Effect of Deamidation of Asparagine 146 on Functional and Structural Properties of Human Lens αB-Crystallin

Ratna Gupta and Om P. Srivastava

PURPOSE. To elucidate the effect of deamidation on the structural and functional properties of human αB-crystallin.

METHODS. Site-directed mutagenesis was used to generate three deamidated mutants of αB-crystallin: N78D, N146D, and N78D/N146D. The mutations were confirmed by DNA sequencing and matrix-assisted desorption ionization-time of flight (MALDI-TOF) mass spectrometry. Recombinant native αB-crystallin (wild type [WT]) and the three mutated αB species were expressed, and each species was purified to homogeneity by ion-exchange chromatography followed by hydrophobic interaction chromatography. The structural and functional properties compared with WT protein were investigated, respectively, by static light scattering (SLS), circular dichroism (CD), and fluorescence spectroscopy and by determining chaperone activity with the use of three substrates.

RESULTS. Native WT and the N78D mutant showed relatively higher chaperone activity compared with the N146D and N78D/N146D mutants with all the substrates. Further, during binding experiments with 1-anilino-8-naphthalenesulfonate (ANS), the WT and N78D mutant showed relatively more binding than the N146D and N78D/N146D mutants. On determining far-UV circular dichroism and tryptophan (Trp) fluorescence spectra, significant secondary and tertiary structural changes were observed in the N146D and N78D/N146D mutants compared with WT and the N78D mutant. The static light scattering data showed a high order of oligomerization in all the three mutants. N146D and N78D/N146D formed the largest oligomers of 750 and 770 kDa, respectively, compared with WT (580 kDa).

CONCLUSIONS. The results show that the deamidation of N 146 but not of N 78 have profound effects on the structural and functional properties of αB-crystallin.

Keywords: α-crystallin, deamidation, chaperone activity, light scattering, far-UV circular dichroism, ANS binding, oligomerization

C rystalins are the structural proteins of mammalian lens and belong to two classes, the α-crystallin and the βγ-crystallin superfamilies. α-Crystallin is one of the most abundant proteins in lens mature fiber cells, constituting approximately 35% of total lens proteins, and exists in vivo as an 800-kDa oligomer of two subunits of αA- and αB-crystallins at a ratio of 3:1.1,2

αB-crystallin is a small heat shock protein (hsp), and is structurally related to other hSHPs including αA-crystallin, hsp27, hsp20, hsp22, and myotonic dystrophy protein kinase binding protein.3 It shares 57% homology with αA-crystallin and plays extralenticular roles in both normal and diseased tissues.4 Reports show an overexpression of αB-crystallin in the development of benign tumors associated with tuberous sclerosis, neuromuscular disorders,1 and other neurologic diseases, such as Alexander’s, Alzheimer’s, and Parkinson’s diseases.5,6

Bovine α-crystallin in a dilute solution at 37°C had a molecular weight (M₀) of approximately 550,000, and its Mₙ in its native state was approximately 700,000.6,7 A recent determination of molar mass distribution by a light-scattering instrument showed polydispersity in α-crystallin, with the average M₀ of 700,000 for bovine α-crystallin and 585,000 for human recombinant αB-crystallin.5 Because the accuracy of the light-scattering system was 3% to 5%, the M₀ was fairly accurate.

It is believed that oligomerization or multimeric assembly is a prerequisite for the molecular chaperone activity of αA- and αB-crystallins, which prevents undesirable protein interactions and their aggregation, and therefore contributes to the maintenance of lens integrity and transparency.4,5,6 This chaperone activity of the crystallins is known to decrease with age and during cataractogenesis.9 According to Horwitz,3 although the questions about the ability of α-crystallin to refold denatured protein remains, several examples show its ability to protect enzymes from denaturation.10,11

Cataractogenesis (development of lens opacity) is believed to be a consequence of accumulation of insoluble aggregates and cross-linked products of α-, β, and γ-crystallins. The insolubilization of crystallins is thought to be initiated by post-translational modifications,12–14 which change their structural and functional properties. During aging and cataract formation, αB-crystallin undergoes numerous in vivo posttranslational modifications, such as phosphorylation of amino acid S, N-terminal acetylation; oxidation of M and C residues; degradation and deamidation of N and Q; and carbamylation of K and R residues.12,15–17 It is speculated that these modifications either act independently or in combination to cause unfolding, aggregation, or cross-linking of proteins, which in turn leads to protein insolubilization and to lens opacity.12,13 However, modification-induced mechanisms are poorly understood.

