, the spectral changes with time are very similar to those observed for the experiments conducted previously on α-lactalbumin in the absence and presence of isolated bovine α-crystallin and will not be discussed in detail here except to emphasize that the molten globule state of apo-α-lactalbumin forms within the dead time of the experiment and gradually builds up to a maximum as all the disulfide bonds are reduced (~ 320 s in the absence of any chaperone protein) [31]. The spectrum is broad because of the dynamic nature of the molten globule state. The interaction of reduced, molten globule apo α-lactalbumin with R120G alphaB-crystallin is enhanced compared to that with wild type αB-crystallin. As we showed previously [31], the resonance decay (i.e. loss of intensity) of reduced α-lactalbumin in the absence of αB-crystallin arises from aggregation of the molten globule state of this protein. In the presence of αB-crystallin, the resonance decay is due to the molten globule state of α-lactalbumin interacting with, and complexing to, αB-crystallin due to the latter's chaperone action. The decay of resonance intensity represents either of these processes and can be quantified by examining the loss of intensity from the isolated resonance at 6.8 ppm arising from the tyrosine (3,5) ring protons of the molten globule state of reduced α-lactalbumin [31]. In both cases, the decay of resonance intensity is first-order.

(Fig. 1)

The time for resonance intensity to build up to its maximum was almost the same in the absence and presence of wild type αB-crystallin, i.e. approximately 320 s, and was very similar to that observed for α-lactalbumin in the absence and presence of α-crystallin [31]. Thus, wild type αB-crystallin and α-crystallin have no effect on the rate of reduction of the disulfide bonds of apo-α-lactalbumin. By contrast, in the presence of R120G αB-crystallin, the time for complete disulfide bond reduction was decreased to around 150 s, implying that R120G αB-crystallin promoted unfolding, and hence disulfide bond reduction, of α-lactalbumin.

Plots of the loss of α-lactalbumin tyrosine (3,5) resonance intensity against time (Figure 2) show that the rate of signal loss in the absence of αB-crystallin (1.509 (± 0.066) x 10^{-3} s^{-1}) is the same as that observed in previous studies [31]. The ability of αB-crystallin, however, to stabilize the molten globule state of α-lactalbumin is decreased slightly (approximately 1.5 fold) compared to that found for α-crystallin (rate = 1.227 (± 0.055) x 10^{-3} s^{-1} and 8.00 (± 0.53) x 10^{-4} s^{-1} respectively [31]). In the presence of R120G αB-crystallin, however, the rate of loss of resonance intensity of α-lactalbumin was 2.404 (± 0.130) x 10^{-3} s^{-1}, i.e. 2.0 times faster than in the presence of wild type αB-crystallin and 1.6 times faster than in the absence of any chaperone. Thus, the interaction of reduced apo-α-lactalbumin with R120G αB-crystallin is enhanced compared to the wild type protein. The interaction of the two proteins leads to a destabilized complex that associates and precipitates [22].

(Fig. 2)

After the NMR experiment had been completed, the solution of R120G αB-crystallin and α-lactalbumin contained a heavy precipitate whereas the mixture of wild type αB-crystallin and α-lactalbumin was clear. The sample of reduced α-lactalbumin, of course, contained precipitated protein. SDS-PAGE of the precipitate in the mixture of R120G αB-crystallin and α-lactalbumin contained both proteins (not shown), as found previously by Bova et al. [22] implying that R120G αB-crystallin bound to reduced α-lactalbumin and the resultant complex precipitated.

^{1}H NMR spectroscopy of R120G αB-crystallin with time.
Over the period of a week, the ^{1}H NMR spectrum of R120G αB-crystallin was monitored. Figure 3 shows the aromatic and NH region of the 1D ^{1}H NMR spectrum and the NH to α,β,γ-CH region of the 2D TOCSY spectrum of R120G αB-crystallin at selected times over this period. Initially, the spectrum contained only the expected resonances and cross-peaks from the highly mobile and unstructured C-terminal extension of αB-crystallin, which encompasses the last 12 amino acids of the protein [32-34]. However, relatively soon after dissolving R120G αB-crystallin in solution (e.g. after 3-4 days), additional resonances and cross-peaks appeared in the NMR spectra which,

from their lack of chemical shift dispersion, arise from relatively unstructured regions of the protein. Thus, R120G αB-crystallin is intrinsically unstable and readily unfolds with time. From the large number of additional cross-peaks observed in the TOCSY spectra of R120G αB-crystallin and their chemical shifts, extensive regions of the protein have conformational mobility and little structure and are exposed to solution. Furthermore, the unfolding caused the protein to be destabilized such that extensive precipitation occurred during the course of the experiment. In comparison, acquiring NMR spectra of the wild type protein over a period of a couple of months showed no evidence of degradation (not shown).

