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Aggregation of deamidated human β B2-crystallin and incomplete rescue by α -crystallin chaperone

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Abstract

Aging of the lens is accompanied by extensive deamidation of the lens specific proteins, the crystallins. Deamidated crystallins are increased in the insoluble proteins and may contribute to cataracts. Deamidation has been shown *in vitro* to alter the structure and decrease the stability of human lens β B1, β B2 and β A3-crystallin. Of particular interest, β B2 mutants were constructed to mimic the effect of *in vivo* deamidations at the interacting interface between domains, at Q70 in the N terminal domain and at Q162, its C terminal homologue. The double mutant was also constructed. We previously reported that deamidation at the critical interface sites decreased stability, while preserving the dimeric 3D structure. In the present study, dynamic light scattering, differential scanning calorimetry and small angle X-ray scattering were used to investigate the effect of deamidation on stability, thermal unfolding and aggregation. The bovine β Lb fraction was used for comparative analysis. The chaperone requirements of the various samples were determined using bovine α -crystallins as the chaperone. Deamidation at both interface Gln residues or at Q70, but not Q162, significantly lowered the temperature for unfolding and aggregation, which was rapidly followed by precipitation. This deamidation-induced aggregation and precipitation was not completely prevented by α -crystallin chaperone. A potential mechanism for cataract formation *in vivo* involving accumulation of deamidated β -crystallin aggregates is discussed.

Keywords

lens; β -crystallins; deamidation; α -crystallin chaperone; protein aggregation; cataracts

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1. Introduction

Lens crystallins act as structural proteins, which are able to retain life-long transparency in the absence of protein turnover. Lens aging, however, is accompanied by post-translational modifications of the lens specific proteins, the crystallins, leading to their aggregation, resulting in loss of lens optical properties and eventually cataract (Hains & Truscott, 2007). In the last ten years two-dimensional gel electrophoresis and mass spectrometry revealed a large number of deamidated species (Wilmarth et al., 2007; Lampi et al., 1998). Deamidation introduces a negative charge at physiological pH by replacing an amide with a carboxyl group, and also causes some isomerization (Robinson and Robinson, 2004). Such changes may disrupt the normal crystallin structure and short range order that ensure lens refractive index gradient and transparency (Delaye and Tardieu, 1983). In the lens, the α -crystallins, which belong to the sHSP family, may prevent aggregation and opacities by acting as molecular chaperones of modified crystallins. The purpose of this study was to determine the effect of deamidation on β B2-crystallin aggregation.

Crystallins in the β/γ -family consist of an N-terminal domain (N-td) and a C-terminal domain (C-td) linked by a connecting peptide (cp). N-td and C-td are built from one double Greek key domain (D1 in N-td, D2 in C-td), (Bloemendal et al., 2004), and either a N-terminal extension (N-te) or a C-terminal extension (C-te); D1 and D2 are homologous domains, belonging to the same structural superfamily. The vertebrate β/γ -crystallin family evolved from a single domain protein present in urochordates (Shimeld et al., 2005). In solution, γ -crystallins are monomers, whereas β -crystallins assemble into dimers and higher oligomers. Yet, all 3D structures have a “conserved domain pairing interface” between D1 and D2. The pairing is intramolecular in the monomeric γ -crystallins and intermolecular in the case of β B2-crystallin. However, the pairing is intramolecular in the β B1 crystal structure where the formation of higher oligomers creates a new interface for assembly (van Montfort et al., 2003; MacDonald et al., 2005; Smith et al., 2007). Interactions at the interface were investigated with a series of mutations that disrupted the β -strands of the Greek key motifs (Liu and Liang, 2006). β -strands critical for dimerization were identified, including β 5 and β 12 that contain the sites of interest in this present study. Therefore, the integrity of the interface is essential for dimer formation.

The β/γ -crystallins are exceptionally stable proteins that do not unfold and aggregate *in vitro* until 60°C or higher (Bloemendal et al., 2004). Dimeric rat β B2 was shown to unfold by either a three state or a two-state model, depending upon the concentration (Wieligmann et al., 1999). The N-td is the less stable (as observed by Mills et al. (2007) by comparing stability of the isolated N-td and C-td of human γ D), and unfolded first. Similar studies on human dimeric β B2-crystallin and on the more stable monomeric γ C (Fu and Liang, 2002) and γ D crystallins (Flaugh et al., 2006) also concluded that unfolding could be fit to a three state model with a partially unfolded monomer as an intermediate.

Deamidation at the predicted interacting interface between domains at homologous sites in β B1, β B2, and β A3 has been extensively studied and found to decrease the heat and urea stability of the dimers (Kim et al., 2002; Lampi et al., 2006; Takata et al., 2007, 2008). Deamidation at the homologous sites also destabilized γ D and lowered the kinetic barrier to unfolding of the N-td, but not the C-td (Flaugh et al., 2006). The β B2-crystallin is of particular interest because it is the major β -crystallin in the mammalian lens, is a component of both the β_{LOW} and β_{HIGH} oligomers isolated from the lens, and helps to solubilize other β -crystallin subunits (Bateman and Slingsby, 1992). We have previously investigated deamidation at the dimer interface in β B2 by replacing Q70 in the D1 and Q162, its D2 homologue, with E residues (Lampi et al., 2006). Mutant proteins were analyzed by light scattering and other spectroscopic methods, which indicated that the dimeric quaternary structure had been preserved, although the secondary and tertiary structure had been altered, particularly of the doubly deamidated

mutant (DM), Q70EQ162E. Chemical-induced unfolding of WT β B2 was monophasic suggesting highly cooperative unfolding. Deamidation shifted the equilibrium of unfolding in association with a loss of cooperativity, indicating decreased stability. Further decreased stability was noted for the DM.

