3-Hydroxykynurenine Oxidizes α-Crystallin: Potential Role in Cataractogenesis†

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ABSTRACT: The α-, *β*-, and *γ*-crystallins are the major structural proteins of mammalian lenses. The human lens also contains tryptophan-derived UV filters, which are known to spontaneously deaminate at physiological pH and covalently attach to lens proteins. 3-Hydroxykynurenine (3OHKyn) is the third most abundant of the kynurenine UV filters in the lens, and previous studies have shown this compound to be unstable and to be oxidized under physiological conditions, producing H_2O_2. In this study, we show that methionine and tryptophan amino acid residues are oxidized when bovine α-crystallin is incubated with 3-hydroxykynurenine. We observed almost complete oxidation of methionines 1 and 138 in αA-crystallin and a similar extent of oxidation of methionines 1 and 68 in αB-crystallin after 48 h. Tryptophans 9 and 60 in αB-crystallin were oxidized to a lesser extent. αA-Crystallin was also found to have 3OHKyn bound to its single cysteine residue. Examination of normal aged human lenses revealed no evidence of oxidation of α-crystallin; however, oxidation was detected at methionine 1 in both αA- and αB-crystallin from human cataractous lenses. Age-related nuclear cataract is associated with coloration and insolubilization of lens proteins and extensive oxidation of cysteine and methionine residues. Our findings demonstrate that 3-hydroxykynurenine can readily catalyze the oxidation of methionine residues in both αB- and αA-crystallin, and it has been reported that α-crystallin modified in this way is a poorer chaperone. Thus, 3-hydroxykynurenine promotes the oxidation and modification of crystallins and may contribute to oxidative stress in the human lens.

The human lens contains a high concentration of proteins called crystallins which are the main contributors to the refractive index of the lens. The crystallins can be separated into three distinct families, the α-, *β*-, and *γ*-crystallins, on the basis of their oligomeric size. α-Crystallin, which exists as a polydisperse, multimeric protein with an average molecular mass of ∼700 kDa (*1*), can also function as a molecular chaperone (*2*) and has two subunits, αA and αB, each with a mass of approximately 20 000 Da.

The human lens also contains a number of UV-absorbing compounds derived from the metabolism of tryptophan (*3*). The most abundant of these UV filters is 3-hydroxykynurenine glucoside (3OHKynG),[1] which is found at levels of up to 500 nmol/g in lens tissue (*4*). Kynurenine (Kyn) and 3-hydroxykynurenine (3OHKyn) are also detected in human lens extracts at levels approximately 1 and 2 orders of magnitude lower, respectively.

Previous studies have shown that at neutral pH, UV filters can readily undergo side chain deamination and covalently attach to proteins in the lens (*5*, *6*). In vitro, cysteine and histidine have been shown to be the major sites of modification of the lens protein by Kyn and 3OHKynG at physiological pH (*7*, *8*), while in vivo, Cys, His, and Lys have been identified as residues to which Kyn and 3OHKynG are bound (*9*, *10*). 3OHKyn is the only UV filter with a free aromatic hydroxyl group; thus, it can readily participate in complex oxidative processes (*11*). In the normal older lens, 3OHKyn would also be expected to deaminate and bind to lens proteins through its side chain, and work in our laboratory has confirmed that this is the case (*12*).

With age, levels of the major lens antioxidant, glutathione (GSH), decrease significantly, particularly in the nucleus (*4*), and at levels below ∼1 mM GSH, the likelihood of 3OHKyn oxidation is greatly increased (*13*). Furthermore, the production of H_2O_2 as a result of 3OHKyn autoxidation (*14*) could augment the intracellular oxidative processes, leading to oxidation of amino acid residues. It has been reported that oxidation of methionine and Trp residues in both bovine and human α-crystallin occurs in vivo (*15*−*21*). Oxidation at the same residues could be duplicated in vitro using Fenton chemistry (*22*, *23*), implying that H_2O_2 and/or hydroxyl radical was the oxidizing species. Oxidation of α-crystallin has been demonstrated to cause a significant reduction in the chaperone efficacy of α-crystallin, suggesting that oxidation may have important consequences for protein aggregation in the lens (*24*).

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[1] Abbreviations: GSH, glutathione; 3OHKyn, 3-hydroxykynurenine; 3OHKynG, 3-hydroxykynurenine glucoside; Kyn, kynurenine; M_{ox}, methionine sulfoxide; Msr, Met sulfoxide reductase; W_{ox}, oxidized tryptophan; TFA, trifluoroacetic acid.

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In this study, we aimed to identify the products formed when bovine α-crystallin was incubated with 3OHKyn in vitro. Furthermore, we proposed to use the tryptic peptides identified from these modified proteins as standards to search for analogous in vivo modifications in the normal and cataractous human lens.

EXPERIMENTAL PROCEDURES

Materials. All organic solvents and acids were HPLC grade (Ajax, Auburn, Australia). Milli-Q water (purified to 18.2 MΩ/cm^2) was used in the preparation of all solutions. Formic acid, HCl, horseradish peroxidase, 3OHKyn, *o*-phenylenediamine, trifluoroacetic acid (TFA), thiourea, Tris-HCl, and urea were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Trypsin was from Promega.

Incubation of α-Crystallin with 3OHKyn. Bovine α-crystallin (20 mg) and 3OHKyn (4 mg) were dissolved in 100 mM phosphate buffer (pH 7.2, 4 mL), and chloroform (20 μL) was added to inhibit bacterial growth. The solution was bubbled with argon (5 min), sealed, wrapped in foil, and incubated at 37 °C for 48 h. Control incubations of α-crystallin in the absence of 3OHKyn were performed in parallel. An aliquot was taken at time zero for mass spectral analysis. After incubation for 48 h, an equal volume of 1% (v/v) formic acid was added to the remaining mixture prior to reversed phase HPLC analysis.

Measurement of Oxygen Levels. Oxygen levels were measured using a Strathkelvin (Glasgow, U.K.) oxygen electrode in a 1 mL cell at 37 °C, connected to a Strathkelvin 781 oxygen meter with electrode output coupled to a MacLab instrument (ADI Instruments, Sydney, Australia).