(Fig. 3)

2. Size exclusion chromatography (SEC) and light scattering data on R120G αB-crystallin.

The size of the R120G and wild type αB-crystallin oligomers was investigated by SEC and dynamic light scattering (Figure 4a). Light scattering data for freshly prepared solutions of these proteins, monitored as they eluted from a size exclusion column, showed that the wild type protein had a mass of 560 kDa at its maximum elution position whereas R120G αB-crystallin had a mass of 1000 kDa. As expected, both proteins were highly heterogeneous with the mass range of R120G αB-crystallin being much greater than that of the wild type protein: i.e. 570 kDa compared to 180 kDa. Previous studies [23, 24] had qualitatively indicated this behaviour from size exclusion profiles. With time, SEC of a sample of R120G αB-crystallin left at room temperature indicated that it progressively aggregated and, after 13 days, a reduced amount of soluble protein was present (Figure 4b). Mass spectra characterization of these samples also indicated a progressive increase in oligomer size of the R120G αB-crystallin [10] (not shown). The loss of solubility of R120G αB-crystallin is consistent with the results from monitoring of the NMR spectrum of the protein with time (Figure 3). In a control experiment, no change in the oligomer size of wild type αB-crystallin occurred, as monitored by electrospray MS (not shown).

(Fig. 4)

3. Mass spectrometry of R120G αB-crystallin.

To examine the primary sequence changes of R120G αB-crystallin, it and wild type αB-crystallin were incubated at 25°C for up to 16 days and aliquots were sampled at regular intervals for analysis by MS and SDS-PAGE. No degradation of wild type αB-crystallin was observed after 9-day incubation, as evidenced by the large single peak at 20.16 kDa in the transformed mass spectrum (Fig. 5a). This spectrum was identical to that obtained from wild type αB-crystallin immediately after dissolution (not shown). R120G αB-crystallin, however, was degraded rapidly, whereby significant proteolysis had occurred after five days of incubation (Fig. 5c). In fact, only C-terminal truncation products of R120G αB-crystallin were present after 9 days of incubation, with no full-length protein remaining (Fig. 5c,d). Thus, the mutant was highly susceptible to degradation, possibly autolysis.

Individuals who carry the R120G mutation in their αB-crystallin gene are heterozygous, and αB-crystallin isolated from their muscle cells contains equal amounts of wild type and R120G αB-crystallin [11]. Therefore, the stability with time of a 1:1 mixture of wild type and R120G αB-crystallin, incubated at 25°C for 16 days, was examined. As for pure wild type αB-crystallin, no detectable degradation occurred of the mixture over this period (data not shown). The implication is that wild type αB-crystallin stabilized R120G αB-crystallin, most likely by protecting it from unfolding and proteolysis.

The absence of contaminating protease activity was evident from the high purity of the samples (MS and SDS-PAGE data not shown), and further assured by the use of wide-range protease inhibitors throughout the purification and experimental procedures. Also, the presence of a unique unknown protease in the R120G αB-crystallin sample is highly unlikely, as the same expression strain and purification procedure was used for the wild type protein, and from the absence of degradation in the mixture of wild type and R120G αB-crystallin.

(Fig. 5)

4. Chaperone ability and stability to urea of R120G αB-crystallin.

Previous studies have shown that R120G αB-crystallin is a poorer chaperone than the wild type protein with respect to a diversity of target proteins under various stress conditions [24]. Figure 6 compares the chaperone ability of freshly prepared solutions of R120G and wild type αB-crystallin in the presence of heated βL-crystallin and reduced insulin. For the first target protein, the chaperone ability of R120G αB-crystallin was reduced significantly compared to the wild type protein (Fig. 6a; a complete suppression of aggregation was obtained at 0.12:1.0 ratio wild type αB-crystallin:βL-crystallin, but only 77% suppression with the same amount of R120G αB-crystallin; while for 0.06:1.0 ratio of the chaperone to βL-crystallin, 81% and 17% suppression, respectively, were achieved). However, R120G αB-crystallin was a comparable chaperone to the wild type protein in its ability to prevent the precipitation of the B chain of insulin at 37°C (Fig. 6b).

