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Abstract

Purpose: To determine how deamidation and partial loss of the N- and C-terminal extensions alter the heat stability of βB1-crystallin.

Methods: Human lens βB1, a deamidated βB1, Q204E, and αA-crystallins were expressed. Truncated βB1 was generated by proteolytic removal of part of its terminal extensions. The aggregation and precipitation of these proteins due to heating was monitored by circular dichroism and light scattering. The effect of heat on the stability of both monomers and oligomers was investigated. The flexibility of the extensions in wild type and deamidated βB1 was assessed by ^{1}H NMR spectroscopy.

Results: With heat, deamidated βB1 precipitated more readily than wild type βB1. Similar effects were obtained for either monomers or oligomers. Flexibility of the N-terminal extension in deamidated βB1 was significantly reduced compared to the wild type protein. Truncation of the extensions further increased the rate of heat-induced precipitation of deamidated βB1. The presence of the molecular chaperone, αA-crystallin, prevented precipitation of modified βB1s. More αA was needed to chaperone the truncated and deamidated βB1 than deamidated βB1 or truncated βB1.

Conclusions: Deamidation and truncation of βB1 led to destabilization of the protein and decreased stability to heat. Decreased stability of lens crystallins may contribute to their insolubilization and cataract formation.

Keywords

b1, human, crystallins, decreased, increased, stability, modified, requirement, chaperone, heat, beta

Disciplines

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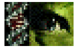 *Molecular Vision* **2002; 8:359-66** <http://www.molvis.org/molvis/v8/a43>
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Decreased heat stability and increased chaperone requirement of modified human βB1-crystallins

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Purpose: To determine how deamidation and partial loss of the N- and C-terminal extensions alter the heat stability of βB1-crystallin.
Methods: Human lens βB1, a deamidated βB1, Q204E, and αA-crystallins were expressed. Truncated βB1 was generated by proteolytic removal of part of its terminal extensions. The aggregation and precipitation of these proteins due to heating was monitored by circular dichroism and light scattering. The effect of heat on the stability of both monomers and oligomers was investigated. The flexibility of the extensions in wild type and deamidated βB1 was assessed by ^{1}H NMR spectroscopy.
Results: With heat, deamidated βB1 precipitated more readily than wild type βB1. Similar effects were obtained for either monomers or oligomers. Flexibility of the N-terminal extension in deamidated βB1 was significantly reduced compared to the wild type protein. Truncation of the extensions further increased the rate of heat-induced precipitation of deamidated βB1. The presence of the molecular chaperone, αA-crystallin, prevented precipitation of modified βB1s. More αA was needed to chaperone the truncated and deamidated βB1 than deamidated βB1 or truncated βB1.
Conclusions: Deamidation and truncation of βB1 led to destabilization of the protein and decreased stability to heat. Decreased stability of lens crystallins may contribute to their insolubilization and cataract formation.

Crystallins are the major structural proteins in the lens and form complex protein-protein interactions with each other. α-Crystallins form large assemblies predicted to contain as many as 33 subunits and act as molecular chaperones to prevent the aggregation and precipitation of the crystallins [1]. β-Crystallins contain aggregates ranging in size from dimers to octomers, while γ-crystallins exist as monomers. βB1-crystallin is a major subunit of the β-crystallins and comprises 9% of the total soluble crystallins in the human lens [2]. In the newborn, βB1 is predominantly found in the βHigh fraction, comprised of tetramers and octomers, after isolation by size exclusion chromatography [3]. Interaction of βB1 with other β-crystallins is likely to contribute to the stability of the βHigh assembly [4], and thus, the stability and packing of crystallins in the lens.

βB1, like all the basic β-crystallins, is characterized by having N- and C-terminal extensions. The N-terminal extension of βB1 is the longest of any crystallin, 57 amino acids. During aging, βB1 is extensively truncated [3,5]. Initial cleavage within the first year of life removes the first 15 residues from the N-terminal extension. Several additional cleavages occur between residues 33 and 41 [6]. Analysis of the water-insoluble proteins from older lenses suggests the presence of even more extensively degraded forms of βB1 [7]. During aging, increasing amounts of truncated βB1 are found in the βIntermediate and βLight assemblies, therefore, loss of the N-terminal extension of βB1 may cause dissociation of β-assemblies [4].

Extensive deamidation (conversion of Gln/Asn to Glu/Asp) has also been reported in crystallins from older lenses [3,5]. The introduction of these negative charges into the crystallins during aging would be expected to destabilize native structure and possibly increase susceptibility to aggregation. This hypothesis is supported by the finding of an increased amount of deamidation in γS-crystallin in human cataractous lenses [8]. Our earlier studies used two-dimensional electrophoresis and mass spectrometry to identify several distinct forms of deamidated βB1 in 55-year-old donor lenses [3]. Deamidation was also recently identified at Asn 157 in water-insoluble βB1 [7]. With recent improvements in sensitivity of mass spectrometry, confirmation and identification of more sites is anticipated. A useful strategy to determine the effect of these deamidations on protein structure and stability is to use mutagenesis to engineer them into recombinantly expressed proteins. While the effect of the sites of deamidation in βB1 have yet to be determined, a site at Gln 204 [3,9], in a hydrophobic region of βB1, did not significantly alter the secondary structure of the protein but did affect the shape of the βB1 dimer [10].

We hypothesize that the post-translational modifications, deamidation and truncation, change the conformation of βB1. Indeed, in this study we use NMR spectroscopy to demon-

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strate that the N-terminal extension of deamidated βB1 has significantly reduced flexibility compared to the wild type protein. The changes in the structure, and hence stability of βB1 and its aggregates, may lead to insolubilization and cataract formation. Thus, the purposes of the present study were to extend our earlier work by first determining the stability of the βB1 monomers and homodimers to heat, and then to examine how post-translational modifications altered this stability. The effect of deamidation on the stability of crystallins has never been measured. We found that deamidation had a profound effect on βB1 stability and that truncation within the N- and C-terminal extensions further decreased stability. Truncation alone had little effect. Heating deamidated and/or truncated βB1s with an equal molar amount of αA resulted in precipitation of both proteins. However, additional αA prevented the precipitation. The role of hydrophilicity of βB1 in maintaining stability of βB1 and its complexes with αA-crystallin is discussed.