Deamidation of crystallins is a nzenzymatic process and is one of the most prevalent posttranslational modifications that occur during aging and cataract formation.1,2 Both N and Q amino acids have been shown to undergo deamidation,12 which results in the introduction of negative charge at the site of modification and thus may alter the protein’s tertiary structure and affect its biological properties. Because deamidation changes the native conformation, it is believed to change the protein’s three-dimensional structure.12,13 This has been shown in several studies; however, we still poorly understand the exact role of deamidation in protein destabilization during aging and cataractogenesis and have not identified a definitive cause-and-effect relationship between deamidation and chaperone activity. Recent studies have shown a correlation between two posttranslational modifications (oxidation and phosphorylation) and the chaperone activity of αB-crystallin.9,18,19 However, presently, no correlative study exists in the extension of the study.20

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literature that shows the effect of deamidation on the chaperone activity of αA- and αB-crystallins. The in vivo deamidation of α, β, and γ-crystallins has been shown in several studies.\textsuperscript{12,13,15,16,20–22} A recent study from our laboratory has shown deamidation of N\textsubscript{146} in αB-crystallin of normal and cataractous human lenses.\textsuperscript{16} Similarly, the increased deamidation of N\textsubscript{78} in γ-crystallin\textsuperscript{20,22} and N\textsubscript{101} in human αA-crystallin and their aggregation in high molecular weight species in normal human lenses have been reported.\textsuperscript{21}

In addition, Hanson et al.\textsuperscript{22} have reported that deamidation is significantly greater than other modifications in crystallins in lenses from 50- to 60-year-old donors. Similarly, Lampi et al.\textsuperscript{23} have shown that deamidation and truncation of human βB1-crystallin lead to structural destabilization and decreased stability in higher temperatures. In a later study, they reported that deamidation alters the structure of the βB1 dimer.\textsuperscript{24}

As stated, we identified deamidation of N\textsubscript{146} in a fragment of human αB-crystallin isolated from normal and cataractous lenses of 60- to 80-year-old donors.\textsuperscript{16} Such deamidation of N\textsubscript{146} has also been reported in bovine αB-crystallin,\textsuperscript{17} but the effect of deamidation on structure and functional properties of the crystallin is presently unknown. One way to study this is to use site-specific mutagenesis to generate deamidated αB-crystallin mutants and determine their structural and functional properties. Therefore, in this study, these mutants were used to determine the effects of deamidation on conformation, the microenvironment of various amino acids, and the chaperone activity of αB-crystallin, to gain an insight into the structural and functional properties of the crystallin.

In the present study, three mutant forms of human αB-crystallin (N78D, N146D, N78D/N146D) were generated and comparative analyses were performed to determine their chaperone activities and structural properties by static light-scattering (SLS), circular dichroism (CD), and fluorescence spectroscopy. The results show that deamidation at N\textsubscript{78} in αB-crystallin has relatively less effect on functional and structural properties than does deamidation at N\textsubscript{146}.

**Materials and Methods**

The restriction endonucleases Neol and EcoRV and the molecular weight protein markers and DNA markers were purchased from Amersham Biosciences (Arlington Heights, IL) and Promega (Madison, WI), respectively. T7 promoter, T7 terminator, and other primers used in the study were obtained either from Invitrogen (Carlsbad, CA) or from the University of Alabama at Birmingham’s Oligo Synthesizing Core Facility. Molecular biology-grade chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. All chemicals for two-dimensional (2-D) gel electrophoresis were from Amersham Biosciences or BioRad (Hercules, CA). Diethylaminoethyl (DEAE)-Sephacel and butyl-Sepharose were obtained from Amersham Biosciences.

**Bacterial Strains and Plasmids**

The bacterial strain *Escherichia coli* BL21 (DE3)pLysS was transformed with mutant amplicons according to a standard procedure.\textsuperscript{26} The proteins were overexpressed by addition of isopropylthiogalactoside (IPTG; final concentration of 1 mM) and the cultures were incubated further at 37°C for 3 hours. Bacterial cells were lysed in 50 mM Tris-HCl buffer (pH 7.5) containing 0.5 mM EDTA and 0.3 mM NaCl (TEN buffer). The isolation and purification of the mutant proteins was performed as described by Merck et al.\textsuperscript{27} and Pasta et al.,\textsuperscript{28} with a few modifications, as follows. The soluble fraction was dialyzed against 50 mM Tris-HCl buffer (pH 7.0) containing 0.5 M (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, and the bound proteins were eluted with a gradient of 0 to 0.5 M NaCl in TEN buffer. Next, the fractions containing αB-crystallin were identified by SDS-PAGE (15%), pooled, and subjected to hydropathic interaction chromatography using a butyl-Sepharose column. During the chromatography, the equilibration buffer contained 50 mM phosphate buffer (pH 7.0) containing 0.5 mM EDTA and 1 mM dithiothreitol (DTT; TED buffer) and subjected to DEAE-Sepacel ion-exchange chromatography. The bound proteins were eluted with a gradient of 0 to 0.5 M NaCl in TED buffer. Next, the fractions containing αB-crystallin were dialyzed against water at 5°C for 24 hours. The purity of the wild-type (WT) and mutant αB-crystallin species was examined by SDS-PAGE and 2-D gel electrophoresis. The concentrations of the proteins were determined by using a protein determination kit (Pierce, Rockford, IL) and by absorbance at 280 nm, as required.

