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Identification of tryptophan oxidation products in bovine α-crystallin

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Abstract

Oxidation is known to affect the structure, activity, and rate of degradation of proteins, and is believed to contribute to a variety of pathological conditions. Metal-catalyzed oxidation (MCO) is a primary oxidizing system in many cell types. In this study, the oxidative effects of a MCO system (the Fenton reaction) on the structure of the tryptophan residues of α-crystallin were determined. Tandem mass spectrometry (MS/MS) was utilized to identify specific tryptophan and methionine oxidation products in the bovine α-crystallin sequence. After oxidative exposure, α-crystallin was digested with trypsin, and the resulting peptides were fractionated by reverse-phase HPLC. Structural analysis by mass spectrometry revealed that tryptophan 9 of αA- and tryptophan 60 of αB-crystallin were each converted into hydroxytryptophans (HTRP), N-formylkynurenine (NFK), and kynurenine (KYN). However, only HTRP and KYN formation were detected at residue 9 of αB-crystallin. Oxidation of methionine 1 of αA- and methionine 1 and 68 of αB-crystallin was also detected. The products NFK and KYN are of particular importance in the lens, as they themselves are photosensitizers that can generate reactive oxygen species (ROS) upon UV light absorption. The unambiguous identification of HTRP, NFK, and KYN in intact α-crystallin represents the first structural proof of the formation of these products in an intact protein, and provides a basis for detailed structural analysis of oxidized proteins generated in numerous pathological conditions.

Keywords: α-crystallin, Fenton reaction, mass spectrometry, *N*-formylkynurenine, oxidation, tryptophan

Oxidation of cellular components is believed to play a major role in destruction of cellular integrity, and has been demonstrated to cause damage to specific cellular components including lipids, DNA, and proteins (Thomas et al., 1985; Imlay & Linn, 1988; Stadtman, 1990). Protein oxidation, in particular, has been reported to alter susceptibility to proteolysis and decrease enzymatic activity, which certainly has profound physiological implications (Stadtman, 1990, 1992). These studies, and others that indicate accumulation of oxidized proteins with age (Berlett & Stadtman, 1997), have led to the hypothesis that protein oxidation is an important contributor to the aging process (Gershon & Gershon, 1970; Oliver et al., 1987a, 1987b; Smith et al., 1991). Furthermore, protein oxidation products have been shown to be present in significant quantities, suggestive of a deleterious role, in numerous diseases including ischemia–reperfusion injury, rheumatoid arthritis, muscular dystrophy, emphysema, atherosclerosis, Alzheimer's disease, and senile cataract (Aruoma & Halliwell, 1989; Chapman et al., 1989; Murphy & Kehrer, 1989; Garland, 1990; Oliver et al., 1990; Dean et al., 1997).

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Metal-catalyzed oxidation (MCO) is believed to be a primary cause of biomolecular oxidation occurring in many cells. A variety of MCO systems have been shown to oxidize proteins including both enzymatic and nonenzymatic systems (Stadtman, 1993). Because MCO components are prevalent in biological systems, it is important to understand their effects on biological structure and function. The amino acids that are particularly susceptible to oxidation include methionine, cysteine, histidine, and tyrosine; however, other products have also been observed for proline, lysine, and arginine (Amici et al., 1989; Stadtman, 1990). Tryptophan residues can also be oxidized by ionizing radiation, but are typically not expected to be oxidized by MCO systems because tryptophan is not likely a site for metal ion binding (Stadtman, 1993). Several tryptophan oxidation products including hydroxytryptophans (HTRP), *N*-formylkynurenine (NFK), kynurenine (KYN), and 3-hydroxykynurenine (3OH-KYN) have been identified by characteristic absorbance and fluorescence spectra (van Heyningen, 1971; Holt et al., 1977; Maskos et al., 1992; Sen et al., 1992); however, no direct structural information has been reported for tryptophan oxidation products in intact peptides or proteins.

Tryptophan oxidation in the lens is particularly important for several reasons. First, the lens is under nearly constant UV exposure that can result in photoexcitation of tryptophan leading to the formation of the indoyl radical, which can subsequently react to

form several tryptophan oxidation products (Grossweiner, 1984; Spector, 1995). Furthermore, tryptophan oxidation products are themselves photosensitizers capable of generating reactive oxygen species (ROS) and can, therefore, perpetuate the oxidation of lens protein residues (Walrant & Santus, 1974; Pileri et al., 1976; Reszka et al., 1996). In addition to photooxidative stress, all of the components for nonenzymatic metal-catalyzed oxidation are present in the lens (Garland, 1990), including both iron and hydrogen peroxide at micromolar concentrations. Increases in hydrogen peroxide levels (24 μM in noncataractous lenses to 69 μM in cataractous lenses) and lens protein carbonyl content have been observed with age and with senile cataract formation (Spector & Garner, 1981; Garland et al., 1988), which has led to the hypothesis that lens protein oxidation is believed to play a role in senile cataractogenesis (Garland, 1990; Spector, 1995). The identification of tryptophan oxidation products and their specific structures is an important step toward understanding the roles of protein oxidation in lens disease.

