Deamidation Affects Structural and Functional Properties of human αA-Crystallin and its Oligomerization with αB-Crystallin

R. Gupta and O. P. Srivastava
Department of Physiological Optics
University of Alabama at Birmingham, Birmingham, AL

Correspondence address: O. P. Srivastava
Department of Physiological Optics,
Worrell Bldg. 924 South 18th Street
University of Alabama at Birmingham
Birmingham, AL, 35294-4390
Email: srivasta@uab.edu
Phone number: (205) 975-7630

Running Title: Deamidation affects α-crystallin properties

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Abbreviations

AIAS: 4-acetamido-4'-[(iodoacetyl)amino]stilbene-2,2'-disulfonic, disodium salt, ANS: 1-anilino-8-naphthalenesulfonate, CD: circular dichroism, DTT: Dithiothreitol, FRET: Fluorescence Resonance Energy Transfer, LYI: lucifer yellow iodoacetamide, PCR: polymerase chain reaction.
SUMMARY

To determine the effects of deamidation on structural and functional properties of alpha A-crystallin, three mutants (N101D, N123D and N101D/N123D) were generated. Deamidated alpha B-crystallin mutants (N78D, N146D and N78D/N146D), characterized in a previous study (Gupta R, Srivastava O. P., Invest Ophthalmol Vis Sci., 2004; 206-214), were also used. The biophysical and chaperone properties were determined in: (a) homoaggregates of alpha A-mutants (N101D, N123D and N101D/N123D), and (b) reconstituted heteroaggregates of alpha-crystallin containing: (i) WT-alpha A:WT- alpha B crystallins (ii) Individual alpha A-deamidated mutants: WT-alpha B crystallins [iii] WT-alpha A:individual alpha B-deamidated mutant crystallins. Compared to the WT-alpha A, the three alpha A-deamidated mutants showed reduced levels of chaperone activity, alterations in secondary, and tertiary structures, and larger aggregates. These altered properties were relatively more pronounced in the mutant N101D compared to the mutant N123D. Further, compared to heteroaggregates of WT-alpha A and WT-alpha B, the heteroaggregates containing deamidated subunits of either alpha A- or alpha B-crystallins, and their counterparts WT proteins, showed higher molecular mass, altered tertiary structures, lower exposed hydrophobic surfaces and reduced chaperone activity. However, the heteroaggregate containing WT-alpha A and deamidated alpha B-subunit showed lower chaperone activity, smaller oligomers, and three fold lower subunit exchange-rate than heteroaggregate containing deamidated alpha A- and WT-alpha B subunits. Together, the results suggested that: (a) Both Asn residues (N-101 and N-123) are required for the structural integrity and chaperone function of alpha A-crystallin. (b) Presence of WT-alpha B in the alpha-
crystallin heteroaggregate leads to packing-induced structural changes that affects the oligomerization and modulates chaperone activity.

INTRODUCTION

Alpha-crystallin, the most abundant protein in lens mature fiber cells constitutes approximately 35% of the total lens protein. \textit{In-vivo}, the \(\alpha\)A- and \(\alpha\)B- subunits at a ratio 3:1 form an oligomer of 800 kDa oligomer. Both \(\alpha\)A- and \(\alpha\)B-crystallins are small heat shock proteins (Hsp), and show molecular chaperone activity to protect proteins from aggregation in the eye lens (1-2). Because of this property, \(\alpha\)-crystallin is believed to play a crucial role in maintaining the lens transparency.

Like other small heat shock proteins, \(\alpha\)-crystallin also contains a highly conserved sequence of 80-100 residues (residue no. 62 to 143 in \(\alpha\)A- and no. 66 to 147 in \(\alpha\)B-crystallin) called \(\alpha\)-crystallin domain (3, 4). Based on similarities with the structure of other Hsps, it is believed that the N-terminal region (Residue no. 1-62 in \(\alpha\)A- and no. 1 to 66 in \(\alpha\)B-crystallin) of \(\alpha\)-crystallin forms an independently folded domain whereas the C-terminal (referred as the C-terminal extension; residue no. 143-173 in \(\alpha\)A- and no. 147 to 175 in \(\alpha\)B-crystallin) is flexible and unstructured (4). Previous reports show that removal of N-terminal residues (56 residues) and C-terminal extensions (32-34 residues) of \(\alpha\)A- and \(\alpha\)B-crystallin lead to improper folding, reduced chaperone activity and formation of trimers or tetramers (5-9). The \(\alpha\)-crystallin domain is believed to be engaged in the subunit-subunit interactions, but the individual amino acids in subunit interactions and chaperone activity have not been fully identified. Nevertheless, two disease-related point mutations of a highly conserved Arg at equivalent positions in \(\alpha\)A
(R116C) and αB (R120G) caused structural changes that lead to hereditary cataracts (10, 11).

During cataract development, the conformational changes, unfolding and subsequent cross-linking of crystallins are believed to lead to accumulation of insoluble cross-linked product of α-, β- and γ-crystallins. Present literature suggests a multifactorial mechanism for the development of cataract-specific cross-linked species, which might be driven by post-translational modifications. These included disulfide bonding (12), glycation (13), oxidation of Trp and His residues (14, 15), deamidation (16), and transglutaminase-mediated cross-linking (17). However, despite identification of these modifications in crystallins, their exact roles in the mechanism of crystallin cross-linking remain poorly understood.

Age-related deamidation of crystallins seems to be one of the major post-translational modifications that occur frequently. In-vivo the deamidation of α-, β-, and γ-crystallins has been shown in several studies (18-21). In αA-crystallin, greater deamidation of Q-50 and N-101 has been reported compared to Q-6 and N-123 in high molecular weight- and water insoluble-proteins (18, 22-23). Likewise, in αB-crystallin deamidation of Q and N residues have been shown (23, Srivastava, unpublished results).

Because deamidation introduces a negative charge, it is believed to cause alterations in protein tertiary structure, affecting their structural and functional properties. However, the effects of deamidation on structure and function of αA- and αB-crystallins have not been investigated until recently. Our recent report showed that the deamidation of N-146 residue and not N-78 residue in human αB-crystallin resulted in
significant changes in its structural and functional properties (24). Although both N-78 and N-146 residues are present in the conserved regions of the αB-crystallin, the dramatic effects of deamidation of N-146 residue, which is close to the C-terminal region, was surprising. A similar investigation of effect of deamidation of N-101 and N-123 residues (also present in the conserved region) of αA-crystallin on its structural and functional properties has been lacking in the literature. Additionally, although interaction between αA- and αB-crystallins in their oligomeric form is needed for the molecular chaperone activity, and for the maintenance of lens transparency (25-28), the effect of deamidation on subunit interactions and chaperone activity is also presently unknown. However, another intriguing question is how deamidation in αA- and αB-subunits affects the formation of the α-crystallin heteroaggregate, and affects its structural and functional properties.

It has been reported that the αA- and αB-crystallin subunits constantly undergo rapid exchange, which might be responsible for oligomer stability and suppression of the non-specific aggregation of crystallins (28). Consistent with this observation was a recent report (29) showing that mutation of R116C in αA-crystallin resulted in altered subunit composition with WTαB- in the heteroaggregates, and increased oligomer size compared to the oligomers of WTαA- and WTαB- subunits. Similarly, the mutation of Arg to Asp residue in αA-crystallin resulted drastic change in protein structure (30). This is consistent with Studer’s study which showed that chaperone activity is coupled to multimerization in bacterium α-HSP protein from Bradyrhizobium japonicum (31).