**Expression and Purification of Wild-Type and Deamidated Proteins**

*E. coli* BL21 (DE3)pLysS was transformed with mutant amplicons according to a standard procedure.\textsuperscript{26} The proteins were overexpressed by addition of isopropylthiogalactoside (IPTG; final concentration of 1 mM) and the cultures were incubated further at 37°C for 3 hours. Bacterial cells were lysed in 50 mM Tris-HCl buffer (pH 7.5) containing 0.5 mM EDTA and 0.3 mM NaCl (TEN buffer). The isolation and purification of the mutant proteins was performed as described by Merck et al.\textsuperscript{27} and Pasta et al.,\textsuperscript{28} with a few modifications, as follows. The soluble fraction was dialyzed against 50 mM Tris-HCl buffer (pH 7.9) containing 0.5 mM EDTA and 1 mM dithiothreitol (DTT; TED buffer) and subjected to DEAE-Sepacel ion-exchange chromatography. The bound proteins were eluted with a gradient of 0 to 0.5 M NaCl in TED buffer. Next, the fractions containing αB-crystallin were identified by SDS-PAGE (15%), pooled, and subjected to hydropathic interaction chromatography using a butyl-Sepharose column. During the chromatography, the equilibration buffer contained 50 mM phosphate buffer (pH 7.0) containing 0.5 M (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, and the bound proteins were eluted with a decreasing (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} concentration (i.e., 0.5–0.0 M). Again, the αB-crystallin-containing fractions were analyzed by SDS-PAGE, concentrated by lyophilization, and dialyzed against water at 5°C for 24 hours. The purity of the wild-type (WT) and mutant αB-crystallin species was examined by SDS-PAGE and 2-D gel electrophoresis. The concentrations of the proteins were determined by using a protein determination kit (Pierce, Rockford, IL) and by absorbance at 280 nm, as required.

**2-D Gel Electrophoresis**

The protein samples were mixed with resolubilization buffer (5 M urea, 2 M thiourea, 2% [3-C-cholamidopropyl]dimethylammonio-1-propanesulfonate (CHAPS), 2% cetylpyridinium betaine 3:10, 2 mM tri-n-butyl phosphate, and 40 mM Tris (pH 8.0)) in a 2:1 ratio.\textsuperscript{29} Each sample was subjected to 2-D gel electrophoresis (isoelectric focusing [IEF] in the first dimension and SDS-PAGE in the second dimension). IEF separation was performed (Immobiline DryStrips; pH range, 3.0–10.0), by following the manufacturer’s instructions (Amersham Biosciences). SDS-PAGE in the second dimension was performed with a 15% polyacrylamide gel.

**Chaperone Activity Assays**

The chaperone activity of the WT and the deamidated αB-crystallin species was studied by using three assay methods, in which the

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**Table 1. Oligonucleotide Primers Used in Mutagenesis of N\textsubscript{146}, N\textsubscript{78}, and N\textsubscript{78}/N\textsubscript{146} Residues in Human Lens αB-Crystallin**

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Primers</th>
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<tbody>
<tr>
<td>N146D</td>
<td>Forward: 5’GGGGTCTCCTACCTGTTGGACGGAACAAAGACAGG3’</td>
</tr>
<tr>
<td>N78D</td>
<td>Forward: 5’GACAGGTTTCCTGCTGACCTGATGTTCATGACACGCAC3’</td>
</tr>
</tbody>
</table>

Mutated bases are set in bold.

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**Effect of Deamidation of N\textsubscript{146} on Human αB-Crystallin**

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aggregation of the target protein was induced either by thermal or nonthermal means. In these analyses, the aggregation of the target proteins was monitored at 360 nm (due to light-scattering) as a function of time (60 minutes) using a scanning spectrophotometer (model UV-2101 PC; Shimadzu, Columbia, MD) equipped with a six-cell positioner (model CPS-260; Shimadzu) and a temperature controller (model CPS-260; Shimadzu). With insulin as a substrate, the aggregation was determined at 25°C. Aggregation of insulin (1 μM in 10 mM phosphate buffer [pH 7.4] containing 100 mM NaCl) was initiated by 20 mM DTT at different chaperone to target protein molar ratios (αB-crystallin to insulin) as described in the Results section. During the thermal aggregation assay, 1 μM citrate synthase (CS, in 50 mM phosphate buffer [pH 7.8] containing 150 mM NaCl and 2 mM EDTA) was incubated at 45°C with various concentrations of αB species to obtain the desired chaperone-to-target protein molar ratios. In the third assay, aggregation of human αD-crystallin was monitored at 65°C. Human recombinant αD-crystallin was purified as previously described by Srivastava and Srivastava. Recombinant human αD-crystallin (1 μM) in phosphate buffer was incubated in the absence or presence of various concentrations of the αB species, and the identical assay was performed for each concentration.