METHODS

Protein expression: Human recombinant wild type (wt) βB1, a deamidated mutant (Q204E) βB1, and wild type αA crystallins were expressed and purified as previously described [10]. Briefly, an RT-PCR product generated using gene-specific primers was inserted into pCR T7/CT TOPO vector (Invitrogen, San Diego, CA) and transformed into the cloning cells, Top 10F' (Invitrogen). Positive clones were transformed into the expression cells, BL21(DE3) Star (Invitrogen). Use of donor tissue for these experiments were approved by the Oregon Health and Science University institutional review board.

Proteins were purified as previously described [10]. Briefly, cell pellets were lysed by freeze-thawing and sonication in a phosphate buffer, pH 6.8. Cell lysates were centrifuged and the supernatants applied to a SP Sepharose Fast Flow cation exchange column (Amersham Biosciences, Piscataway, NJ). The purity of βB1 by this method has been previously demonstrated [10] and was checked by mass spectrometry. For NMR studies, βB1 was further applied to a size exclusion column (3x80 cm S-300 HR Sephacryl column, Amersham Biosciences) at a flow rate of 0.4 ml/min in 29 mM Na_2HPO_4, 29 mM KH_2PO_4, 100 mM KCl, 0.7 mM EDTA, 1 mM DTT, pH 6.8. αA was lysed from *E.coli* cell pellets and purified by size exclusion chromatography as described for βB1. This was followed by ion exchange (2.5x12 cm Macro Prep DEAE column, BioRad, Hercules, CA, USA) at a flow rate of 3 ml/min in 20 mM Tris/HCl, pH 7.4, 0.16 mM EDTA, 1 mM EGTA. Proteins were eluted using a linear 0-500 mM NaCl gradient.

Truncated βB1s were generated by cleaving purified wt and Q204E βB1s with recombinant calpain (Calbiochem, La Jolla, CA) at a ratio of 4 Units of Calpain (2.3 μg) per mg βB1. Proteolysis was initiated by adding 2 mM calcium and the reaction was allowed to continue for 1-2 h at 37 °C. Calpain removes 47 amino acids off the N-terminus and 5 amino acids off the C-terminus of wt and Q204E βB1 ([10] and unpublished data). The reaction was stopped by removing the calcium by buffer exchange at 4 °C.

Protein identity and purity were confirmed by mass spectrometry using an electrospray ionization mass spectrometer (ThermoQuest, San Jose, CA, USA) to compare predicted and calculated masses. Mass accuracy of the measurements was 0.01% as confirmed using a myoglobin standard. The purity of the preparations was also examined by electrophoresis using pre-cast polyacrylamide Nupage mini-gels (Novex, San Diego, CA). Proteins were visualized by staining with Coomassie blue G-250.

Circular dichroism: Initial denaturation experiments were performed at a rate of heating of 0.75 °C/min for wt βB1 from 30 to 80 °C. Due to precipitation of the protein at higher temperatures, no signal was recovered upon cooling. The CD signal was measured at 215 nm with an AVIV Circular Dichroism spectrophotometer, model 202 (Protein Solutions, Lakewood, NJ). A temperature below the temperature at which loss of secondary structure/precipitation occurred was chosen for the experiments below. The fraction unfolded protein to precipitated was calculated as the difference in signals (ellipticity) at a specified temperature and the minimum signal divided by the difference in minimum and maximum signals. Circular dichroism was performed at 0.1 and 0.4 mg/ml in 1 mm cells. Samples were dialyzed exhaustively against 10 mM phosphate buffer, pH 6.8, and 100 mM NaF. Protein concentrations were confirmed by amino acid analysis. Representative CD spectra from 180 to 260 nm for both proteins were previously published [10].

Light scattering: Light scattering was determined indirectly by measuring absorbance at 405 nm. Two different protocols were used, each optimized to investigate the effect of heat on either monomers or oligomers of βB1s. Based on previous data, a 0.1 mg/ml concentration contained predominantly monomers of βB1, while higher concentrations were predominantly dimers with small amounts of higher-ordered oligmers [10]. The samples at 0.1 mg/ml were heated at 55 °C for 725 min in a thermal jacketed cuvette with constant stirring (Cary 4 Bio UV-Visible spectrophotometer, Varian, Palo alto, CA). Incubations were performed in the same buffer as for circular dichroism, except the NaF was replaced by NaCl. No observable precipitate or change in protein concentration was seen following heating of wt βB1 under these conditions. Higher concentrations or temperatures resulted in precipitation of wt βB1.

Heat induced aggregation of βB1 was also measured in the wells of microtiter plates. Samples at 1.5 mg/ml concentration were heated in a programmable thermal controller to 55°C and the temperature monitored with a probe. Samples were placed in the following buffer after chromatography by repeated concentration: 20 mM phosphate buffer, pH 6.8, 75 mM NaCl, 1 mM DTT, 0.5 mM EGTA. At set intervals, each tube was vortexed and 50 μl transferred to a 96 well plate. Samples were shaken and absorbance read at 405 nm on a microtiter plate reader. All samples used in light scattering experiments were freshly prepared and stored in a protease inhibitor cocktail (Complete Inhibitor Tablets, Boehringer Mannheim GmbH, Mannheim, Germany).

Protein assays: Protein content was assayed by the Coomassie Plus-200 assay (Pierce, Rockford, IL) following manufacturer's instructions.

NMR spectroscopy: ^{1}H NMR spectra were acquired at 500 MHz and 25 °C using a Varian Inova-500 spectrometer (Varian, Inc., Palo Alto, CA). The acquisition conditions are outlined elsewhere [11]. Mixing times of 70 ms and 100 ms were used in the TOCSY and NOESY spectra respectively. In all experiments, WET methods were used for suppression of the large water resonance [12].