Several studies have examined tryptophan oxidation products in intact lens proteins utilizing primarily protein absorbance and fluorescence measurements. In whole lens homogenates and in the intact human lens, NFK and KYN, as well as other kynurenine derivatives, have been detected (Pirie, 1971; van Heyningen, 1971; Sen et al., 1992). Although NFK has been reported to be a minor constituent of the endopeptidase-resistant fraction from human cataracts, oxindolyl alanine and KYN were found to be present in higher abundance based on fluorescence and absorbance measurements (Dillon et al., 1984). The exposure of calf lens α-crystallin to ozone has been reported to result in characteristic NFK absorption, fluorescence, and phosphorescence (Fujimori, 1982). In calf lens γ-crystallin and human lens α-crystallin, fluorescence attributed to NFK has been reported after exposure to UV irradiation (Andley & Clark, 1989a, 1989b; Walker & Borkman, 1989) and the detection of ROS production from these UV-irradiated proteins provided further indication of NFK formation (Andley & Clark, 1989a, 1989b). Based on data from these and other studies, NFK and KYN are presently considered, in the lens, to be endogenous, in vivo photosensitizers (Zigler & Goosey, 1981; Roberts & Dillon, 1989), which are produced from the oxidation of free tryptophan. However, neither the identification of specific modified tryptophan structures in lens proteins nor the locations of specific modified tryptophan residues in those proteins have been unequivocally demonstrated.

Our previous work identified the sites of photooxidation in bovine α-crystallin (Finley et al., 1997). In this study, the ability of the Fenton reaction, an MCO system, to oxidize tryptophan residues in intact lens α-crystallin, and the susceptibility of the three tryptophans to oxidation have been investigated. Mass spectrometric analysis of the structure of oxidized α-crystallin indicates that various HTRP, NFK, and KYN products are formed in the intact protein molecule by the Fenton reaction and all of the tryptophan residues of α-crystallin are susceptible to oxidation. The possible functional implications of oxidation of these tryptophan residues in α-crystallin are discussed.

Results

Tryptophan oxidation products

A variety of tryptophan oxidation products has been identified including HTRP, NFK, KYN, and 3OH-KYN. The molecular weights and structures of these residues are shown in Figure 1. The molecular weight differences of 16, 32, and 4 relative to tryptophan for HTRP, NFK, and KYN, respectively, allow for their identification in mass spectrometric analyses. The molecular weight of tryptophan is 186 Da while the various HTRP (2-, 4-, 5-, 6-, and 7-) are 16 Da higher (MW = 202). NFK (MW = 218) is formed by the addition of two oxygen atoms followed by cleavage of the indole ring and KYN (MW = 190) results from hydrolysis of NFK and loss of the carbonyl group.

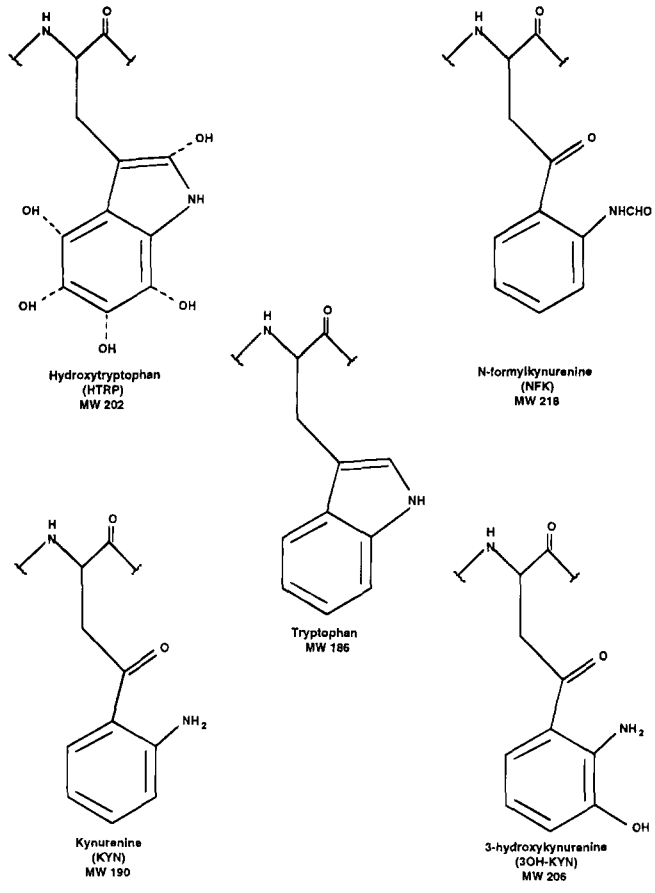

Fig. 1. The structures of tryptophan, hydroxytryptophan, *N*-formylkynurenine, kynurenine, and 3-hydroxykynurenine residues along with the corresponding molecular weights are shown.