The ability of the α -crystallin chaperone to rescue modified and unfolded β -crystallins may be an important protective mechanism in the lens. Recent spectroscopic analyses suggest “that the aggregation-prone pathway involved a non-native low-energy intermediate state which is recognized and bound by α -crystallin prior to aggregation” (Evans et al., 2008). We have demonstrated that the association of α -crystallin with $\beta_{\text{LOW}}/\gamma$ -crystallins undergoing denaturation required an activated state of the α -crystallins using a combination of FRET and X-ray analysis (Putilina et al., 2003). Furthermore, the α -crystallin subunit exchange was slowed down upon association with the β/γ substrate.

In the present study, we sought to address if deamidation at the critical interface increased susceptibility to heat-induced aggregation and if this could be prevented by α -crystallin chaperone. Dynamic light scattering (DLS), differential scanning calorimetry (DSC), and small angle X-ray light scattering (SAXS) were used to analyze human β B2 (h β B2) and its deamidated mutants: Q70E, Q162E and the DM. The dimer enriched bovine β_{LOW} fraction (β Lb) was analyzed for comparison. The chaperone requirements of the various samples were investigated using readily available bovine α -crystallins, made of α A- and α B subunits in a 3/1 ratio, as the native chaperone (α N).

Unfolding can induce a variety of processes, which are potentially detrimental to the cells. In the lens of the eye aggregation is particularly dangerous, because large size aggregates efficiently scatter light, altering transparency. The availability of chaperones and their ability to bind unfolded entities and prevent aggregation is therefore of particular importance.

2. Materials and methods

2.1. Expression and purification of the crystallins

Human β B2-WT and mutants were expressed as previously described in Lampi et al (2006). The expression plasmid, pET 3a containing the WT betaB2 sequence was kindly provided by Drs. Nicolette Lubsen (University of Nijmegen) and Orval Bateman (University of London). Deamidations were mimicked by replacing glutamines with glutamic acids using site-directed mutagenesis. Plasmids were transformed into the *Escherichia coli* strain BL21(DE3) (Novagen, Madison, WI). Cells were lysed and proteins were purified by successive ion-exchange chromatography. Mutations and purity of proteins were confirmed by SDS-PAGE and mass spectrometry. The α N and β Lb were purified from young calf lenses as described previously (Putilina et al., 2003). Briefly, the cortex was separated from the nucleus by a gentle stirring of the lens in a 150 mM phosphate buffer, pH 6.8 (22 mM Na₂HPO₄, 28 mM KH₂PO₄, 70 mM KCl, 1.3 mM EDTA, 3 mM NaN₃, 3 mM DTT) for 15-30 minutes at 6 °C. Crystallins were prepared from the clear supernatant fraction of the cortical extracts by gel filtration using a Superdex S-200 PG column. The β_{LOW} fraction eluted in two peaks, β La and β Lb. The latter corresponds to 65% β B2, 23% β B3 and 12% β A3 (Slingsby and Bateman, 1990). When necessary, the fractions were concentrated by ultrafiltration. The purified proteins were stored at 4°C and were never freeze dried. Concentrations were determined with a NanoDrop-1000 UV/Vis spectrophotometer (Thermo Scientific, Wilmington, DE).

2.2. Multiple sequence alignment and contact analysis

Regions of amino acid sequences of h β B2-WT and of all the bovine β -crystallins were aligned by combining Mafft results (Katoh et al., 2002) with the analysis of superimposed 3D

structures. In order to identify homologous amino acid sites, sequences for the two homologous domains, indicated as D1 for the N-td and D2 for the C-td, were aligned together. The N-te and C-te and the connecting peptides were aligned separate from the domains. The N-td extensions are highly variable both in length and in amino acid composition, and therefore, in order to build their alignment, more sequence information was taken into account, including the β - N-td extensions of 23 vertebrate species. The atomic contacts, either intra or intermolecular, made by Q70 and Q162 were determined from *in silico* analysis (CCP4); the distance cut-off was set to 3.65 Å for all contacts, and the hydrogen bonds were detected according to angle and atom type. A similar approach was used to compute the intramolecular contacts within each of the two homologous domains (D1 and D2) of the human β B2 3D structure. These atomic contacts were classified as follows: common to D1 and D2 (if observed in both of them, i.e. involving homologous sites) or specific to one of these domains. We further analyzed the available 3D structures of β - and γ -crystallins in order to evaluate the superfamily conservation of the D1 and D2 specific contact patterns.

The 3D structures analyzed included: human β B2 (PDB code: 1YTQ); bovine β B2 (1BLB, 2BB2); mouse β B2 (1E7N); human β B1 (1OKI); bovine γ S (1A7H); mouse γ S (1ZWM, 1ZWO, 2A5M); human γ S (1HA4); bovine γ B (1AMM, 1GAM, 1I5I, 1GCS, 4GCR); mouse γ C (2V2U); human γ D (1HK0, 2G98); bovine γ D (1ELP); rat γ E (1A5D, 1ZGT, 1ZIE, 1ZIQ, 1ZIR); bovine γ E (1M8U), bovine γ F (1A45).