Fluorescence Studies
All fluorescence spectra were recorded in corrected spectrum mode with a spectrofluorometer (RF-551PC; Shimadzu) with excitation and emission band-passes set at 5 and 3 nm, respectively. The total tryptophan (Trp) intensities of the WT (0.15 mg/mL) or the deamidated αB species (0.15 mg/mL) in 10 mM phosphate buffer (pH 7.4) containing 100 mM NaCl were recorded by excitation at 295 nm.

The binding of a hydrophobic probe, 8-anilino-1-naphthalenesulfonate (ANS) to WT and αB mutants was determined as described previously. To the WT or deamidated αB species (0.15 mg/mL) in 10 mM phosphate buffer (pH 7.4) containing 100 mM NaCl, we added a 0.1-cm-path-length cell of 20,000 of the WT and three deamidated αB-crystallin to insulin used. The percentage protection from aggregation of Site-Specific Mutations in αB-Crystallin Mutants
Human lens αB-Crystallin contains two asparagines (N) at positions 78 and 146. By point mutation, each of the N residues was changed to D in two individual mutants, and both N78 and N146 were changed to D in one mutant. The resultant deamidated proteins are referred to as N78D (N at position 78 changed to D), N146D (N at position 146 changed to D), and N78D/N146D (N at 78/146 positions changed to D) mutants throughout the text.

DNA sequencing data confirmed the mutations at the desired positions in the three mutants (results not shown). To confirm this further, the expressed WT and mutant proteins after trypsin digestion were analyzed by matrix-assisted desorption ionization-time of flight (MALDI-TOF) mass spectrometry. The isotopic distribution of tryptic fragments of αB-crystallin species further confirmed mutation(s) of N to D residue at the desired positions. A trypic peptide with a mass of 921.57 was detected in WT representing residue 75-82 (sequence: RFSVNLVDVK) of αB-crystallin (Fig. 1A). In the mutant N78D, the major peak observed had a mass of 922.56, suggesting a gain of 1 mass unit, due to mutation of N78 to D78 (Fig. 1C). Similarly, a peptide with a mass of 2625, representing residue 124-149 (sequence: RIPADVDPLTITSSLSSDGVLTVNGPR) of αB-crystallin was seen in the WT (Fig. 1B), but a peptide with mass of 2626.08 was relatively higher in the mutant N146D compared with a peptide with mass of 2625 (Fig. 1D). A gain of 1 mass unit in the peptide with a mass of 2625 in the mutant compared with WT suggested mutation of N146 to D146. Similar results (i.e., gain of 1 mass unit) were obtained in two peptides with a mass of 922.56 and 2626.08 from the N78D/N146D mutant compared with the WT, confirming the mutations at both the sites (results not shown).

Expression and Purification of Human Recombinant αB- and Deamidated αB-Crystallin Mutants
The WT and the mutated αB-species were expressed in E. coli. SDS-PAGE analysis and MALDI-TOF analyses showed the expression of full-length recombinant αB-crystallin in the system used. The expressed proteins were purified to homogeneity as described in the experimental procedure in the Methods section, using ion-exchange and hydrophobic chromatography. After purification, SDS-PAGE analysis showed a single protein band of 20,000 of the WT and three deamidated αB mutant species (Fig. 2). During the hydrophobic chromatography, all three mutants were found to be more acidic than the WT species. The observation suggested that the deamidated mutants differ considerably from the WT in columbic interactions.

Comparison of the Effect of Deamidation on the Chaperone Activity of WT and Deamidated αB Mutants
As stated earlier, the chaperone assay was performed using three different substrates at room temperature or at elevated temperatures. Figure 3A, shows chaperone activity of αB-crystallin species on DTT-induced aggregation of insulin at 25°C at various chaperonin-to-insulin ratios. The WT showed a maximum chaperone activity at all the ratios of chaperonin to insulin used. The percentage protection from aggregation (monitored at 360 nm) of insulin increased as the concentrations of WT αB-crystallin were increased. At the 1:2.1:2 molar ratio of insulin to WT αB or mutant, the WT showed 50% protection, whereas the N78D showed 40% and N146D and N78D/N146D mutants showed only 8% to 20% protection. At a higher ratio of chaperonin to insulin (2:1), the activity of the WT increased to 80% and of the N78D mutant to 45%, but it remained only 8% to 10% in the N146D and N78D/N146D mutants.

