Identification of Modified Tryptophan Residues in Apolipoprotein B-100 Derived from Copper Ion-Oxidized Low-Density Lipoprotein†

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ABSTRACT: Oxidative modifications of low-density lipoproteins (LDL) may contribute to the pathogenesis of atherosclerosis. Although the oxidation products of the lipid components of LDL have been studied extensively, less is known about the oxidation products of the apoprotein, apolipoprotein B-100. To identify the specific oxidative modifications, we oxidized LDL in the presence of Cu^{2+}, treated with DNPH, precipitated and delipidated the protein, digested the protein with trypsin, and analyzed the peptides by high-performance liquid chromatography. We isolated nine peptides that exhibited measurable absorbance at 365 nm, which is characteristic of hydrazones derived from DNPH and is not observed in peptides derived from unoxidized LDL. Unexpectedly, we obtained the same peptides with absorbance at 365 nm in Cu^{2+}-oxidized LDL not treated with DNPH. N-terminal sequence analyses and mass spectrometry indicated that the peptides isolated from the Cu^{2+}-oxidized LDL all contained kynurenine residues in place of Trp residues found in the native apoprotein. The product profile we observed in Cu^{2+}-oxidized LDL was remarkably different from the profiles observed in LDL oxidized by HOCl or myeloperoxidase in vitro, and the preferential oxidation of Trp to kynurenine in Cu^{2+}-catalyzed oxidation of LDL contrasts with the products observed following oxidation of LDL with HOCl or myeloperoxidase. Our studies to date support the working hypothesis that the specific products of protein oxidation are sufficiently distinct to be developed as biomarkers of proposed mechanisms of oxidation of LDL and biological molecules in other toxicities and diseases.

Oxidative modifications of low-density lipoproteins (LDL)[1] are believed to contribute to the pathogenesis of atherosclerosis (*1*). The oxidation of LDL in vitro is accelerated significantly by metal ions and is inhibited by chelating agents, whether incubated in the absence or the presence of cells (*2*, *3*). Steinbrecher et al. (*4*) showed that LDL incubated in cell-free F-10 medium supplemented with 5 μM Cu^{2+} became oxidized and exhibited chemical and biological properties similar but not identical to cell-modified LDL. They found appreciable increases in relative electrophoretic mobilities of the apoprotein, greater levels of thiobarbituric reactive substances and lysophosphatides, and increased uptake by macrophages of the oxidized LDL (ox-LDL). Since then, many investigators studying oxidized LDL have used Cu^{2+}-catalyzed oxidation as an experimental model (*5*). Esterbauer et al. (*6*) suggested that lipid hydroperoxides (LOOH) are essential for Cu^{2+} to initiate LDL oxidation ($LOOH + Cu^{2+} \rightarrow LOO\cdot + Cu^{+} + H^{+}$), but the mechanisms involved in metal-induced oxidation of LDL are unclear. The Cu^{2+} oxidation model has been useful in exploring many facets of the LDL oxidation process and may have biological relevance. However, the extent to which LDL is oxidized by Cu^{2+} often has been greater in many of these studies than is probably relevant physiologically, at least for the initial stage of atherogenesis.

Although the oxidation products of the lipid components of LDL have been studied extensively, less is known about the oxidation products of the apoprotein, apolipoprotein B-100 (apo B-100). Metal-catalyzed oxidations (MCO) of other proteins have been studied, and these modifications could be important in pathological processes in addition to atherosclerosis (*7*, *8*). The functional groups formed by MCO that have been characterized to date have been primarily aldehydes and ketones, but hydroxylated phenylalanine, tyrosine, leucine, and valine residues have been reported, along with dityrosine as a product of oxidation (*9*, *10*). Aldehydes and ketones, which are not found in unoxidized proteins, react with DNPH to form hydrazones that exhibit strong absorbances at 365 nm. Stadtman (*11*) proposed that during MCO a redox-active transition metal (e.g., Fe^{2+}/Fe^{3+} or Cu^{+}/Cu^{2+}) binds to a metal-binding site on the protein

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[1] Abbreviations: ox-LDL, oxidized low-density lipoprotein; MCO, metal-catalyzed oxidation; DNPH, 2,4-dinitrophenylhydrazine; HPLC, high-performance liquid chromatography; apo B-100, apolipoprotein B-100; TPCK, *m*-tosyl-L-phenylalanine chloromethyl ketone; NFK, *N*-formylkynurenine; EDTA, ethylenediaminetetraacetic acid; PTH, phenylthiohydantoin; MS, mass spectrometry; MPO, myeloperoxidase.