To answer the questions raised above, the objectives of this study were to determine: (a) the effect of deamidation of Asn on structural and functional properties of
αA-crystallin, and (b) the effect of deamidation of αA- or αB-crystallins on oligomerization with WTαA-/WTαB- subunits, and their structural and functional properties. The results presented show that the deamidation of both N-101 and N-123 in αA-crystallin altered its structural and functional properties, but relatively more so by deamidation of the N-101 residue. Similarly, compared to heteroaggregates of WTαA- and WTαB-crystallins, the heteroaggregates containing deamidated αA- or αB-mutants, and their counterparts WT proteins showed higher molecular mass, altered tertiary structure, lower exposure of hydrophobic surface and reduced chaperone activity. Further, heteroaggregate containing WTαA-crystallin and deamidated αB-mutants showed lower chaperone activity, lower sized oligomers, and three-fold lower subunit exchange rate compared to heteroaggregates containing deamidated αA-mutants and WTαB-crystallin.

**EXPERIMENTAL PROCEDURES**

**Materials:**

The restriction endonucleases *Nco*I and *Eco*RV, the molecular weight protein markers and DNA markers were purchased from Amersham Biosciences (Piscataway, NJ) and Promega (Madison WI), respectively. The T7 promoter, T7 terminator and other primers used in the study were obtained either from Invitrogen (Carlsbad, CA) or from University of Alabama at Birmingham-Oligo Synthesizing Core Facility. Molecular biology grade chemicals were purchased from Sigma (St. Louis, MO), unless stated otherwise. All chemicals for 2D-gel electrophoresis were from Amersham Biosciences (Piscataway, NJ) or BioRad (South San Francisco, CA). DEAE-Sephacel, Agarose gel
A1.5 and Butyl-Sepharose were obtained from Amersham Biosciences. Two fluorescent probes, lucifer yellow iodoacetamide (LYI) and 4-acetamido-4'-(iodoacetyl) amino stilbene-2, 2'-disulfonic, disodium salt (AIAS) were purchased from Molecular Probes (Eugene, OR). Unless indicated otherwise, all other chemicals used in this study were purchased from Fisher (Atlanta, GA) or Sigma (St. Louis, MO) companies.

Bacterial strains and plasmids:

E.coli BL21 (DE3) pLysS bacterial strain was obtained from Promega. The human αB-crystallin cDNA cloned on a plasmid pDIRECT was received from Dr. Mark Petrash, Washington University, St. Louis, MO. Cells were propagated in Luria broth, and recombinant bacteria were selected using ampicillin.

Site-specific Mutagenesis:

Deamidation of Asn (N) to Asp (D) residue at the positions 101 and 123 or both in αA-cDNA was introduced using Quickchange site-directed mutagenesis kit and following the manufacturer’s instructions (Stratagene, La Jolla, CA). Recombinant human αA-crystallin coding sequence was cloned in pDIRECT as described earlier (32). These constructs were used as templates with the mutated primers (where N was replaced by D, Table 1) to generate site-specific mutations. Briefly, 25 ng of template was used, and the PCR conditions were as follows: pre-denaturing at 95°C for 30 s, followed by 16 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 1 min. and extensions at 68°C for 5 min. After digestion with DpnI, 2 μl of the PCR product was
used to transform XL1-Blue supercompetent cells provided with the above Stratagene kit. The sequencing of DNA preparation identified the positive clones.

Expression and purification of wild-type and deamidated mutant proteins:

*E. coli* BL21 (DE3) pLysS was transformed with mutant amplicons using a standard *E. coli* transformation procedure. The crystallin constructs were grown in LB medium to a cell density of ~0.5-0.6 at 600 nm, and induced with 1 mM IPTG at 37°C for 3 h. The purification method for the WT and three αA-mutants was similar to one used previously to purify the WT and deamidated αB-mutants (24). Cells were harvested and after a freeze-thaw cycle, lysed in 50 mM Tris-Cl buffer, pH 7.5 containing 0.5 mM EDTA and 0.3 mM NaCl (TEN buffer). Following centrifugation at 17,000 x g for 20 min., the supernatant fraction was collected and dialyzed against 50 mM Tris-Cl buffer, pH 7.9 containing 0.5 mM EDTA and 1 mM DTT (TED buffer), and subjected to DEAE-Sephacel ion-exchange chromatography using a 3 cm x 30 cm column. The bound proteins were eluted with a gradient of 0 to 0.5 M NaCl in TED buffer. The αA-crystallin species containing fractions, identified by SDS-PAGE using a 15% polyacrylamide gel (33), were pooled and dialyzed against 50 mM phosphate buffer, pH 7.0 at 5°C for 24 h. Next, the preparation was subjected to hydrophobic interaction chromatography using a Butyl-Sepharose column (3 cm x 20 cm). The column-equilibration buffer was 50 mM phosphate buffer, pH 7.0 containing 0.5 M (NH₄)₂SO₄, and the bound proteins were eluted with a decreasing (NH₄)₂SO₄ concentration (i.e., 0.5 M to 0 M). The crystallin containing fractions were identified as above by SDS-PAGE, pooled and concentrated by ultra-filtration using Amicon stirred
cell (Millipore, Billerica, MA). The purity of WT and mutant αA-crystallin species was examined by SDS-PAGE, and two-dimensional (2D)-gel electrophoresis as described below. The protein concentrations were determined with either Pierce protein determination kit or absorbance at 280 nm. The total protein yields of different αA-species were as: WTαA-crystallin: 1 mg/ml, mutant N101D: 1.6 mg/ml, mutant N123D: 1.3 mg/ml, and mutant N101D/N123D: 1 mg/ml.

**Two-Dimensional (2-D) gel electrophoresis:**

The protein samples were mixed with resolubilization buffer (5 M urea, 2 M thiourea, 2% 3-[C3-cholamidopropyl]dimethyl-ammonio-1-propanesulfonat (CHAPS), 2% caprylylsulfobetaine 3-10, 2 mM tri-butyl phosphine, 40 mM Tris, pH 8.0) in the ratio 2:1, respectively (34). Each preparation was subjected to 2-D gel electrophoresis (IEF in the first dimension and SDS-PAGE in the second dimension). IEF separation was carried out using Immobiline Dry Strips (pH range of 3-10) by following manufacturer’s instructions (Amersham Biosciences). SDS-PAGE in the second dimension was performed using a 15% polyacrylamide gel by Laemmli’s method (33).

**Labeling of recombinant crystallins with different fluorescence probes:**

The cysteine residue in recombinant αA-crystallin species was covalently labeled with lucifer yellow iodoacetamide (LYI, 8.4 mM) by following Bova et al. method (28) and manufacturer’s instructions (Molecular Probes, Eugene, OR). The desired crystallin species (1 mg/ml) was dissolved in 20 mM MOPS buffer, pH 7.9 containing 100 mM NaCl. The disulfide bonds in proteins were reduced using 10-fold molar excess of Tris-
(2-carboxyethyl) phosphine) (TCEP, 15 µl of 1 M solution) to liberate free thiol for maximum labeling followed by incubation first at room temperature for 14 h and then at 37°C for 6 h. Upon completion of the reaction, 5-fold excess of β-mercaptoethanol was added to consume excess thiol-reactive reagent. Similarly, using exactly the above conditions, the recombinant αB-crystallin species were labeled with 4-acetamido-4′-[iodoacetyl] amino] stilbene-2, 2′-disulfonic, disodium salt (8.4 mM, AIAS) by incubation at room temperature for 12 h. The extent of labeling was determined spectrophotometrically using molar extinction coefficients of 47 000 mol⁻¹ cm⁻¹ at 336 nm for AIAS, and 11, 000 mol⁻¹ cm⁻¹ at 426 nm for LYI (values given by Molecular Probes), and corrected protein concentration for the contribution of the dye at 280 nm was determined. The unbound (AIAS) dye was then separated from fluorescent-labeled protein by a Sephadex G-25 column chromatography, using 50 mM phosphate buffer pH 7.5 containing 100 mM NaCl, and 2 mM EDTA for column equilibration and sample elution. Similarly, the unbound LYI was separated from the protein preparations by dialysis against 50 mM phosphate buffer pH 7.5 for 24 h at 4°C with three changes of the buffer.