Modified peptides of α-crystallin

Bovine α-crystallin, an aggregate composed of αA and αB subunits, was exposed to the Fenton reaction, digested with trypsin, fractionated by HPLC, and the resulting fractionated peptides were mass analyzed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. To identify the modified peptides, the masses measured by MALDI were compared to the masses from computer-generated tryptic peptide maps of bovine αA- and αB-crystallins. Figure 2 shows portions of MALDI mass spectra (mass range 1,400–1,560) of α-crystallin tryptic peptides that eluted at 27, 28, 30, and 33 min from a C18 HPLC column.

Two tryptic peptides from α-crystallin eluted at 27 min (spectrum A), which gave MALDI molecular ions at m/z 1,484 (peak 1) and m/z 1,496 (peak 2). The peptide at m/z 1,484 (peak 1) is

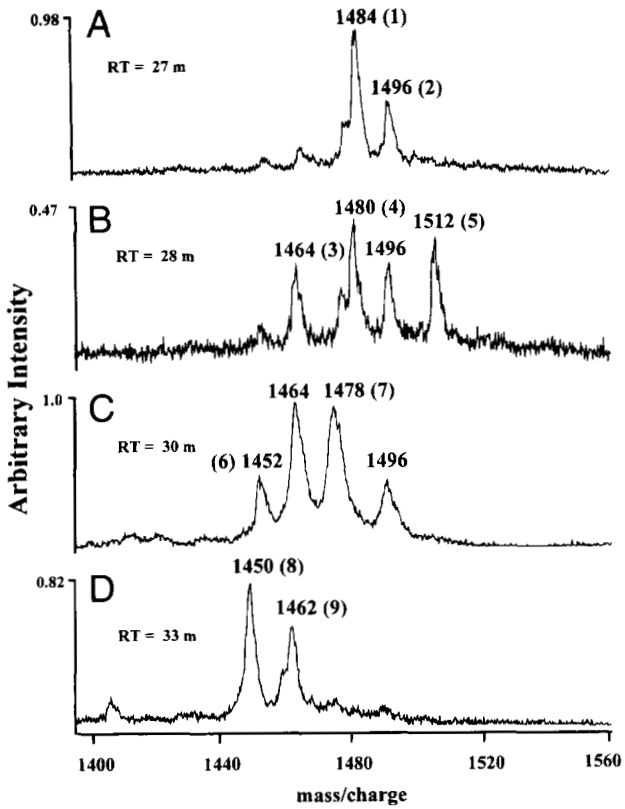

Fig. 2. MALDI spectra (mass range 1,400–1,560) of tryptic peptides of α-crystallin, after exposure to the Fenton reaction (20–100 μL H_2O_2, 0.1 mM $FeCl_2$, 0.3 mM EDTA), which eluted from a C18 column at (**A**) 27 min, (**B**) 28 min, (**C**) 30 min, and (**D**) 33 min are shown. The peaks are labeled with their respective mass-to-charge (m/z) ratios measured by MALDI. The y-axis indicates the intensity of the peaks in arbitrary units normalized to the largest peak in each spectrum.

21 Da higher than the mass of αB 57–69 ($[M+H]^+$ = 1,463), while the peptide at m/z 1,496 (peak 2) is 33 Da higher than the mass of αB 57–69. Three other peptides were observed in the MALDI mass spectrum of the 28 min fraction (spectrum B) at m/z 1,464 (peak 3), 1,480 (peak 4), and 1,512 (peak 5). Peak 3 is 32 Da higher than the mass of αB 1–11 ($[M+H]^+$ = 1,432) and also approximates the mass of αB 57–69. Peaks 4 and 5 are 17 and 49 Da higher than the mass of αB 57–69, respectively. After 30 min, two peptides at m/z 1,452 (peak 6) and 1,478 (peak 7) eluted from the C18 column (spectrum C). The peak at m/z 1,452 (peak 6) is 20 Da higher than the mass of αB 1–11, while the peak at m/z 1,478 (peak 7) is 49 Da higher than the mass of αA 1–11 ($[M+H]^+$ = 1,429). In the HPLC fraction, which eluted at 33 min (spectrum D), two peptides were detected at m/z 1,450 (peak 8) and m/z 1,462 (peak 9). Peak 8 and 9 are 21 and 33 Da higher than the mass of αA 1–11. The mass of peak 9 is also within experimental error of the mass of αB 57–69. The peptides at m/z 1,484 (peak 1), 1,496 (peak 2), 1,480 (peak 4), 1,512 (peak 5), 1,452 (peak 6), 1,478 (peak 7), and 1,450 (peak 8) were only detected in oxidized (Fenton reaction) samples of α-crystallin, and were not found in the control digests (data not shown). Peptides were detected using MALDI in the unfractionated control digest with masses in the vicinity of m/z 1,464 (peak 3) and 1,462 (peak 9), which were presumably due to the unmodified αB 57–69 peptide. By MALDI, peptides were also detected in α-crystallin exposed to the Fenton reaction with masses identical to or 16 Da higher than αA 1–11 and αB 1–11 (data not shown).