2.3. Dynamic Light Scattering (DLS)

DLS measurements were performed on a DynaPro instrument (Wyatt Technology Corp., Santa Barbara, CA) using regularization methods (Dynamics software, version 6). All samples were filtered through 0.22 μ m filters using centrifugation to eliminate large aggregates, dust particles or bubbles. Protein concentrations were measured and adjusted to be in the 0.1 – 2 mg/mL range for sufficient signal to noise ratio while avoiding saturation of the detector. Sample volumes were 50 μ L, the correlation time, τ , was 0.5 μ s, and the acquisition time for one measurement was 10 s. Each experiment consisted of at least 20 and up to 360 measurements. With monomodal solutions of particles, the measurement of the autocorrelation function of the scattered light as a function of time allows determination of the diffusion coefficient of the species in solution, D_T . The hydrodynamic radius, R_h , is then calculated from the Stokes-Einstein equation, in which $R_h = K_B T / 6\pi D_T \eta$, where K_B is the Boltzman constant, T the absolute temperature, and η the viscosity of the solvent. The temperature was changed with a Peltier controller from 4°C up to 80°C. Chaperone activity was assayed by DLS, by monitoring as a function of time the light scattered at 90° by the sample. The scattering of the different β -crystallins, either alone or in the presence of baN, was first measured at $T_0 = 20^\circ\text{C}$ in a separate experiment and shown to remain constant as a function of time. Then, after equilibration of the instrument at 60°C, the cell containing the sample was placed in the sample holder and the total scattered intensity, I_s , was recorded as a function of time over 0.5 or 1 h periods. To facilitate the analysis, the β -crystallin concentration was kept constant and equal to 0.5 mg/mL. To measure chaperone-like activity the baN amount was varied to reach the desired ratio of chaperone to substrate. Chaperone and substrate were mixed at either 4°C or ambient temperature before measuring intensity of scattered light at 60°C. All the experiments were repeated 3 times. The following normalized scattered intensity, I_n , was used: $I_n = [I_s(T) - \langle I_s(T_0) \rangle] / \langle I_s(T_0) \rangle$. For chaperone experiments with h β B2-WT and b β Lb, the scattered intensity difference, ΔI_s , was used: $\Delta I_s = I_n(\text{mixture}) - I_n(\text{baN})$. For chaperone experiments, with the deamidated mutants, the raw intensity, $I_s(60^\circ\text{C})$, measured as a function of time for the different samples (at the same concentrations and chaperone :substrate ratios), was directly compared.

2.4. Small angle X-ray scattering (SAXS)

Data collection was carried out at the ID2 beamline at the European Radiation Synchrotron Facility (ESRF, Grenoble, France). The scattering intensity was measured with a 2D detector, a fiber optically coupled FReLoN CCD based on Kodak image sensor, 4×4 binning mode, as a function of q . The parameter q was defined as $q = 2\pi s = (4\pi/\lambda)\sin\theta$, where s is the amplitude of the scattering vector, λ is the wavelength of the X-rays (0.0995 nm) and $\sin\theta$ the scattering angle. The sample to detector distance was set to 2 m, giving a q range from 0.07354 to 3.8 nm and an increment $\Delta q = 0.0086515$ nm. The values of the intensity at the origin, $I(0)$, and of the radius of gyration, R_g , were inferred from the slope and the intercept, respectively, from the linear fit of $\ln(I(q))$ versus q^2 at low q values, following the Guinier approximation: $I(q) = I(0) \exp(-q^2 R_g^2/3)$. The human β -crystallin concentrations were from 3 to 7 mg/mL, to get a sufficient signal to noise ratio. The sample volume was 40-50 μ L. For the chaperone assays, the scattering curves of h β B2-WT and b α N were first recorded at 15°C. Then, b α N alone or in the presence of h β B2-WT, in a (1:1) ratio, were incubated at 60°C for 30 minutes and the scattering curves recorded.

2.5. Thermodynamic parameters and unfolding temperatures by DSC

Thermal denaturation of proteins was studied by differential scanning calorimetry (DSC). The DSC equipment was a Nano-Differential Scanning Calorimeter III, CSC 6300 (TA Instruments, New Castle, DE). All measurements were performed in a “simplified” 150mM phosphate buffer pH 6.8 (22 mM Na₂HPO₄, 28 mM KH₂PO₄, 70 mM KCl). The proteins were dialyzed just before the DSC experiment, and the dialysis buffer was kept for the reference cell. Each cell volume (reference and protein solution) was 300 μ L. The solutions were heated from 15°C to 95°C at 1°C/min, and the reverse scans were performed from 95°C to 15°C at 2°C/min at a constant pressure of 3 atmospheres. The h β B2WT, b β Lb, Q70E, Q162E and DM concentrations were respectively 1.7, 1.3, 0.53, 2.7 and 0.5 mg/mL. Data treatment was carried out using software provided by the manufacturer (CSC Software package, CpCalc Data Analysis), using a partial specific volume of 0.73 ml/g and graphed using Origin software (Northampton, MA).

3. Results

3.1. Sequence alignment, 3D structure and atomic contacts of Q70 and Q162

The sequence alignment of all the bovine β -crystallins and human β B2-wildtype (h β B2-WT) are shown in Fig. 1. The two homologous domains D1 and D2 are aligned, and the Greek key motifs 1–4 are indicated. Only one glutamine is conserved at homologous positions in the two domains (Bloemendal et al., 2004). This glutamine corresponds to Q70 and Q162 in h β B2 and is also highly conserved within the γ -crystallin sequences.

Since the β B2 3D structure is known (Bax et al., 1990; Lapatto et al., 1991), the location of the conserved Gln was represented in Fig. 1B, with its intra and intermolecular contacts. The glutamines are related by a pseudo two-fold symmetry axis. They are in contact at the interface between the two domains and their other contacts, although involving different amino acids are similar. The intramolecular contacts of Q70 in D1 are with G60, E69, F71 and of Q162 in D2 are with G152, L161, Y163. The intermolecular contacts of Q70 are with Q162, L164, F176 (within D2) and of Q162 with Q70, V72, W84 (within D1). No difference was reported between the atomic pattern of H-bonds involving Q70 and Q162. An additional intermolecular contact is observed for Q162, not present for Q70, between residues Q162 and 86 (D1) within β -crystallins and between Q162 and 85 (D1) within γ -crystallins.

The amino acid composition at sites corresponding to Q70 and Q162 contacts were computed among 96 β -crystallin sequences of 25 vertebrate species, from birds to teleostfishes through

mammals and amphibians (sequences not shown). The composition as follows demonstrates the extremely high conservation at these sites: D1 60 (63.5% G, 35.5% A, 1% S), 69 (52.1% E, 46.9% Q, 1% H), 70 (81.2% Q, 17.8% M, 1% R), 71 (86.5% F, 13.5% Y), 72 (56.3% V, 43.7% I), 84 (96.9% W, 2.1% F, 1% Y), and D2 152 (54.2% G, 33.3% C, 12.5% A), 161 (55.2% Y, 17.7% R, 13.5% F, 12.6% L, 1% N), 162 (99% Q, 1% L), 163 (92.8% Y, 5.2% F, 1% L, 1% H), 164 (37.5% V, 35.5% L, 25% I, 1% A, 1% K), 176 (61.5% W, 32.3% F, 4.2% Y, 1% L, 1% V).