Reconstitution of α-crystallin heteroaggregates:

The purified WT-αA was mixed with purified WT-αB, or the individual purified αB-deamidated mutants (i.e., N78D, N146D or N78D/N146D) at 3:1 ratio. Similarly, purified WT-αB was mixed with purified individual αA-deamidated mutants (i.e., N101D, N123D or N101D/N123D) at 1:3 ratio. The molecular weights of resulting heteroaggregates were determined using Static Light Scattering (SLS) method as described below. The
chaperone activities, surface hydrophobicity and intrinsic tryptophan fluorescence of the heteroaggregates were also determined using the methods described below. The rate of subunit exchange in the heteroaggregates (WT-αA:WT-αB, WT-αA:αB-N146D, αA-N101D:WT-αB, αA-N123D: WT-αB, αA-N101D/N123D: WT-αB) was determined by mixing the AIAS-labeled WT-αB or individual αB-mutants with LYI-labeled WT-αA or individual αA-mutants, and using Fluorescence Resonance Energy Transfer (FRET) method as described below.

In a separate experiment, the formation of heteroaggregates following denaturation of αA or αB species by guanidine hydrochloride (Gdn) and renaturation after removal of Gdn by dialysis was determined. Using this method, the heteroaggregate formation between the following species at the ratio of 3:1 (αA:αB) was examined: (1) WT-αA: WT-αB, (2) αA-N101D mutant: WT-αB, (3) αA-N123D: WT-αB, (4) αA-N101D/N123D mutant: WT-αB, (5) WT-αA: αB-N146D mutant.

The purified WT-αA/deamidated αA mutants were mixed with their purified counterparts in 3:1 ratio (αA:αB) followed by denaturation in 4M Gdn and renaturation as described in the Bera et al. method (30). Briefly, the individual heteroaggregates were mixed to 4M Gdn (final concentration) and incubated at 40°C for 6 h. This was followed by dialysis against 50 mM Tris HCl pH 7.9 at 5°C for 48 h with four changes of the buffer. To ascertain whether the Gdn-denaturation and renaturation treatment provided a functionally active heteroaggregate, their chaperone activity against insulin as a target protein was determined.
Determination of Structural and Functional Properties of WTαA-crystallin and its deamidated mutants:

1. Chaperone Activity Assays:

The chaperone activity was determined by using three target proteins (i.e., insulin, citrate synthase (CS) and recombinant human γD-crystallin) essentially by the methods previously described (24). The aggregation of insulin by 20 mM DTT at 25°C, citrate synthase at 43°C and γD-crystallin at 63°C either in absence or at varying concentrations of different αA-crystallin species (i.e., WT-αA, N101D, N123D and N101D/N123D mutants) was determined. The aggregation was monitored using light scattering at 360 nm with time (60 min) using a Shimadzu UV-VIS scanning spectrophotometer (model UV2101 PC) equipped with a six-cell positioned (Shimadzu model CPS-260) and a temperature controller (Shimadzu model CPS 260).

2. Fluorescence Studies: All fluorescence spectra were recorded in corrected spectrum mode using a Shimadzu RF-5301PC spectrofluorometer with excitation and emission band passes set at 5 and 3 nm, respectively. The total intrinsic Trp fluorescence intensities of the WTαA-, the three deamidated αA-mutants, and the α-crystallin heteroaggregates (0.15 mg/ml each), dissolved in 10 mM phosphate buffer pH 7.4 containing 100 mM NaCl, were recorded with an excitation at 295 nm and emission between 300-400 nm.

The binding of a hydrophobic probe, 8-anilino-1-naphthalenesulfate (ANS) to WTαA-, deamidated αA-mutants and the α-crystallin heteroaggregates was determined by recording fluorescence spectra with excitation at 390 nm and emission between 400-
600 nm. In these experiments, 15 µl of 0.8 mM ANS (dissolved in methanol) was added to the protein preparations (0.15 mg/ml, dissolved in 10 mM phosphate buffer, pH 7.4 containing 100 mM NaCl) and incubated for 10 min at 37°C. In another experiment, the WTαA- and the three αA-mutants were heated with the above described ANS concentration at 43°C for 10 minutes, and fluorescence spectra were determined after cooling the preparations to room temperature.

3. Circular Dichroism Studies: To investigate the conformational changes in the WTαA- and the three deamidated αA-mutants, the CD spectra were determined using JasCo spectropolarimeter model 62DS at the room temperature. The αA-crystallin preparations at 0.1 mg/ml or 1 mg/ml (dissolved in 50 mM potassium phosphate buffer, pH 7.4) were used for recording the far- and near-UV CD spectra, respectively. The path length was 0.1 cm and 1 cm during the far- and near- UV CD spectra determination, respectively. The spectra reported are the average of 5 scans, corrected for buffer blank and were smoothed. Secondary structures were estimated using PROSEC program (35).

4. Static Light Scattering: Determination of molecular weights of WTαA-, the three deamidated αA-mutants, and the α-crystallin heteroaggregates was carried out using a static light scattering instrument (SLS, Precision Detectors, Model 202), which exploits gel-permeation chromatography, coupled to low- and high- angle laser light scattering, and a differential refractive index detector. All proteins were dissolved in 50 mM Tris-HCl, pH 7.9, and preparations were filtered through 0.2 µm filter prior to their analysis.
Results utilized both $90^\circ$ and $15^\circ$ light-scattering detection. To reconstitute $\alpha$-crystallin heteroaggregates (1 mg/ml), the purified preparations of deamidated $\alpha$A- and deamidated $\alpha$B- species were mixed at a ratio of 3:1 ($\alpha$A:$\alpha$B).

5. Measurement of subunit exchange rate: FRET was carried out to determine the subunit exchange rate in $\alpha$-crystallin heteroaggregates containing deamidated $\alpha$A-/\alpha$B- and their counterparts WT protein as described by Bova et al. (28). Briefly, the exchange reaction was initiated by mixing 0.4 mg/ml LYI-labeled WT-$\alpha$A or LYI- labeled individual $\alpha$A-mutants with 0.4 mg/ml AIAS-labeled WT-$\alpha$B crystallin. At desired time intervals (described in figure legends), 20 $\mu$l of the reaction mixture was withdrawn, and diluted 100-fold with the buffer A (50 mM sodium phosphate pH 7.5 containing 100 mM NaCl and 2 mM DTT). The emission spectrum of the aliquots (excitation at 426 nm and emission at 525 nm) was recorded using Shimadzu spectrofluorometer (model RF 5301PC). The rate constant was obtained by fitting the data to the exponential function $F(t)=C_1 + C_2 e^{-kt}$, where $F(t)$ is the fluorescence intensity at 525 nm, and $k$ is the rate constant for subunit exchange. The constants $C_1 + C_2 =1$ at time 0, and $C_1$ is the fluorescence intensity at time $\infty$.

RESULTS

Confirmation of specific mutations at desired sites in $\alpha$A-crystallin:

In two individual $\alpha$A-crystallin mutants, the N-101 or N-123 residues were changed to D residue, and in another double mutant, both N-101 and N-123 were
changed to D residue. The resulting three deamidated proteins are referred as N101D, N123D and N101D/N123D mutants throughout the text.

DNA sequencing results confirmed the mutations of N to D residue at the desired positions in the three mutants. To confirm this further, the expressed WTαA-crystallin and the three mutant proteins were analyzed by MALDI-TOF mass spectrometric method after trypsin digestion. The isotopic distribution of tryptic fragments further confirmed mutation(s) of N to D residue at the desired sites in the three mutant proteins. The tryptic peptides with a mass of 1627.68 (residue no. 100-112, sequence: HNERQDDHGYISREFHR, Fig. 1B) and 2785.2 (residue no. 120-145, sequence: LPSNVDQSALSCSLSADGMLTFCC, Fig. 1C) were detected in the N101D and N123D mutants, respectively. The corresponding comparative peaks showed mass of 1626.5 and 2784.7 in WTαA-crystallin (Fig. 1A). A gain of one mass unit in the mutant proteins compared to WT-αA suggested mutation of both N-101 or N-123 to D residue. Similar results (i.e., gain of one mass unit) was observed in the two tryptic peptides with mass of 1627.5 and 2785.4 from the N101D/N123D mutant compared to WT-αA, confirming double mutations of N-101 and N-123 to D in the protein.