Mass shifts of 16, 32, and 48 Da above the predicted mass of an α-crystallin tryptic peptide and not corresponding to any other tryptic fragment were tentatively assigned as due to oxidation of that peptide. The addition of a single oxygen atom to one amino acid in a peptide would result in a 16 Da mass shift. A mass shift of 32 Da could be due to single addition of an oxygen atom to two different amino acids or could be due to the addition of two oxygen atoms to a single amino acid (e.g., methionine to methionine sulfone or tryptophan to NFK). Oxidation of three different amino acids, as well as formation of methionine sulfone or NFK plus a single oxygen addition to a different amino acid, would result in a mass shift of 48 Da. A mass shift of 20 Da could potentially be due to the addition of a single oxygen atom to one amino acid (16 Da) in the peptide plus formation of KYN (4 Da increase in the mass of tryptophan) or could be due to the formation of 3-hydroxykynurenine.

Oxidized α-crystallin residues

The HPLC fractionated tryptic peptides, which, by their MALDI measured molecular weights, were tentatively assigned as modified, were sequenced by tandem mass spectrometry (MS/MS) to determine the identities and locations of modifications. Peptides that were selected for sequencing had molecular weight shifts of 16, 20, 32, and 48 Da.

Tandem mass spectrometry in an ion trap mass spectrometer employs low-energy collisions of peptide ions with helium to produce fragment ions primarily of two types termed b and y ions (Biemann & Scoble, 1987). These ions result from fragmentation of the peptide amide bonds with the charge remaining either on the N-terminus (b ions) or on the C-terminus (y ions). The masses of these fragment ions correspond to the loss of particular amino acids from the peptide termini. Doubly charged molecular ions are commonly observed for tryptic peptides using electrospray ionization, and the resulting fragment ions from the MS/MS experiment are typically singly charged due to one charge remaining with each fragment upon dissociation. Tandem mass spectra for peptides containing specific tryptophan oxidation products are presented in Figures 3–5. Unmodified and methionine-oxidized αA 1–11, αB 1–11, and αB 57–69 peptides were also sequenced (data not shown), and the spectra were comparable in quality to those shown below.

The tandem mass spectrum of m/z 732 (doubly charged ion of peak 9 in Fig. 2D), which eluted at 33 min, is shown in Figure 3. Based on the MALDI measured molecular weight (1,462), this mass was originally assigned as either unmodified αB 57–69 or αA 1–11, with two additional oxygen atoms. The b_2 (m/z 305), b_3 (m/z 418), b_4 (m/z 489), b_5 (m/z 602), and b_7 (m/z 867) fragment ions from this peptide were identical to those measured in the MS/MS spectrum of the oxidized αA 1–11 peptide (data not shown), which verified that this peptide is αA 1–11. The b_2 ion indicates that one site of oxidation is on the N-terminus and again implicates either methionine 1 or aspartic acid 2 as the site of oxygen addition. The peak at m/z 699 corresponds to the doubly charged molecular ion ($[M+2H]^{2+}$) of m/z 732 after loss of $(CH_3SOH)^{2+}$. The loss of $(CH_3SOH)^{2+}$ from the molecular ion (Lagerwerf et al., 1996), combined with the y_{10} ion (m/z 1,271), confirms that methionine 1 is oxidized, which accounts for 16 Da of the 32 Da mass shift. The mass of the C-terminus containing y_2 ion (m/z 294) in the tandem mass spectrum is identical to the predicted mass for

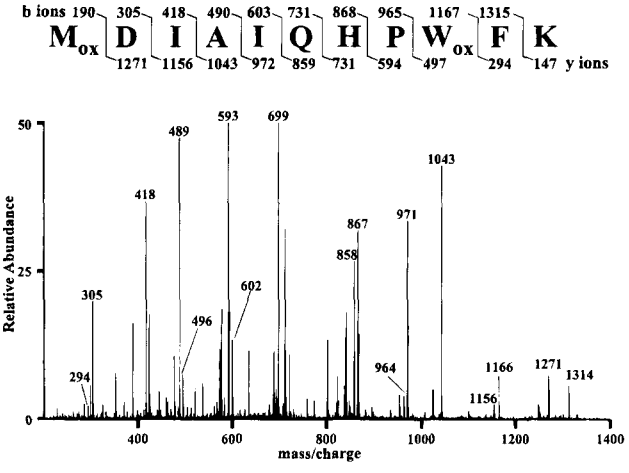

Fig. 3. Tandem mass spectrum of m/z 732, which eluted at 33 min from a C18 column. The sequence of αA 1–11, with methionine sulfoxide designated as M_{ox} and oxidized tryptophan denoted by W_{ox}, and the masses of the predicted fragment ions are shown at the top.