RESULTS

In order to test the stability of βB1-crystallins, recombinant wild type βB1 and a deamidated βB1 mutant (Q204E) were heated from 30 to 80 °C at a rate of 0.75°C per minute (Figure 1). Circular dichroism, followed by the signal at 215 nm, indicated no loss of secondary structure for either protein up to 62 °C. This was followed by irreversible loss of secondary structure and precipitation of the protein by 70 °C. Therefore, temperatures below 60 °C were chosen for experiments below.

As a measure of unfolding and aggregation, changes in absorbance due to light scattering at 405 nm were followed for dilute solutions of βB1 (0.1 mg/ml) upon heating at either 50 or 55 °C (Figure 2A). The molar mass of βB1 determined by multi-angle laser light scattering at this concentration was previously shown to be 30,000 Daltons, suggesting the protein is predominantly monomeric [10]. The deamidated mutant protein showed a significantly greater increase in light scattering at both temperatures, again demonstrating lower thermal stability. At 50 °C, the light scattering of deamidated βB1 increased rapidly with biphasic kinetics. The initial rate at a concentration of 0.1 mg/ml for deamidated βB1 was 0.015 min^{-1}as compared to 0.006 min^{-1} for wild type βB1.

The ability of αA to prevent, via its chaperone action, the aggregation of βB1 upon heating was also tested. The presence of 1:1 molar ratio of αA to wt βB1 significantly diminished the increase in light scattering seen upon heating wt βB1 (Figure 2B). In contrast, a 1:1 molar ratio of αA to deamidated βB1 produced stabilization against precipitation, followed by a sharp, dramatic increase in light scattering (Figure 2C). The sharp increase suggested precipitation of the Q204E βB1 and αA complex as was seen below (Figure 3B). This behavior could be prevented by increasing the concentration of αA. Thus, a 2:1 molar ratio of αA to deamidated βB1 prevented the precipitation of deamidated βB1 to a level similar to that seen for native wt βB1 in the presence of an equimolar amount of αA.

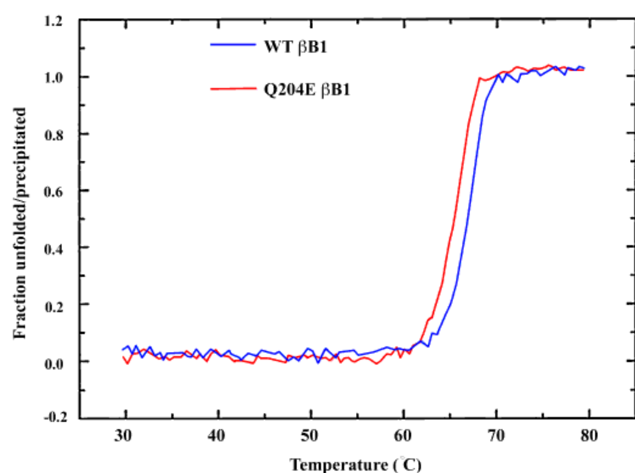

Figure 1. Percent of unfolded wt βB1 and Q204E βB1. Percent of unfolded wt βB1 (red curve) and Q204E βB1 (blue curve) were measured by changes in the CD spectra at 215 nm. Protein concentrations are 0.4 mg/ml in 10 mM phosphate buffer, pH 6.8. Cell path length is 1 mm.

Due to instrumental sensitivity and our interest in the structure of the monomeric protein, the above studies were conducted at a very low concentration of βB1. Because oligomerization of βB1 is concentration dependent [10,13] and oligomer state can influence thermal stability, we also examined heat denaturation at a higher βB1 concentration of 1.5 mg/ml (Figure 4). The results were similar to those observed at 0.1 mg/ml. Deamidated βB1 again exhibited greater light scattering than wt βB1. As a control, heating αA did not result in any changes in absorbance (Figure 4A). Thermal denaturation at the higher concentration resulted in readily observable precipitation, and deamidated βB1 produced more precipitated protein than wt (Figure 3A).

Similar results were obtained after incubating αA with modified βB1s at either 0.1 or 1.5 mg/ml. A 1:1 equal molar ratio of deamidated βB1 with αA did not prevent aggregation; in fact, precipitation was enhanced (Figure 4B). However, adding twice as much αA significantly inhibited precipitation (Figure 4B). SDS-PAGE of the proteins following centrifugation showed that the increased light scattering observed in Figure 4B was due to precipitation of both the deamidated βB1 and αA (Figure 3B, lanes 1 and 2). Additional αA increased the solubility of both proteins (Figure 3B, lanes 4 and 5).

The next set of experiments examined the role of the N- and C-terminal extensions on the heat stability of βB1. Both N- and C-terminal extensions of βB1 and deamidated βB1 were cleaved by calpain. Calpain previously has been shown to remove 47 amino acids from the N-terminus and 5 amino acids from the C-terminus [10]. In contrast to deamidation, little difference in the rate of absorbance increase was observed between wt and truncated wt βB1s during heating (Figure 2A and Figure 5A). However, there was a marked difference in the interaction of αA with the two proteins. A 1:1 ratio of truncated wt βB1 and αA resulted in a lag in the onset of aggregation, followed by a rapid increase in absorbance similar to that seen for deamidated βB1. This was not observed with full-length wt βB1 (Figure 2B). A 2:1 ratio of αA to truncated wt βB1 increased the duration of the initial lag phase (Figure 5B).

Heating the doubly modified truncated, deamidated βB1 led to a much more rapid increase in aggregation than heating deamidated βB1 (Figure 5A). This increase in light scattering did not fit parameters for biphasic kinetics as was observed

for deamidated βB1. Incubating an equal molar amount of αA with the truncated, deamidated βB1 only prevented an increase in light scattering for the first 100 min (Figure 5C). After this, a sharp increase occurred. The greatest and most rapid precipitation due to heating of all the proteins occurred with a 1:1 mixture of truncated, deamidated βB1 and αA. Additional αA at a 2:1 ratio did not prevent this increase (not shown), however a 4:1 ratio of αA:βB1 did. Thus, a greater amount of αA was required to prevent heat-induced aggregation of truncated, deamidated βB1 than for either truncated or deamidated βB1.