**Expression and purification of human recombinant WTαA- and three deamidated αA-mutants:**

The WT-αA crystallin and the three mutated αA-species were expressed in *E.coli*. The SDS-PAGE and MALDI-TOF mass spectrometric analyses showed the expression of full-length recombinant αA-crystallin. The expressed proteins were purified to homogeneity by a combination of methods as described in the Experimental
Procedures, and each species on SDS-PAGE analysis showed a single ~20 kDa protein band (Figure 2).

**Effect of deamidation of N-101 and N-123 on structural and functional properties of αA-crystallin:**

1. **Comparison of effect of the deamidation on the chaperone activity of WT-αA and deamidated αA-mutants:** The chaperone activity of WT-αA and αA-mutant species was determined using three target proteins (insulin, citrate synthase, and γD-crystallin) at three varying ratios of chaperone: target protein (i.e., 0.5:1, 1:1 and 2:1). With insulin as a target protein, no chaperone activity in the N101D mutant was observed at the ratio of 0.5:1 (crystallin: target protein, Fig. 3A), but the other two mutants (N123D, N101D/N123D) showed relatively higher activity, although the levels were significantly lower than the WT-αA-crystallin. At the higher ratios of chaperone: insulin (1:1), the N101D mutant again lacked any chaperone activity, but the activity was observed at the 2:1 ratio, although it was lowest compared to the other two mutants (N123D and N101D/N123D). Together, the data of chaperone activity towards insulin suggested that the mutation of N101D resulted in relatively greater loss (50-100%) of chaperone activity compared to the other two mutations (N123D, N101D/N123D).

With CS and recombinant γD-crystallin as target proteins, the N101D mutant again showed relatively lower chaperone activity (~ 50%) compared to the WT-αA crystallin and the other two mutants (i.e., N123D and N101D/N123D; Figures 3B and 3C). Further, the N123D mutant also showed a lower chaperone activity compared to WT but it was relatively greater than N101D mutant. Additionally, although the
N101D/N123D mutant showed a lower chaperone activity than the N123D mutant with insulin and CS as target proteins; its activity was almost at the same level as in the other two mutants with γD-crystallin as the target protein. Together, the data suggested a significant loss of chaperone activity in αA following deamidation of N-101 residue compared to deamidation of N-123 residue, and a progressive decrease in chaperone activity was observed in the following order: WT-αA>αA N123D>αA N101D/N123D>αA N101D.

2. Surface hydrophobicity of WT-αA and αA-deamidated mutants: Because past studies have suggested that the interaction between the chaperone molecule (α-crystallin) and target protein largely involves hydrophobic residues, the fluorescence spectra of ANS-bound to WT-αA crystallin and the three deamidated mutants were determined (Figure 4). ANS fluoresces at about 512 nm, but on binding to WT-αA, it showed fluorescence at 482 nm (Figure 4A). The three αA-mutants showed a decrease in ANS-fluorescence intensity compared to WT and also a shift from 482 (seen in WT-αA) to 396-480 nm. Furthermore, compared to WT-αA, the decrease in the fluorescence intensity was highest in the N101D mutant (32%) followed by N123D (19%) and N101D/N123D (10%) mutants. Together, the results suggested a deamidation-induced decrease in available hydrophobic surface area in the following order: WT-αA>αA N101D/N123D>αA N123D>αA N101D. The decrease in surface hydrophobicity was in parallel with a decrease in chaperone activities of the αA-species (see above).

Because αA-crystallin is shown to be a better chaperone at higher temperatures, the ANS-binding of WT-αA and the three mutants were determined at 43°C (Figure 4B).
The fluorescence intensity was 20-30% higher in all protein species compared to the levels observed at 37°C. Although the relative hydrophobicity of WT-αA and the mutants showed temperature- dependence, the order of the decrease in fluorescence intensities at 43°C were different than seen at 37°C, and was as follows: WT-αA>N101D>N123D>N101D/N123D>N123D.

3. Analysis of secondary and tertiary structures of WT-αA and αA-deamidated mutants: Compared to the highest fluorescence intensity of Trp at 336 nm of WT-αA crystallin, the N101D mutant showed an emission at 332 nm. The N123D and N101D/N123D mutants also showed similar shift in the emission maxima with slight difference in fluorescence intensity (Figure 5). Together, the results suggested that deamidation of N-101 and N-123 residues caused altered microenvironment of the Trp residue in the αA- mutants compared to WT-αA.

The far and near UV-CD spectra of WT-αA and the three αA-mutants are shown in Figures 6A and 6B, respectively. The far UV-CD spectrum of WT-αA crystallin was similar as reported previously (36) with minimum around 218-220 nm, suggesting that the protein is in β-sheet conformation and is properly folded. However, the mutants exhibited varied profiles (Figure 6A). The levels of secondary structures were estimated using PROSEC program and are shown in Table II. The content of α-helix (11%), β-sheet (44%) and β-turns (17%) and random coil (28%) in WTαA- were consistent with the previous data (36). Compared to WT-αA, the β-sheet and β-turn contents were reduced to 50% in the N123D mutant, and 70-80% in the N101D and N101D/N123D mutants, with significant increase in levels of α-helix and random coil conformation.
The near UV-CD spectrum of a protein is mainly a representation of the local environments of the aromatic amino acid residues (Trp, Phe and Tyr). The near UV spectra of the three mutants did not overlap with the WT-αA (Figure 6B). Like previous data (36), the near UV spectra of the WT-αA also showed five distinct maxima around 259, 265, 273, 279 and 287 nm, and five distinct minima around 262, 268, 275, 284 and 292 nm. The peak signal caused by Phe residue in the 250-270 region showed little alteration in the three mutants compared to WT-αA. However, the peaks beyond 270 nm in all the three mutants were markedly different in their intensity compared the WT-αA (Figure 6B). Indeed, the spectra beyond 270 nm of the three deamidated αA mutants were very different from the spectrum of WT-αA, which confirmed differences in microenvironment of Tyr and/or Trp following deamidation.

4. Determination of quaternary structures of WT-αA and the deamidated mutants:

To compare the quaternary structures of the homoaggregates of WT-αA and the deamidated αA mutants, their molecular mass were determined by static light scattering method using a TSK G-4000 PWXL column (TOSO Haas, Montgomeryville, PA). The estimated molecular mass is shown in Table III. The three αA-mutants showed significant difference in oligomer sizes compared to WT-α-crystallin, i.e., N101D/N123D formed the largest oligomers of 823 kDa followed by N123D oligomers of 809 kDa, N101D of 608 kDa, and WT-αA of 670 kDa.

Characterization of reconstituted heteroaggregates containing WT-αA: WT-αB, WT-αA: deamidated-αB and deamidated-αA: WT-αB subunits:
1. **Molecular mass of reconstituted heteroaggregates:** The molecular mass of reconstituted α-crystallin containing WT-αA: WT-αB, WT-αA: deamidated αB-mutants (N78D, N146D or N78D/N146D), and WT-αB: αA-mutants (N101D, N123D or N101D/N123D) at 3:1(αA: αB) ratio were determined using the SLS instrument (Table III). While the mass of the oligomers of WT-αA:WT-αB was 745 kDa, the reconstituted oligomers with deamidated proteins showed higher mass, i.e., the mass of the oligomers containing WT-αB and deamidated αA-mutants ranged between 878 to 1867 kDa, and that of WT-αA:deamidated αB-mutants between 728-1121 kDa. Additionally, the results showed that compared to N101D mutant, the N123D mutant produced heteroaggregates of larger sizes with WT-αB subunit, suggesting deamidation of Asn affected the oligomerization process. The fact that the deamidated N101D/N123D mutant produced even larger oligomers with WT-αB subunit suggested that both Asn residues might be involved in maintaining the quaternary structure, and also in the subunit interaction with αB-crystallin. However, while the size of the heteroaggregate of αB N78D mutants: WT-αA was almost the same as that of WT-αA: WT-αB, the oligomers produced by αB N146D mutants: WT-αA was of larger sizes suggesting that N-146 plays a role in subunit interaction with the αA-crystallin. Taken together, the deamidation in αA- and αB-subunits resulted generally in loosely organized oligomer structures.