(Fig. 6)

The intensity of tryptophan fluorescence of the native state was lower (by ~15%) for R120G compared to wild type αB-crystallin, implying a more unfolded conformation in the N-terminal domain. However, the unfolding of R120G αB-crystallin in urea (in 50 mM phosphate buffer pH 7.4 at 25°C), as measured by tryptophan fluorescence wavelength maximum, did not differ from that of wild type αB-crystallin (not shown), i.e. both proteins were half unfolded at a concentration of ~3.2 M urea. Furthermore, this unfolding occurred at a lower concentration than bovine α-crystallin, which is consistent with previous studies [35]. Thus, freshly made-up solutions of R120G and wild type αB-crystallin have similar stabilities to denaturing agents in their N-terminal region. The N-terminal domain is not as exposed to solution as the C-terminal domain (refs 38 and 40 in original version of paper – our review articles), which may explain the similar susceptibility to urea of the two proteins, implying that the observed structural differences between the two proteins arise predominantly from their C-terminal regions.

Discussion

It is apparent from our real-time NMR studies of the structural changes following reduction of α-lactalbumin that R120G αB-crystallin promotes the unfolding of apo-α-lactalbumin so that its disulfide bonds are more accessible to the reducing agent. Thus, in contrast to αB-crystallin and α-crystallin [29-31], R120G αB-crystallin did not stabilize the molten globule state of reduced α-lactalbumin. The destabilized structure of R120G αB-crystallin compared to the wild type protein facilitates its ready interaction with unfolding α-lactalbumin such that the resultant aggregated complex is not stable and subsequently precipitates [22]. The ability of R120G αB-crystallin to promote the unfolding of reduced α-lactalbumin may arise because the destabilized structure of the chaperone causes greater exposure of its chaperone binding site(s) which most likely comprise, at least in part, the region from residues 74 to 92 in the C-terminal (α-crystallin) domain of αB-crystallin [36]. Mchaourab and co-workers [37, 38] have proposed that binding of T4 lysozyme mutants to αA- and αB-crystallin occurs through two modes which have affinity for differently structured T4 lysozyme species. The destabilized structure of R120G αB-crystallin may promote one of these modes of binding over the other, leading to the rapid association of reduced α-lactalbumin with R120G αB-crystallin.

Even though the R120G mutation had no significant effect on the chaperone activity of αB-crystallin towards reduced insulin, it resulted in a significant destabilization of reduced α-lactalbumin in our NMR studies. The marked difference in the chaperone activity of R120G αB-crystallin in the presence of these two target proteins under reduction stress was observed by Bova et al. [22] and was attributed to a possible target protein-specificity of R120G αB-crystallin which may be present in wild type αB-crystallin but is enhanced by the structural alterations in the mutant [22]. Furthermore, the different conformational states of reduced α-lactalbumin and the B chain of insulin may be important factors in this variation in affinity. The small insulin B chain is likely to have little secondary structure compared to the molten globule state of the much-

larger, more structured, reduced α-lactalbumin. A differential mode of binding of α-crystallin to proteins, reflecting their free-energy of unfolding, has been shown [38]. Thus, the insulin B chain would favor interaction with the low-affinity binding site of αB-crystallin whereas the α-lactalbumin (molten globule) intermediate would interact with the high affinity site.