Figure 3B shows the chaperone activity of WT and the three αB-mutants against heat-induced aggregation of CS at 43°C. Again, the WT showed increasing chaperone activity with increase in concentration. Further, whereas the N78D mutant showed almost the same level of chaperone activity as the WT,

Static Light-Scattering
The light-scattering experiments were performed on an SLS instrument (Model 202; Precision Detectors, Bellingham, MA). Protein samples in 50 mM Tris-HCl (pH 7.9) were filtered through a 0.2-μm filter before analysis. Results used both 90° and 15° light-scattering detection.

RESULTS
Confirmation of Site-Specific Mutations in αB-Crystallin Mutants
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the N146D and N78D/N146D mutants showed very little chaperone activity (5%–8%) compared with WT and the N78D mutant at all the ratios.

Figure 3C shows the chaperone activity of WT and deamidated αB mutants during heat-induced denaturation at 63 °C of human recombinant γD-crystallin at different molar ratios of γD and WT or mutant proteins. The WT showed maximum chaperone activity compared with the three mutants; however, the protection of denaturation of γD-crystallin was only 40% compared with the activity observed during the two assays. All three mutants showed very little chaperone activity at the two ratios of 0.3:1 and 0.6:1 (chaperonin to γD-crystallin), but, at the higher molar ratio of 1.2:1.2, the mutant N78D showed relatively higher chaperone activity compared with the other two (N146D and N78D/N146D) mutants. Together, the results show that although the chaperone activity of WT and the three deamidated αB mutants varied in the three assay systems, typically the order of chaperone activity remained the same (i.e., WT > N78D > N146D > N78D/N146D). Further, the chaperone activity of N146D and N78D/N146D mutants was considerably reduced compared with WT and N78D, suggesting the relatively greater role of N146 compared with N78 in the maintenance of chaperone function of αB-crystallin. These findings are supported by the earlier reports, which showed that the difference in stoichiometry of chaperone to target protein affects chaperone activity.4,28

Surface Hydrophobicity of WT and αB Deamidated Mutants

ANS (a hydrophobic probe) is nonfluorescent in aqueous solution and becomes fluorescent when bound to the hydrophobic residues on the surface of a molecule. Figure 4A shows the fluorescence intensity of WT and the mutant proteins after binding with ANS at 37 °C. At 12 μM ANS concentration, the fluorescence intensity of ANS bound to WT was at its maximum compared with the mutants. At an identical concentration, the fluorescence intensity of ANS-bound to N78D, N146D, and N78D/N146D was 85%, 60%, and 25%, respectively, compared with WT. The data suggest that after mutation of the N146 residue, the hydrophobicity of the mutants compared with WT was significantly reduced, but similar deamidation of N78 caused little change in hydrophobicity.

Figure 4B shows the surface hydrophobicity of WT and mutant proteins after ANS binding at 43 °C. In this experiment, the samples were heated to 43 °C with ANS and the fluorescence intensity of each sample was measured after cooling to room temperature. The fluorescence intensity of ANS bound to WT and N78D at 43 °C was 10% higher than the intensity observed at the physiological temperature (37 °C). In contrast, the fluorescence intensity of ANS bound to the mutants N146D and N78D/N146D at 43 °C decreased significantly compared with ANS-binding at the physiological temperature (37 °C). Together, the data suggest that the relative surface hydrophobicity of WT and mutants were dependent on temperature, but in general the hydrophobicity of the N146D and N78D/N146D mutants was lower compared with the WT and the N78D mutant.

Secondary and Tertiary Structure Analyses

Comparative analyses of secondary and tertiary structure of WT and the three deamidated αB-mutants were performed by determining their CD spectra and Trp fluorescence, respectively. Tryptophan fluorescence data reflect a protein’s gross positioning of Trp residues. Proteins that differ in the exposure of their...
Trp residues may differ in the wavelength of illumination at which fluorescence emission maxima are obtained (\( \lambda_{\text{max}} \)). In our study, the fluorescence emission spectrum of WT had the highest fluorescence intensity and a \( \lambda_{\text{max}} \) at 358 nm (Fig. 5). The N78D mutant showed 50% fluorescence intensity and an emission maximum at 340 nm, indicating an insignificant change in its local environment of the Trp residue compared with WT. The N146D and N78D/N146D mutants showed an emission maximum at 330 and 332 nm, respectively, the Trp fluorescence was highly quenched in these two mutants compared with the WT, suggesting substantial changes in the microenvironment around the Trp in the N146 mutant.

