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Deamidation alters the structure and decreases the stability of human lens β A3-crystallin

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Abstract

According to the World Health Organization, cataracts account for half of the blindness in the world, with the majority occurring in developing countries. A cataract is a clouding of the lens of the eye due to light scattering of precipitated lens proteins or aberrant cellular debris. The major proteins in the lens are crystallins and they are extensively deamidated during aging and cataracts. Deamidation has been detected at the domain and monomer interfaces of several crystallins during aging.

The purpose of this study was to determine the effects of two potential deamidation sites at the predicted interface of the β A3-crystallin dimer on its structure and stability. The glutamine residues at the reported *in vivo* deamidation sites of Q180 in the C-terminal domain and at the homologous site Q85 in the N-terminal domain were substituted with glutamic acid residues by site-directed mutagenesis. Far UV and near UV circular dichroism spectroscopy indicated that there were subtle differences in the secondary structure and more notable differences in the tertiary structure of the mutant proteins compared to wild type β A3-crystallin. The Q85E/Q180E mutant also was more susceptible to enzymatic digestion, suggesting increased solvent accessibility. These structural changes in the deamidated mutants led to decreased stability during unfolding in urea and increased precipitation during heat-denaturation. When simulating deamidation at both residues, there was a further decrease in stability and loss of cooperativity. However, multiangle-light scattering and quasi-elastic light scattering experiments showed that dimer formation was not disrupted, nor did higher-order oligomers form. These results suggest that introducing charges at the predicted domain interface in the β A3 homodimer may contribute to the insolubilization of lens crystallins or favor other, more stable, crystallin subunit interactions.

Cataracts account for half of all blindness according to the World Health Organization (1). The greatest incidence is in developing countries. A cataract is opacity within the lens of the eye due to scattering of light by precipitated proteins or by aberrant cellular debris. The major proteins within the lens belong to the α and β/γ -crystallin families. Protein concentrations can reach 400 mg/mL and above in the center of the lens, and it is their ordered packing that is necessary for transparency (2). There is an extremely low rate of turnover of crystallins in differentiated lens cells. Thus, crystallins accumulate modifications due to environmental and metabolic damage during an individual's entire lifetime. This makes the lens an easily accessible tissue to study the effects of post-translational modifications on protein unfolding and aggregation.

The major post-translational modifications in lenses are truncation, methylation, oxidation, disulfide bond formation, advanced glycation end-products and deamidation (3–12). Of these

modifications, deamidation has been difficult to quantitate because it results in only a single dalton change. Recently, Wilmarth et al have identified deamidation to be the most prevalent of these post-translational modifications in lens protein fractions from adult and cataractous lenses (11,13). This report counted the number of times tryptic peptides triggered spectra during chromatography and mass spectrometry and is biased towards peptides that have the least losses during these procedures. Nonetheless, the majority, 66%, of the tryptic peptides identified by mass spectrometry to be modified was deamidated. For β A3-crystallin nearly half the potential sites of deamidation have been confirmed to date with several sites deamidated between 35–43% (3, 77).

Given the long life span of the proteins in the adult lens, the large number of reported deamidation sites is not surprising. Robinson and Robinson have reviewed the mechanism of deamidation (14). Gln and Asn residues tend to form rings with the backbone nitrogen of the carboxyl side residue in a general base catalyzed reaction. This is followed by rapid hydrolysis on either side of the α carbon hydrogen to generate isomers. Alternatively, an imide can form between the amide nitrogen and the backbone carboxylic carbon of Asn, followed by cleavage. All amides have an intrinsic, genetically determined rate of deamidation and cleavage. Robinson and Robinson have proposed that deamidation sites are programmed into proteins to act as clocks to time molecular events (14). Deamidation has been demonstrated in other proteins to trigger turnover (15). Furthermore, specific Gln residues may be deamidated by transglutaminase that catalyzes crosslinking between Lys and Gln, but can also catalyze deamidation of the Gln (16). At a few sites, an Asn or Gln in one β -crystallin subunit is the corresponding acid at the homologous site in another subunit dictated by the gene sequence.

The accumulation of modified crystallins is associated with changes in their aggregation properties (7,17–20). The seven β -crystallin subunits form dimers and complex hetero-oligomers that change in size and relative amounts during aging (7,18,21–23). Modified β -crystallins were found to form complex mixtures that were difficult to separate (3). Altered crystallin interactions may lead to insolubilization. More significantly, there was the greater number of deamidated peptides identified in the insoluble proteins by Wilmarth and co-workers (11).

How deamidation may alter crystallin interactions and cause protein insolubilization is not known. Deamidation introduces a negative charge at physiological pH by replacing an amide with a carboxyl group. A potential mechanism may be in disruption of a critical structural region in the protein.

The crystallins in the β/γ -crystallin superfamily are highly homologous, containing a common polypeptide chain fold characterized by the “Greek key motif”. Individual monomers have two domain regions linked by a connecting peptide that is either bent as in β B1-crystallin (24) or extended as it is for β B2-crystallin (25). The β -crystallins also have N-terminal or N- and C-terminal extensions. The role of the extensions and connecting peptide in oligomer formation remains speculative and may differ depending on the crystallin subunit (26–30). From the X-ray crystal structures, there are hydrophobic interactions and H-bonds across the domain interfaces that most likely influence formation of the complex β -crystallin oligomers (31,32). Surface charges at the interface may also be important (33).