Previous ^{1}H NMR spectroscopic studies of other β-crystallin subunits, i.e. βB2, βA3, have shown that their N- and C-terminal extensions have great conformational flexibility compared to the domain core and adopt little or no preferred conformation (summarized in reference 13). In order to gain insight at the molecular level into the structural changes associated with deamidation of Q204 in βB1, ^{1}H NMR spectra were acquired of wt and deamidated βB1. Figure 6A shows the TOCSY NMR spectrum of cross-peaks arising from the NH protons of wt βB1 due to spin-spin coupled protons within the same amino acid. From analysis of this spectrum and a NOESY spectrum giving through-space connectivities between NH protons and the preceding α-CH protons, many of the cross-peaks in the TOCSY spectrum could be assigned. From these data, it was apparent that these cross-peaks arise predominantly from the N- and C-terminal extensions of wt βB1 crystallin. The strong similarity of their chemical shifts to those for random coil peptides and hence lack of dispersion particularly in the α-CH chemical shifts, imply that the extensions have no ordered structure and have great conformational flexibility. The flexibility of the terminal extensions does not exclude the possibility of structure induced upon interaction with other proteins in the lens cell as would be suggested by NMR data on crystallin homogenates [14]. This lack of dispersion and the large number of residues in both extensions led to significant spectral overlap and hence the inability to assign all cross-peaks from the extensions of βB1.

The TOCSY spectrum of deamidated βB1 (Figure 6B) shows far fewer cross-peaks than in the spectrum of the wild type protein. Interestingly, cross-peaks from the C-terminal extension of βB1 were present in this spectrum but, in the main, those from the N-terminal extension were absent. Thus, introduction of the negative charge at residue 204, well distant from the N-terminal extension, leads to a significant loss of conformational mobility in the long N-terminal extension but does not affect flexibility of the much shorter C-terminal extension.

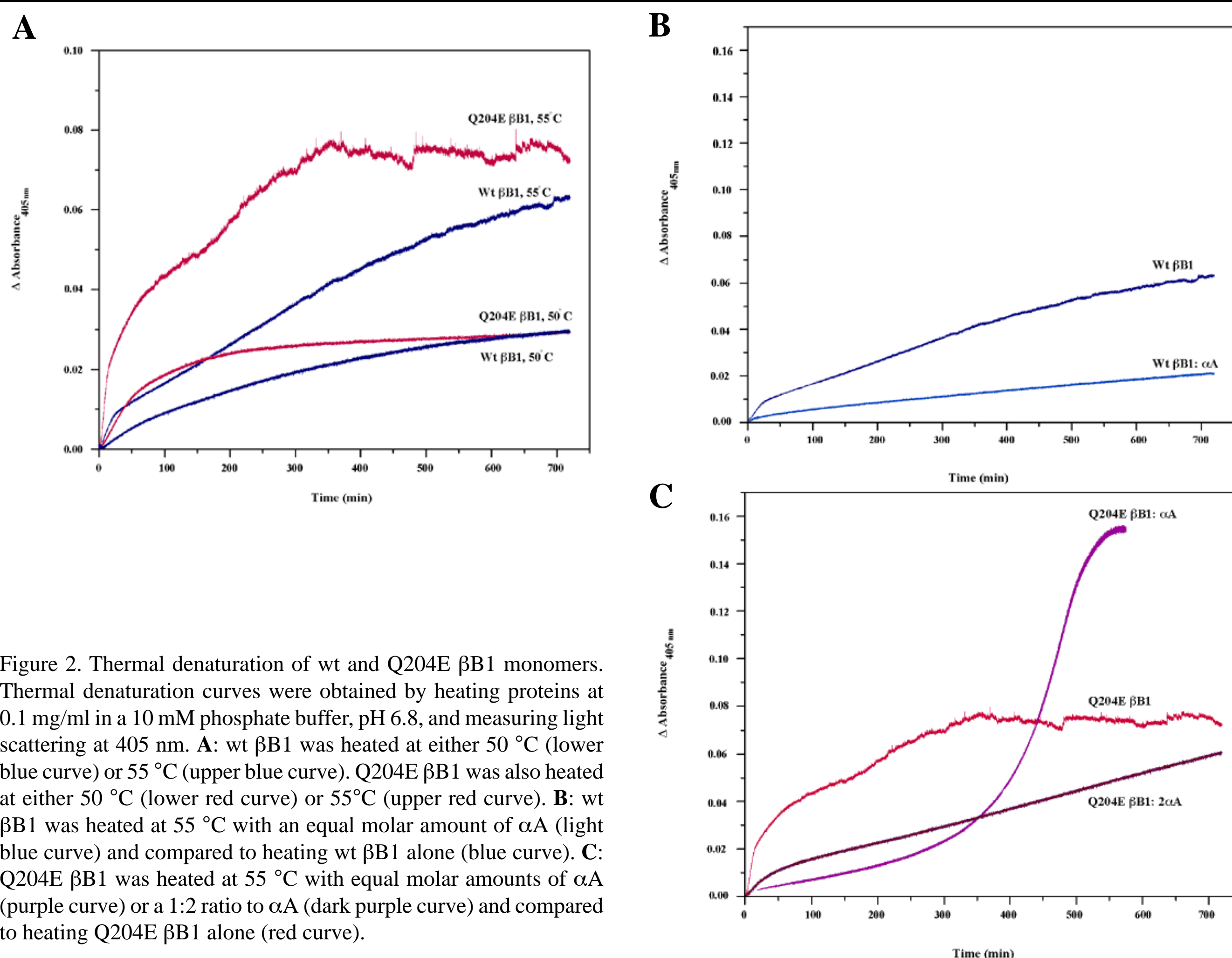

Figure 2. Thermal denaturation of wt and Q204E βB1 monomers. Thermal denaturation curves were obtained by heating proteins at 0.1 mg/ml in a 10 mM phosphate buffer, pH 6.8, and measuring light scattering at 405 nm. **A**: wt βB1 was heated at either 50 °C (lower blue curve) or 55 °C (upper blue curve). Q204E βB1 was also heated at either 50 °C (lower red curve) or 55°C (upper red curve). **B**: wt βB1 was heated at 55 °C with an equal molar amount of αA (light blue curve) and compared to heating wt βB1 alone (blue curve). **C**: Q204E βB1 was heated at 55 °C with equal molar amounts of αA (purple curve) or a 1:2 ratio to αA (dark purple curve) and compared to heating Q204E βB1 alone (red curve).