Hydrogen Peroxide (H_2O_2) Assay. A horseradish peroxidase (HRP)/*o*-phenylenediamine (OPD) assay was used to measure the H_2O_2 concentration. A sample of the reaction mixture (10 μL), Milli-Q water (10 μL), 200 mM phosphate buffer (pH 5.8) containing HRP (50 μL), and OPD (5 mg/mL, 100 μL) were added to a microtiter plate, mixed, and allowed to react for 5 min at room temperature, and the reaction was quenched by the addition of 1 M HCl (30 μL). The absorbance was measured at 490 nm, and the concentration of H_2O_2 was calculated by referencing a standard curve.

HPLC Purification of Modified α-Crystallin. Reversed phase HPLC was performed on a Shimadzu system. For analytical scale separations, a Phenomenex column (Jupiter, 5 μm, C18, 300 Å, 250 mm × 4.6 mm) was used with the following mobile phase conditions: solvent A [aqueous 1% (v/v) formic acid] and solvent B [80% (v/v) acetonitrile/H_2O and 1% (v/v) formic acid] using a linear gradient of from 0 to 100% B over the course of 40 min at a flow rate of 1 mL/min. The elution products were detected using the UV absorbance at 280 nm and freeze-dried directly after collection.

Purification of 3OHKyn-Modified α-Crystallin Tryptic Peptides. Trypsin (20 μg) was added to unmodified αA (1 mg), unmodified αB (1 mg), modified αA (1 mg), or modified αB (0.5 mg) in 50 mM ammonium bicarbonate (pH 8, 1 mL). The solution was bubbled with argon, sealed, wrapped in foil, and incubated at 37 °C for 3 h. HPLC separation of the tryptic digestion products was performed on the same system and column described above, and fractions were collected manually and lyophilized. The following mobile phase conditions were used: solvent A [aqueous 0.1% (v/v) TFA] for 5 min followed by a linear gradient of from 0 to 50% solvent B [80% (v/v) acetonitrile/H_2O and 0.1% (v/v) TFA] over the course of 50 min followed by a linear gradient from 50 to 100% B over the course of 15 min. The flow rate was 0.5 mL/min, and peptides were detected for collection using an absorbance wavelength of 229 nm.

Mass Spectrometry. Electrospray ionization (ESI) mass spectra were acquired on a quadrupole time-of-flight Q-ToF2 hybrid mass spectrometer (Waters Micromass, Manchester, U.K.) in positive ion mode. Samples collected from the HPLC separation were dissolved in 50% (v/v) aqueous acetonitrile and 0.2% (v/v) formic acid and analyzed using the nanospray source. The cone voltage ranged from 20 to 40 V, and the mass spectrometer was calibrated with (Glu^1)-fibrinopeptide B (0.5 pmol/μL). Ions observed in the spectra were cross-referenced to a database of predicted masses for tryptic peptides of bovine αA- and αB-crystallin that had been modified by 3OHKyn. Ions that matched masses in the database were further analyzed using tandem MS to confirm the identity of the modified peptide. Crystallin sequences were imported from the ExPASy proteomics server, Swiss-Prot database (http://www.expasy.ch/sprot/).

Acid Hydrolysis of Modified αA-Crystallin. Modified αA-crystallin (2 mg) was hydrolyzed in an evacuated hydrolysis tube with 6 M HCl (1 mL), thioglycolic acid (5%, v/v), and phenol (1%, w/v) for 24 h at 110 °C. The hydrolysate was freeze-dried and dissolved in 0.1% (v/v) aqueous TFA and examined by HPLC, using HPLC conditions identical to those for trypsin digestion. The UV detector was set at 360 nm.

Analysis of Human Lens Samples. Normal aged human lenses (two lenses, both 73 years of age) were obtained from the Sydney Eye Hospital Lions Eye Bank (Sydney, Australia). The cataractous lenses [type III/IV, Pirie classification (*25*)], 50 and 80 years of age, were obtained from K. T. Sheth Eye Hospital (Rajkot, India). Each lens was extracted with 7 M urea, 2 M thiourea, and 50 mM ammonium bicarbonate (pH 8) to give a concentration of approximately 46 mg/mL. An aliquot (∼4.6 mg) was taken and diluted to ∼0.9 M urea, 0.25 M thiourea, and 50 mM ammonium bicarbonate (pH 8) containing trypsin (4.4 μg). This was bubbled with argon, sealed, wrapped in foil, and incubated at 37 °C for 8 h. After this time, an additional 4.4 μg of trypsin was added to the sample, which was again bubbled with argon and incubated for a further 16 h.

Thiourea was used to protect lens proteins from artifactual oxidation, specifically, to prevent artifactual Met oxidation to Met sulfoxide (M_{ox}). The efficacy of this technique was evaluated by incubating M_{ox} or Met under conditions identical to those of the lens extracts and measuring the levels, using LC−MS, of each after the incubation period. Thiourea fully protected Met from oxidation, and there was no evidence of M_{ox} converting to Met as can occur if DTT or 2-mercaptoethanol is used to prevent oxidation (*26*−*29*).

HPLC of Human Lens Digests. HPLC was performed on the same HPLC system and column described above. The following mobile phase conditions were used: solvent A [aqueous 0.1% (v/v) TFA] for 5 min followed by a linear gradient from 0 to 10% solvent B [80% (v/v) acetonitrile/H_2O and 0.08% (v/v) TFA] over the course of 5 min followed