2. **Chaperone activity of reconstituted heteroaggregates:** The chaperone activities of the reconstituted α-crystallin heteroaggregates were determined using two target proteins, i.e. insulin and recombinant γD-crystallin. Compared to the chaperone activity
of the oligomer of the WT-αA:WT-αB, the oligomers containing either deamidated αA- or deamidated αB-mutants and their counterpart WT proteins, showed about 50% lower chaperone activity (Figure 7A and 7B). The only exception was the heteroaggregates of WT-αA: αB N78D mutant, which showed a higher chaperone activity than all the other heteroaggregates. In general, the decrease in chaperone activity in the heteroaggregates showed the following order; WT-αA: WT-αB>WT-αA: αB-N78D > αB-WT: αA101D/N123D>αB-WT: αB-N123D>αB-WT: αA-N101D> WT-αA: αB-N78D/N146D >WT-αA: αB-N146D.

Figure 7C shows the chaperone activity of the heteroaggregates formed after denaturation with guanidine hydrochloride and renaturation after dialysis. With insulin as a target protein, the results were similar to the reconstituted α-crystallin heteroaggregates formed by mixing purified non-denatured subunits as shown in Fig. 7A. As shown in Fig. 7C, the progressive decrease in chaperone activity in the heteroaggregates showed the following order; WT-αA: WT-αB > αA-N123D:αB-WT > αA-N101D: WT-αB > αA101D/N123D: WT-αB> αA-WT: αB-N146D.

Together, the results suggested that deamidation of Asn residues in both αA- and αB-crystallins affected the chaperone activity of the heteroaggregate, and this could lead to higher molecular weight aggregates seen during aging or cataract development.

3. Hydrophobicity of the reconstituted heteroaggregates: As stated above, because chaperone activity is believed to be partly due to the interactions of hydrophobic patches of α-crystallin with a target protein (2), a hydrophobic probe such as ANS could provide information as it fluoresces upon binding to apolar surfaces. As
shown in Figure 8, the fluorescence intensities of ANS bound to oligomers containing deamidated αA- or αB-mutants and their counterparts WT proteins differed significantly. Compared to the heteroaggregates of WT-αA: WT-αB, the exposed hydrophobic surface area was reduced in all the oligomers. The ANS-binding results showed a progressive decrease in the available hydrophobic surface area was in the following order: WT-αA: WT-αB > WT-αA:αB-N78D > αA-N123D:αB-WT > WT-αB:αA-N101D > WT-αB: αA-N101D/N123D > WT-αA:N146D > WT-αA:αB-N78D/N146D. Together, the results suggested that the deamidation of N-101 in α-crystallin heteroaggregates (WT-αB:αA-N101D) showed relatively greater exposed surface hydrophobic area compared to heteroaggregate with deamidated N-146 (WT-αA:αB-N146D).

5. Intrinsic Trp fluorescence of reconstituted heteroaggregates: The Trp fluorescence of different reconstituted α-crystallin heteroaggregates was determined with excitation at 295 nm and emission in between 300-400 nm. The heteroaggregates containing WT-αA:WT-αB showed the emission maxima at 336 nm whereas the heteroaggregate containing WT-αA:deamidated αB-mutants or WT-αB:deamidated αA-mutants showed differences in fluorescence spectra. Compared to α-crystallin heteroaggregate containing WT-αA:WT-αB, the heteroaggregates containing WT-αA: αB-N78D/N146D mutant, and WT-αB:αA-N101D/N123D mutant, showed maximum quenching in fluorescence intensity with a blue shift to 331-333 nm (Figure 9). The heteroaggregates containing WT-αA:αB-N78D mutant or WT-αA:αA-N123D mutant exhibited greater fluorescence intensity with the same λ<sub>max</sub>. The heteroaggregates containing WT-αB:αA-N101D mutant or WT-αA:αB-N146D mutant showed intermediate
fluorescence intensities with a shift to 337 nm. Together, the results suggested that the microenvironment of the Trp residues of the reconstituted crystallin heteroaggregates containing deamidated mutant proteins was altered.

6. Rate of subunit-exchange in heteroaggregates:

The WT-αA, WT-αB, αA-mutants (N101D, N123D and N101D/N123D), and αB-mutant (N146D) were labeled with fluorescent probes and their subunit-exchange rates were determined in the heteroaggregates by the FRET method. The cysteine residue of WT-αA and αA-deamidated mutants were labeled with LYI (a sulfhydryl-specific fluorophore), and the WT-αB and the αB-deamidated mutant were labeled with AIAS fluorophore as described in the Experimental Procedures. The emission maxima of LYI-labeled WT-αA/αA-mutants (excitation at 426 nm) and AIAS-labeled WT-αB/αB- mutants (excitation at 336 nm) were at 525 nm and 415 nm, respectively. The overlap of the emission spectrum of AIAS fluorophore with the absorption spectrum of the LYI-fluorophore suggested that energy was transferred between the donor and acceptor fluorophores during FRET (data not shown). Following the mixing of the AIAS-labeled WT-αB or N146D mutant (donor), or the LYI-labeled WT-αA or deamidated αA-mutants (acceptor) in 3:1 ratio (αA:αB), the subunit exchange rate was determined. A quenching of donor fluorescence at 426 nm concomitantly occurred with an increase in acceptor fluorescence at 525 nm in all the heteroaggregates. Emission spectra of one such example is shown in Figure 10A and other heteroaggregates also showed similar spectrum. The heteroaggregates containing WT-αA and WT-αB showed changes in fluorescence intensity until 120 min, but the quenching of donor fluorescence continued.
until the 240 min in the heteroaggregates containing WT-αA and deamidated αB-mutants, and also in the heteroaggregates containing deamidated αA and WT-αB subunits. The acceptor fluorescence intensities at 525 nm for all the heteroaggregates are plotted against time in Fig. 10B. The subunit exchange rate from the increase in acceptor fluorescence intensity were calculated by fitting the data to the exponential function $F(t)/F(0) = A_1 + A_2 e^{-kt}$ where ‘k’ is the subunit exchange rate constant. The subunit exchange rate was found to be 0.051 min$^{-1}$ for heteroaggregate containing WT-αA and WT-αB, which is in agreement with the previously published results (5, 26). Compared to heteroaggregates containing WT-αA:WT αB-, the heteroaggregates containing deamidated αA-mutants: WT-αB showed relatively lower subunit exchange rate i.e., WT-αB: αA-N101D, k=0.041 min$^{-1}$; WT-αB: αA-N123D, k=0.0393 min$^{-1}$; WT-αB: αA-N101D/N123D, k=0.0417 min$^{-1}$. The heteroaggregates containing WT-αA and deamidated αB-mutants showed a nearly 3-fold reduced subunit exchange rate (WT-αA: αB-N146D, k=0.016 min$^{-1}$) suggesting that deamidation of N residues in αA-/αB-crystallins altered the electrostatic interactions, more so by N-146 in αB-crystallin.

Together, the results suggested that electrostatic interaction, which the first step in subunit-subunit interactions, was followed by non-covalent interactions occurred during the heteroaggregate multimeric assembly.

The heteroaggregate containing deamidated αA-mutants: WT-αB also formed larger oligomers, exhibited relatively greater chaperone activity and more surface hydrophobicity compared to heteroaggregates containing WT-αA and deamidated αB-mutants. The data suggested that presence of WT-αB in the α-crystallin heteroaggregate increased the surface area, which most likely favor subunit exchange
and unmask the chaperone sites eventually regulating both the structural and functional properties.