DISCUSSION

Proteolysis and deamidation are major post-translational modifications of lens crystallins in the aging human lens [3,5,7,15,16]. We previously reported that introducing a deamidation at Gln 204 in the C-terminus or truncation of the extensions of βB1 causes a change in the shape of the protein as evidenced by multi-angle laser light scattering [10]. The NMR data reported herein are consistent with this observation. The N-terminal extension in deamidated βB1 has reduced flexibility compared to its wild type counterpart possibly due to electrostatic interactions between one or more of its positively charged residues (K5, K21 and K23), and the introduced negative charge at residue 204. In this study we explored whether these modifications also decreased the heat stability of βB1.

We found that human recombinant βB1 was very heat stable, and retained its native secondary structure up to 62-65 °C (Figure 1). The stability of the βB1 secondary structure was most likely responsible for the resistance of the protein to heat-induced aggregation. Deamidation decreased the thermal-stability of βB1 as measured by changes in light scattering, while truncation of the extensions alone did not effect the heat stability of βB1.

Deamidation decreased the heat-stability of both the monomer and oligomer forms of βB1. Previous light scattering measurements have estimated the size of βB1 at the concentrations used in this study to be a monomer at 0.1 mg/ml and an oligomer of predominately dimers at 1.5 mg/ml [10,13]. Similarly, at the relatively high concentrations used for the NMR studies, βB1 is present as an oligomer. These concentrations are considerably lower than the high concentrations found in the lens, but allow for comparison of the behavior of modified proteins. By homology to the βB2 X-ray crystal structure [17], the site of deamidation in βB1 is predicted to be at the interface between the two subunits of the dimer. Deamidation alters the shape of the dimer [10] by reducing

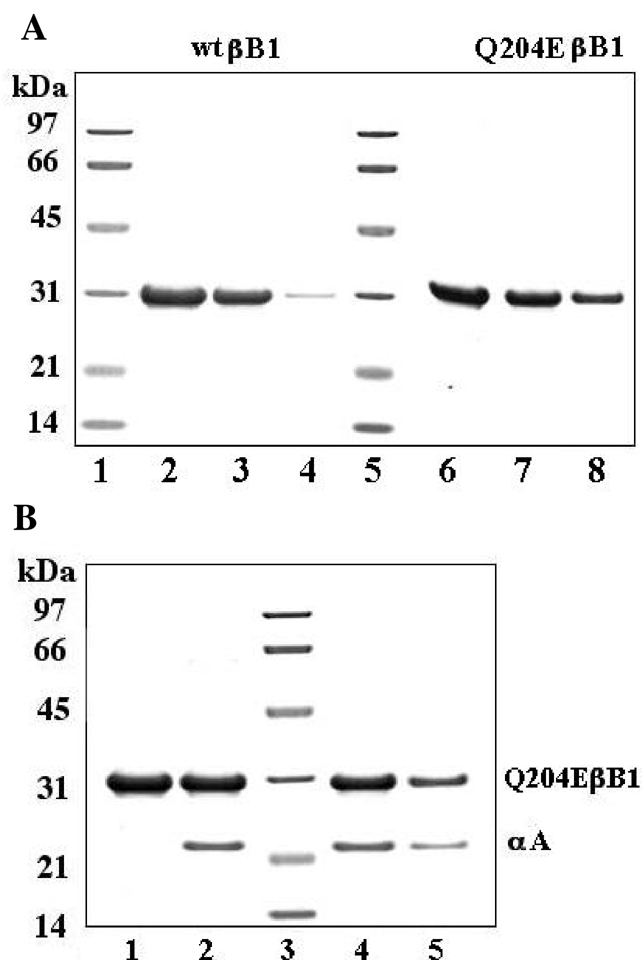

Figure 3. SDS-PAGE of βB1 samples from Figure 4. SDS-PAGE of wt βB1 and Q204E βB1 samples from Figure 4. **A**: Soluble and precipitated proteins were separated by centrifugation after heating for 180 min. Lanes 1 and 5 show molecular weight markers. Lanes 2 and 6 show wt βB1 and Q204E βB1 before heating. Lanes 3 and 7 show soluble wt βB1 and Q204E βB1. Lanes 4 and 8 show precipitated wt βB1 and Q204EβB1. **B**: Soluble and precipitated Q204E βB1 after heating in the presence of αA. Lanes 1 and 4 show soluble Q204E βB1 and αA after heating at equal molar concentrations (lane 1) and a 1:2 ratio of Q204E βB1 to αA (lane 4). Lanes 2 and 5 show precipitated Q204E βB1 and αA after heating at equal molar concentrations (lane 2) and 1:2 ratio of Q204E βB1 to αA (lane 5). Lane 3 shows molecular weight markers.