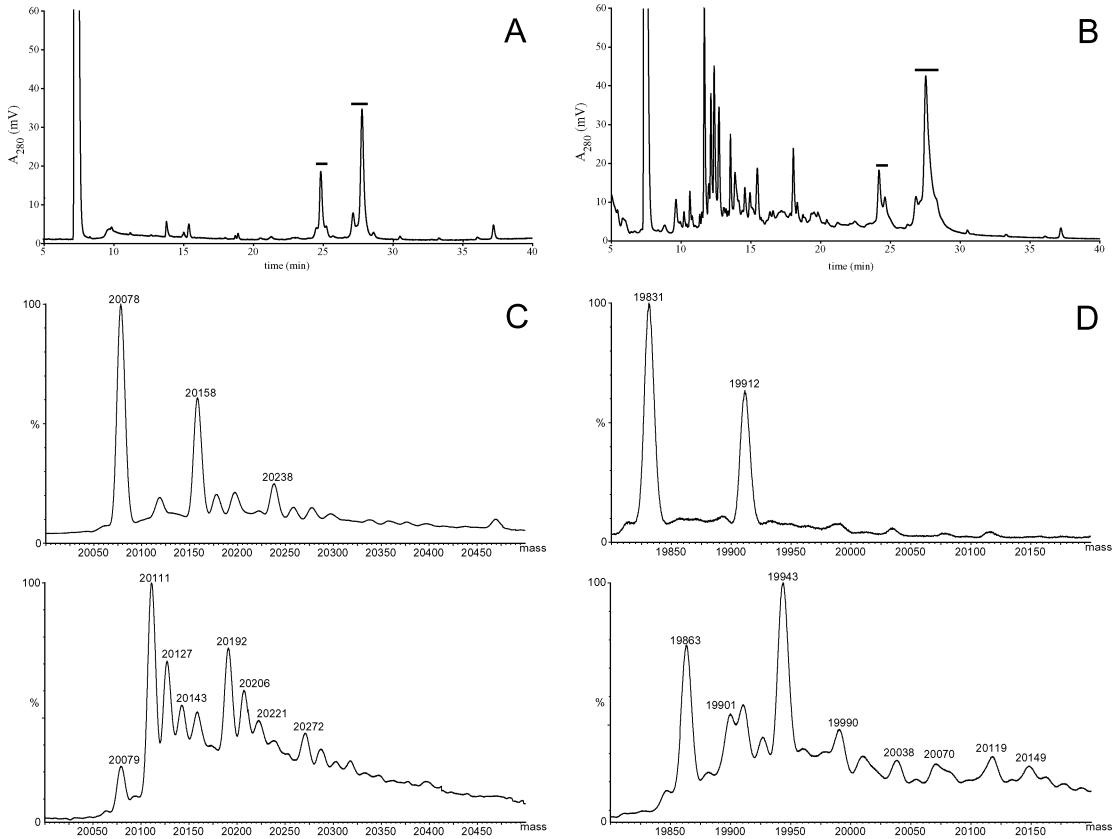

FIGURE 1: α-Crystallin was modified with 3OHKyn at 37 °C for 48 h. (A) RP-HPLC chromatogram of the initial aliquot of the reaction mixture at time zero. (B) RP-HPLC chromatogram of the reaction mixture after incubation for 48 h. Bars represent the actual range of fraction collection for each peak. (C) Transformed mass spectra of native bovine αB-crystallin (top) and modified bovine αB-crystallin (bottom). (D) Transformed mass spectra of native bovine αA-crystallin (top) and modified bovine αA-crystallin (bottom).

by a linear gradient from 10 to 60% B over the course of 65 min followed by a linear gradient from 60 to 100% B over the course of 20 min and re-equilibration in the aqueous phase for 5 min. The flow rate was 0.5 mL/min. The UV detector was set at 214 nm.

Quantification of Human Lens Peptides. Selected ion recording (SIR) was performed at unit resolution on a Quattro micro mass spectrometer (Waters Micromass) to determine the relative quantities of oxidized and normal peptides in aged normal and cataractous lenses. Peak fitting settings were as follows: smoothing (Savitzky Golay) with a window size of three scans; peak detection, valleys 30% above baseline; peak tailing, 5% wider than the leading edge; baseline, 10% peak height (maximum); peak separation, 90% above baseline; detect shoulder peaks selected, 95% of maximum; threshold, relative height of 2. Peptides were first identified by analysis on the Q-ToF2 instrument, and then these masses were employed for SIR of the $(M + 2H)^{2+}$ and $(M + 3H)^{3+}$ charge states on the Quattro micro system. Peptides were chromatographed on a Phenomenex column (Luna, 3 μm, C18, 100 Å, 150 mm × 1.0 mm) using a Waters 2695 HPLC system to create a gradient identical to that used for the human lens digests described earlier, except that 0.1% (v/v) formic acid was used in both buffers and the flow rate was 50 μL/min.

RESULTS

Modification of α-Crystallin by 3OHKyn. Bovine α-crystallin was incubated with 3OHKyn at pH 7.2, under low oxygen tension. This was achieved by bubbling the solution with argon. After the solution had been bubbled with argon for 5 min, the oxygen level had fallen to 40 mmHg as measured with an oxygen electrode. An initial aliquot was taken at time zero for mass spectral analysis, and after incubation for 48 h, the remaining mixture was separated into its component products by HPLC. The HPLC chromatogram (Figure 1A) of the sample recorded at time zero contained three major peaks. The product eluting in the first peak at 7.5 min gave rise to a molecular ion at *m*/*z* 225, corresponding to unmodified 3OHKyn. The second and third peaks eluted considerably later, at 24.8 and 27.3 min, respectively. The transformed mass spectrum (Figure 1C, top) of the peak at 24.8 min contained three major species with observed masses of 20 078 (native αB-crystallin), 20 158 (phosphorylated αB-crystallin), and 20 238 Da (doubly phosphorylated αB-crystallin). The transformed mass spectrum (Figure 1D, top) of the peak eluting at 27.3 min in the HPLC chromatogram gave rise to two peaks with observed masses of 19 831 (native αA-crystallin) and 19 911 Da (phosphorylated αA-crystallin).