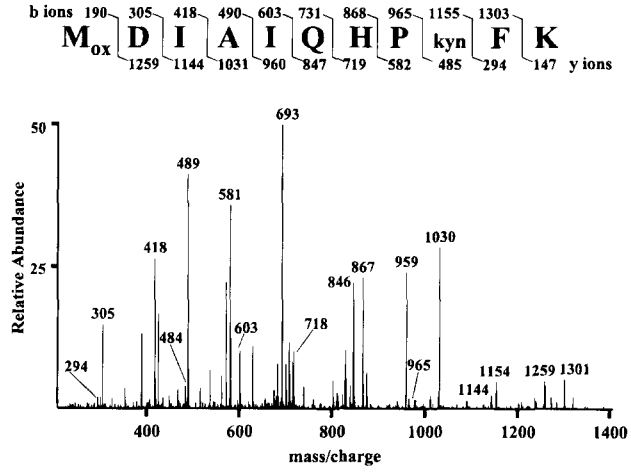

Fig. 5. Tandem mass spectrum of m/z 725, which eluted at 32 min from a C18 column. The sequence of αA 1–11, with methionine sulfoxide designated as M_{ox} and kynurenine denoted by KYN, and the masses of the predicted fragment ions are shown at the top.

this fragment ion, which indicates that the second site of oxidation is not on phenylalanine 10 or lysine 11 residues. The y_3 ion at m/z 496 is shifted by 16 Da higher than the predicted mass for the unmodified peptide (data not shown), providing evidence that the site of oxidation is tryptophan 9. In addition, the y_4–y_{10} ions are also shifted by 16 Da. Furthermore, the b_9 (m/z 1,166) ion, but not the b_8 ion, is shifted by 32 Da higher than the predicted fragment ion masses (16 Da due to Met 1 oxidation), proving that tryptophan 9 is the site of the second oxygen addition in this peptide. The amino acid sequence of αA 1–11 with methionine 1 and tryptophan 9 singly oxidized and the corresponding fragment ions are displayed at the top of the figure.

Figure 4 shows the tandem mass spectrum of the peak at m/z 739.5 (doubly charged ion of peak 7 in Fig. 2C), which eluted at 30 min. Based on the MALDI measured molecular weight, this

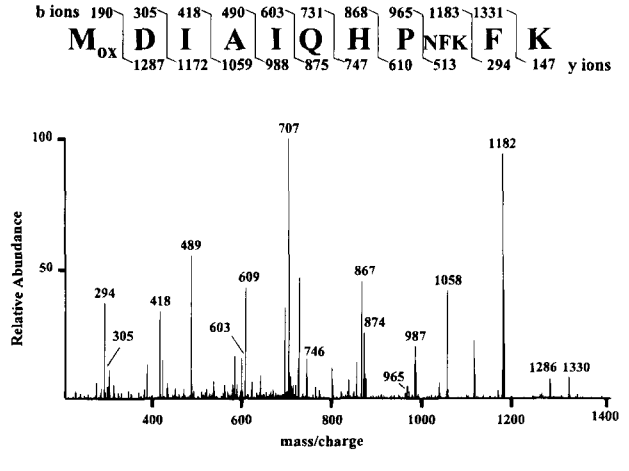

Fig. 4. Tandem mass spectrum of m/z 739.5, which eluted at 29 min from a C18 column. The sequence of αA 1–11, with methionine sulfoxide designated as M_{ox} and *N*-formylkynurenine denoted by NFK, and the masses of the predicted fragment ions are shown at the top.

mass was determined to be shifted 48 Da from αA 1–11 and was tentatively assigned as this peptide with addition of three oxygen atoms. The peak at m/z 707 corresponds to the mass of the doubly charged molecular ion ($[M+2H]^{2+}$) after loss of $(CH_3SOH)^{2+}$, indicating methionine 1 oxidation. A comparison of the b ion masses detected with the b ion masses for unmodified αA 1–11 and with the b ion masses from the singly oxidized αA 1–11 peptide indicates that the masses of the b_2 (m/z 305), b_3 (m/z 418), b_4 (m/z 489), b_5 (m/z 603), b_7 (m/z 867), and b_8 (m/z 965) ions are shifted by 16 Da higher than the unmodified peptide fragments. Identification of methionine 1 as a site of addition of one oxygen atom accounts for 16 Da of the 48 Da shift in the mass of this peptide. The C-terminus containing y_4 (m/z 609), y_5 (m/z 746), y_6 (m/z 874), y_7 (m/z 987), y_8 (m/z 1,058), and y_{10} (m/z 1,286) masses were shifted 32 Da higher than the masses of the y ions from the unmodified αA 1–11 peptide, which indicates that the modification exists in the portion of the peptide containing the sequence PWFK. Because the mass of the y_2 ion (m/z 294) is identical to the mass of this ion in the unmodified peptide, the 32 Da modification is not on the C-terminal phenylalanine or lysine, which confines this mass shift to the proline or tryptophan residues. The b_8 (m/z 965) ion, which contains the proline residue, is shifted by 16 Da not 48 Da while the b_9 (m/z 1,182) and b_{10} (m/z 1,330) ions are shifted by 48 Da. The detection of these ions implicates tryptophan 9 as the site of the addition of two oxygen atoms (32 Da), which indicates conversion of this residue to NFK. The MS/MS fragmentation pattern (predicted ions shown at top of figure) confirmed that this peptide corresponds to the amino acid sequence of αA 1–11 with methionine 1 oxidized and tryptophan 9 converted to NFK.