Numerous deamidations have been reported in the crystallins, with differences in both the sites and levels of detection (3,11,13). Deamidation in β B1 can have differing effects depending on the deamidation site (21,34). Our research and that of others has focused on deamidations that occur at the domain interfaces (34–37). Since, deamidation at Q180 which lies at the predicted interface in β A3-crystallin has been reported to increase during aging (3), studies were conducted to determine the effect of deamidation at this key structural region. The level of

deamidation at Q180 were estimated to be as high as 14% in the lens of a 28-year-old donor, but has not been reported at the homologous residue in the N-terminal domain (3). This site, Q85, is in a long tryptic peptide and may not have been detected by current methods. We have previously reported that deamidation at the domain interface in the β B1-crystallin dimer and the subunit interface in the β B2-crystallin dimer decreased stability of the dimer (35,38). Similar results have been reported at the intradomain interface of the γ D-crystallin monomer (36,37). While, the crystal structures for the above proteins are known, the crystal structure for β A3-crystallin is not. Therefore, the purpose of this study was to probe interactions across the predicted interface of the β A3-crystallin dimer by determining the effects of two potential deamidation sites and to compare these results with changes in stabilities reported for the other crystallins.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Proteins

Wild type human β A3-crystallin (WT) was recombinantly expressed as previously described (34). Briefly, total RNA was extracted from lens tissue from an anonymous human donor less than a year old (Lions Eye Bank of Oregon). RNA was transcribed into cDNA. The forward, C CAT ATG GAG ACC CAG GCT GAG and reverse, AGG ATC CAT CAG CTACTG TTG GAT TC of WT gene specific primers (GenBank accession No. NM 005208) with added restriction enzyme sites were used to generate a PCR fragment. This was inserted into the pCR-Blunt II-TOPO vector by blunt-end ligation (Invitrogen, Carlsbad, CA) and then transformed into TOP 10 *Escherichia coli* (*E. coli*) cells. The sequence containing the full-length WT was then subcloned into pET-3a vector (Novagen, San Diego, CA) using the restriction enzyme sites.

In vivo deamidations were mimicked at glutamines 85 and 180 in β A3-crystallin by replacing the glutamine with glutamic acid residues by site directed mutagenesis using internal primers containing the desired mutation (QuikChange Mutagenesis, Stratagene, Cedar Creek, TX) as previously described for β B1- and β B2-crystallins. The forward primers contained the sequence TCT GTG GGC AAG AGT TTA TCC and TCG TGG GTA TGA GTA TAT CTT GG to generate β A3-crystallin mutants Q85E and Q180E, respectively. The mutation was introduced into the WT β A3 sequence in the same pCR-Blunt II-TOPO vector described above and then subcloned into the pET-3a vector (Novagen). The double mutant (Q85E/Q180E) was made with the primer introducing the Q180E mutation into the vector containing the Q85E mutation. Clones containing the correct insert were confirmed by sequencing (Nevada Genomics Center, Reno, NV).

Recombinant β A3-crystallins were expressed using the *E. coli* strain BL21 (DE3) *pLysS* (Invitrogen). Cells were grown in LB culture medium containing 1% D-glucose. To express these recombinant proteins, cells were induced with 0.4 mM IPTG when cultures reached an OD of 0.4 at 600 nm. After four hours of culturing, cells were harvested and freeze-thawed three times, followed by vortexing in first-step chromatography buffer containing protease inhibitor cocktail (Roche, Mannheim, Germany).

All proteins were purified from *E. coli* by successive ion-exchange chromatography. All cell lysates were applied to a DEAE Sepharose Fast Flow anion exchange column (Amersham Biosciences, Piscataway, NJ) equilibrated in a Tris buffer (pH 7.8) for the first step of purification and a SP Sepharose cation exchange column (Amersham Biosciences, Uppsala, Sweden) equilibrated in a MES buffer (pH 5.6) for the second step. A 50 mM Tris buffer with 2 mM EDTA, and 1 mM DTT was used for an anion exchange column, and a 20 mM MES buffer containing 2 mM EDTA and 1 mM DTT was used for cation exchange chromatography. Each protein was eluted with a 0–500 mM NaCl gradient for first and second steps. After

purities of the proteins were checked by electrophoresis, mass spectrometry (LTQ, ThermoFinnigan, San Jose, CA) was performed to confirm the deamidation sites, as previously described (21,34).

Circular Dichroism Measurements

Circular Dichroism Measurements in the near-UV, and far-UV range were obtained using a JASCO J-810 spectropolarimeter (JASCO, Easton, MD). Samples were exhaustively dialyzed into 5 mM NaH₂PO₄, 5 mM Na₂HPO₄ (pH 6.8), containing 100 mM NaF, and measured in a 1.0 cm cell for near-UV and 0.1 cm for far-UV. Experiments were performed at 4 °C for better signal to noise ratios. This was particularly important below 200 nm. Spectra scans were done in triplicate at 1 nm resolution. Experiments were repeated on three different protein preparations except for the double mutant, which was repeated on two different protein preparations. Protein concentrations were 0.2–0.3 mg/mL for near UV-CD, and 0.1–0.15 mg/mL for far UV-CD. Concentrations were determined by UV absorbance at 280 nm either in buffer or in 6 M GuHCl or by amino acid analysis, with good agreement between the methods. Spectra were processed using CDtool from Birkbeck College, London (39). The percent secondary structure was calculated using DICHROWEB website located at (<http://www.cryst.bbk.ac.uk/cdweb/html/home.html>) which is part of the BBSRC Centre for Protein and Membrane Structure and Dynamics (40–42). A modification of the Variable Selection method, CDSSTR, was used to analyze data from 270 to 185 nm(41,43). The mean square error of the algorithm is 4 % (41).