The atomic contacts are also highly conserved within the available 3D structures between the β - and γ -crystallins. Interactions between residues homologous to 84 (D1) and Q162 and between residues homologous with 176 (D2) and Q70 were observed within the β - crystallins, whereas an additional intramolecular contact was occasionally observed within the γ -crystallins (with the homologous residues 59 (D1) for Q70, and 151 (D2) for Q162). Based on the above sequence and 3D structures, Q70 and Q162 are homologous between domains and within the β/γ -crystallins and have conserved atomic contacts.

3.2. 3D structure and intramolecular contacts within D1 and D2 domains

The analysis of the atomic contacts within each homologous domain (D1 and D2) were performed for the 3D structure of h β B2 (PDB code: 1YTQ). These two domains share 114 homologous contacts (i.e. involving homologous sites, according to the alignment of the D1 and D2 sequences); in addition, 27 contacts are specific for D1, and 39 are specific for D2. These contacts share the same domain specificity within other available 3D structures of β -crystallins. The D2 specific contacts mainly involved distinct secondary structures, and may contribute to its stability; in particular, the residue Y163 (next to Q162 and located within the B12 β -strand) interacts with the residues Q154 (within the B11 β -strand) and P179 (between the H4 helix and the B14 β -strand). Among the 27 and 39 contacts that are specific for h β B2 D1 and D2, respectively, 19 and 33 share the same domain specificity within 3D structures of γ -crystallins. However, the D2 specificity of the interaction between the Y163 and P179 residues is only observed within the h β B2 and b β B2 3D structures.

3.3. Assembly state, polydispersity and temperature induced aggregation of the β samples by DLS

The hydrodynamic radii (R_h) and the percentages of polydispersity (% Pd) were determined by DLS at ambient temperature and are listed in Table 1. For h β B2-WT and its deamidated mutants, Q70E, Q162E and the DM, the R_h was equal to 3.1 ± 0.1 nm, indicating that only dimers were present in the solutions. The polydispersity remained small, around 20%, in agreement with what was expected for monodisperse, yet non spherical, particles.

The onset of temperature induced aggregation of the samples were measured by first equilibrating the samples at 37°C for 15 minutes, then measuring the total intensity of scattered light as a function of temperature for 200 s at every 2°C increase (Fig. 2). This method allowed for rapid detection of aggregate formation. For all samples, the normalized scattered intensity remained unchanged until the unfolding temperature and then increased immediately as aggregates formed with a size > 100 nm. Since the scattered intensity is proportional to the molecular weight, even a few percent of large particles was immediately detected. Aggregation was defined as greater than 10% aggregates in a sample and results were compared to the native bovine b β Lb, fraction.

Aggregation of the proteins occurred at different temperatures (Fig. 2). The onset of aggregation was at 43°C for the DM mutant, 51°C for both b β Lb and Q70E, 55°C for Q162E and 61°C for h β B2-WT. Samples were also incubated for longer times at lower temperatures. After one hour, even a few percent of aggregates were detected at 37°C for the DM mutant,

49°C for b β Lb, 46°C for Q70E, 51°C for Q162E and 55°C for h β B2-WT. All temperatures were within $\pm 0.5^\circ\text{C}$. Aggregate formation was not reversible and precipitation rapidly occurred following aggregation. Despite the similarity in atomic contacts, Q70E and Q162E aggregated at different temperatures. For all samples, the R_h values listed in Table 1 remained constant until the onset of aggregation. The R_h values were identical for h β B2-WT and the mutants, being equal to 3.1 ± 0.1 nm, and were slightly lower for b β Lb at 2.9 ± 0.1 nm.

The contribution of the aggregates to the intensity of the scattered light overwhelmed the signal from the dimers, precluding detection of possible dimer unfolding intermediates. Since the b β Lb sample was a mixture of different β -subunits that may aggregate at different temperatures, aggregates that formed at 51°C were isolated by centrifugation. These aggregates accounted for about 10% of the initial protein content. The remaining soluble sample was reanalyzed as a function of temperature. This fraction indeed started to unfold and aggregate later, at about 60°C.

3.4. Thermal denaturation of the β samples by DSC

For all samples, DSC detected a main transition peak, accompanied by more or less pronounced shoulders on either side, with the whole transition occurring over about 10°C. These temperature induced transitions were not reversible. The shoulders indicated that substates were involved in the unfolding process, with most likely one major and one or more minor intermediates. Therefore, the analysis was made assuming a maximum of 3 peaks, with gaussian shapes, i.e. at least 2 intermediate states. The temperatures of half transition, T_t , and the fractions corresponding to each peak, are given in Table 2. The T_t corresponding to the main peaks were 64.4°C, 56.7°C, 66.1°C, 53.0°C for h β B2-WT, Q70E, Q162E and DM, respectively with error $\pm 0.1^\circ\text{C}$. These values are only 3–10°C higher than the values determined by DLS above. The speed of the temperature scans were similar at 2°C/200 s for DLS and at 1°C/60 s for DSC. Since DLS measures aggregation and DSC measures unfolding, these results confirm that aggregation and unfolding occur at similar times, with aggregation preceding complete unfolding.

The calorimetric thermodynamic parameters of the system (the enthalpy ΔH and the entropy ΔS) were calculated from the area under the peak of the molar heat capacity curves. In contrast to the transition temperature, these values are highly sensitive to the shape and the baseline of the molar heat capacity curves (not shown). In the case of h β B2-WT1, DM and b β Lb, the enthalpy and the entropy values were found similar, respectively around 180 kcal/mol and 0.55 kcal/(K.mol), but they were probably under estimated because the baselines were difficult to define. For the Q70E mutant, it was not possible to calculate these calorimetric values. For the β B2-WT2 and the Q162 mutant, $\Delta H = 225$ and 240 kcal/mol, and $\Delta S = 0.67$ and 0.70 kcal/(K.mol).