Figure 5 shows the tandem mass spectrum of m/z 725 (doubly charged ion of peak 8 in Fig. 2D), which eluted at 33 min. From MALDI molecular weight analysis, this peptide was predicted to be αA 1–11, with an additional 20 Da that could correspond to one oxidized amino acid plus KYN or 3-hydroxykynurenine. The fragmentation pattern again verified this peptide as αA 1–11. The peak at m/z 693 is the doubly charged molecular ion ($[M+2H]^{2+}$) after loss of $(CH_3SOH)^{2+}$, which indicates that the methionine 1 resi-

due is oxidized in this peptide. The masses of the b_2–b_9 ions (m/z 305, 418, 489, 603, 867, 965) are 16 Da higher than the b ion masses of the unmodified peptide and are identical to the b ion masses for the singly oxidized αA 1–11 peptide. This 16 Da addition on methionine 1 does not account for the 20 Da shift in mass measured in the MALDI mass spectrum; therefore, an additional 4 Da shift, postulated to be KYN, remains to be identified. The y_3 (m/z 484), y_4 (m/z 581), y_5 (m/z 718), y_6 (m/z 846), y_7 (m/z 959), y_8 (m/z 1,030), y_9 (m/z 1,144), and y_{10} (m/z 1,259) ions are shifted higher by 4 Da compared to the y ion masses for the unmodified peptide. The 4 Da modification must then be contained in the C-terminus of the peptide. The y_2 ion (m/z 294) mass is identical to that measured for the unmodified αA 1–11 peptide indicating that the modification is not on the phenylalanine 10 or lysine 11 residues. The presence of the b_9 (m/z 1,154) and b_{10} (m/z 1,301) ions, which are shifted by 20 Da and contain the tryptophan 9 residue, in combination with the y_3 (m/z 484) ion that also contains tryptophan 9 identifies this residue as the site of the 4 Da modification. This modification results from the conversion of tryptophan 9 to KYN. The amino acid sequence of αA 1–11 with methionine 1 oxidized and tryptophan 9 converted to KYN and the predicted b and y ion fragment masses is displayed at the top of the figure.

A summary of the tryptophan modifications identified in oxidized peptides from bovine α-crystallin exposed to the Fenton reaction is presented in Table 1. No αA 1–11, αB 1–11, or αB 57–69 peptides were sequenced, which contained tryptophan oxidation products without oxidation of a methionine (1 or 68) residue. Also, no αB 1–11 peptides containing NFK were detected. With the exception of the product at m/z 1,510, the formation of all of the tryptophan oxidation products in αA 1–11, αB 1–11, and αB 57–69 occurred at all concentrations of hydrogen peroxide (1–5%) tested. Although the formation of KYN in αB 57–69 was detected at all concentrations of hydrogen peroxide, the formation of NFK (m/z 1,510) in this peptide was only observed at the highest concentration tested (5%).

Discussion

Protein oxidation has been shown to result in altered protein degradation, loss of enzymatic activity, and possibly disease progression (Stadtman, 1990, 1992; Dean et al., 1997) and, therefore, represents an important physiological and biochemical phenomenon that must be understood to prevent functional impairment. Because of the strong evidence that oxidative stress plays a role in the development of senile cataract (Garland, 1990; Spector, 1995), our work focuses on lens protein oxidation; however, the identification of specific oxidized residues in lens α-crystallin has broader implications in several regards. First, the unambiguous identification of oxidized tryptophan products provides a basis for their identification in other proteins exposed to oxidative stress. In addition, the tryptophan oxidation products identified in this work have also been identified as photosensitizers capable of producing reactive oxygen species (Walrant & Santus, 1974; Pileri et al., 1976; Reszka et al., 1996). Because the lens is exposed to large amounts of UV radiation, formation of photosensitizers in the lens proteins could exacerbate damage to lens protein structure and function via photooxidative processes. Finally, because α-crystallin has been found in other tissues including heart, striated muscle, spleen, lung, and brain (Bhat & Nagineni, 1989) and because of its possible role as a molecular chaperone (Horwitz, 1992), any modifications to its structure could have a significant impact on its function throughout the body.