**Discussion**

Both \( \alpha \)A- and \( \alpha \)B-crystallins, either as homoaggregates or heteroaggregate, show chaperone activity, and this property is believed to prevent aggregation of unfolded proteins and thus prevent cataract development (37). A previous study has correlated the significance of oligomeric state, different regions and specific amino acids in both crystallins to their functional, structural and oligomerization properties (38). However, this structure-function relationship is not yet fully understood. Similarly, the effect of *in vivo* post-translational modifications of specific amino acids on the structure-function relationship of both \( \alpha \)A- and \( \alpha \)B- crystallins is also poorly understood. Although deamidation of \( \alpha \)A- and \( \alpha \)B-crystallins is one of the most frequent *in-vivo* modifications during aging and cataract development (see Introduction), the effects of deamidation on its structural and functional properties have not been examined except in one of our recent report (24). This study showed that the deamidation of N-146 and not N-78 (the two deamidation sites) in \( \alpha \)B-crystallin caused significant changes in structural and functional properties of the crystallin. Because both N-78 and N-146 residues resides are in the conserved region (\( \alpha \)-crystallin domain, residue no. 66 to 147) of the \( \alpha \)B-crystallin (3, 4), the vicinity of the N-146 residue towards the conserved C-terminal region (residue no. 147-175) might be responsible for the above effects. The \( \alpha \)A-crystallin also contains two potential deamidation sites (i.e. N-101 and N-123) in the conserved region (\( \alpha \)-crystallin domain, residue no. 62 to 143), and are also in the
vicinity of the conserved C-terminal region (i.e., residue no. 143-173; 3, 4). Because no information exists in the literature regarding effects of deamidation of these two residues on the structural and functional properties of \(\alpha\)-crystallin, the present study was undertaken. First, we examined the effects of deamidation of N-101 or N-123 residues, or both on the structural and functional properties of \(\alpha\)-homoaggregates. Next, we reconstituted heteroaggregates using deamidated \(\alpha\)/\(\alpha\)-mutants and their counterpart WT proteins, and determined their structural and functional properties.

The major findings during the study of \(\alpha\)-homoaggregates were: (1) Deamidation of either N-101, N-123 or both residues in \(\alpha\)- resulted in altered secondary and tertiary structures, oligomerization properties, and reduced levels of the chaperone activity with three target proteins (insulin, citrate synthase and human recombinant \(\gamma\)-crystallin). (2) The N101D mutant exhibited the lowest chaperone activity, and maximum change is secondary, tertiary and quaternary structures, compared to the other two deamidated \(\alpha\)-mutants. Therefore, the N-101 residue is of relatively greater importance compared to N-123 in maintaining the structural and functional integrity of \(\alpha\)-crystallin.

The major findings during the investigation of reconstituted heteroaggregates were: (1) Relative to reconstituted heterooligomers of WT-\(\alpha\) and WT\(\alpha\)-crystallins, the oligomers of larger sizes were generated in heteroaggregates of deamidated \(\alpha\)- or \(\alpha\)-mutants and their counterparts WT proteins. However, heteroaggregates containing deamidated \(\alpha\) mutants and WT-\(\alpha\)- were of larger sizes than heteroaggregates of deamidated \(\alpha\)- mutants and WT- \(\alpha\)-crystallin. (2) Compared to the heteroaggregates containing WT-\(\alpha\) and WT-\(\alpha\), the heteroaggregates containing deamidated \(\alpha\)-
mutants and WT-αB exhibited higher chaperone activity, altered tertiary structures and lower subunit-exchange rate. The heteroaggregate containing WT-αA and deamidated αB mutants showed three-fold lower subunit-exchange rate, suggesting that WT-αB in the α-crystallin heteroaggregate modulates its dynamic structural and functional properties.

Together, the above findings suggest that deamidation of Asn in αA- and αB-crystallins have major impact on structural and functional properties of their homoaggregates and heteroaggregates. The results also suggest relative impact of deamidation of N-101 in αA-, and N-146 in αB-crystallin is far greater than the deamidation of N-123 in αA-, and N-78 in αB-crystallin. Further, the negative charges generated due to the conversion of N to D residue at the above sites apparently changed the conformation of crystallins, and in turn their chaperone activity. The two-deamidation sites (N-101 and N-123) in the αA-crystallin, like other small heat shock proteins, fall within the highly conserved sequence of α-crystallin domain (i.e., residue no. 62 to 143) [3, 4]. Therefore, the major question is why will the deamidation of these two residues in αA-crystallin cause the above changes. It is more intriguing by the fact that between the two N residues of αA-crystallin, only the N-123 but not the N-101 has been conserved in the mammalian species (39). Because a frequent in-vivo deamidation of N-101 and not of the N-123 residue has been reported in past studies (18, 40), our above findings suggest that deamidation of N-101 would compromise the structural and functional properties of the crystallin in-vivo during aging.

Our studies show that both chaperone activity and exposed hydrophobic regions were affected following deamidation at both sites in αA-crystallin. This finding is
important because previous studies have shown that the hydrophobic regions of \( \alpha \)-crystallin are involved in binding with substrate proteins during chaperone activity (41-43). The residue no. 71-78 of \( \alpha \)-crystallin represents the most hydrophobic region of the molecule (44), which is also an ANS- binding site. However, such binding caused a decrease in chaperone activity (42). Recent studies in \( \alpha \)-crystallin have suggested that factors other than hydrophobicity might also play important role in the chaperone activity (45-47). The observation that the hydrophobicity is not the sole determinant of chaperone activity was also supported by our findings. The \( \alpha \)-N101D mutant did not show parallel coherence between chaperone activity and hydrophobicity with an increase in temperature. Although this mutant exhibited increased surface hydrophobicity at elevated temperature (43\(^0\)C), it did not exhibit equivalent increase in chaperone activity compared the WT-\( \alpha \)-A. An explanation for this anomaly might be that ANS (a hydrophobic probe), binds to all the exposed hydrophobic patches in the N101D mutant at elevated temperature because of a change in conformation, but all these binding sites might not be large enough to bind to the target protein (CS and recombinant \( \gamma \)-crystallin), which resulted in reduced chaperone activity.

Because buried charged amino acids generally pair with amino acids of opposite charges and often have functional importance (48), and therefore an unpaired negatively charged residue following deamidation at N-101 position would perturb the tertiary structure. This might have caused the formation of homoaggregates of a larger size in the deamidated mutants. Additional reports of loss of chaperone activity in both \( \alpha \)-A and \( \alpha \)-B on mutation of charged residues (10, 49-51) further argue for the \( \alpha \)-A- and \( \alpha \)-B-crystallins to conserve their net charge through evolution (52). Our finding that an
additional negatively charged residue due to deamidation resulted in changed tertiary structure, and relatively larger oligomers compared to WT-αA crystallin was supported by additional results. The results of far UV CD spectra clearly showed that the homoaggregates of deamidated αA-crystallin adopted more of a α-helical conformation compared to WT-αA crystallin. The near UV spectra, which provide information regarding the aromatic environment in the tertiary structure of a protein and therefore, also represents packing of a polypeptide to form tertiary structures, confirmed changed conformation of the deamidated αA-crystallin homoaggregates compared to that of WT-αA crystallin. The three mutants showed peaks beyond 270 nm that were markedly different in their intensity compared the WT-αA crystallin, suggesting differences in their tyrosine and/or tryptophan microenvironments.

In mammalian lens, α-crystallin exists as a heteroaggregate of αA- and αB-subunits, and are present at a molar ratio of 3:1 although previous reports indicated that various ratio can be formed in vitro (53, 54). The interaction between αA- and αB-crystallins has been investigated in several past studies. It has been shown that the αA and αB-crystallin complex has greater thermal stability than either protein alone (55), suggesting that greater potential of the complex to protect proteins under stress. In the quaternary structure of αA-crystallin, the small multimers of αA- subunits remains in dynamic equilibrium with oligomeric complex (5). This study further showed that αB-crystallin readily exchange with subunit of αA-crystallin but not with other proteins unrelated to Hsp.