Associative Behavior Analysis of Expressed Proteins

Samples were concentrated to between 0.4 and 15 mg/mL using 10,000 MWCO cellulose centrifuge spin filters (Amicon, Millipore, Billerica, MA) and filtered to remove particulate matter. Samples were analyzed at each concentration step before concentrating higher to avoid dilution effects later. Size-exclusion chromatography (SEC) was performed in-line with multi-angle laser (MALS) and quasi-elastic (QELS) light scattering (miniDAWN, Wyatt Technology, Santa Barbara, CA), to determine the association state of β A3-crystallins by measuring molar masses and sizes (27). A 50 μ L sample was injected onto a Superose 12 10/300 GL column (Amersham Biosciences) equilibrated in buffer containing 29 mM Na₂HPO₄, 29 mM KH₂PO₄, 100 mM KCl, 1 mM EDTA, 1 mM DTT (pH 6.8) with a flow rate of 0.4 mL/min.

The weight-averaged molar masses were calculated with software provided by the manufacturer (ASTRA IV, Wyatt Technology) as previously described (21). The software reported the radius of hydration, R_H , assuming a spherical conformation. The frictional coefficient, f , was determined from this value using Stokes's Law, $f=6\pi\eta R_H$, where η is the viscosity of the solvent. The frictional coefficient for β A3-crystallins, f_0 , was compared to the theoretical frictional coefficient of β A3-crystallins, f_0 , in a spherical conformation, and the predicted axial ratio was estimated (21). Assuming a sphere, the volume of β A3 can be estimated and used to extrapolate its radius, R_S , using $R_S = (3M_W v/4 \pi N_A)^{1/3}$, where v is specific volume, 0.72 cm³/g plus the specific volume of water, 0.3 cm³/g (44).

Stability of Expressed Proteins

Stability of deamidated mutants relative to WT β A3 was measured by fluorescence spectrometry during protein unfolding/refolding in urea. A stock solution of 9.07 M urea in phosphate buffer was prepared according to the method of Pace (45). An Abbe refractometer was used to measure the urea concentration (VEE GEE Scientific, Inc., Kirkland, WA). The fluorescence buffer (pH 7.0) contained 50 mM Na₂HPO₄, 50 mM NaH₂PO₄, 5 mM DTT, and 2 mM EDTA. Proteins at 1 μ M were incubated in urea for 24 h at 22 °C. Initial timed fluorescence experiments determined that the proteins completely unfolded in 8 M urea after

5 h. For refolding experiments, proteins were thus incubated in 8 M urea for 5 h, then diluted to the desired urea concentration and incubated for a total 24 h.

Fluorescence intensities were measured on a Photon Technology International QM-2000-7 spectrometer using the manufacture's supplied software, FeliX (Photon Technology International, Lawrenceville, NJ). Emission spectra were recorded between 300 and 400 nm with an excitation wavelength at 285 nm or 295 nm. Slit widths were set to 2 nm. Emission spectra were corrected for the buffer signal.

The extent of unfolded protein was calculated from the normalized intensities (FI 360/320). In order to determine the free energy of unfolding, the system must achieve reversible equilibrium at each stage of unfolding. Relative stabilities were determined in this study by fitting the data to a two-state model and measuring the denaturant concentration at the transition midpoint, C_m , and approximating the apparent free energy, apparent ΔG_D . The following two-state model was employed:

$$K_D = f_D / f_N$$

Where f_N is the fraction on native protein, f_D is the fraction of denatured protein. The apparent ΔG_D , at any denaturant concentration is Apparent

$$\text{Apparent } \Delta G_D = -RT \ln K_D$$

T is the absolute temperature, and R is the universal gas constant in $\text{kcal mol}^{-1} \text{K}^{-1}$. In the case of urea denaturation, apparent ΔG_D is linearly related to the urea concentration in the transition points such that Apparent

$$\text{Apparent } \Delta G_D = \text{Apparent } \Delta G_D^0 - mc$$

where the apparent ΔG_D^0 is the reference-state apparent Gibbs free energy of unfolding without urea. The apparent m value is the slope of the line relating unfolding to denaturant concentration. The midpoint of the transition, C_M is calculated from apparent $\Delta G_D^0 = mC_M$ and provides the urea concentrations at the midpoint of denaturation.

Heat Solubility Analysis

Heat induced precipitation of $\beta A3$ -crystallins were measured in a microtiter plate reader at various times. Following chromatography, samples were buffer exchanged into 10 mM Tris buffer (pH 7.1) containing 1 mM DTT and concentrated to 0.5 mg/ml. Aliquots of 100 μL were placed into wells and the plate heated at 55 °C with continuous shaking at 110 rpm. Absorbances were read at 405 nm. Experiments were performed in triplicate. In a separate experiment proteins were heated at 55°C in a thermocycler for 180 min and then centrifuged at $15,000 \times g$ to fractionate the precipitated proteins. Equal proportions of supernatant and pellet were analyzed.

Enzyme Accessibility Analysis of Expressed Proteins

Protein accessibility to solvent was determined by digesting proteins with trypsin (Promega, Madison, WI). The buffer conditions used for tryptic digestion were the same as those described for CD analysis, except 0.1 M NaHCO_3 was added to maintain trypsin activity. Reactions were stopped by adding 5% formic acid. Mass spectrometry was used to determine the extent of trypsin digestion (LCQ, ThermoFinnigan).