The native β -crystallin, b β Lb, contains a high percentage of β B2 (65%, Slingsby and Bateman, 1990), and was therefore, compared to h β B2-WT. The DSC scan of b β Lb gave 2 peaks with the main peak at 62.4°C, in agreement with a previous analysis (Khanova et al., 2005). The higher stability of the h β B2 as compared with b β Lb was in agreement with the reported higher stability of the human β B2 compared to the bovine β B2 (Evans et al. 2008).

3.5. Assembly state of the β samples by SAXS

The SAXS intensity curves of the h β B2-WT, deamidation-mimicking mutants, and b β Lb were recorded as a function of temperature from ambient to 60°C or 55°C for the Q70E mutant (ambient, 37°C, 45°C, 48°C, 55°C, 60°C with temperatures within 0.5°C). The scattering curves at 37°C are shown in Figure 3A. The shapes of the scattering curves were similar for all the samples and were consistent with the presence of only dimers in solution. The similarity

of the scattering curves confirmed that the mutations were not sufficient to disrupt dimer formation and did not introduce large reorganization of the dimer structure. The radii of gyration (R_g) calculated from the Guinier approximation were 2.7 ± 0.1 nm for h β B2-WT and b β Lb, and were close to 3.0 ± 0.1 nm for the mutants (Fig. 3B). The higher values for the mutants could originate from a few % of aggregated material. Minor differences observed at higher angles around the secondary maximum may correspond to differences in flexibility of the various dimers.

The scattering curves, recorded as a function of increasing temperature (not shown), remained unchanged until a transition temperature was reached. In all cases, transitions were characterized by an increase of the intensity only at the smallest angles, and a shift (decrease) of the whole scattering curve, both owing to the formation of aggregates. These aggregates became larger with increased temperature, until they started to precipitate out of the X-ray beam as seen with the decrease of the zero-angle extrapolated intensity I_0 (Fig. 3C). The remaining soluble material was still significantly structured, as the shape of the curves of the portion that remained soluble until 60°C (55°C for the Q70E mutant) was similar to those of the native samples shown in Fig. 3A at ambient temperature. This is illustrated with the radii of gyration (R_g), which remained almost constant as a function of temperature (Fig. 3B).

At the end of the experiment at 60°C or 55°C, about 50% of the h β B2-WT sample had precipitated and at least 70% for the mutants (Fig. 3C). While the onset of aggregate formation could not be precisely determined by these SAXS experiments, the data indicated that precipitation started at or near the following temperatures: 60°C for h β B2-WT, 55°C for Q162E, 48°C for Q70E and 43°C for DM.

The SAXS data therefore confirmed that precipitation occurred at temperatures close to temperatures for unfolding and aggregation and that the Q70E and the Q162E mutations were not equivalent.

3.6. Chaperone-like activity of α -crystallin toward b β Lb and h β B2-WT and size of the b α N-h β B2-WT/b β Lb assemblies by DLS

Chaperone assays were performed at 60°C with the native bovine α -crystallin, b α N, which contains both α A- and α B-crystallin subunits, as the molecular chaperone. The physiological substrate in the eye lens, b β Lb, was used as a reference. Indeed, both b β Lb and h β B2-WT unfold around 60°C, and could not be rescued by human α B-crystallin, which also unfolds and aggregates at 60°C (data not shown). The chaperone assays were performed with DLS in order to distinguish between chaperone-substrate association and aggregation. Indeed, the R_h values of the β - and α -crystallins and of their complexes remain lower than 20 nm. Instead, as soon as large soluble aggregates are formed, R_h values larger than 50 nm are measured and the scattered intensity rapidly increases at the same time. The chaperone activity of b α N toward b β Lb and h β B2-WT was investigated as a function of the chaperone:substrate ratio, from 2:1 up to 1:8. Both total scattered intensities and R_h values were recorded. The results are shown in Fig. 4. The evolution as a function of time over a one hour period of the normalized scattered intensities demonstrated that b α N totally prevented aggregation of both b β Lb (Fig. 4A) and h β B2-WT (Fig. 4B) up to a 1:2 ratio of chaperone to substrate. At lower chaperone to substrate ratios, less aggregation was prevented.

The R_h of the b α N:b β Lb and b α N:h β B2-WT mixtures in Fig. 4A-B were determined and are plotted in Figure 4C-D. Similar results were obtained with b β Lb or h β B2-WT. As expected from our previous report (Skouri-Panet et al. 2006), b α N increased in size at 60°C, the major increase occurring in the first 20 minutes, from 8.5 to 10.4 nm.

Since, the measured R_h values are a weighted average of all the species in a sample, at time zero, the R_h values were a weighted average of α N and β -crystallin R_h values. With increasing β concentration, R_h values decreased from approximately 8.5 to 6.0 nm reflecting the greater contribution of the smaller β -dimer than the α -multimer. Upon mixing of α N with either β Lb or β B2, there was an increase in the R_h values with time, which could be attributed to either the doubling in size of the α N upon activation and/or formation of the β -crystallin: α N complex assemblies. However, the association of either β Lb or β B2 induced an increase in R_h in addition to what was observed for α N alone, confirming an association between the chaperone and the substrate. This increase was more pronounced at the higher β -crystallin concentrations. The final R_h values at a (1:4) ratio were smaller for β B2-WT than for β Lb, possibly owing to a closer association of the β B2-WT with the α N assemblies. These data confirm that the ability of α N to prevent heat-induced precipitation of the β B2 proteins in Fig. 4 was due to an association between α N and the substrates.