The stabilization of the reduced, molten globule state of α-lactalbumin by wild type αB-crystallin was not as effective as that by α-crystallin (Figures 1 and 2) [31]. The difference in stabilization rates may reflect the differences in structural arrangement and/or subunit exchange rates of the two chaperones with reduced α-lactalbumin. Both of these factors are crucial for interaction of the two proteins and subsequent complexation. The implication is that, under these conditions, αB-crystallin is not as good a chaperone as α-crystallin. The chaperone ability of the individual αA- and αB-crystallin subunit oligomers varies markedly depending on the solution conditions, i.e. temperature, type of stress and target protein [39]. For example, the chaperone ability of αB-crystallin with reduced α-lactalbumin as a target protein, does not depend greatly on temperature, whereas the chaperone ability of αA-crystallin improves markedly at higher temperatures [39]. However, no direct comparisons have been made between the chaperone ability of αB-crystallin and α-crystallin with the same target protein. The ratio of the two subunits in the lens is ~ 3:1. Hybrid oligomers of 3:1 w:w αA:αB-crystallin mixtures are more stable to temperature than their individual subunit counterparts [7] which, from the previous discussion, implies a better chaperone performance of α-crystallin compared to αB-crystallin, particularly at elevated temperatures.

With time, the SEC data indicated that R120G αB-crystallin aggregated to an even greater extent than the very large species present initially (Figures 4 and 5). The MS spectra also showed the appearance of C-terminally truncated monomer species with time. The relatively unfolded, truncated, monomeric R120G αB-crystallin is most likely to be highly destabilized and therefore a precursor to the highly aggregated species. In

addition, this time-dependent proteolysis of R120G αB-crystallin would promote unfolding leading to the observation of resonances in the NMR spectrum from unstructured regions, according to their chemical shift, along with resonances from the peptide fragments.

A previous study [23] found that R120G αB-crystallin was more susceptible to chymotryptic digestion than the wild type protein, which is consistent with our observations of increased proteolytic sensitivity in the mutant. The proteolysis from the C-terminus of R120G αB-crystallin and the concomitant unfolding are consistent with the solubilizing role that this flexible region plays in the structure of the protein and its importance in maintaining the structural integrity of the well-ordered domain core of the protein (e.g. the C-terminal α-crystallin domain). Thus, in previous studies on mouse Hsp25, a related sHsp, we observed that removal of the highly mobile C-terminal 18 amino acids caused a major structural change (unfolding) in the protein and reduction in its chaperone activity [40]. Furthermore, other studies have shown that sHsps with deletions from the C-terminus have reduced chaperone ability [41-44] and swapping of the C-terminal extensions between the two α-crystallin subunits has a significant effect on the chaperone ability of each protein, in addition to causing structural changes in the domain core of the proteins [45]. Finally, extensive C-terminal truncation of both subunits of α-crystallin occurs *in vivo* with age [46], which is consistent with the tendency for flexible regions of proteins to be susceptible to proteolysis [47]. In the case of R120G αB-crystallin, proteolysis from the C-terminus may exacerbate the tendency for the already destabilized protein to unfold and hence promote its association and aggregation, eventually leading to significant precipitation.

The arginine residue at position 116 in αA-crystallin is conserved across 28 mammalian species in addition to chicken and frog [48] and the equivalent residue in αB-crystallin, R120, is present in the sequences of αB-crystallin from twelve species of vertebrates [11]. These arginine residues are positioned within the highly conserved α-crystallin domain, which is widely considered to be critical for chaperone function in many sHsps [49]. The α-crystallin domain is believed to play a role in subunit-subunit interaction

[50] and, as discussed above, contains the putative chaperone-binding region. The R112 and R116 residues are buried within the protein, indicating the existence of "buried salt bridges in the core of the αA-crystallin oligomer and/or the subunits" [51]. The α-crystallins have maintained their net charge through the course of evolution [48] and disruption of this preserved charge balance may lead to major structural change [12]. This in turn may lead to impaired chaperone function and destabilization of the protein, implicated in cataract [12].

Studies on homologous proteins have also indicated the importance of the corresponding residue in subunit interactions for Hsp16.3 from *Mycobacterium tuberculosis* and human Hsp27 [51], or structural stabilization through formation of hydrogen bond in Hsp16.5 from *Methanococcus jannaschii* [52]. Previous studies, in addition to the results presented herein, show that R116C αA-crystallin and R120G αB-crystallin have disrupted secondary, tertiary and quaternary structures [22, 24, 25, 28].