After incubation for 48 h, the control samples showed no change in either their HPLC profiles or the mass spectra of the separated αA-crystallin and αB-crystallin species (not shown). The HPLC chromatogram (Figure 1B) of the α-crystallin/3OHKyn reaction mixture, however, exhibited numerous minor peaks eluting between 7.5 and ~18 min. The peak at 7.5 min was due to 3OHKyn, and most of the minor peaks were identified as oxidation products of 3OH-

Table 1: Modifications Observed by Whole Protein Mass Spectrometry Analysis of Bovine α-Crystallin after Incubation for 48 h in the Presence of 3-Hydroxykynurenine

crystallin	proposed modification[a]	expected mass (Da)	observed mass (Da)
αB	$2X_{ox}$	20 111	20 110
αB	$3X_{ox}$	20 127	20 127
αB	$4X_{ox}$	20 143	20 143
$αB_p$	$2X_{ox}$	20 191	20 192
$αB_p$	$3X_{ox}$	20 207	20 206
$αB_p$	$4X_{ox}$	20 223	20 221
$αB_{2p}$	$2X_{ox}$	20 271	20 272
αA	$2X_{ox}$	19 864	19 863
αA	$3X_{ox} + Na^+$	19 902	19 901
αA	3OHKyn	20 039	20 038
αA	$2X_{ox}$ + 3OHKyn	20 071	20 070
$αA_p$	X_{ox}	19 928	19 927
$αA_p$	$2X_{ox}$	19 944	19 943
$αA_p$	3OHKyn	20 119	20 119
$αA_p$	$2X_{ox}$ + 3OHKyn	20 151	20 149

[a] X_{ox} is methionine sulfoxide or oxidized tryptophan.

Kyn (*11*). Peaks that could not be readily identified were not further investigated.

Two major products also eluted close to the retention times of the unmodified α-crystallin species; however, the peaks were noticeably broader (Figure 1B). The transformed mass spectrum (Figure 1C, bottom) of the product eluting at 24.4 min contained a number of species with a mass greater than that of native αB-crystallin. Previous work has shown that, at pH 7, 3OHKyn undergoes facile loss of ammonia from the amino acid side chain, yielding deaminated 3OHKyn (*30*). The deaminated 3OHKyn contains an α,*β*-unsaturated moiety that is subject to nucleophilic attack. The predicted mass of bovine αB-crystallin modified with one deaminated 3OHKyn is 20 285 Da. Only a very minor peak among the spectrum noise was observed at this mass, whereas the major species that were observed had masses of 20 110 and 20 192 Da (Table 1). These masses are 32 Da higher than the mass of native αB-crystallin and that of phosphorylated αB-crystallin, respectively. Analysis of the smaller peaks in the spectrum revealed that further incremental mass increases of 16 Da had occurred, up to a total of 64 Da, for both native and phosphorylated αB-crystallin (Table 1). This result suggested that oxidation of Met and/or Trp residues had occurred in the presence of 3OHKyn, whereas no such oxidation was observed in the control experiment.

The transformed mass spectrum (Figure 1D, bottom) of the product eluting at 27.3 min also exhibited numerous peaks. The predicted mass of bovine αA-crystallin modified with 3OHKyn is 20 038 Da (Table 1). A peak at this mass can be seen in the transformed mass spectrum, as well as a peak corresponding to 3OHKyn-modified phosphorylated αA-crystallin at 20 118 Da; however, as observed for αB-crystallin, they are not major components of the spectrum. Once again, the two dominant products resulting from the incubation of αA-crystallin with 3OHKyn were observed to be double oxidation of the native and phosphorylated chains. A list of all modified species for both αA- and αB-crystallins is shown in Table 1.

Tryptic Digestion of Modified αB-Crystallin. To confirm that oxidation was responsible for the 16 Da mass increases observed in the transformed mass spectrum of 3OHKyn-modified αB-crystallin, the protein, purified by HPLC, was digested with trypsin. The HPLC chromatogram of the modified αB-crystallin digest products is shown in Figure 2. The masses of all of the predicted native and modified tryptic peptides were calculated and subsequently used to identify modified species in the digest mixture.

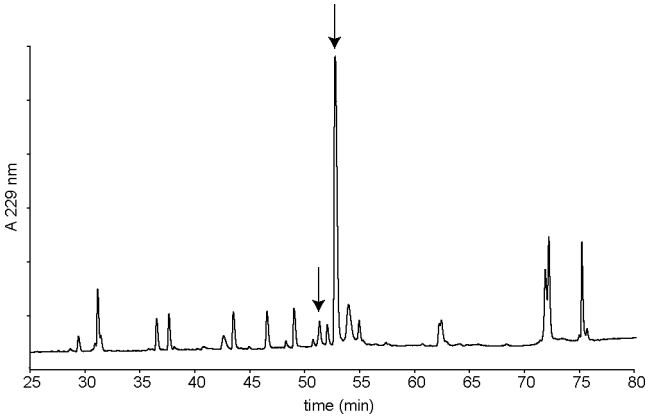

FIGURE 2: RP-HPLC chromatogram of tryptic digest products of αB-crystallin after modification with 3-hydroxykynurenine. Arrows indicate peaks which contained peptides with oxidatively modified amino acid residues.

A major peak in the HPLC chromatogram eluted at 52.8 min (Figure 2). Analysis of this fraction using nanoelectrospray ionization mass spectrometry (nanoESI-MS) showed that there was a doubly charged $(M + 2H)^{2+}$ molecular ion of *m*/*z* 723.85, which corresponded to peptide Ac-M_{ox}-DIAIHHPWIR (Table 2) of αB-crystallin in which Met had formed M_{ox} (spectrum not shown). Tandem MS (MS/MS) was used to confirm the sequence of this peptide, and the location of the modification giving rise to an additional 16 Da (Figure 3A). The series of b and y ions were consistent with the N-terminal tryptic peptide of αB-crystallin with M_{ox} at the N-terminus. This was further confirmed by the observation of a neutral loss of 64 Da (CH_3SOH) for all of the b ions, characteristic of M_{ox}, indicating that the N-terminal residue was indeed oxidized.

Another peptide, giving rise to an $(M + 2H)^{2+}$ ion of *m*/*z* 731.85, eluted at 51.2 min on the HPLC chromatogram (Figure 2). This ion corresponded to peptide Ac-M_{ox}-DIAIHHPW_{ox}IR (Table 2) in which it appeared that the M_{ox} modification was accompanied by oxidation of Trp (W_{ox}), i.e., the addition of one oxygen atom. Assignment of b and y product ions in the MS/MS spectrum (Figure 3B) confirmed Trp9 to be the residue where oxidation had occurred. A neutral loss of 64 Da was observed for the ions b_{1-8}; however, neutral losses for b_9 and b_{10} were not observed.