3.7. Chaperone-like activity of α -crystallin with deamidated β B2 substrates by DLS

The ability of the α -crystallin chaperone to rescue the deamidated mutants was analyzed at two chaperone:substrate ratios, 1 :1 and 1 :2. The raw scattered intensities observed for β B2 and its deamidated mutants are shown in Figure 5. Similar to β B2-WT, Q162E was rescued by α N at a 1:1 ratio (Fig. 5). It was also rescued at a 1:2 ratio, yet less efficiently (not shown). The α N was also able to rescue the DM, but to a lesser degree than it was able to rescue WT or Q162E. In addition, in the presence of DM, the light scattering intensity was rather unstable (Figs 5). Such instability could reflect the formation of rather heterogeneous complexes, including a significant percentage of large-sized ones, followed by precipitation as a function of time.

In contrast to Q162E, no chaperone activity was demonstrated with Q70E as the substrate (data not shown). Since the Q70E mutant unfolds around 50°C (Fig. 2 and 3C), the chaperone assay was repeated at 55°C. We had previously shown that α N required activation at 60°C to associate with physiological substrates (Putilina et al. 2003). Therefore, α N was incubated at 60°C for 15 minutes before mixing with Q70E at 55°C. Similar results were obtained as α N, although pre-activated, did not rescue the Q70E mutant. This suggests that most of the Q70E mutant may be in the aggregated state, which may be unavailable for interactions with the chaperone.

3.8. Chaperone-like activity of α -crystallin with β B2 by SAXS

The scattering curves of α N, β B2-WT, and a 1:1 ratio of α N: β B2-WT incubated at 15°C were also recorded at 15°C. The scattering curve of the α N: β B2 mixture was identical to that of the sum of the curves of α N and β B2-WT confirming there was no association at 15°C. Next, samples were incubated at 60°C. The α N and a 1:1 ratio of α N: β B2-WT were incubated for 30 minutes at 60°C and the scattering curves recorded at 15°C (Fig. 6, dark grey and black curves, respectively). The scattering curve of β B2-WT at 15°C was added to the curve of α N at 60°C (light grey curve in Fig. 6) and compared to the curve of the α N: β B2 mixture at 60°C (black curve in Fig. 6). Both curves coincide at high angles ($q > 2 \text{ nm}^{-1}$) indicating that no protein loss had occurred during the incubation of the mixture. Furthermore, the curves differed at low angles ($q < 0.5 \text{ nm}^{-1}$), indicating that complexes had formed. The intensity at the origin of the incubated mixture was roughly twice that obtained by the addition of the scattering curves of the individual components. Since the intensity at the origin is proportional to the molecular weight of the particles in solution, the greater intensity indicates all the β -crystallins in the incubated mixture were associated to the chaperone to form complex assemblies. The measured R_g for the α N and the mixture incubated at 60°C were 7.3 and 8.1nm, respectively. The 7.3 nm for α N is consistent with a doubling in size of the α N, with a 6.0nm R_g at ambient temperature (Skouri-Panet et al., 2006). The increased R_g value for the

b α N:h β B2-WT mixtures were larger than the b α N assemblies alone, in agreement with the DLS observations and further supporting the formation of complexes.

4. Discussion

The complementary techniques of DLS, DSC, and SAXS were used to study the effect of deamidation on stability, formation of intermediates and aggregation of β B2-crystallin. These techniques were used, because of the different sensitivity of the various techniques to the presence of intermediates and aggregates. The major findings of this study were that mimicking deamidation at the interface in the β B2- dimer led to unfolding and aggregation upon heating, rapidly followed by precipitation. Deamidation had a greater affect at Q70 in the N-td than at Q162 in the homologous C-td. The native α -chaperone, active against its physiological β/γ -crystallin substrates, was only able to partially rescue this precipitation.

4.1. Structure and stability of WT and deamidated h β B2

The thermal unfolding of human β B2 measured by DLS and SAXS is in agreement with previous reports (Evans et al., 2008) and is similar to the unfolding temperature of h β B1 (Lampi et al., 2002). Deamidation, while preserving the dimeric structure of β B2 in solution, resulted in destabilization, as evidenced by a significant shift in the mid-point of thermal unfolding for Q70E and DM. Deamidation had less of an effect at Q162. Since the *in silico* analysis of the available 3D structures revealed no significant difference between the atomic contacts of Q70 and Q162 it was, therefore, unexpected that the Q70E and Q162E mutants were not equivalent. However, the differences between the patterns of intramolecular contacts of D1 and D2 (i.e. the higher number of contacts, mostly contributing to domain stabilization within D2 rather than within D1) might explain the greater stability of D2 compared to D1. In particular, some stabilizing D2-specific contacts are located near the residue Q162.

4.2. Unfolding and aggregation of deamidated β B2

From the DSC data, the thermal unfolding of h β B2-WT, the deamidated mutants, and b β Lb was at least three-states (or sub-states). A three-state model was determined for the unfolding of rat β B2 according to N2 $\leftrightarrow$ 2I $\leftrightarrow$ 2U where unfolding of the native protein (N) to the unfolded protein (U) occurs through a concentration-dependent intermediate (I) (Weilgmann et al., 1999). In this model, the monomeric intermediate state consisted of an unfolded N-td and folded C-td. Similarly, unfolding of h β B2 was also found to have an unfolded N-td and a structured C-td (Evans et al., 2008).

Deamidation appears to have enhanced destabilization, leading to formation of the intermediate or the aggregate, and is consistent with our previously published results in which an intermediate was observed for the urea-induced unfolding of the double deamidated h β B2 (Lampi et al., 2006). Homologous deamidated mutants of γ D were populated by partially unfolded intermediates that likely had structured C-td and less structured N-td leading to aggregation (Flaugh et al., 2006).

All the β -crystallins under study were found to aggregate at the onset of their thermal destabilization. Deamidation decreased the onset of temperature-induced aggregation. The Q162E mutant was less destabilized by temperature, whereas DM was the most easily destabilized and rapidly aggregated. Dynamic light scattering is sensitive to even small amounts of aggregates and aggregates were already visible by DLS after 2 minutes. The aggregation was quickly followed by precipitation.