Molecular Modeling

Since β A3-crystallin has previously been modeled as “opened” and “closed” monomers or as a dimer (pdb: 1BLB, (29)), homology modeling was used here to predict the effects of introducing a negative charge across the interface of both conformations. The human β A3-crystallin sequence was modeled using either the bovine β B2-crystallin dimer structure (pdb: 1BLB) as an open dimer model (29) or the human β B1-crystallin (pdb: 1OKI) as a close dimer model. The models were refined by energy minimization with MOE software (Chemical Computing Group Inc., Montreal, Canada). Both the highly variable N- and C-terminal extensions were omitted from the modeling. Another non-homologous region, S100-A104, resulted in a loop not seen in either β B2- or β B1-crystallin, and was deleted from the model. The models are represented in ribbon representation using DS ViewerPro software (Figure 1, Accelrys Inc, San Diego, CA). Solvent accessible surface area (SASA) of equivalent positions of Q85 and Q180 in the β A3 model was calculated by the software GETAREA1.1 (46).

Electrophoresis

Electrophoresis was performed using precast, 1.0 mm thick 8×8 cm, polyacrylamide NuPAGE 10 % Bi-Tris gels (Invitrogen, Carlsbad, CA). Proteins were visualized by staining with SimplyBlue SafeStain (Invitrogen).

Protein Concentration

Concentrations were calculated from the UV absorbance at 280 nm and an extinction coefficient for β A3-crystallin of $2.62 \text{ (mg/mL)}^{-1}\text{cm}^{-1}$ or a molar extinction coefficient of $65,290 \text{ (M}^{-1}\text{cm}^{-1})$ (www.scripps.edu/cgi-bin/cdputnam/protcalc3). Where noted, amino acid analysis was performed (Molecular Structure Facility, UC Davis).

RESULTS

The Effect of Deamidation on the Structure of β A3-Crystallin

Homology modeling was done to predict the effects of deamidation on the β A3-crystallin structure (Figure 1). A crystal structure for β A3 is not known. Therefore, β A3 was first modeled using either a β B1-crystallin-like closed (Figure 1A–C) or a β B2-crystallin-like open dimer (Figure 1D–F) for a template, as was previously demonstrated by Sergeev et al (29). The sequence homology of the globular domain regions between β A3 and β B1 is 46% and between β A3 and β B2 is 42%. Despite these high homologies, the WT β A3 model had less defined structure than either β B1 (Figure 1A and B) or β B2 (Figure 1D and E), including an extra loop between S100 and A104. This region did not lie near the region of interest for this study and is not shown in Figure 1. Solvent accessibility measurements were made to approximate the accessibility of Q85 and Q180 using GETAREA 1.1. Both side chains were buried between 81 and 97%, with the more buried Glu side chains in the closed model. This was in good agreement with the values calculated for the homologous residues in β B1 (Q204, 99%) and β B2 (Q70, 86% and Q162, 87%). When Glu were introduced at these residues, there was a shift in the orientation of the side chains and a small increase in the distance between the domains (Figure 1C and F). In β B1, there was H-bonding across the domain interface between Q204 and I117 that was maintained in the closed β A3 model, but not when Glu residues were introduced. The H-bonding across the interface in β B2 was not maintained in the open β A3 model.

WT β AS and deamidated mutants were expressed in *E. coli* in the soluble proteins. All recombinant proteins were isolated by the same methods with similar yields. After purification, only a single protein band was observed by SDS-PAGE electrophoresis indicating high purity (Figure 2). The presence of Glu at residues 85, 180, and at both residues in the double mutant

was confirmed by mass spectrometry (Figure 3). Mass spectrometry also did not detect any other proteins present in significant amounts.

Circular dichroism measurements of WT β A3 and the deamidated β A3 mutants showed typical β -strand rich structures characteristic of β -crystallins with strong maxima at 198 nm and minima at 214 nm in the far-UV range (Figure 4A). The slight minimum at 230 nm, unique to β A3 among the β -crystallins (47), was also observed. Wild type β A3 folded with 41% total β -strand content (Table 1). Differences in the spectra of the singly and doubly deamidated mutants below 195 nm, resulted in decreases in the estimated helical contents of the mutants, which corresponded to increases in β -turns and other structure as reported in Table 1. These changes were consistent for all three mutants. The near-UV CD spectra of the four mutants differed with the double mutant having the greatest difference (Figure 4B).

The Effect of Deamidation on the Association of β A3-Crystallin

Introducing a negative charge across the domain interface of the β A3 did not alter the dimer formation (Figure 5). At all concentrations tested between 0.5 and 12 mg/ml, WT and the deamidated β A3 mutants formed oligomers, with masses suggesting dimer formation (Figure 5A) and with a radius of hydration, R_h , between 3.0 and 3.4 nm (Figure 5B).

To determine the effect of deamidation on the overall shape of β A3, the frictional coefficients, f , were determined from the R_h (Table 2). Taking an R_h for β A3 as 3.2 nm (Figure 5B) with a weight-averaged molar mass of 49 kDa (Figure 5A) gave a calculated f by the equation of Stokes's Law of 5.4×10^{-11} kg/sec. The ratio of this f value to that predicted for a sphere of the same M_w , f_0 , would give f/f_0 of 1.17. The results matched those of the previously published value of 1.13 for β -Low crystallin (22). This frictional ratio of β A3 would predict an axial ratio of 3.0–4.0 for a prolate structure (Table 2).

These experiments were repeated without salt to avoid the potential for the salt to mask the effects of the deamidations (Figure 6). While, there was a greater polydispersity reflected by the lower molar masses at the tailing edge of the peak, the molar masses were between 45–50 kD, suggesting predominantly dimer formation.