It is believed that the interactions between the subunits involve ionic as well as hydrophobic interaction, because reduced subunit-exchange rates were observed under
either very high or very low salt concentrations (28, 56). Because deamidated forms of αA- or αB- or both might exist in-vivo, it is logical to study structural and functional properties of the heteroaggregates containing deamidated αA- or αB-mutants with their counterparts WT proteins. As stated in results, α-crystallin was reconstituted using deamidated subunits αA-/αB- and their counterpart WT proteins at a ratio of 3:1. Our results show relatively reduced levels of the subunit exchange and chaperone activity in heteroaggregates containing deamidated αA-/αB-crystallins with their counterpart WT-proteins compared to similarly reconstituted aggregates with WT-αA and WT-αB. The reduced chaperone activity in these heteroaggregates could be due to change in tertiary structure, and heterologous subunit packing upon deamidation. This argument was supported by the observed blue shift to 331-333 nm during intrinsic Trp fluorescence measurement of heteroaggregate of deamidated αA-/αB- and their counterparts WT protein compared to 336 nm peaks in the oligomers of WT-αA and WT-αB crystallin.

The larger size oligomers in the above heteroaggregates containing either deamidated αA or αB with their counterpart WT protein further support the above argument. Compared to the heteroaggregates of WT-αA and WT-αB, a 3X decrease in the subunit-exchange rate in the heteroaggregates containing deamidated αB and WT-αA was observed, but the relative rate was only slightly lower in the aggregates of deamidated-αA and WT-αB. Similar differences were observed when oligomer sizes and chaperone activity were compared.

Together, the above data suggested that mutation of Asn in αA- or αB-crystallins in the conserved region resulted in modified structure and function, possibly due to a different mode of subunit assembly. This effect was greater in the heteroaggregates
containing deamidated αB compared to those containing deamidated αA-subunit. The α-crystallin heteroaggregates containing deamidated αA mutant and WT-αB oligomers (i.e., αA N123D:WT-αB and αA N101D/N123D:αB-WT) also showed high oligomer sizes, and slight increase in chaperone activity compared to other mutants suggesting that presence of WT-αB modulates the chaperone activity in these oligomers. Apparently, the WT-αB in the α-crystallin heteroaggregate modulates the formation of oligomeric complex, their flexible building units, extended surface area, and the chaperone activity in these oligomers either directly or by inducing packing changes in the heteroaggregate.

In our study, the presence of αB-crystallin in the multimeric α-crystallin complex (containing deamidated-αA mutant and WT-αB) affects the subunit exchange, increases the chaperone activity and oligomer sizes. How the oligomeric structure favors free and rapid subunit-exchange and relative increase in chaperone activity remains largely unexplored. Probably, optimum surface area facilitates free subunit exchange and leads to the formation of stable quaternary structure. The time-dependent structural and functional changes with these oligomers may elucidate a possible mechanism for chaperone activity. We are currently attempting to answer these questions.

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Figure Legends

Figure 1: MALDI-TOF spectra of WT-αA and deamidated mutants. (A) Tryptic fragments peaks of WT-αA with mass of 1400.6 (residues no. 1-11, sequence: MDVTIQHPWFK), 1626.4 (residues no. 100-112, sequence: HNERQDDHGYISREFHR), 1654.1 (residues no. 158-173, sequence: AIPVSREEKPTSAPSS) and 2784.9 (residues no. 120-145, sequence: LPSNVDQSAALSCSLADGMLTFCC). (B) Tryptic fragment peaks of the αA-N101D mutant with mass of 1400.6 (residues no. 1-11 [N-terminal region], sequence: MDVTIQHPWFK), 1627.4 (residues no. 100-112, sequence: HNERQDDHGYISREFHR) showing a gain of one mass unit due to deamidation at N-101 to D residue (compare peak with mass of 1626.4 in WT in A to 1627.4 in the mutant in B). The peak with mass of 1654.1 represents the residues no. 158-173 (the C-terminal region) with the sequence of AIPVSREEKPTSAPSS. (C) Tryptic fragment peaks of αA-N123D mutant with mass of 1400.6 (residues no. 1-11, sequence: MDVTIQHPWFK), 1654.1 (residues no. 158-173, sequence: AIPVSREEKPTSAPSS) and 2785.7 (residues no. 120-145, sequence: LPSNVDQSAALSCSLADGMLTFCC). The mutant showed a gain of one mass unit due to deamidation at N-123 to D residue (compare peak with mass of 2784.9 of WT in A to 2785.7 in the mutant in C). The N101D/N123D mutant showed a mass of 1627.6 and 2785.9 as shown in (B) and (C) after deamidation of N-101 and N-123 residues (results not shown).

Figure 2: SDS-PAGE analysis of purified WT-αA crystallin and the three αA-deamidated mutants. Purification of the WT and the three αA-crystallin mutants was performed by a combination of methods (see Experimental Procedures).

Figure 3: Comparison of chaperone activities of the αA- WT crystallin and the three αA-deamidated mutants. The chaperone activity assays were performed with three target proteins.
and at three different temperatures. The micromolar concentrations of the $\alpha$A$\alpha$-WT and the three $\alpha$A mutants were based on the aggregate molecular mass of individual proteins, as determined by light-scattering (see Table III). Protein aggregation was determined by monitoring light scattering at 360 nm. Chaperone activity is represented as the percent protection provided by the WT and mutant proteins. The % protection provided by the WT$\alpha$A- is considered as 100 % protection. (A) Aggregation of insulin B chain by reduction with DTT in the presence of different chaperonin-to-insulin ratios at 25°C. The concentration of insulin was 100 µg. (B) Aggregation of CS in the presence of different chaperonin to CS ratios at 43°C. The concentration of CS was 100 µg. (C) Aggregation of human recombinant $\gamma$D-crystallin in the presence of different chaperonin to $\gamma$D-crystallin ratios at 63°C. The concentration of recombinant $\gamma$D crystallin was 100 µg.

**Figure 4: Fluorescence spectra of WT-$\alpha$A and three deamidated $\alpha$A-mutants following ANS binding.** Fluorescence spectra of ANS-bound to WT-$\alpha$A and to the three deamidated mutants at: (A) 37°C and (B) 43°C. The concentration of each $\alpha$A species was 0.15 mg/ml, and that of ANS 12 µM. All the protein preparations were heated with ANS at 43°C for 10 min and fluorescence spectra determined after cooling the preparations to the room temperature. Fluorescence spectra of the preparations were recorded excitation at 390 nm and emission between 400 to 600 nm.

**Figure 5: Intrinsic Trp fluorescence spectra of WT-$\alpha$A and three $\alpha$A-deamidated mutants.** The preparations were excited at 295 nm and fluorescence spectra were recorded with emission between 300 and 400 nm. The concentration of each crystallin species was 0.15 mg/ml.
Figure 6: (A) Far-UV CD spectra of WT-αA and three deamidated αA mutants. The spectra were recorded at the concentration of 0.1 mg/ml of each species with a cell path length of 0.1 cm. The spectra reported are the average of five determinations. (B) Near-UV CD spectra of WT-αA and three αA-deamidated mutants. The spectra were determined at 1 mg/ml of each preparation with a cell with a 1 cm path length. The reported spectra are the average of five determinations of each sample, and are corrected for the cell blank, and smoothed.