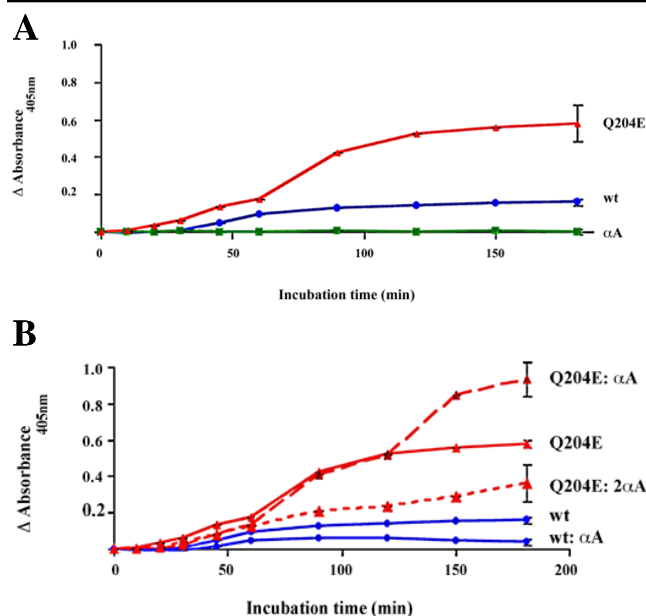

Figure 4. Thermal denaturation of wt and Q204E βB1 oligomers. Thermal denaturation of wt and Q204E βB1 oligomers was assessed at 55 °C. Thermal denaturation curves were obtained by heating proteins at a concentration of 1.5 mg/ml in a 20 mM phosphate buffer, pH 6.8, and measuring light scattering at 405 nm. Error bars are standard deviation, N=4. **A**: wt βB1 (blue curve), Q204E βB1 (red curve), and αA (green curve) were heated at a concentration of 1.5 mg/ml for wt and Q204E βB1 and 1.1 mg/ml for αA. **B**: Wt βB1 (blue curves) was heated with or without an equal molar amount of αA. Q204E βB1 (red curves) was heated with or without equal molar amounts of αA or a 1:2 ratio to αA.

the flexibility of the N-terminal extension (Figure 6). As a result, the stability of the dimer is lowered under heating stress. At the low concentrations used for monomer studies it is not possible to study the protein using NMR spectroscopy to ascertain whether the N-terminal extension has reduced flexibility in monomeric deamidated βB1. The reduced temperature stability of the mutant would imply, however, that similar structural features are present in both monomeric and oligomeric deamidated βB1.

Heating each of the expressed βB1 proteins examined here resulted in an increase in light scattering due to protein aggregation. At longer times, higher temperatures, and higher concentrations, heat-induced aggregation resulted in extensive precipitation that was clearly observed after centrifugation. The molecular chaperone, αA-crystallin, was added to determine if it could prevent the heat-induced aggregation and precipitation of the βB1-crystallins. A 1:1 molar ratio of αA was able to chaperone wt βB1 at 55 °C at both high and low βB1 concentrations (Figure 2). However, after a significant lag period, heating a 1:1 ratio of deamidated βB1 to αA resulted in an accelerated precipitation of both proteins. αA-crystallin is a very efficient chaperone at these elevated temperatures, which is most likely related to its enhanced subunit exchange rate [18]. The poor chaperoning ability of αA with deamidated βB1 must therefore arise from the structural changes in deamidated βB1 compared to the wt protein. αA and the destabilized βB1 proteins form a complex as a result of the former's chaperone action. However, at stoichiometric amounts of both proteins, the complex containing deamidated βB1 eventually becomes insoluble as the hydrophobic deamidated βB1 saturates the binding sites on αA. Similar behavior is observed during the chaperone action of destabilized forms of αB-crystallin [19] and a related small heat-shock protein, Hsp25 [20]. Thus, R120G αB is a poor chaperone because it has a disordered structure. Interaction with a target protein leads to complexation and rapid precipitation of both proteins [19]. For Hsp25, deletion of its flexible and polar C-terminal extension leads to similar behavior when it complexes to a target protein, α-lactalbumin [20]. It would seem, therefore, that for deamidated βB1, the reduction in flexibility of its N-terminal