The Effect of Deamidation on the Susceptibility of β A3-Crystallin to Enzymatic Digestion

Tryptic digestion was performed to investigate the solvent accessibility of WT β A3 and Q85E/Q180E. Trypsin cleaved the first 17 amino acids from the N-termini of both proteins (Figure 7A, B). This was confirmed by mass spectrometry. Further digestion of WT occurred slowly with a visible loss of protein at 8 hr. In contrast, Q85E/Q180E was degraded after 2 hr. Deamidation increased the susceptibility of β A3 to further trypsin digestion, suggesting an increased solvent accessibility.

The Effect of Deamidation on the Stability of β A3-Crystallin

The relative stabilities of the deamidated mutants were compared with WT β A3 during unfolding in urea. Protein unfolding and refolding were detected by measuring fluorescence emission due to aromatic residues dominated by nine tryptophan residues in β A3 of which only four are buried (47). As denaturation of β A3 progressed in increasing urea concentrations, the fluorescence peak increased and shifted from 338 to 353 nm (data shown for WT β A3 Figure 8A). The proteins completely unfolded at urea concentrations ≥ 5.0 M. The emission spectra for all of the proteins were indistinguishable in the absence of urea with excitation at either 285 nm (Figure 8B) or 295 nm (Figure 8C). In order to compare to previously published results with β B2, 285 nm was used to monitor unfolding.

Unfolding of WT β A3 showed high cooperativity, without an observable transition in the unfolding curve. From the concentrations under the eluting peaks in the above MALS experiments, β A3 was predicted to be predominantly a dimer at the concentrations used for unfolding. Data were first fit to a three state model depicting the dimer dissociating to two monomers before unfolding. However, these calculations showed large uncertainties. For comparison, midpoints of transition curves were determined in order to rank stability. Apparent free energies were approximated from the unfolding and refolding curves and by assuming two states, native and denatured (Figure 9 and Table 3). A midpoint of transition of 4.5 M urea was observed for WT β A3 (Figure 9A). Deamidated β A3-crystallins unfolded at lower urea concentrations (Table 3). The relative apparent ΔG_D^0 's and the unfolding curves of Q85E and Q180E were indistinguishable, but lower than for WT β A3 (Figure 9A). There was a further decrease in stability for Q85E/Q180E, which had a midpoint of unfolding of 3.7 M urea and a loss of cooperativity (Figure 9B and Table 3).

Unfolding was reversible down to 2 M urea for WT and the mutants and refolding proceeded along similar paths to that observed during unfolding for all (Figure 9C). At urea concentrations < 2 M urea, there was a small increase in intensity due to light-scattering aggregates, indicating incomplete refolding.

The Effects of Deamidation on the Solubility of β A3-Crystallin during Heating

Wild type β A3 and Q85E/Q180E were heated at 55°C and the change in turbidities measured at 405 nm (Figure 10A). Experiments were performed at 55°C, because it had previously been reported that the majority of β A3 precipitated by 60°C (47). A lower temperature was chosen to distinguish differences between proteins and for comparison with our previously published data of WT β B1, which loses secondary structure at 60-65°C (27). Upon prolonged heating, WT β A3 had a greater turbidity than WT β B1 (Figure 10A). Deamidation significantly increased the turbidity of β A3 with the greatest increase occurring rapidly between 30 and 90 min. In a separate experiment, proteins were heated for 180 min and the precipitated protein visualized by SDS-PAGE. The same proportion of pellet was analyzed. A strong protein band was detected from the pellet of the Q85E/Q180E (Figure 10B, compare lanes 8 to 9).

DISCUSSION

The amount of deamidation in the normal human adult and cataractous lens is striking (7,11, 13). Yet, the significance of this modification in the lens is unknown. Deamidation may contribute to compromised function of the lens or simply accumulate due to the long life span of the lens proteins. The major results from this study found that deamidation in a highly conserved sequence and a predicted interface in β A3 decreased the stability without disrupting the dimer, suggesting an altered function of β A3.

Effect of Deamidation on β A3-Crystallin Structure

Wild type β A3 folded with the expected secondary structure similar to previous reports for β A3 and with a similar β -strand content to the β -Low fraction from bovine lenses (27,41,47–49). The maximum fluorescence intensity at 338 nm of WT was similar to previously reported values of 332–340 nm (47,50), reflecting two exposed and three partially exposed Trp. The intensity from excitation at 295 nm was 80% of the intensity from excitation at 285 nm. Thus, Trp emission strongly contributed to the emission at 285 nm (Figure 8). The maximum intensity for β B1 was at 336 nm and for β B2 at 227 nm, reflecting the fewer exposed Trp (35,38,47), with PB2 having only one exposed and one partially buried Trp (47).

Simulating deamidation *in vitro* at the predicted domain interface of β A3 did not dramatically alter secondary structure as indicated by only slight differences in the far-UV CD. There were

no differences in the predicted β -strand content, but there was an 8–9% decrease in helical content and a 4–5% increase in other structure of the mutants. This could be explained by a possible loss of order or denaturation of the N-terminal extension without complete unfolding due to a loss of order of the β -strands comprising the Greek key motifs. Due to the uncertainty in predicting secondary structure of beta sheet proteins, the significance of these differences is unclear.

A lack of differences in the fluorescence emission, likewise suggested that the globular domains of all the proteins had folded with similar structure. Since, a strong Tyr emission at 285 nm can mask changes in the Trp emission, experiments were also performed at 295 nm and gave similar results. This suggested that the buried Trp were not different between WT and the mutants or that the emission of the exposed Trp dominated the emission. Near-UV CD spectroscopy was more sensitive to the tertiary structural changes than fluorescence emission was, particularly between WT and Q85E/Q180E. Taken together the CD and fluorescence results suggest that deamidation at the interface slightly altered the structure of β A3.