Figure 7: Comparison of chaperone activities of reconstituted α-crystallins heteroaggregates. The chaperone activity assays were performed with two target proteins at different temperatures. (A) Aggregation of insulin B chain (100 µg) by reduction with DTT in the presence of different chaperonin to insulin ratios at 25°C. (B) Aggregation of human recombinant γD-crystallin (100 µg) in the presence of different chaperonin to γD crystallin ratios at 63°C. Protein aggregation in each assay was determined by monitoring using light-scattering at 360 nm. Chaperone activity is represented as the percent protection provided by the heteroaggregates containing WT and mutant proteins. (C) Chaperone activity assay of α-crystallin heteroaggregates formed after denaturation (Gdn) and renaturation (dialysis). Aggregation of insulin B chain (100 µg) by reduction with DTT in the presence of chaperonin to insulin ratio (0.5:1) at 25°C.

Figure 8: Fluorescence spectra of reconstituted α-crystallin heteroaggregates following ANS binding. Fluorescence spectra of different heteroaggregates (0.15 mg/mL) bound to ANS bound at 37°C. Fluorescence spectra of the samples were recorded following excitation at 390 nm and emission between 400 to 600 nm. The concentration of ANS was 12 µM.
Figure 9: Intrinsic Trp fluorescence spectra of reconstituted α-crystallin heteroaggregates. The preparations were excited at 295 nm and fluorescence spectra were recorded with emission between 300 and 400 nm. The concentration of each preparation was 0.15 mg/ml.

Figure 10: Subunit exchange rates between heteroaggregates containing WTαA-/deamidated αB- and deamidated αA-/WTαB-crystallins. (A) Time dependant FRET due to subunit exchange of AIAS-labeled WTαB- and LYI-labeled WTαA-, excited at 426 nm and emission at 525 nm). The emission spectra were recorded at 0, 15, 30, 60, 120, 180 and 240 min. after mixing of labeled LYI- and AIAS- crystallins at 3:1 ratio with a final concentration of 1 mg/ml. The other heteroaggregates (αA-N101D: WTαB-, αA-N123D: WTαB-, αA-N101D/N123D: WTαB-, WTαA-:αB-N146D) showed a similar pattern (B) Time dependant increase in emission intensity due to subunit exchange. Increase in relative fluorescence intensity at 525 nm due to fluorescence resonance energy transfer (FRET) from the AIAS-labeled crystallin to the LYI-labeled WTαA- or deamidated αA-crystallin species. Each curve represents the best statistical fit of the data to the exponential function \( F_t/F_0 = C_1 + C_2 e^{-kt} \) where ‘k’ is the subunit exchange rate. The subunit exchange rate of the heteroaggregates containing deamidated αA- and WTαB- was higher than WTαB- heteroaggregates containing WTαA- and deamidated αB-crystallin.
Figure 2
Figure 4

Surface hydrophobicity - ANS binding assay

(A) Relative Fluorescence Intensity

Surface hydrophobicity - ANS binding assay at 43°C

(B) Relative Fluorescence Intensity
Figure 5

Relative Fluorescence Intensity

Wavelength (nm)

αA-crystallin

N123D

N101D/N123D

N101D
Figure 6

(A) Mean residue ellipticity as a function of wavelength (nm) for αA-crystallin, N101D, N123D, and N101D/N123D.

(B) Mean residue ellipticity as a function of wavelength (nm) for αA-crystallin, N101D, N123D, and N101D/N123D.
Figure 7

(A) chaperone: insulin (wt/wt) % protection

- aA-WT:aB-WT
- N101D:aB-WT
- N123D:aB-WT
- N101D/N123D:aB-WT
- aA-WT:N78D
- aA-WT:N146D
- aA-WT:N78D/N146D

Chaperone: Insulin (wt/wt)

(B) chaperone: recombinant gD (wt/wt) % protection

- aA-WT:aB-WT
- N101D:aB-WT
- N123D:aB-WT
- N101D/N123D:aB-WT
- aA-WT:N78D
- aA-WT:N146D
- aA-WT:N78D/N146D

Chaperone: Recombinant gD (wt/wt)
Figure 7c

% Protection

Alpha crystallin heteroaggregates (wt/wt)

- Alpha A:alphaB
- Alpha A N101D:alphaB
- Alpha A N123D:alphaB
- Alpha A N101D/N123D:alphaB
- Alpha A:alphaB N146D
Figure 8

Wavelength (nm)

Relative Fluorescence Intensity

WTαA::WTαB

WTαA:N78D

WTαA:N146D

WTαA:N78D/N146D

N101D:WTαB

N123D:WTαB

N101D/N123D:WTαB

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Figure 9

The graph shows the relative fluorescence intensity as a function of wavelength (nm) for various mutants. The wavelengths range from 300 nm to 400 nm.

- WTαA:WTαB
- WTαA:N78D
- WTαA:N146D
- WTαA:N78D/N146D
- N101D:WTαB
- N123D:WTαB
- N101D/N123D:WTαB
Figure 10

(A) Relative Fluorescence Intensity vs. Wavelength (nm)

(B) F(t)/F(0) at 525 nm vs. Time (min.)

- N123D:αB-WT
  - k = 0.0393 min⁻¹
- WTαA:αB-WT
  - k = 0.051 min⁻¹
- N101D:αB-WT
  - k = 0.041 min⁻¹
- αA-WT:N146D
  - k = 0.016 min⁻¹
- N101D/N123D:αB-WT
  - k = 0.0417 min⁻¹
Table I. Oligonucleotide primers used in the site-specific mutagenesis of N-101, N-123 and N-101/123 residues to D residue in human lens αA-crystallin. The mutated bases are highlighted.

| Constructs | Primers |
|------------|---------|
| N101D      | Forward 5’ ATCCACGAAAGCACGACGAGCGCCAGGACGACC3’ |
|            | Reverse 5’ GGTCGTCCTGGCGCTCGTCGTGCTTTCCGTGGAT3’ |
| N123D      | Forward 5’ GCTACCGCCTGCCGTCCGACGTGGACCAGTCCGCCC |
|            | Reverse 5’ GGCCGACTGGTCCACGTCGGACGGGAGGGCGGTAGC |
Table II: The levels (%) of secondary structure contents in WT-αA and the three αA-deamidated mutants. The secondary structure contents of the protein species were determined from the far UV-CD spectra as shown in Figure 6A.

|               | WT-αA | N101D | N123D | N101D/N123D |
|---------------|-------|-------|-------|-------------|
| α-helix       | 11    | 47    | 33    | 38          |
| β-sheet       | 44    | 22    | 19    | 19          |
| β-turn        | 17    | 4     | 13    | 4           |
| Random coil   | 28    | 31    | 41    | 39          |
Table III: Determination of molecular mass by static light scattering method of oligomers of WT-αA, WT-αB, three deamidated αA-mutants, three deamidated αB-mutants and the reconstituted α-crystallin heteroaggregates containing WT-αA:WT-αB, WT-αA:αB-deamidated mutants (N78D, N146D or N78D/N146D), and: αA-mutants (N101D, N123D or N101D/N123D): αB-WT at 3:1 ratio. The static light measurements were carried out by both 15° and 90° detectors.

| Crystallin oligomers | Molecular mass (kDa) |
|---------------------|---------------------|
| WTαA-              | 670,000             |
| αB-WT              | 580,000             |
| αA-N101D mutant    | 608,000             |
| αA-N123D mutant    | 809,000             |
| αA-N101D/N123D mutant | 823,000          |
| αB-N78D mutant     | 670,000             |
| αB-N146D mutant    | 780,000             |
| αB-N78D/N146D mutant | 820,000       |
| WTαA-:αB WT        | 745,000             |
| WTαA-:αB-N78D mutant | 728,000       |
| WTαA-:αB-N146D mutant | 1121,000         |
| WTαA-:αB-N78D/N146D mutant | 758,000      |
| αB-WT:αA-N101D mutant | 878,000         |
| αB-WT:αA-N123D mutant | 1007,000        |
| αB-WT:αA-N101D/N123D mutant | 1867,000    |
Deamidation affects structural and functional properties of alpha A-crystallin and its oligomerization with alpha B-crystallin
Ratna Gupta and Om P. Srivastava

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