The second methionine residue in bovine αB-crystallin is Met68 within the tryptic peptide of residues 57−69 (APSWIDTGLSEMR), which also contains a tryptophan residue, Trp60. A peptide giving rise to an $(M + 2H)^{2+}$ ion of *m*/*z* 739.85 eluted at 51.2 min (Figure 2), corresponding to a single-site oxidation of the APSWIDTGLSEMR peptide. Oxidation of Met68 was confirmed from the MS/MS spectrum, as a neutral loss of 64 Da was observed for all of the y ions (y_{2-12}) except for y_1 (spectrum not shown). Coeluting in the same HPLC peak (51.2 min, Figure 2) was an $(M + 2H)^{2+}$ ion of *m*/*z* 747.83. This ion corresponded to a peptide with a mass 16 Da greater than that arising from the *m*/*z* 739.85 ion described above, suggesting an additional site of oxidation. This was confirmed by the MS/MS data

Table 2: Oxidative and Covalent Modifications Identified in α-Crystallin and Human Cataractous Lens Protein after Tryptic Digestion[a]

peptide	modification	sequence	measured $(M + 2H)^{2+}$ (m/z)
		Bovine	
αA 1−11	M_{ox}	Ac-M_{ox}DIAIQHPWFK	722.10
αA 120−145	M_{ox}	LPSNVDQSALSCSLSADGM_{ox}LTFSGPK	1321.5
αA 120−145	M_{ox}	LPSNVDQSALSC*SLSADGM_{ox}LTFSGPK	1425.0
αB 1−11	M_{ox}	Ac-M_{ox}DIAIHHPWIR	723.85
	M_{ox}, W_{ox}	Ac-M_{ox}DIAIHHPW_{ox}IR	731.85
αB 57−69	M_{ox}	APSWIDTGLSEM_{ox}R	739.85
	M_{ox}, W_{ox}	APSW_{ox}IDTGLSEM_{ox}R	747.83
		Human Cataract	
αA 1−11	M_{ox}	Ac-M_{ox}DVTIQHPWFK	730.42
αB 1−11	M_{ox}	Ac-M_{ox}DIAIHHPWIR	723.87

[a] Oxidation of methionine and tryptophan residues of bovine α-crystallin resulted directly from co-incubation with 3-hydroxykynurenine. 3-Hydroxykynurenine was also found attached to the single cysteine residue of αA-crystallin. M_{ox} is methionine sulfoxide. W_{ox} is oxidized tryptophan. C* is 3OHKyn-modified cysteine.

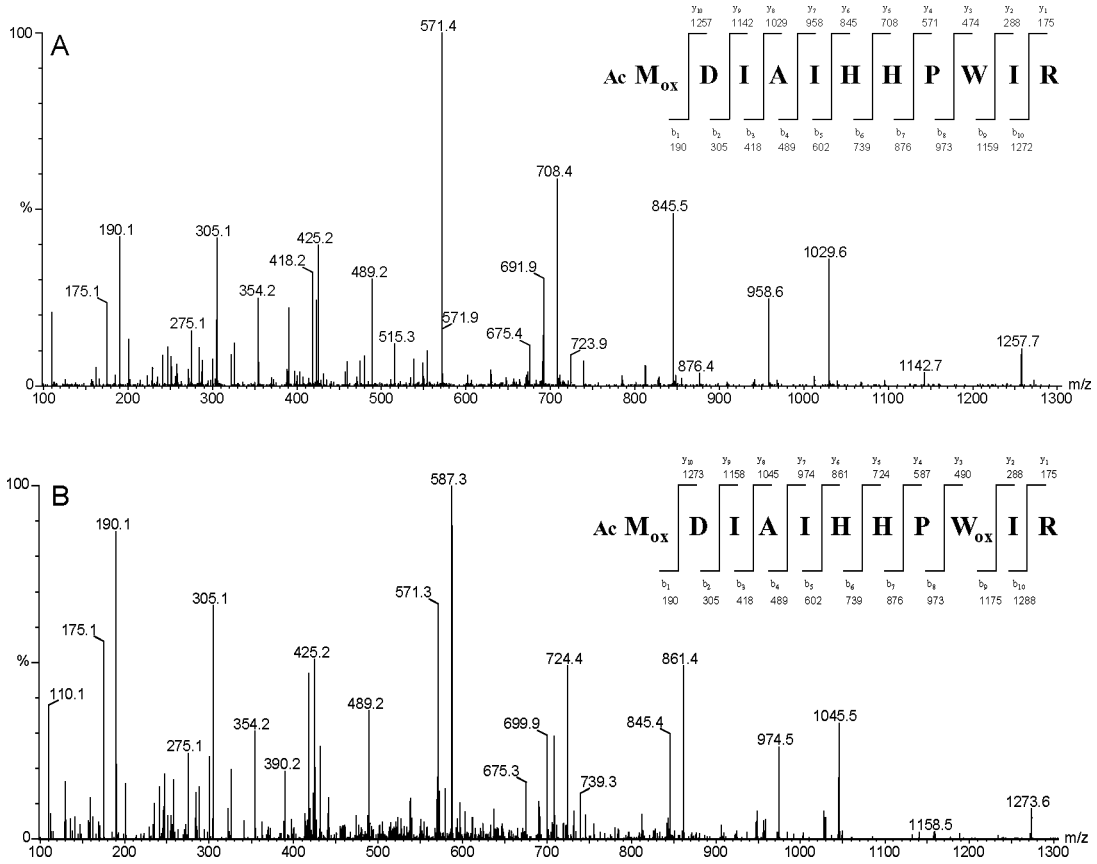

FIGURE 3: MS/MS spectra of a modified αB-crystallin peptide, residues 1−11, showing (A) oxidation at Met1 and (B) oxidation at Met1 and Trp9.

which yielded product ion series consistent with oxidation of both Met68 and Trp60 (Table 2, spectrum not shown).