Wild type β A3 formed a dimer with weight averaged molar masses between 47–52 kDa at all concentrations tested. Sergeev et al have reported a monomer-dimer equilibrium that was dependent on concentration and temperature (29). Since, a monomer was not detected in our studies, even at the lowest concentrations, it is more likely that the differences were due to differences in salt and ionic strengths of the buffers used. Indeed when experiments were repeated without salt, masses were lower than the predicted dimer mass under the tailing edge of the peak where the concentrations would also be less. The methods used in both studies, MALS and sedimentation equilibrium are sensitive to buffer conditions (51). The difference may also reflect the three additional Cys residues in the murine β A3 that replace Ser residues in the human.

Deamidation did not disrupt homodimer formation. Even mimicking 100% deamidation did not dissociate the homodimer or cause higher-order oligomerization. Maintaining the neutral charge of these Gln residues at the interface appeared not to be critical for the folding of the domains or for dimer formation. Similar results were obtained for β B2 (35,38). For β B1, deamidation in the N-terminal domain resulted in a slightly higher molar mass and an earlier elution on SEC, suggesting a much larger oligomer size than what was determined by MALS (35,38). These differences in elution and behavior between the β -subunits, may partially be explained by a greater difference in secondary structure between WT β B1 and β B1 Q204E that was not detected in the other β -subunits. Recently, it has been reported that disruption of an ion pair across the dimer interface in the β B2 E74R mutant resulted in a more monomer-like protein with a drastically reduced solubility (52). This supports that ion pairs stabilize the hydrophobic interactions across the interface, while the conserved Gln residues fine-tune these interactions.

Comparison of the measured frictional coefficients to those calculated for spheres of the same molecular weights resulted in similar axial ratios using either a predicted oblate or prolate ellipsoid. A prolate would be more descriptive of the open β B2 model and an oblate more descriptive of a closed β B1 model. Other methods will be needed to distinguish possible shape differences between the two different models.

The Q85E/Q180E mutant was more susceptible to enzymatic digestion than WT, suggesting an increase in solvent accessibility. Further supporting structural differences particularly of Q85E/Q180E was the observation that the Q85E/Q180E eluted later on a cation exchange column suggesting a more exposed charge. This may be similar to reports that introducing a charge at the interface did not change the overall fold of γ B-crystallin similar to what was seen here for β A3, but created a cavity filled with water molecules (32).

The above results are consistent with the modeled structure of deamidated β A3 and with what is known about β B1 and β B2 from their crystal structures (24,25,53). The side chains of Q85 and Q180 in β A3 and the homologous residues in β B1 and β B2 are at least partially buried and matched with what was reported for the homologous residues in γ D-crystallin (54). These residues are involved in H-bonding across the domain or monomer interface. Hydrophobic residues also surround them. Introducing charged groups from Glu residues did not alter the secondary structure, in agreement with our experimental data. In the Q85E/Q180E model, there was a slightly greater distance across the interface, with the charges oriented away from each other. In the closed monomer model of WT β A3, the side chain of Q180 H-bonded with I87 across the domain interface. This was lost in the Q85E/Q180E model. The loss of H-bonds and the potential charge repulsions at the interface of the Q85E/Q180E, appear not to be great enough to dissociate the dimer, suggesting that the hydrophobic interactions may be able to counter these effects. The subtle structural changes due to deamidation are consistent with our experimental data and can explain the effect on stability described below.

Deamidation of β A3-Crystallin Decreases Stability

The urea stability of β A3 was intermediate between what we have reported before for β B1 and β B2 (35,38). The urea concentrations for half unfolding of β B2, β A3, and β B1 were 2.9 M, 4.5 M, and 5.9 M, respectively.

The unfolding mechanisms for several members of the β/γ crystallin family have been hypothesized to contain several intermediates. Sergeev et al have described the dissociation of the β A3 dimer as a fast reversible monomer-dimer equilibrium with the intermediate containing both stable closed monomers and unstable open monomers (29). Even the unfolding of the monomer, γ D, was best fit by three transitions with the first and second transition unfolding of the N-terminal domain and the third transition unfolding of the C-terminal domain (54). However, fitting the data to a three state model resulted in large errors of at least one of the transitions for β B2 (35), γ D (37), and for β A3 in this study. Silent intermediates have been hypothesized for small globular proteins, where the intermediates are less stable than the unfolded state (55). Only when mutations were made in the wild type proteins were inflections in the unfolding curves present (35,50,54), suggesting that the intermediates had been stabilized. With Q85E/Q180E there was a loss of cooperativity, but not a plateau in the unfolding/ refolding curves. Other methods, such as CD, may be needed to detect the intermediates.

Deamidation in β A3 decreased its stability during urea-induced denaturation and heat-induced precipitation. The urea unfolding/refolding curves for Q85E and Q180E were shifted to lower urea concentrations. Similar results were obtained at the homologous residues in β B1 and β B2. Recently, the N- and C-terminal domains of β A3 were expressed independently and the C-terminal domain was determined to be less stable (50). In contrast, the N-terminal domain of β B2 is less stable than the C-terminal domain (56). Yet, with both β B2 and β A3, the single deamidated mutants were similarly destabilized regardless of the domains in which the deamidation was introduced ((35) and Figure 9A). This suggests that the effects of deamidation at these sites are at the interface rather than in the domains directly.