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and the Fe^{2+}− or Cu^{+}−protein complex reacts with H_2O_2 to produce reactive oxygen species that resemble HO•. The reactive oxygen species react with molecular structures in the immediate vicinity of the metal-binding site, resulting in the formation of aldehyde or ketone derivatives. These transformations can change the biological functions of the proteins and mark the proteins for degradation by proteases (*11*).

The initial intent of the present studies was to characterize the specific DNPH-reactive modifications of apo B-100 introduced during the Cu^{2+}-catalyzed oxidation of LDL, using the absorbance at 365 nm of the DNPH-derived hydrazones. However, we identified nine peptides from ox-LDL in which the tryptophan (Trp) residues were converted to the respective kynurenines, which also absorb at 365 nm. Trp residues can be oxidized by ionizing radiation, ozone, singlet oxygen, or metal ion-catalyzed oxidation, which has been followed most commonly by loss of fluorescence in the oxidized proteins (*12*−*14*). Oxidative cleavage of the indole ring of the Trp residue forms *N*-formylkynurenine, which hydrolyzes to kynurenine, which both exhibit significant absorbance at 365 nm. In our previous studies of the oxidation of LDL by reagent HOCl in vitro (*15*), we observed oxidation of Trp residues, but these products did not appear to be the corresponding kynurenines in that they did not exhibit detectable absorbances at 365 nm without reaction with DNPH, and N-terminal sequence analyses did not yield peaks comigrating with the phenylthiohydantoin (PTH) derivative of kynurenine.

MATERIALS AND METHODS

Materials. DNPH and kynurenine were purchased from Sigma Chemical Co. (St. Louis, MO), and *m*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin was purchased from Worthingon Biochemicals (Freehold, NJ). Acetonitrile, methanol, and other solvents for high-performance liquid chromatography (HPLC) were from Burdic and Jackson (Muskegon, MI). Vydac C_{18} columns were from The Separations Group (Hesperia, CA). Reagents and solvents for the automatic gas-phase sequencer were purchased from Applied Biosystems (Foster City, CA). All other chemicals used were of the highest grade available.

Isolation of LDL. Plasma was obtained from healthy human volunteers after 12 h of fasting. Aprotinin (0.06 TIU/mL), NaN_3 (0.02%), and EDTA (0.3 mg/mL) were added to the plasma. The LDL was separated from the plasma by sequential ultracentrifugation within a density cutoff of 1.020−1.063 g/mL, followed by ultracentrifugation to a density cutoff of 1.063 g/mL. The LDL were then dialyzed in the dark for 24 h at 4 °C against at least four changes of vacuum-degassed 0.05 M phosphate buffer, pH 7.4, which contained 0.16 M NaCl.

LDL Oxidation. LDL was oxidized as described previously (*16*). Briefly, LDL (2 mg/mL) in phosphate-buffered saline (PBS) at a volume of 6 mL was transferred to a dialysis bag (Spectrum, # 2). The bag was then immersed in 18 mL of 6.67 μM $CuSO_4$ and PBS. Oxygen was bubbled through a 50 mL plastic tube into the mixture outside of the dialysis bag for 24 h while incubating at 37 °C.

DNPH Labeling of ox-LDL. DNPH was reacted with ox-LDL as described previously (*15*). Briefly, the DNPH was added to 2 M HCl to a final volume of 10 mL of ox-LDL, or unmodified LDL as a control, mixed with an equal volume of the DNPH solution, and incubated at room temperature for 30 min, at which time an equal volume of 20% TCA was added to the reaction mixture. The mixture was then centrifuged at 5000 rpm for 10 min. The precipitate was washed 4 times with EtOH/EtOAc (1:1), and the resulting pellet was dried under a stream of nitrogen gas.