The important light scattering from the soluble temperature induced aggregates prevented detection by DLS of light scattering from the remaining soluble monomers or dimers. The rapid

thermal denaturation of h β B2 masked the detection of any intermediate state, between native and unfolded proteins, before aggregation. However, scattering curves were obtained by SAXS of the fraction remaining in solution at 60°C, since the material precipitating was no longer in the path of the laser beam. The shapes of the resulting scattering curves were consistent with the presence of both small aggregates and globular particles, either monomers, or dimers, or mixtures of both. Thus, the material remaining in solution was still partially structured.

4.3. Intermediate states and chaperone requirements

The chaperone requirements for β B2 and its deamidated mutants correlated with the stability of the proteins. The b α N chaperone was able to rescue the relatively stable h β B2-WT, preventing aggregation with only half as much chaperone as substrate. However, b α N was less efficient to rescue the less stable b β Lb, Q162E, was only able to partially rescue DM, and was unable to rescue Q70E.

In the three state model of the h β B2-WT, the presence of a partially structured monomer was found important for recognition by the α -chaperone, as the monomeric intermediate state was proposed to both aggregate and associate with the chaperone (Evans et al., 2008). The more protein present in the intermediate state such as may result from deamidation (Lampi et al., 2006), the more α -chaperone would be required to prevent aggregation leading to precipitation. In the case of Q70E, interactions with α -crystallin may be limited possibly because less Q70E protein is in the intermediate state, where the chaperone could bind it, than the aggregated state where the chaperone does not. It is possible that the surfaces in the Q70E intermediate state necessary for interaction with the chaperone were not presented to the chaperone and/or that aggregation was kinetically preferred to formation of the heterocomplex between the deamidated h β B2 and α -crystallin chaperone. Either of these possibilities could be explained by a greater disruption of the C-td in Q70E than in Q162E or DM that was predicted by the greater solvent accessibility detected by hydrogen/deuterium exchange with mass spectrometry (personal communication, Dr. Takumi Takata, Oregon Health and Science University).

4.4. Complexes formed between β samples and α -chaperone

We have previously shown that the mechanism for the chaperone-like activity of b α N towards the physiological substrate b β L involves the activation of the chaperone by the doubling of its size simultaneously with the formation of the b α N-b β L hetero-complexes (Putilina et al. 2003). In this study, analysis of the R_h of hetero-complexes formed at 60°C, for different ratios of b α N with either b β Lb or h β B2, showed a further increase in size, indicating that the β -crystallins were not simply filling the holes of the α -crystallin assemblies, as proposed in the literature (Regini et al., 2007). It remains unknown, however, whether the β -crystallins were simply covering the α -crystallin assembly or a more mixed assembly was formed through subunit exchange.

4.5. Model for cataracts

Taken together, the DLS, SAXS and DSC data reported in this study strongly suggest that the partially unfolded intermediate states of h β B2 are prone to aggregate. During normal aging, crystallins in the lens of the eye are extensively deamidated. Deamidation detected at the interface in h β B2 from cataractous lenses (Tsur et al., 2005), significantly decreased the unfolding temperature and enhanced rapid precipitation during thermal unfolding of the protein. These *in vitro* studies suggest a potential mechanism for deamidation-induced cataracts. Many of the modifications observed *in vivo* in aged lenses are the same as in cataractous lenses. The mechanism of unfolding, soluble aggregation, and rapid precipitation may be initiated *in vivo* by additional modifications or by reaching a threshold level of deamidation. Furthermore, we have shown that the α -crystallin chaperone is only able to partially rescue the deamidated protein from precipitating.

In studies of other destabilized mutants of h β B1 and h β B2, yet remaining soluble and being partially reversible at $\leq 37^\circ\text{C}$ with no high molecular weight aggregates formed, it has been reported that the destabilized mutants marginally bind h α B-crystallin, with increased binding for the more destabilized ones, suggesting that the substrate was in a nonnative, excited state or folded intermediate (Sathish et al., 2004). Destabilized h β B1 mutants were found to bind h α A with a decrease in binding at higher h β B1 concentrations (McHaourab et al., 2007). The higher concentrations may have favored a dimer intermediate with lower affinity to h α A than the monomer intermediate. In both of these studies, large α -crystallin to substrate ratios was used. While the mechanism is different from the chaperone-like activity explored in the present study, it suggests that the nature and the amount of the intermediate is important for recognition by the chaperone.

In conclusion, aggregation of deamidated h β B2 may have led to intermediates not as readily recognized by α -crystallin chaperone or occurring before interaction with α -crystallin chaperone could occur. Additionally, saturation might occur at lower α -crystallin to h β B2 substrate ratios for the deamidated mutants as was observed for deamidated mutants of h β B1 (Lampi et al., 2002).

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Abbreviations

IPTG	isopropyl α -D-thiogalactoside
PMSF	phenyl-methyl-sulfonyl fluoride
DLS	dynamic light scattering
R_h	hydrodynamic radius
Pd	polydispersity
SAXS	small angle X-ray scattering
R_g	radius of gyration
DSC	differential scanning calorimetry
MW	molecular weight
MM	molecular mass
SDS-PAGE	sodium dodecyl sulfate -polyacrylamide gel electrophoresis
FRET	Förster resonance energy transfer
3D	three dimensional