In this work, mass spectrometric analysis has revealed three α-crystallin tryptic peptides that are oxidized upon exposure to the products of a nonenzymatic, metal-catalyzed oxidation system, the Fenton reaction. More specifically, a number of different oxidized structures have been identified, including peptides with oxidized methionine residues and a variety of tryptophan products. The unambiguous identification of HTRP, NFK, and KYN in intact α-crystallin represents the first structural proof of the formation of these products in an intact protein. Every tryptophan residue in αA-crystallin (tryptophan 9) and αB-crystallin (tryptophan 9 and 60) was found to be converted to HTRP and KYN. While tryptophan 9 in αA and tryptophan 60 in αB were also found in the NFK form. These data suggest that the tryptophan 9 in αB is present in a slightly different environment in the α-crystallin aggregate. These results differ from experiments performed on UV-irradiated α-crystallin (Finley et al., 1997). In that study, there was little or no evidence for formation of tryptophan oxidation products in the intact α-crystallin protein by UV light.

Oxidation of proteins via the Fenton reaction depends on the ability of the protein to bind iron; that is, proteins with high affinity for iron are readily oxidized (Samuni et al., 1983; Stadtman, 1992). The presence of an EDTA–Fe complex prevents oxidation of proteins that bind free iron (Stadtman, 1990, 1992); however, the complex can enhance oxidation in proteins that bind EDTA (Amici et al., 1989). In the case of α-crystallin, recent work (Smith et al., 1997) identified oxidative changes upon exposure to Fenton chemistry in the absence of EDTA and no tryptophan oxidation was reported. Oxidative changes identified in this work suggest that the protein can bind the EDTA–Fe complex specifically in the region of tryptophan residues. This is not surprising, given the fact that α-crystallin can chaperone a large number of very different proteins (Horwitz, 1992). Based upon the generality of α-crystallin chaperoning properties, it is conceivable that α-crystallin could bind a range of different iron-containing complexes that are presumably present in the lens, as evidenced by very low concentrations of free iron. The differential oxidation of tryptophan 9 in αB could be due to differential binding of the EDTA–Fe complex in the α-crystallin aggregate.

Table 1. *Oxidized peptides sequenced from bovine α-crystallin after exposure to Fenton reaction products*

Peptide	Amino acid sequence	Measured m/z[a,b]	Peak number[b]	Modification
αA 1–11	Ac-MDIAIQHPWFK	1,429		
		1,450	8	$M1_{ox}$, KYN9
		1,462	9	$M1_{ox}$, HTRP9
		1,478	7	$M1_{ox}$, NFK9
αB 1–11	Ac-MDIAIHHPWIR	1,431		
		1,452	6	$M1_{ox}$, KYN9
		1,464	3	$M1_{ox}$, HTRP9
αB 57–69	APSWIDTGLSEMR	1,463		
		1,484	1	$M68_{ox}$, KYN60
		1,496	2	$M68_{ox}$, HTRP60
		1,512	5	$M68_{ox}$, NFK60

[a]MALDI measured $[M+H]^+$ molecular ion.
[b]From Figure 2.

The formation of tryptophan oxidation products in α-crystallin may have important consequences in vivo. Although Andley et al. have shown that the tryptophans of α-crystallin are not essential for the chaperone activity (Andley et al., 1996), the destruction of these residues by UV irradiation has been correlated with a decrease in activity (Schauerte & Gafni, 1995). These results suggest that the tryptophan residues may not be essential for chaperone activity but, if these residues are oxidized into tryptophan oxidation products, a decrease in activity may result. Loss of chaperone activity due to tryptophan oxidation product formation could be caused by destabilization of the protein (Okajima et al., 1990; Kitano et al., 1995) or by ROS produced by these photosensitizers. These ROS could further oxidize α-crystallin resulting in denaturation or disruption of protein/protein interactions followed by a loss of activity. Lens pigmentation, which has been determined to increase with age (Pirie, 1968; Bando et al., 1975), is thought to be partially due to the accumulation of NFK (Pirie, 1968; Sen et al., 1992). This study demonstrates that NFK and KYN can be formed in intact α-crystallin, which may be contributing to the observed in vivo fluorescence of human lenses (Sen et al., 1992). Because hydrogen peroxide increases in the lens with cataract formation (Spector & Garner, 1981), the MCO-mediated formation of these fluorescent compounds (NFK and KYN) may be one mechanism contributing to the increased fluorescence of cataracts (Pirie, 1968).