In conclusion, our work has provided further evidence that the naturally occurring R120G mutant of αB-crystallin is intrinsically unstable which is likely to be due to removal of the positive charge of R120 leading to structural alteration and unfolding of the protein. This unfolding most likely leads to exposure of hydrophobic regions, which facilitates its self-aggregation (as demonstrated by SEC), and aggregation with a target protein (as demonstrated by real-time NMR spectroscopy with reduced α-lactalbumin). It is possible that the ability of R120G αB-crystallin to promote the unfolding of α-lactalbumin arises from one of its exposed regions being the chaperone binding site(s). In addition, R120G αB-crystallin is susceptible to truncation from the C-terminal extension which leads to unfolding of the protein, a decrease in its overall solubility and has detrimental effects on chaperone function by perturbing the putative role of the C-terminal extension in maintaining the integrity of the α-crystallin domain. It is possible, therefore, that a combination of the above factors contributes to the disease states that result from expression of R120G αB-crystallin, and, by inference, R116C αA-crystallin.

Materials and methods

Bovine milk α-lactalbumin (calcium-depleted) and insulin from bovine pancreas were purchased from Sigma. Deuterated d_{10}-dithiothreitol (DTT) and D_2O were obtained from Cambridge Isotope Laboratories, Inc., USA. Complete protease inhibitor cocktail tablets were purchased from Roche.

<u>Expression and purification of wild type and R120G αB-crystallin.</u>
The expression vector pET24d(+) containing the gene for human wild type αB-crystallin was a gift from Prof. W. de Jong (Nijmegen, Netherlands). The expression vector PET20b(+) (constructed by Prof. J. Horwitz) contained the gene for the R120G mutant of human αB-crystallin.

The plasmid DNA for expression of αB-crystallin was transformed into *E. coli* BL21(DE3) strain prior to expression. Expression and purification of αB-crystallins were performed according to the protocol of Horwitz et al. [53] with minor changes. Transformed cells were grown on LB media containing ampicillin (100 μg/ml) to select for pET20b(+)-αB-crystallin-R120G, or 50 μg/mL kanamycin to select for pET24d(+)-αB-crystallin. Protein expression was induced with 0.5 mM isopropyl-beta-D-thiogalactopyranoside. Cells were harvested by centrifugation and pellets lysed by a single freeze-thaw cycle, followed by incubation with lysozyme, then deoxycholic acid and DNAse I [53]. Dithiothreitol (DTT) and polyethyleneimine were then added to final concentrations of 10 mM and 0.12% (v/v), respectively. The lysate was then allowed to incubate at room temperature for 10 min before being centrifuged for 10 min at 17 000 g and 4°C. The resulting supernatant was filtered through 0.2 μm Sartorius Minisart filters and loaded onto a column containing DEAE-Sephacel (Sigma-Aldrich, St. Louis, U.S.A) with a 90 mL bed volume. Anion-exchange chromatography was performed at 4°C. Recombinant αB-crystallins were eluted in the first peak with 0.1M NaCl (in a buffer of 20 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, 0.02% NaN_3, pH 8.5) as monitored by absorbance at 280 nm.

Fractions from ion-exchange chromatography were concentrated and DTT was added to a final concentration of 50 mM. The sample was then allowed to incubate at room temperature for 30 min before being loaded onto a Sephacryl S300HR size-exclusion column (2.6 cm x 100 cm). Gel filtration was performed at temperatures below 10°C. Recombinant αB-crystallins were eluted as the first peak, as monitored by absorbance at 280 nm, with a 50 mM Tris-HCl buffer containing 1 mM EDTA, 0.02% (w/v) NaN$_3$, pH 8.5, at a flow rate of 0.3 mL/min. During the purification procedure all buffers contained 0.2 mM PMSF and protease inhibitor cocktail (Roche).

Purification of bovine βL-crystallin

βL-crystallin was isolated from calf lenses and separated from other crystallin fractions using size-exclusion chromatography on a Sephacryl S-300HR column (Amersham), as described previously [54].