When simulating deamidation at both residues, there was a further decrease in stability and loss of cooperativity. The loss of cooperativity was most notable at lower urea concentrations. However, a distinct plateau in the transition for Q85E/Q180E was not observed as had been for Q70E/Q162E β B2 (35) and for Q204E β B1 (38), suggesting deamidation may have affected the unfolding of β B1 and β B2 differently than for β A3.

The difference in the apparent ΔG_D^0 s between WT β A3 and the deamidated mutants is consistent with a loss of H-bonding observed in the Q85E/Q180E model. Charge repulsion

and dissociation of H-bonding are enough to account for decreasing stability without dramatic structural changes.

In summary, simulating deamidation across the domain interface in β A3-crystallin led to subtle changes in secondary and tertiary structure without disrupting dimer formation. However, these structural changes were enough to decrease stability upon denaturation in urea or heat. Deamidation at the interface alone in β A3 or as previously reported in β B1 and β B2 was not enough to trigger spontaneous unfolding or precipitation. However, the accumulation of many deamidations over a long period of time such as occurs *in vivo* may lead to deamidation-mediated decrease in protein solubility. Of the reported deamidation sites to date in β A3, eight sites were more abundant in the insoluble proteins from aged lenses (11). While deamidation at Q85 or Q180 were not increased in the insoluble proteins, deamidation at these sites may contribute to decreased solubility by increasing the susceptibility of proteins to other modifications.

Alternatively, in other tissues deamidation has been proposed to have specific functions (14). The Gln 85 and 180 in β A3 are conserved among all the human β -crystallins, except for in β B1 where a Met replaces the Gln in the N-terminal domain (7,57) and are located in highly conserved sequences of 10-15 residues, suggesting biological function. Based on their primary sequences, their deamidation rates would be predicted to be low and are in agreement with the limited data available on their *in vivo* rates (3,14). This may imply controlled deamidation at these sites.

The observed changes in structure and stability may facilitate hetero-oligomer interactions by weakening the interface interactions of the homodimer. This “breathing” at the interface has recently been suggested as a potential mechanism by which β B2 forms complex hetero-oligomers and may also be important for β A3 (52,58). Considering the low level of deamidation at these sites, this may be a fine-tuning mechanism to maintain crystallin order later in life. This would not exclude the possibility that the accumulation of large amounts of deamidation could eventually trigger precipitation. Future studies comparing deamidation sites in different regions should shed light on these different possibilities.

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ABREVIATIONS

BBSRC	Biotechnology and Biological Sciences Research Council
DTT	dithiothreitol
EDTA	ethylendiaminetetraacetic acid
FI	fluorescence intensity

GuHCl	guanidine hydrochloride
IPTG	isopropyl-beta-D-thiogalactopyranoside
MES	2-(N-morpholino)ethanesulfonic acid
MWCO	Molecular weight cut off
PDB	Protein Data Bank
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

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Figure 1.

Three-dimensional model structure of deamidated β A3-crystallin. (A) The β B1-crystallin crystal structure used as the template (PDB: 1OKI). (B) The WT β A3 “closed” monomer model from the β B1-crystallin. (C) The Q85E/Q180E of β A3 “closed” monomer model. (D) The β B2-crystallin crystal structure used as the template (PDB: 1BLB). (E) The WT β A3 “open” dimer model from the β B2-crystallin dimer. (F) The Q85E/Q180E of β A3 “open” dimer model. The Q85E and Q180E at the interface are shown in ball and stick.

Figure 2. Gel electrophoresis of purified recombinant β A3-crystallins. Molecular weight markers (MW); WT (Lane 1); Q85E (Lane 2); Q180E (Lane 3); Q85E/Q180E (Lane 4). One microgram of each protein was visualized with Coomassie stain on a 1.0 mm thick, 10% Bis/Tris gel.

Figure 3. Mass spectrum of peptide 178–193 from recombinant β A3-crystallin containing the Q180E mutation. The parent ion of peptide 178–193 was doubly charged with a measured monoisotopic m/z of 978.4 (unmodified peptide monoisotopic m/z is calculated to be 977.9).

Figure 4. (A) Far-UV CD spectra and (B) Near-UV CD spectra of WT, Q85E, Q180E, and Q85E/Q180E of β A3-crystallin. Samples contained 10 mM sodium phosphate and 100 mM NaF (pH 6.8) and were measured in a 0.1 cm cell for far-UV, and 1.0 cm for near-UV at 4 °C.

Figure 5.

(A) Molar mass and (B) radius of hydration of the WT ($\circ$), Q85E (Δ), Q180E ($\times$), and Q85E/Q180E ($\square$) of β A3-crystallins determined by SEC in line with MALS or QELS. Column was equilibrated in 58 mM Na/K phosphate (pH 6.8), 100 mM KCl, 1 mM EDTA, 1 mM DTT with a flow rate of 0.4 mL/min. Predicted molar mass for the WT β A3 dimer is 50,250 Da. Molar masses were determined on three different sample preparations and on two different sample preparations for R on WT at 4 mg/mL. Error bars are shown.

Figure 6.

Chromatogram of molar masses of WT ($\circ$), Q85E (Δ), Q180E ($\times$), and Q85E/Q180E ($\square$) of β A3-crystallin determined by SEC-MALS in 100 mM sodium phosphate, 5 mM DTT, and 2 mM EDTA (pH 7.0). The line tracing represents the signal from the UV detector. The individual data points represent the molar masses in a narrowly eluting volume. A 50 μ L sample of 3.6–4.0 mg/mL was analyzed for each protein.

Figure 7.