Structural Analysis. The DNPH-modified and unmodified delipidated LDL were digested with TPCK-treated trypsin (enzyme:substrate ratio = 1:50) at room temperature for up to 24 h as described previously (*17*). The digested apo B-100/peptide mixtures were analyzed with a Hewlett-Packard 1090 liquid chromatography system, with a Vydac C18 column (250 × 4.6 mm) eluted with a TFA/acetonitrile gradient. The major peptide peaks that were detected by absorbance at 365 nm were collected manually and purified by using sequential chromatography (*17*). The purified peptides were sequenced by using a gas-phase automated sequencer (Applied Biosystems) equipped with an on-line 120A PTH-Analyzer. The retention time of PTH-kynurenine was determined by analysis of authentic kynurenine through the Applied Biosystem gas-phase sequencer.

Electrospray Mass Spectrometry. The purified peptides were analyzed in a Finnigan MAT TSQ 700 mass spectrometer onto which was mounted a modified Vestec electrospray source. The electrospray source was optimized for nanoliter flow rates (200−900 nL/min) such that the eluant was sprayed directly from a fused silica capillary needle. Dry ultrapure nitrogen was used as a carrier gas. For the ox-LDL peptides, a 50 μm (inner diameter) "spray" needle was packed with 1 cm of reverse-phase liquid chromatography packing to act as a desalting and preconcentration device. The integration of sample processing with the electrospray source allowed us to analyze small volumes of dilute solutions. All the samples were diluted 200−400-fold with a mixture of water/methanol/formic acid (50:50:0.1; v/v/v) and were infused through a silica capillary needle. The product ion spectra of the chosen precursor ions were obtained by using a Sciex API-III Plus triple-quadrupole mass spectrometer (PE-Sciex, Norwalk, CT). Collision energies of 20−30 eV were used, and the signals recorded on about 100 scans were averaged.

RESULTS

Purification and Identification of Oxidized Peptides from LDL Treated with DNPH. To identify the DNPH-reactive peptides of apo B-100 derived from Cu^{2+}-oxidized LDL, the ox-LDL were treated with DNPH before being delipidated, digested with trypsin, and purified by HPLC. As expected, the unoxidized DNPH-treated LDL yielded no peaks with absorption at 365 nm (data not shown) (see ref *18*). Twenty-three fractions were collected from the tryptic digests (Figure 1). Following the procedures described previously (*15*), the peptides detected by absorbance at 365 nm were collected and purified further by HPLC (data not shown).

From the fractions collected, 10 peptides were isolated and identified. The sequences of the peptides, the locations of these peptides on apo B-100, and the retention times of each peptide eluted with the respective buffer systems are listed in Table 1. Sequencing and amino acid analyses indicated

Table 1: Structure Information of Purified Cu^{2+}-Oxidized LDL Tryptic Peptides[a]

peptide	sequence	position on apo B-100	modified residue	RT, in % B of TFA system	RT, in % B of phosphate system	RT, in % B of NH_4OAc system	remark
(D)10-1-3	VA**W**HYDEEK	1138−1146	W^{1140}	22.5	17	19	TR
(A)5-2-3	SEILAH**W**SPAK	1108−1118	W^{1114}	28	27	32.4	MX
(D)12-1-9				28	27	32.5	
(D)12-1-10	IYSL**W**EHSTK	3532−3541	W^{3536}	28	28.8	34	MX
(A)5-2-4	**W**NFYYSPQSSPDK	3995−4007	W^{3995}	28	29.8		
(A)6-2-1				28.6	27		MX
(D)12-1-11				28	28.8		
(A)8-2-1	NLQNNAE**W**VYQGAIR	4080−4094	W^{4087}	30	25	30.5	between TR regions
(D)13-1-8				29.6	25	28.2	
(A)13-2-2	IVQILP**W**EQNEQVK	550−563	W^{556}	34	29.2	37.2	TR
(D)16-1-8				34.5	30	37.5	
(D)23-2-2				62	30	38	
(A)13-2-1	NLTDFAEQYSIQD**W**AK	2533−2548	W^{2546}	34	26	34.2	TN
(A)14-2-1				34.2	26	34.2	
(D)16-1-5				34.5	27	34.2	
(A)16-2-1	VN**W**EEEAASGLLTSLK	4029−4044	W^{4031}	36.5	27	34	TR
(D)18-1-6					26	33.8	
(A)17-2-1	TIDQMLNSELQ**W**PVPDIYLR	2648−2667	W^{2659}	39.5	29.5	39	MX
(D)19-1-10				39.5	30	39.5	