**Far UV CD of WT and the Deamidated Mutants**

The changes in secondary structure in WT and the three mutants were examined by determining their far-UV CD spectra. Thevariation of CD as a function of wavelength is shown in Figure 6. The CD spectrum of WT showed a minimum around 210 to 212 nm, unlike the typical 218-nm band for \( \beta \)-sheet conformation. The results presented herein are similar to those in a previous study, which showed a difference in \( \lambda_{\text{max}} \) in the negative band in \( \alpha \)-A and \( \alpha \)-B-crystallins.34 The present study used the PROSEC program to deconvolute the CD spectra. The estimates of secondary structure obtained indicated that the conformation of the WT consisted primarily of \( \beta \)-sheet (58%–65%) with some \( \alpha \)-helix, which is consistent with the previously published data.3,4 No significant change in CD spectra was observed in the N78D mutant compared with the N146D and N78D/N146D mutants (Fig. 6). Although the...
The intensity of the negative ellipticity did not significantly change in the case of the mutants N146D and N78D/N146D, a shift in $\lambda_{\text{max}}$ in comparison to WT was observed. The mutants showed maximum ellipticity at 207 to 210 nm, indicating the occurrence of conformational transitions. All the three mutants showed decreased $\beta$-sheet content (both $\beta$-sheet and $\beta$-turn combined were 55%–60%) compared with WT. Because the native $\alpha$B-crystallin has predominantly $\beta$-sheet conformation, the induced conformational changes appear to be a consequence of deamidation of the N residue at position 146 in the mutant.

**Quaternary Structure of WT and the Deamidated Mutants**

To compare the quaternary structure of the WT and the mutants, the molecular weights of these species were determined by an SLS instrument as described in the experimental procedures. The method is reliable because it uses gel-permeation chromatography coupled to low- and high-angle laser light-scattering and a differential refractive index detector to determine the molecular weight. Figure 7 shows the elution profile intensity of the negative ellipticity did not significantly change in the case of the mutants N146D and N78D/N146D, a shift in $\lambda_{\text{max}}$ in comparison to WT was observed. The mutants showed maximum ellipticity at 207 to 210 nm, indicating the occurrence of conformational transitions. All the three mutants showed decreased $\beta$-sheet content (both $\beta$-sheet and $\beta$-turn combined were 55%–60%) compared with WT. Because the native $\alpha$B-crystallin has predominantly $\beta$-sheet conformation, the induced conformational changes appear to be a consequence of deamidation of the N residue at position 146 in the mutant.

### Figures

**Figure 4.** Fluorescence spectra of WT and three deamidated mutants of $\alpha$B-crystallin after ANS binding. Fluorescence spectra of ANS bound to WT and the three deamidated mutants at (A) $37^\circ$C and (B) $43^\circ$C. The concentration of each $\alpha$B-crystallin species was 0.15 mg/mL, and that of ANS was 12 $\mu$M. WT and mutated protein preparations were heated with ANS at $43^\circ$C for 10 minutes and fluorescence spectra determined after cooling them to room temperature. Arrows: WT $\alpha$B-crystallin and the three deamidated mutants.

**Figure 5.** Intrinsic Trp fluorescence spectra of WT and three deamidated mutants of $\alpha$B-crystallin. The preparations were excited at 295 nm and fluorescence spectra were recorded between 300 and 400 nm of emission. The concentration of each crystallin species was 0.15 mg/mL. Arrows: WT $\alpha$B-crystallin and the three deamidated mutants.

**Figure 6.** Far-UV circular dichroism spectra of WT and three deamidated mutants of $\alpha$B-crystallin. The spectra were recorded at the concentration of 1 mg/mL of each $\alpha$B species and a cell path length of 0.1 cm. Arrows: WT $\alpha$B-crystallin and the three deamidated mutants.

**Figure 7.** SLS measurements of WT and three deamidated mutants of $\alpha$B-crystallin. Elution profile and molecular mass of oligomers formed by WT and the deamidated mutants (arrows). The concentration of the $\alpha$B-crystallin species was 1 mg/mL.
The static light measurements were made by both 15° and 90° detectors.

and the molecular mass of WT and the three mutants. Based on the difference of elution of WT and the mutants from an SLS column (TSK G-4000 PWXL, Toso Haas, Montgomeryville, PA) and their size detection by the light-scattering instrument, the sizes of the oligomeric proteins were determined. The mutant N78D/N146D (770,000) eluted last followed by N146D (750,000) and N78D (670,000) just ahead of WT αβ-crystallin (580,000). Together, the data suggest that the mutants formed larger oligomers than the native αβ-crystallin (Fig. 7). The molecular weight at the peak obtained from the light-scattering measurements is shown in Table 2. The observed molecular weight of αβ-crystallin oligomer was found to be 580,000, which matched with an earlier reported study.3