(A) Trypsin digestion of WT and Q85E/Q180E β A3-crystallins. WT digested for 2, 4, 6, and 8 h (Lanes 1–4); Q85E/Q180E digested for 2, 4, 6, and 8 h (Lanes 5–8). (B) WT and Q85E/Q180E of β A3 incubated for 8 h without trypsin. WT (Lane 1); Q85E/Q180E (Lane 2), molecular weight markers (MW).

Figure 8.

(A) Urea-induced denaturation of WT β A3 as measured by fluorescence spectrometry at 285 nm. A 1 μ M sample of WT was incubated in 0 M, 4 M, 4.25 M, 5 M, and 7 M urea for 24 h at 22 °C. (B) Fluorescence emission at 285 nm and (C) 295 nm of the 1 nM sample of WT (○), Q85E (Δ), Q180E (×), and Q85E/Q180E (□) of β A3-crystallins were incubated in 0 M urea for 24 h at 22 °C.

Figure 9.

(A) Equilibrium unfolding of WT (○), Q85E (△), Q180E (×), and Q85E/Q180E (□) of β A3-crystallins in 0–8 M urea at 285 nm excitation. Proteins were analyzed at 1 μ M concentration. Samples were excited at 285 nm and emission intensities recorded as described in the text. Data were analyzed as the ratio of fluorescence intensities at 360/320 nm (FI 360/320 nm). (B) Equilibrium unfolding of WT (○), and Q85E/Q180E (□) of β A3 in 2.5–5.5 M urea at 285 nm excitation. (C) Equilibrium unfolding of WT (○), and refolding of WT (●) of β A3 in 0–8.0 M urea at 285 nm excitation. Samples were incubated in urea buffer at 22 °C, of 5 h, then diluted to refold as described in the text.

Figure 10.

(A) Thermal aggregation/precipitation curves. Turbidity of WT β B1-crystallin (Δ), WT β A3-crystallin ($\circ$), and Q85E/Q180E β A3-crystallin ($\square$) at 55 °C and a concentration of 0.5 mg/ml. The change in turbidity was measured at 405 nm on a microtiter plate reader. Error bars are standard derivations, N=3. (B) Samples heated for 180 min were also separated into supernatant and pellet and visualized by SDS-PAGE. Molecular weight marker (MW), WT β B1 before heating, supernatant, and pellet after heating (Lanes 1, 2, and 3); WT β A3 before heating, supernatant, and pellet after heating (Lanes 4, 5, and 6); and Q85E/Q180E of PA3 before heating, supernatant, and pellet after heating (Lanes 7, 8, and 9).

Table 1

Secondary Structure Prediction of WT β A3-crystallin, Q85E, Q180E, and Q85E/Q180E as Determined by DIHCROWEB (42,43).

mutant	percent of structure (%)			
	helix	sheet	turn	other
WT β A3	15	41	19	25
Q85E	6	40	23	30
Q180E	7	40	23	29
Q85E/Q180E	7	41	21	30

Table 2
Molar Mass, Size, and Predicted Axial Ratios of Recombinant β -crystallins.

Protein	MW (kDa)	R (nm)	$f(\times 10^{-11} \text{ kg/sec})$	flf_0	Predicted Axial Ratio
WT β A3	49 $\pm$ 2	3.2 $\pm$ 0.2	5.4 $\pm$ 0.3	1.17 $\pm$ <0.1	3.0–4.0
Q85E	49 $\pm$ 2	3.2 $\pm$ 0.2	5.4 $\pm$ 0.3	1.17 $\pm$ <0.1	3.0–4.0
Q180E	50 $\pm$ 2	3.1 $\pm$ 0.2	5.2 $\pm$ 0.3	1.13 $\pm$ <0.1	3.0–4.0
Q85E/Q180E	50 $\pm$ 2	3.1 $\pm$ <0.1	5.2 $\pm$ 0.2	1.13 $\pm$ <0.1	3.0–4.0
WT β B1 ^a	52 $\pm$ 4	3.4 $\pm$ <0.1	5.7 $\pm$ <0.1	1.22 $\pm$ <0.1	4.0–5.0
WT β B2 ^b	45 $\pm$ 3	2.8 $\pm$ <0.1	4.7 $\pm$ <0.1	1.06 $\pm$ <0.1	2.0–3.0

^aFrom reference (21)
^bFrom reference (35).

Table 3Apparent Free Energy of unfolding for WT, Q85E, Q180E, and Q85E/Q180E β A3-crystallin.

Protein	^a C _M	^b Δ C _M	^c Apparent Δ G _D ⁰	^d Apparent $\Delta\Delta$ G _D ⁰
WT β A3	4.5 $\pm$ 0.1	–	14 $\pm$ 2.0	–
Q85E	4.2 $\pm$ 0.1	–0.3 $\pm$ 0.1	10 $\pm$ 0.1	–4.0 $\pm$ 2.1
Q180E	4.1 $\pm$ 0.1	–0.4 $\pm$ 0.1	9.6 $\pm$ 0.8	–4.4 $\pm$ 1.9
Q85E/Q180E	3.7 $\pm$ 0.1	–0.8 $\pm$ 0.1	4.4 $\pm$ 0.4	–9.6 $\pm$ 2.4

^aThe midpoints of unfolding transitions for mutants from extrapolation method.^b Δ C_M=C_Mmutant–C_Mwildtype in units of M.^cThe Apparent Δ G⁰ (kcal/mol), of unfolding based on urea curves. $RT \ln K$ values were calculated for $K = f_D/f_N$ from the transition region of the urea denaturation curves.^dApparent $\Delta\Delta$ G_D⁰= Δ G_D⁰mutant– Δ G_D⁰wild type in units of kcal/mol.