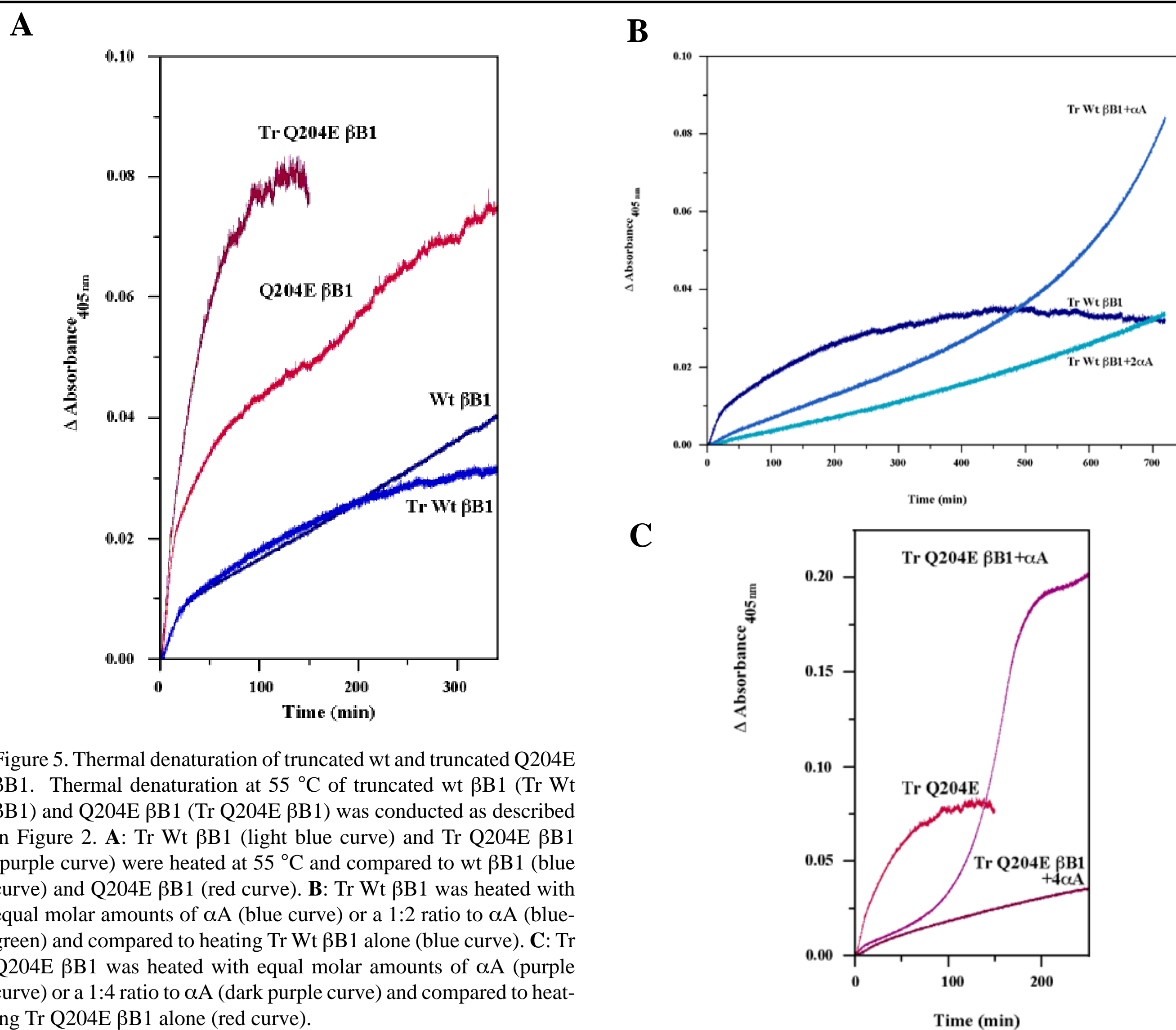

Figure 5. Thermal denaturation of truncated wt and truncated Q204E βB1. Thermal denaturation at 55 °C of truncated wt βB1 (Tr Wt βB1) and Q204E βB1 (Tr Q204E βB1) was conducted as described in Figure 2. **A**: Tr Wt βB1 (light blue curve) and Tr Q204E βB1 (purple curve) were heated at 55 °C and compared to wt βB1 (blue curve) and Q204E βB1 (red curve). **B**: Tr Wt βB1 was heated with equal molar amounts of αA (blue curve) or a 1:2 ratio to αA (blue-green) and compared to heating Tr Wt βB1 alone (blue curve). **C**: Tr Q204E βB1 was heated with equal molar amounts of αA (purple curve) or a 1:4 ratio to αA (dark purple curve) and compared to heating Tr Q204E βB1 alone (red curve).

extension and potentially the subtle structural changes associated with deamidation, lead to a protein that is unable to be chaperoned efficiently by αA. If deamidation results in partial unfolding of the core domains, this would then lead to a more hydrophobic protein. However, in the case of wt βB1, the hydrophobicity of the domain core of the protein is counteracted by the flexibility of its charged extensions to the extent that, under heating stress, the complex it makes with αA remains soluble.

Truncation of N- and C-terminal extensions did not affect the thermal stability of βB1 (Figure 5), implying that they are not required to be fully intact in order to maintain solubility of the protein under heating stress. However, when interacting with αA, the truncation of the extensions has a similar effect to that of deamidation, i.e. the protein is more hydrophobic, which leads to precipitation of the complex between both proteins. Thus, removal of a portion of the flexible extensions in βB1 makes the complex with αA less stable. Other studies have shown the importance of the flexible terminal extensions of α-crystallin and other sHsps in maintaining solubility of the chaperone-target protein complex [21,22]. The results presented herein suggest that hydrophobicity, and possibly flexibility in the target protein, in this case βB1, are also important in solubilization of the complex. The extensions have been suggested to be important in stabilizing the interactions of βB1 with other βsubunits [4]. This is supported by our previous report of a decreased heat stability when the βHigh-crystallins from bovine lenses were proteolyzed [23]. The extensions may function to allow oligomerization with other β subunits instead of increasing the stability of their secondary structure.

The combined effect of truncation and deamidation leads to an enhanced rate of precipitation under heat stress (Figure 5C), which is also consistent with the notion that increasing the hydrophobicity of the βB1 molecules promotes their mutual association. Furthermore, the results are consistent with the NMR data implying an interaction of the N-terminal extension with the introduced charge at Q204E. Coupled with this, deamidation could cause βB1 to unfold more readily with the resulting partly folded protein being less soluble without its hydrophilic extensions. Interestingly, in vivo, truncation and deamidation of βB1 occur together during aging [3,5].

In summary, we have shown that a deamidated mutant of βB1 crystallin, Q204E, displays decreased thermal stability compared to wt βB1. This decrease in thermal stability was observed both at concentrations where the proteins are predominantly monomers or dimers. NMR spectroscopy indicates that the deamidated mutant has a marked reduction in flexibility of its N-terminal extension. The structural alteration in the protein leads to a reduction in its thermal stability. Both deamidated βB1 and truncated βB1 have altered conformations and interaction with αA-crystallin, compared to the wt protein, requiring higher concentrations of this important chaperone to maintain solubility upon heat stress. The combination of truncation and deamidation diminished thermal stability to a greater degree than either modification alone. The decreased ability of αA to act as a chaperone of deamidated βB1 and truncated, deamidated βB1 may be another mechanism whereby deamidation and proteolysis could increase susceptibility of crystallins to insolubilization contributing to cataract formation.