### DISCUSSION

In a previous study,16 we showed that N146 residue in human αβ-crystallin undergoes in vivo deamidation, and several fragments containing this modification were found in both water-soluble and -insoluble protein fractions of normal and cataractous human lenses. The fact that only the deamidation of N146 residue was the major posttranslational modification in the αβ fragments suggested that the deamidation may cause structural changes in αβ-crystallin, leading to water insolubilization. The deamidation of crystallins increases with age,12,21,22 and it is more prevalent in crystallins present in the water-insoluble fraction.12,13 Further, deamidation of N143 in S-crystallin has shown that deamidation is influenced by surface exposure (i.e., minimal deamidation for residues with accessibility number 8 nm).35 Therefore, the relatively lower rate of deamidation at N78 in comparison to N146 in αβ-crystallin is probably due to relatively lower accessibility of the N78 residue than of the N146 residue during deamidation. As stated earlier, in a recent study we found deamidation at N146 but not at N78 in αβ fragments.16 Together, apparently the deamidation at N78 in αβ-crystallin is not naturally favorable, and as described earlier, the deamidation at this position only moderately alters the chaperone function of the crystallin.

Interaction between the chaperone activity and the target binding sites involves hydrophobic patches in α-crystallin, but these are not the sole determinant.36 A previous report has shown that at physiological temperature (37°C), αα- and αβ homopolymers show almost the same levels of hydrophobicity. However, in another study, αα-crystallin was shown to be a better chaperone at higher temperature, due to conformational changes that exposed additional hydrophobic sites, whereas no such transition occurred in αβ-crystallin.36

To gain an insight into the differences in chaperone activity of the WT and mutants of αβ-crystallin, we compared structural properties of these species. A monomeric dye, ANS, is known to bind to solvent-exposed hydrophobic residues and become highly fluorescent. Both bis-ANS and ANS have been used to probe hydrophobicity of αα and αβ-crystallins.36 The fluorescence spectra of the probe bound to WT and the N78D mutant showed a moderate increase (10%) at 43°C compared with that shown at physiological temperature (37°C). However, N146D and N78D/N146D mutants showed a significant decrease in the fluorescence intensity at 43°C compared with that at 37°C, indicating lesser accessible hydrophobic residues at the elevated temperature. This finding was further supported by the results of intrinsic Trp fluorescence spectra, which showed a decrease in maximum emission in the above two mutants compared with WT. Together, the data suggest exposure of a relatively lesser number of hydrophobic residues in the mutants compared with WT, which may also explain the lower chaperone activity in the two mutants at 43°C with Cs as a substrate. Apparently, when the temperature was increased from 37°C to 43°C, the two deamidated mutants (N146D and N78D/N146D) underwent conformational changes that led to burying of certain hydrophobic residues and in turn a reduction in the sites available for substrate binding during chaperone activity. In contrast, the deamidation of N78 in the αβ

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**Table 2. SLS Determination of Molecular Weights of Oligomers of WT αβ-Crystallin and Three Deamidated Mutants (N78D, N146D, and N78D/N146D)**

<table>
<thead>
<tr>
<th>αβ-Crystallin Species</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>580,000</td>
</tr>
<tr>
<td>N78D</td>
<td>670,000</td>
</tr>
<tr>
<td>N146D</td>
<td>750,000</td>
</tr>
<tr>
<td>N78D/N146D</td>
<td>770,000</td>
</tr>
</tbody>
</table>

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**Chaperone activity of N78D and N146D varied in the three assays. During the assay with CS, the N78D mutant showed almost the same level of chaperone activity as did the WT at four stoichiometric ratios of CS to αβ species. With this system, the N146D and N78D/N146D mutant showed almost no chaperone activity. During the assay with insulin at various stoichiometric ratios, again the N146D and N78D/N146D mutants showed little chaperone activity (i.e., only 10%–20% of WT activity), but the N78D mutant had almost 50% to 75% of the activity observed with the WT. In the assay with γδ-crystallin, all three mutants showed very little chaperone activity. In a comparison of the chaperone activity at a 1:2.1.2 ratio (substrate to WT or mutants of the αβ species) during the three assays, the order of increasing activity was identical (WT>N78D>N146D>N78D/N146D).**
mutant had minimal effect on chaperone activity because of the hydrophobic residues having same level of exposure as in the WT.

The fluorescence characteristics of a Trp residue are dependent on its microenvironment. αβ-crystallin contains two Trp at positions 9 and 60. The local environment of Trp was examined by the intrinsic Trp fluorescence. The fluorescence emission varies from 320 nm in an apolar solvent to 350 nm in water. The λmax therefore throws some light on the polarity of the Trp residues—that is, the greater the emission the higher the levels of free Trp residue accessible in water. Because the N146D and N78D/N146D mutants showed a λmax at 330 to 332 nm, which is lower than the WT (339 nm), apparently the deamidation at N146 changes the microenvironment around Trp in the two mutants compared with WT. However, the exact organization of these aromatic residues will be known once the crystallographic structure of αβ-crystallin is available.