^{1}H NMR spectroscopy

^{1}H NMR spectra were acquired at 500 MHz on a Varian Inova-500 spectrometer. For the real-time experiments on the interaction between wild type or R120G αB-crystallin with reduced target protein, 2 mg/mL bovine apo-α-lactalbumin and 2 mg/ml of either wild type αB-crystallin or R120G αB-crystallin were dissolved in 10 mM sodium phosphate buffer pH 7.0, containing 10% D$_2$O, 0.1 M NaCl, 2 mM EDTA, 0.02% NaN$_3$. Control samples without αB-crystallin were also prepared in the same buffer. Deuterated DTT (0.4 M), freshly dissolved in the same buffer, was added to give a final concentration of 20 mM, immediately prior to data acquisition. A series of 1D ^{1}H NMR spectra were collected in real time at 37°C with a spectral width of 6000.6 Hz and 16 scans per spectrum resulting in an acquisition time of 46 s for each spectrum. WET methods [55] were used to suppress the residual water signal. Chemical shifts were referenced with respect to the residual water resonance at 4.67 ppm. The height of the isolated resonance arising from the tyrosine (3,5) ring protons of the reduced, molten globule state of apo-α-lactalbumin was measured in each spectrum relative to the first spectrum acquired after the addition of deuterated DTT.

The degradation of R120G αB-crystallin was monitored by ^{1}H 1D and 2D NMR experiments at 25°C over a period of eight days. A freshly prepared sample of R120G αB-crystallin was dissolved in 10 mM sodium phosphate, pH 7.0 in 10% D$_2$O with 0.02% NaN$_3$, at a concentration of 1 mM. WET 1D NMR spectra were acquired with time as described above. ^{1}H-^{1}H Watergate TOCSY [56] spectra were acquired over a spectral width of 5497.5 Hz with 256 t$_1$ increments, 48 transients per increment and a 65 ms mixing time.

Spectra obtained from 1D and 2D NMR experiments were processed using Varian VNMR software (version 6.1c). The decay of resonance (at 6.8 ppm) intensity with time in the real-time 1D ^{1}H NMR data, was analysed using SigmaPlot software (Version 8.0).

Size exclusion chromatography (SEC)
SEC of R120G αB-crystallin was performed on a Precision Superose-6 column (300 x 3.0 mm, Amersham Pharmacia) at 5°C. R120G αB-crystallin (17.8 mg/mL) was incubated in phosphate buffered saline at 25°C for two weeks. Aliquots of 25 μL were removed at 0 hr, 48 hr, 7 day and 13 day intervals, and loaded onto the SEC column. Proteins were eluted with 200 mM ammonium acetate buffer, pH 7.0 at a flow rate of 70μL/min with a detection wavelength of 280 nm.

Determination of molecular mass of R120G aB-crystallin.
Measurement of the molecular mass of wild type and R120G αB-crystallin was performed using SEC and light scattering, as described previously [57].

Mass spectrometry (MS) of wild-type and R120G αB-crystallin
To investigate the stability of wild type and R120G αB-crystallin over time, samples identical to those analysed by 2D NMR, as well as a mixture containing equal amounts of R120G and wild type αB-crystallin, were incubated at room temperature for 16 days in the presence of a complete protease inhibitor (Roche). Aliquots were removed over this period and analysed for degradation by SDS-PAGE [58] (days 0, 2, 5, 9 and 16), and MS

(days 0 and 9). Prior to MS analysis, 20 μl samples were exchanged into 200 mM ammonium acetate, pH 7.0, using Micro Bio-Spin 6 columns (Bio-Rad). The exchanged solutions were diluted fourfold with 50% acetonitrile, 1% acetic acid for analysis using nanoelectrospray MS on a Q-ToF2 hybrid quadrupole time-of flight instrument (Waters/Micromass). Conditions were as follows: capillary voltage 1.5 kV, cone gas 150 Lh^{-1}, sample cone 35 V, extractor cone 4 V, and base pressures throughout. Spectra were acquired over the range m/z 500 to m/z 3000 and the resultant data was transformed to a mass scale using an algorithm in the MassLynx software (Waters/Micromass).

Chaperone activity of R120G αB-crystallin

Reduction assay. 45μM bovine pancreas insulin was incubated in the presence of increasing amounts of wild type or R120G αB-crystallin in 50 mM phosphate buffer, pH 7.5, 20 mM DTT. The progress of insulin B chain precipitation was measured by light scattering at 360 nm recorded at 37οC by a Spectramax 250 multi-well plate reader (Molecular Devices, Sunnyvale, U.S.A.).

Heat-stress assay. The chaperone action of wild type and R120G αB-crystallin was examined with 0.14 mg/mL bovine βL-crystallin, a natural lens target protein for αB-crystallin, in 50 mM sodium phosphate buffer, 0.02% NaN_3, pH 7.5, at 56°C. A Hewlett-Packard 3485 Diode-array UV-Vis spectrophotometer (Palo Alto, U.S.A) connected to a B. Braun Thermomix temperature controller (Melsungen, Germany) was used to monitor protein precipitation via light scattering at 360 nm.