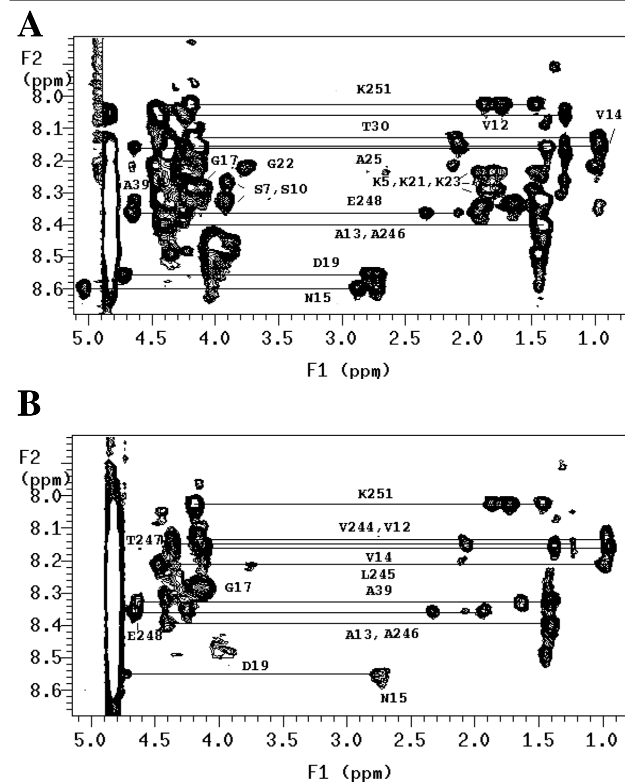

Figure 6. TOCSY NMR spectra of βB1 crystallins. TOCSY NMR spectra of (**A**) wt βB1 and (**B**) deamidated βB1. The spin lock mixing period in each experiment was 70 ms. Cross-peaks arising from the NH protons are shown. Those cross-peaks that could be assigned from NOESY spectra are indicated. **A**: **B**:

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REFERENCES

1. Horwitz J, Bova MP, Ding LL, Haley DA, Stewart PL. Lens alpha-crystallin: function and structure. Eye 1999; 13:403-8.
2. Lampi KJ, Ma Z, Shih M, Shearer TR, Smith JB, Smith DL, David LL. Sequence analysis of betaA3, betaB3, and betaA4 crystallins completes the identification of the major proteins in young human lens. J Biol Chem 1997; 272:2268-75.

3. Lampi KJ, Ma Z, Hanson SR, Azuma M, Shih M, Shearer TR, Smith DL, Smith JB, David LL. Age-related changes in human lens crystallins identified by two-dimensional electrophoresis and mass spectrometry. Exp Eye Res 1998; 67:31-43.
4. Ajaz MS, Ma Z, Smith DL, Smith JB. Size of human lens beta-crystallin aggregates are distinguished by N-terminal truncation of betaB1. J Biol Chem 1997; 272:11250-5.
5. Ma Z, Hanson SR, Lampi KJ, David LL, Smith DL, Smith JB. Age-related changes in human lens crystallins identified by HPLC and mass spectrometry. Exp Eye Res 1998; 67:21-30.
6. David LL, Lampi KJ, Lund AL, Smith JB. The sequence of human betaB1-crystallin cDNA allows mass spectrometric detection of betaB1 protein missing portions of its N-terminal extension. J Biol Chem 1996; 271:4273-9.
7. Hanson SR, Hasan A, Smith DL, Smith JB. The major in vivo modifications of the human water-insoluble lens crystallins are disulfide bonds, deamidation, methionine oxidation and backbone cleavage. Exp Eye Res 2000; 71:195-207.
8. Takemoto L, Bolye D. Increased deamidation of asparagine during human senile cataractogenesis. Mol Vis 2000; 6:164-8 .
9. Zhang Z. Identification and characterization of post-translational modifications in human lens beta crystallins during the aging process [dissertation]. Lincoln (NE): University of Nebraska-Lincoln; 2001.
10. Lampi KJ, Oxford JT, Bachinger HP, Shearer TR, David LL, Kapfer DM. Deamidation of human beta B1 alters the elongated structure of the dimer. Exp Eye Res 2001; 72:279-88.
11. Werten PJ, Carver JA, Jaenicke R, de Jong WW. The elusive role of the N-terminal extension of beta A3- and beta A1-crystallin. Protein Eng 1996; 9:1021-8.
12. Smallcombe SH, Patt SL, Keifer PA. WET solvent suppression and its application to LC NMR and high-resolution NMR spectroscopy. Journal of Magnetic Resonance, Series A 1995; 117:295-303.
13. Bateman OA, Lubsen NH, Slingsby C. Association behaviour of human betaB1-crystallin and its truncated forms. Exp Eye Res 2001; 73:321-31.
14. Cooper PG, Aquilina JA, Truscott RJ, Carver JA. Supramolecular order within the lens: 1H NMR spectroscopic evidence for specific crystallin-crystallin interactions. Exp Eye Res 1994; 59:607-19.
15. Zhang Z, David LL, Smith DL, Smith JB. Resistance of human betaB2-crystallin to in vivo modification. Exp Eye Res 2001; 73:203-11.
16. Lund AL, Smith JB, Smith DL. Modifications of the water-insoluble human lens alpha-crystallins. Exp Eye Res 1996; 63:661-72.
17. Nalini V, Bax B, Driessen H, Moss DS, Lindley PF, Slingsby C. Close packing of an oligomeric eye lens beta-crystallin induces loss of symmetry and ordering of sequence extensions. J Mol Biol 1994; 236:1250-8.
18. Bova MP, Ding LL, Horwitz J, Fung BK. Subunit exchange of alphaA-crystallin. J Biol Chem 1997; 272:29511-7.
19. Bova MP, Yaron O, Huang Q, Ding L, Haley DA, Stewart PL, Horwitz J. Mutation R120G in alphaB-crystallin, which is linked to a desmin-related myopathy, results in an irregular structure and defective chaperone-like function. Proc Natl Acad Sci U S A 1999; 96:6137-42.
20. Lindner RA, Carver JA, Ehrnsperger M, Buchner J, Esposito G, Behlke J, Lutsch G Kotlyarov A, Gaestel M. Mouse Hsp25, a small shock protein. The role of its C-terminal extension in oligomerization and chaperone action. Eur J Biochem 2000; 267:1923-32.
21. Carver JA. Probing the structure and interactions of crystallin proteins by NMR spectroscopy. Prog Retin Eye Res 1999; 18:431-62.
22. Carver JA, Lindner RA. NMR spectroscopy of alpha-crystallin. Insights into the structure, interactions and chaperone action of small heat-shock proteins. Int J Biol Macromol 1998; 22:197-209.
23. Shih M, David LL, Lampi KJ, Ma H, Fukiage C, Azuma M, Shearer TR. Proteolysis by m-calpain enhances in vitro light scattering by crystallins from human and bovine lenses. Curr Eye Res 2001; 22:458-69.

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