To determine the cause of reduced chaperone function in the mutants compared with WT, the structural changes because of the mutation were investigated. The far UV CD spectra revealed that the structure of αβ-crystallin was not affected on deamidation at N78 because negative ellipticity at 210 to 212 nm was observed, although the intensity was lower compared with that in WT. The N146D and N78D/N146D mutants showed a negative band at 207 to 210 nm compared with the 210-212-nm bands for WT, indicating the induction of helical conformation. Because αβ-crystallin has a predominantly β-conformation, the results suggest that the conformational transition may be induced by deamidation at N146.

Previous studies of CD spectra and Fourier transform infrared measurements have shown that the secondary structures of both αα- and αβ-homopolymers are similar, with a slightly higher content of a β-sheet structure (and lower proportion of α-helix) in αβ-crystallin. This study also concluded that the thermotropic changes in the secondary structures of αα- and αβ-crystallins were identical and could not account for the heat-induced increase in the chaperone activity in αα-crystallin. In contrast, as shown earlier, differences in the CD spectra of the WT and mutant αβ species were seen in this study. This suggests that deamidation has a profound effect on the structure of αβ-crystallin.

Our studies of hydrophobic site-binding with ANS, the Trp fluorescence, and far UV-CD spectra indicate the introduction of a negative charge after deamidation at N146 alters further conformational changes and results in loss of chaperone function. Because αβ-crystallin exists as a multimer, and it has been shown that oligomerization is a prerequisite for chaperone activity, quaternary structure of the deamidated mutants was compared with the WT. The SLS data showed that the N146D and N78D/N146D mutants formed the largest oligomer of 750,000 and 770,000, respectively, compared with N78D (670,000) and WT (580,000). The alterations in secondary structures also caused changes in the oligomerization property of the αβ-mutants. As stated earlier, the mutants with relatively higher oligomers also exhibited the lower chaperone activity, lower fluorescence intensity, and lower hydrophobicity (i.e., Trp spectra and ANS binding) compared with WT, indicating that introduction of negative charge on deamidation at N146 results in an inefficient packing (loosely organized) of the structure, destabilizes the protein structure, and hence leads to an increase in oligomer size.

Similar to other small heat shock proteins (sHSPs), αα-crystallin also contains a highly conserved sequence of 80 to 100 residues (residue 62-143 in αα-crystallin and 66-147 in αβ-crystallin) called the αα-crystallin domain. Based on similarities with the structure of other HSPs, it is believed that the N-terminal region (residue 1-62 in αα-crystallin and 1-66 in αβ-crystallin) of αα-crystallin forms an independently folded domain, whereas the C-terminal (referred as the C-terminal extension; residues 143-173 in αα- and 147-175 in αβ-crystallin) is flexible and unstructured. Both deamidation at N78 and N146 are within the αα-crystallin domain region (residue 66-147) of αβ-crystallin. The αα-crystallin domain is engaged in the subunit–subunit interactions, because recombinant αβ-crystallin containing only the αα domain region forms a dimer.40 Both N78 and N146 are important for subunit interaction and chaperone activity. Between the two, the N146 residue is relatively critical to maintenance of chaperone activity and for proper oligomer sizes of αβ homopolymers. How deamidation of either site (N78 and N146) would affect interaction of αα- and αβ-subunits remains to be determined. By and large, attempts to identify individual amino acids in subunit interactions and chaperone activity have been unsuccessful, because site-directed mutagenesis did not cause extensive perturbation in the crystallin structure. However, two disease-related point mutations of a highly conserved Arg at equivalent positions in αα (R116G) and αβ (R120G) cause structural changes that lead to hereditary cataracts.44 Deletion of the last 17 amino acids from human αβ-crystallin causes precipitation, with reduced chaperone activity, and a deletion of 25 residues from the C-terminus in Xenopus Hsp30c reduces its solubility and impairs chaperone activity.46

Together, results of these studies have shown that N- and C-terminal regions are essential for proper folding of αα-crystallin, subunit interactions between αα- and αβ-crystallins, and chaperone activity. However, the C-terminal regions seem to be needed to preserve the native structure of the molecule.46 It is presently unknown whether deamidation of N78, and/or N146 affects the role of N- and C-terminal extensions of αα-crystallin. Further, as stated earlier, deamidation may serve as a signal for proteolysis. Whether this signal is used during age- and cataract-related truncations of αα- and αβ-crystallins and other crystallins remains to be determined. We are presently attempting to find answers to these questions.

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