Tryptic Digestion of Modified αA-Crystallin. 3OHKyn-modified αA-crystallin, purified by HPLC, was digested with trypsin, and the resulting peptide mixture was analyzed directly by nanoESI-MS (Figure 4A). The abundant $(M + 2H)^{2+}$ ion at m/z 722.1 and the abundant $(M + H)^{+}$ ion at m/z 1443.7 corresponded to the Ac-MDIAIQHPWFK peptide, the first 11 residues of αA-crystallin, in which the Met had been oxidized to M_{ox}. The MS/MS spectrum of this peptide is shown in Figure 4B. The majority of the predicted b and y ions for the peptide with N-acetylated M_{ox} at residue 1 were observed in the spectrum. This modification was further confirmed by a characteristic neutral loss of 64 Da from M_{ox} for all of the b ions (b_{1-10}).

The mass spectrum of intact 3OHKyn-modified αA-crystallin indicated that oxidation of two residues was the major modification (Figure 1D, bottom); thus, we searched the peptide mixture for evidence of a second oxidized residue. Tryptic peptide 120−145, containing the only other Met residue in bovine αA-crystallin, has a predicted mass of 2624.3 Da. We identified a doubly charged ion in the MS spectrum of the αA-crystallin digest at m/z 1321.5 (Figure 4A, inset), which corresponds to peptide residues 120−145, containing oxidized Met138. The MS/MS spectrum of this peptide confirmed that this was the correct peptide and that Met138 was indeed oxidized (Figure 4C). Thus, Met oxidation was the major modification caused by incubation of αA-crystallin with 3OHKyn. This tryptic peptide also contains the only Cys residue in αA-crystallin

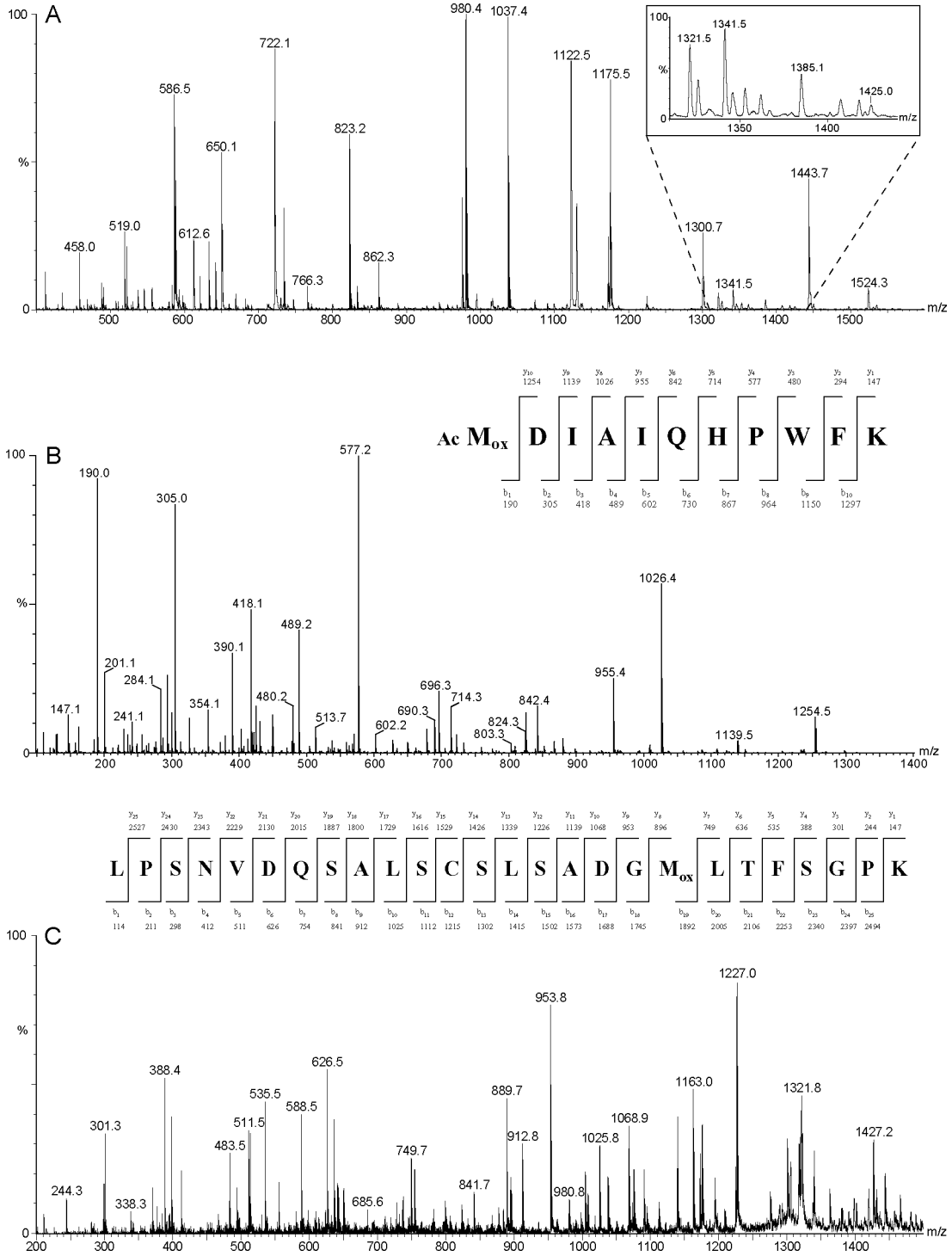

FIGURE 4: (A) NanoESI spectrum of tryptically digested, 3-hydroxykynurenine-modified αA-crystallin. (B) MS/MS spectra of an oxidized αA-crystallin peptide, residues 1−11, showing oxidation at Met1. (C) MS/MS spectra of an oxidized αA-crystallin peptide, residues 120−145, showing oxidation at Met138.

and, consequently, was the major predicted site of the covalent attachment of deaminated 3OHKyn (*7*). Closer inspection of the MS spectrum inset in Figure 4A revealed that an ion was present at *m*/*z* 1425.0, corresponding to peptide 120−145 with both oxidized Met (+16 Da) and covalently attached 3OHKyn (+207 Da). This ion was not sufficiently abundant to obtain useful MS/MS data; thus, acid hydrolysis was employed to confirm the site and relative proportion of modification by 3OHKyn.