Unfolding of wild type and R120G αB-crystallin in urea

Freshly prepared solutions of wild type and R120G αB-crystallin (0.133 mg/ml in 50 mM sodium phosphate buffer pH 7.4) were titrated with 10.7 M urea (Ajax Chemicals) at 25°C. The solutions were stirred for 10 minutes after each addition of urea aliquot. The rate of protein unfolding was monitored as Trp fluorescence intensity and λ_{max} using Hitachi F-4500 fluorescence spectrophotometer using 3.5 ml quartz cuvettes, pathlength 1.0 cm (Sterna, Sydney) with excitation at 295 nm and emission range 300-400 nm.

Acknowledgments

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Figure legends

Figure 1. Aromatic and NH region of the 1D ^{1}H NMR spectra of reduced apo α-lactalbumin at 37°C and pH 7.0 in the (a) absence, (b) presence of 1:1 subunit molar ratio of wild type αB-crystallin and (c) presence of 1:1 subunit molar ratio of R120G αB-crystallin at the times indicated after the addition of 20 mM DTT. The observed resonances after DTT addition arise from the partially folded, molten globule state of α-lactalbumin.

Figure 2. Plots of resonance intensity versus time for the resonance at 6.8 ppm from the tyrosine (3,5) ring protons in the real-time ^{1}H NMR spectra of reduced apo α-lactalbumin at 37°C and pH 7.0 in the (a) absence, (b) presence of 1:1 subunit molar ratio of wild type αB-crystallin and (c) presence of 1:1 subunit molar ratio of R120G αB-crystallin. The apparent rate constants for the exponential curves are 1.509 ($\pm$ 0.066) x 10^{-3} s^{-1} (a), 1.227 ($\pm$ 0.055) x 10^{-3} s^{-1} (b) and 2.404 ($\pm$ 0.130) x 10^{-3} s^{-1} (c).

Figure 3. Changes in the ^{1}H NMR spectra of R120G αB-crystallin with time at 25°C and pH 7.0. (a) 1D spectra of the aromatic and NH region, (b) cross-peaks from the NH to α,β,γ-CH protons in TOCSY spectra. The increasing complexity of the spectra with time corresponds to the progressive unfolding of the protein.

Figure 4. (a) Dynamic light scattering profile of freshly prepared R120G and wild type αB-crystallin, showing a higher average mass and larger mass range for the mutant protein. (b) SEC elution profiles (absorbance at 280 nm) monitoring changes in oligomerization of R120G αB-crystallin by SEC, at the times indicated, over a 13-day period. A shift to earlier elution times indicated that the average mass of the protein increased, and that this was accompanied by aggregation to species of higher mass and a decrease in the amount of soluble protein.

Figure 5. Degradation of R120G αB-crystallin with time at room temperature. (a) Transformed mass spectrum of wild type αB-crystallin after 9 day incubation showed no

change in the monomeric mass of 20.16 kDa, indicating no proteolysis had occurred (b) Spectrum of R120G αB-crystallin immediately after dissolution is consistent with its calculated mass of 20.06 kDa. (c) Spectrum of R120G αB-crystallin after 9 day incubation showed that the protein had undergone extensive proteolysis at the C-terminus, with no full length protein remaining. The truncated species identified are labelled. (d) SDS-PAGE of wild type and R120G αB-crystallin after incubation for the days indicated. While wild type protein remained intact for the duration of the experiment, significant truncation was observed in R120G αB-crystallin from day 5 onwards.

Figure 6. Chaperone ability of R120G αB-crystallin with (a) heated βL-crystallin at 56°C and (b) reduced insulin at 37°C. Legends show molar ratios of target protein: wild type and R120G αB-crystallin on a subunit basis. Upon stress, the level of light scattering by protein aggregates increases to maximum, after which a decrease occurs as a result of sedimentation of precipitated aggregates. Aggregation of target proteins is suppressed by higher ratios of αB-crystallin. R120G αB-crystallin provided less protection for the heat-stressed βl-crystallin than the wild type protein (A), while for reduced insulin the difference was minimal (B).