In summary, this study shows that, upon exposure to the Fenton reaction, HTRP, NFK, and KYN are formed from tryptophan 9 of αA- and tryptophan 60 of αB-crystallin, while only HTRP and KYN are produced from oxidation of tryptophan 9 of αB-crystallin. These results represent, to our knowledge, the first identification of specific tryptophan oxidation products at specific sites in the intact α-crystallin protein by an MCO system (Fenton reaction). Further studies are needed to determine the effect of tryptophan oxidation product formation on chaperone activity and the effect of age/cataract formation on the in vivo generation of these compounds in the human lens. By identifying tryptophan oxidation products in the intact α-crystallin molecule after exposure to the Fenton reaction products, another possible mechanism has been identified for the in vivo formation of UV photosensitizers. These compounds could be responsible for generating ROS in close proximity to susceptible amino acids in α-crystallin or in other neighboring proteins.

Materials and methods

Purification, oxidation, and enzymatic digestion of α-crystallin

Bovine lenses (approximately 6 months old) were obtained from the abattoir. After dissection, the outer cortex was stirred under nitrogen in 50 mM Tris, 0.2 M NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, pH 7.4 at 4 °C until dissolution of the lens. The suspension was homogenized and centrifuged at 105,000 × *g* for 45 min. The supernatant was loaded onto a 5 × 100 cm Sepharose CL-6B gel filtration column and eluted with 50 mM Tris, 0.2 M NaCl, 1 mM EDTA, 2 mM dithiothreitol, pH 7.4. Protein elution was monitored by absorption at 280 nM. Fractions containing α-crystallin were then dialyzed against water, lyophilized, and stored at −20 °C.

Purified bovine α-crystallin was exposed to the Fenton reaction in which α-crystallin (2 mg/mL) in 10 mM sodium phosphate, 0.1 mM $FeCl_2$, and 0.3 mM EDTA, pH 7.4 was reacted with 1, 3, or 5% hydrogen peroxide (≤30 s). Control and oxidized α-crystallin (2 mg) was digested with a 20:1 w/w (α-crystallin:enzyme) ratio of modified sequencing-grade trypsin in 0.1 M N-ethylmorpholine, pH 8.0, at 37 °C for approximately 18 h. Digests were then dried by centrifugation under vacuum and stored at −20 °C.

HPLC Separation

After digestion, the tryptic peptides produced from 2 mg of the oxidized α-crystallin were resuspended in 800 μL of 98% water/2% acetonitrile/0.5% trifluoroacetic acid, and injected (12.5 μmol) onto an Applied Biosystems (San Jose, California) Brownlee Aquapore C18 column (10 cm × 2.1 mm). Separation was accomplished on a Rainin (Woburn, Massachusetts) Dynamax SD-200 HPLC system using a linear gradient from 98% water/2% acetonitrile/0.1% trifluoroacetic acid to 10% water/90% acetonitrile/0.1% trifluoroacetic acid over a period of 75 min with a flow rate of 0.5 mL/min. Elution of peptides was monitored by measuring absorbance at 214 nM and fractions were collected at 1-min intervals. Peptide fractions were dried by centrifugation under vacuum at −20 °C, resuspended in 50% acetonitrile, and analyzed by MALDI and electrospray mass spectrometry.

MALDI-TOF mass spectrometry

An aliquot (1 μL) of each HPLC fraction collected from the oxidized α-crystallin was mixed with a 50 mM solution of α-cyano-4-hydroxycinnamic acid (ACHCA) dissolved in 70% acetonitrile (1:5 v/v protein:matrix) and 1 μL of this mixture was placed on a gold-coated, stainless steel sample plate followed by air drying. A PerSeptive Biosystems (Framingham, Massachusetts) Voyager-DE MALDI time-of-flight mass spectrometer equipped with a 337 nM nitrogen laser and a delayed-extraction source was operated in linear mode (1.2 m ion flight path, 20 kV accelerating voltage) to acquire the mass spectra. Between 100 and 120 mass scans were averaged to obtain one mass spectrum. Instrumental resolution in linear mode with delayed extraction was approximately 700 (full width at half maximum) at m/z 1,297.5. External mass calibration was performed using angiotensin I (MW 1,297.5) and ACTH clip 7-38 (MW 3,660.2) as standards. Mass accuracy was ±2 Da.

Electrospray mass spectrometry

Mass spectra and tandem mass spectra were acquired on a Finnigan (San Jose, California) MAT LCQ ion-trap mass spectrometer equipped with an electrospray source. MS/MS experiments were performed on the HPLC fractions that were dried by centrifugation under vacuum, resuspended in 30 μL of 47% water/47% methanol/6% acetic acid, and injected into a stream of the same solvent flowing into the mass spectrometer at 50 μL/min. A mass spectrum was acquired first to accurately determine the molecular weights of the modified and unmodified peptides. Peptides of interest were mass selected (mass window of 2 Da) for subsequent dissociation, and after activation/dissociation an MS/MS scan was performed. Mass accuracy was ±0.5 Da. An average of 26 mass scans were combined to generate one tandem mass spectrum. Calibration was performed using caffeine, a peptide with the sequence MRFA, and ultramark 1621 (PCR Incorporated, Gainesville, Florida). In the MS/MS spectra (Figs. 3–6), the fragment ion masses were rounded to the nearest integer.

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