Acid Hydrolysis of Modified αA*-Crystallin.* αA-Crystallin, which had been modified with 3OHKyn and purified by HPLC, was hydrolyzed with HCl containing antioxidants. Under these conditions, the recovery of the 3OHKyn-modified amino acids was as follows: 87% 3OHKyn-Cys, 95% 3OHKyn-Lys, and 78% 3OHKyn-His. The HPLC chromatogram (not shown) of the α-crystallin hydrolysate exhibited a doublet peak eluting at 28.4 min, the retention time corresponding to 3OHKyn-Cys. Mass spectrometry of these two peaks revealed a prominent ion at *m*/*z* 329, and MS/MS confirmed it to be 3OHKyn-Cys (*10*). Quantification of material eluting in this doublet showed that there was 0.089 mol of 3OHKyn-Cys per mole of αA-crystallin; i.e.,

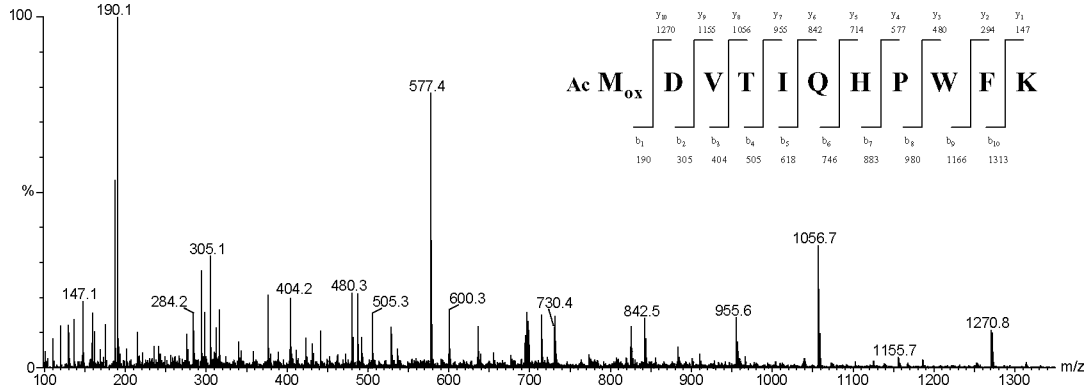

FIGURE 5: MS/MS spectrum of an oxidized human αA-crystallin peptide, residues 1−11, from a cataractous lens showing oxidation at Met1.

8.9% of the protein was modified by 3OHKyn. This value corresponds to the relative abundances of the 3OHKyn-modified and unmodified αA-crystallin peaks observed in the mass spectrum (Figure 4A, inset).

Analysis of Human Lenses. Human lens nuclei from normal aged lenses and from cataract lenses were extracted using urea and thiourea. The inclusion of thiourea was found to prevent the artifactual oxidation of Met residues during extraction and digestion. The proteins were then digested with trypsin and examined by LC−MS using a Quattro micro mass spectrometer. The SIR function was set up to detect the doubly charged $(M + 2H)^{2+}$ ions at *m*/*z* 730.42 and 722.87 corresponding to peptides Ac-M$_{ox}$DVTIQHPWFK in αA-crystallin (residues 1−11) and Ac-M$_{ox}$DIAIHHPWIR in αB-crystallin, respectively (Table 2). SIR was also used to monitor the native (i.e., unoxidized) 1−11 peptides of αA- and αB-crystallin.

Proteins from normal aged lenses were found to contain the native αA-crystallin peptide Ac-MDVTIQHPWFK (residues 1−11) and native αB-crystallin peptide Ac-MDIAIHHPWIR (residues 1−11) (spectra not shown) following tryptic digestion; however, peptides containing oxidized Met residues could not be detected. Analysis of cataractous lens nuclei also showed the presence of the native 1−11 peptides; however, an additional peptide giving rise to a doubly charged $(M + 2H)^{2+}$ molecular ion at *m*/*z* 730.42 was also present, corresponding to peptide Ac-M$_{ox}$DVTIQHPWFK (residues 1−11) in αA-crystallin (Table 2). The MS/MS spectrum of this peptide is shown in Figure 5. Similarly, a doubly charged molecular ion at *m*/*z* 723.87, corresponding to peptide Ac-M$_{ox}$DIAIHHPWIR of αB-crystallin (residues 1−11), was observed (Table 2) where the Met had oxidized to form M$_{ox}$.

Relative quantification of the peptides in cataractous lens nuclei showed that the ratio of native peptide (Ac-MDVTIQHPWFK) to oxidized peptide (Ac-M$_{ox}$DVTIQHPWFK) for peptide 1−11 in αA-crystallin was 27:1. The ratio of native peptide (Ac-MDIAIHHPWIR) to oxidized peptide (Ac-M$_{ox}$DIAIHHPWIR) for peptide 1−11 in αB-crystallin was 19:1. Of course, these values should be treated with some caution since the effects of ion suppression in a complex mixture of tryptic peptides are well-known. The overall yields of peptides derived from cataract proteins were consistently lower than those from an equal mass of normal human lens proteins, as determined by comparison of the 214 nm absorbance of 10 equivalent peaks present in normal and cataractous lenses with a ratio of 3.4:1 ± 2.8 for normal-to-cataractous lenses (data not shown). The ratio of native αA- and αB-crystallin in normal aged lenses and cataractous lenses was found to be 4.8:1 as determined by SIR. This result is consistent with the overall decline in the level of peptides present in cataractous lenses relative to that in normal lenses and is presumably due to a restricted access of trypsin to cleavage sites as a result of extensive post-translational modification of the crystallins or precipitation of the protein.

DISCUSSION

The UV filters 3OHKynG and Kyn spontaneously deaminate and covalently attach to the nucleophilic residues of lens proteins in an age-dependent manner, a process which contributes to the yellowing of the lens (*31*). This process occurs to a much greater degree after middle age, during which a barrier circumscribing the nucleus of the lens is formed (*32*). 3OHKyn is the other UV filter in the kynurenine pathway and, unlike 3OHKynG and Kyn, is an *o*-aminophenol and therefore prone to oxidation in the absence of a sufficient concentration of reductants. In this study, we used mass spectrometry to investigate the impact of 3OHKyn modification of bovine α-crystallin at physiological pH.