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Fig. 1.
(A) Multiple sequence alignment of the bovine β -crystallins and of h β B2. The N-terminal domain (Ntd) comprises the N-terminal extension (Nt) and the first homologous domain (D1). The C-terminal domain (Ctd) comprises the second homologous domain (D2) and the C-terminal extension (Ct). The two domains are connected by the connecting peptide (cp). The Greek key motifs 1-4 are indicated. Note that the sequence numbering usually adopted, and that has been used here, does not include the initial methionine. The secondary structures according to the h β B2 3D structure (pdb code 1YTQ), are indicated by B for β -strands or H for helices. The Q and N residues are represented in bold. Amino acids at positions 70 and 162 are represented by white letters. The amino acid positions corresponding to their intra and intermolecular contact sites in the h β B2 3D structure are highlighted in magenta and green, respectively. (B) 3D representation of h β B2. Amino acids Q70 and Q162 that are involved in the two equivalent dimeric interfaces are represented as spheres; their intra and intermolecular contacts are in magenta and green,

respectively; and the A and B chains are colored in light blue and dark blue, respectively. The right side of the figure focuses on one dimeric interface between D1 of the chain B and D2 of the chain A, with a stick representation of the atoms at the contact sites. The intramolecular contact sites of Q70 (D1) and Q162 (D2) are indicated in light and dark magenta for chains A and B, respectively: G60, E69, F71 (within D1), and G152, L161, Y163 (within D2). The intermolecular contact sites of Q70 (D1) and Q162 (D2) are indicated in light and dark green for chains A and B, respectively: Q162, L164, F176 (within D2), and Q70, V72, W84 (D1).

Fig. 2.

DLS temperature scans of β -crystallins. The scattered intensity was recorded for 200s (20×10 s) every 2°C for β Lb (circles), β B2 (squares), Q70E (diamonds), Q162E (triangles), and DM (upside down triangles). β -crystallin concentrations were 0.5 mg/ml. Detector saturation was 100%. At the onset of aggregation the scattered intensity increased sharply and the experiment for that sample was stopped.

Figure 3A

Figure 3B

Figure 3C

Fig. 3.

(A) SAXS intensity curves for β -crystallins recorded at 37°C. β -crystallins were designated as in Fig. 2. The following concentrations were used: h β B2-WT at 7.2 mg/mL; Q162E at 3.8 mg/mL; Q70E at 6 mg/mL; DM at 3.5 mg/mL; and b β Lb at 60 mg/mL. (B) R_g values calculated from Guinier plots as a function of temperature. Note that for b β Lb the R_g is an apparent R_g , due to the high protein concentration. (C) SAXS intensity extrapolated to zero angle, I_0 , calculated from the Guinier approximation. Its variation is representative of the loss of the total scattering intensity due to the decrease of concentration, because of the formation of large aggregates that precipitated out of the X-ray beam. (The last point for the Q70E mutant is missing because no scattering curve where recorded at 60°C.)

Figure 4A

Figure 4B

Figure 4C

Figure 4D

Fig. 4.

Chaperone assays measured by DLS. Scattered intensity was recorded as a function of time for α N:b β Lb (A) and α N:h β B2 (B), Ratios of chaperone to substrate in A were 0:1, 1:8, 1:4, 1:2, 1:1, and 2:1 and in B were 0:1, 1:2 and 1:1 (Key for both A and B is from solid line to increasing smaller dashes, as shown in figure). The scattered intensity was recorded for 3600 s (360×10 s) at 60°C. Detector saturation was 10% and the β -crystallin concentration was always set at 0.5 mg/mL. The curves were normalized as indicated in Methods. Sizes of chaperone-substrate complexes measured by DLS : variation of the R_h as a function of time for different ratios of bovine α N to b β Lb (C) and to h β B2 (D). Ratios are indicated in (A) and (B) with the addition of a 1:0 ratio.

Fig. 5.

Chaperone assays measured by DLS with β Lb, β B2 and the mutants. Ratios of chaperone to substrate were 1 :1. The scattered intensity was recorded as a function of time for 3600 s (360×10 s) at 60°C . Detector saturation was 10% and the β -crystallin concentration was always set at 0.5 mg/mL. The data displayed here are raw data.

Fig. 6. Chaperone assays measured by SAXS. Comparison of the scattering intensity by αN alone (dark grey curve) and by the $\alpha\text{N}:\beta\text{B2}$ mixture in a 1:1 ratio (black curve) after 30 min incubation at 60°C . Scattering intensities were recorded at ambient temperature. The sum of the intensity scattered by αN at 60°C and βB2 at 15°C has been plotted for the sake of comparison (light grey curve).

Table 1

DLS data												
bβLb			hβB2-WT			Q70E		Q162E		DM		
temperature (°C)	23	37	23	37	23	23	37	23	37	23	23	37
R _h (nm)	2.9 (0.1) ^a	2.9 (0.1)	3.1 (0.1)	3.1 (0.1)	3.1 (0.2)	3.1 (0.2)	3.0 (0.2)	3.1 (0.1)	3.1 (0.1)	3.2 (0.1)	3.2 (0.1)	3.1 (0.1)
Pd (%)	19.3 (1.0)	19.2 (1.1)	16.2 (1.3)	14.7 (2.0)	16.5 (1.7)	16.5 (1.7)	21.0 (1.2)	20.2 (1.4)	19.0 (2.1)	20.9 (2.7)	20.9 (2.7)	25.3 (1.8)

^aThe standard errors are given in parentheses.

Table 2

DSC data.						
	T1 (°C)	T2 (°C)	T3 (°C)	C (mg/mL)	ΔH (kcal/mol)	ΔS (kcal/(K mol))
h9B2-WT1	59.8 °C (23%) ^a	64.3 °C (74%)	68.1 °C (3%)	1.65	183	0.54
h9B2-WT2	58.0 °C (42%)	64.5 °C (44%)	68.0 °C (14%)	0.7	225	0.67
Q70E	52.7 °C (60%)	56.7 °C (35%)	58.6 °C (5%)	0.5		
Q162E	61.7 °C (57%)	66.1 °C (27%)	68.5 °C (16%)	2.7	240	0.70
DM	47.8 °C (54%)	53.0 °C (31%)	68.2 °C (3.2)	0.5	184	0.57
b9Lb	57.0 °C (29%)	62.4 °C (71%)		4	184	0.55

^aThe percentages of material corresponding to each transition are given in parentheses.