Mass spectra of the modified intact subunits of α-crystallin indicated that 3OHKyn was bound to only αA-crystallin at an appreciable level. Acid hydrolysis of the modified αA-crystallin confirmed that 3OHKyn was attached to 8.9% of the protein and that the site of modification was the single Cys residue in αA-crystallin at position 131. This result is in agreement with data on the relative rates of reaction of 3OHKyn with Lys, His, and Cys (unpublished) and with a similar study with Kyn, in which Cys131 was identified as the initial and major site of modification at physiological pH (*7*). αB-Crystallin does not contain a Cys residue; thus, we did not expect to observe any modifications of this protein within the time frame of the reaction. Although modification at His83 of αB-crystallin by Kyn has been previously observed (*8*), Kyn is more stable than 3OHKyn, and this modification was detected after a longer period of incubation (14 days vs 48 h).

The dominant effect observed as a result of 3OHKyn incubation with bovine α-crystallin was the oxidation of Met residues to M$_{ox}$. Indeed, α-crystallin polypeptides in which both Mets had been oxidized to M$_{ox}$ were by far the major species observed after 48 h. Smaller levels of oxidation of

Trp to W_{ox} were also detected. Tryptic digests and MS/MS sequencing confirmed that Met1 and Met138 of αA-crystallin and Met1, Met68, Trp9, and Trp60 of αB-crystallin were oxidized in the presence of 3OHKyn.

The oxidation of Met and Trp residues by 3OHKyn may have important consequences for the aging lens. It is known that H_2O_2 is formed during 3OHKyn oxidation and that this process occurs rapidly in the absence of a reducing agent (*11*). Furthermore, it has been suggested that H_2O_2 is possibly the major oxidant found in the lens and that the concentration of H_2O_2 increases in cataract lenses (*33*). The reasons for this increase are not clear; however, the fact that oxygen is present in the lens (*34*, *35*), as are low levels of 3OHKyn (*4*), provides an ideal environment for the generation of H_2O_2 as the level of the major lens antioxidant, GSH, diminishes with age. In model studies in which intact bovine lenses (which lack UV filters) were incubated with 3OHKyn, the levels of GSH were found to decrease markedly (*36*). This suggests that 3OHKyn can function as an effective prooxidant within the environment of the lens. In the experiments described herein, the amount of H_2O_2 measured after incubation of 3OHKyn with α-crystallin for 48 h was 0.83 μmol per 0.89 μmol of 3OHKyn, corresponding to a H_2O_2 concentration of 200 μM. This was sufficient to totally oxidize the Met residues of α-crystallin. While 3OHKyn readily autoxidizes to yield H_2O_2, other molecules in the lens, such as ascorbate, may also react with oxygen to produce H_2O_2. Thus, the finding of oxidized residues on crystallins does not necessarily implicate 3OHKyn in this process.

Unlike in the case of age-related nuclear cataract lenses in which oxidation of Cys or Met residues is progressive and extensive (*37*, *38*), it would appear that there is little significant oxidation in human lens proteins with age (*39*, *40*). Oxidation of Met and Trp has, however, recently been reported at three sites in α-crystallin separated from a 25-year-old human lens (*20*). These oxidized species were present in covalent multimers, suggesting a possible role for such modifications in cross-linking. If such oxidations have not been induced during isolation and digestion, the presence of M_{ox} in young human lens proteins indicates that Met sulfoxide reductases (Msrs) may have limited activity or are lacking in activity in the lens interior. Msrs are able to reduce M_{ox} and protect lens cells against oxidative stress, evidenced by the onset of stress-induced cell death upon deletion of their genes (*41*). Oxidation of Met has been shown to inactivate the biological function of some proteins (*42*), and the observed decrease in α-crystallin chaperone efficacy upon Met oxidation by H_2O_2 is consistent with this (*24*).

Recently, it was reported that a monoclonal antibody raised against 3OHKyn-modified keyhole limpet hemocyanin reacted with epitopes on proteins associated with the human lens fiber cell plasma membrane (*43*). Although the modified membrane-associated protein was not identified, α-crystallin has long been known to interact with lens plasma membranes, with the suggested involvement of the integral membrane protein aquaporin (*44*, *45*). Furthermore, the levels of α-crystallin associated with the plasma membrane in cataractous lenses are significantly greater when compared with normal levels (*46*). These observations suggest that α-crystallin could be one of the 3OHKyn-modified membrane-associated proteins reported by Staniszewska and Nagaraj.

Approximately half of all Met residues are oxidized in the lenses of advanced nuclear cataract patients (*37*), and as much as 60% of the total Met is oxidized in membrane-bound proteins (*38*). In this study, we found that 3OHKyn was oxidized to produce approximately equimolar amounts of H_2O_2 that presumably then oxidized the Met residues, and to a lesser extent Trp residues, of α-crystallin. Tryptic peptides containing M_{ox} were detected in nuclear cataract but not normal lens digests. In this study, relatively high concentrations of 3OHKyn were used; however, we have observed significant oxidation of Met-containing peptides using 50 μM 3OHKyn, a level approaching those found in the lens (5−10 μM). It is likely that the oxidized Mets found in nuclear cataract lens proteins are derived from exposure to similarly low H_2O_2 levels over long time periods (e.g., years).

In the human lens, 3OHKyn may perform a dual role of oxidation and covalent modification of nucleophilic residues. For example, in the center of nuclear cataractous lenses, where oxidative conditions prevail, both free and protein-bound 3OHKyn may catalyze the formation of H_2O_2, potentially leading to the loss of chaperone activity of α-crystallin and protein aggregation. Protein-bound 3OHKyn can also undergo further reactions which result in the formation of intermolecular cross-links (*13*, *47*, *48*). Covalent cross-linking of polypeptides is another feature that is characteristic of age-related nuclear cataracts.

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