[a] The tryptic peptides detected at 365 nm were separated by HPLC and identified as Trp-containing peptides that Trp converted to kynurenine. (A) denotes peptides that were obtained by tryptic digestion of apo B-100 without prior treatment with DNPH; (D) denotes peptides that were obtained by tryptic digestion of samples following exposure to DNPH. The amino acid residues that were confirmed by automatic sequencing are underlined. The PTH derivatives that did not exhibit the PTH-Trp derivative at the expected retention time are boldfaced. The location of each peptide on the apo B-100 structure (*15*) is given. TR, trypsin-releasable peptides; TN, trypsin-nonreleasable peptides; MX, peptides isolated from both the TN and TR fractions; RT, retention time.

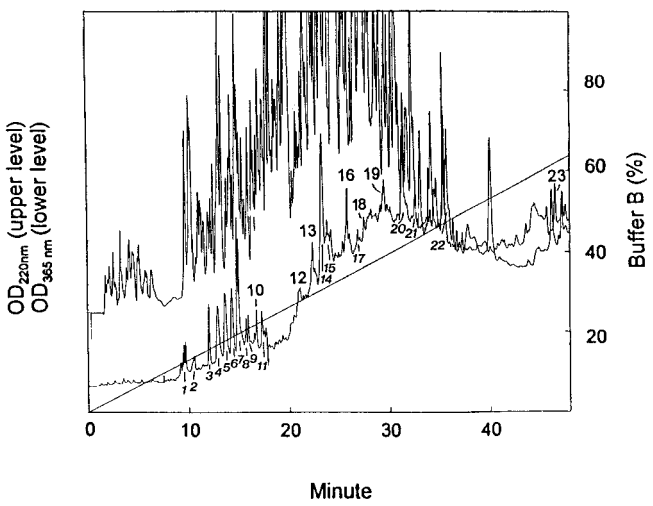

FIGURE 1: HPLC of Cu^{2+}-oxidized LDL treated with DNPH. Tryptic digests of ox-LDL were separated on a Vydac C18 column by using the TFA buffer system with an acetonitrile gradient from 0% buffer B to 67% buffer B in 50 min. Fractions exhibiting absorbance at 365 nm were collected and characterized. Fractions absorbing at 365 nm that contained peptides, as determined by gas-phase N-terminal sequence analysis, are labeled at the top of each peak. Fractions that did not contain detectable peptides are labeled in italic at the bottom of each peak. The absorbance units full scale (AUFS) at 220 and 365 nm were 1.0 and 0.03, respectively.

that the other fractions that are labeled on the bottom of the corresponding peaks in Figure 1 did not show amino-terminal sequence data or amino acid residues. Peptides (D)16-1-8 and (D)23-2-2 were found to have the same N-terminal sequence (IVQILP[kynurenine]EQNEQVK) and the same retention times with the phosphate and ammonium acetate buffer systems (*15*), even though they were isolated from the TFA system with markedly different retention times (34.5% and 62%, respectively, of mobile phase B in the TFA system).

Purification and Identification of Oxidized Peptides from LDL Not Treated with DNPH. Although in our previous studies of LDL oxidation by HOCl or myeloperoxidase in vitro we observed no distinguishable peptide products absorbing at 365 nm in samples not treated with DNPH, with Cu^{2+}-oxidized LDL, a number of such peptides were observed (Figure 2). The 17 fractions that exhibited absorption at 365 nm were collected; 3 of the fractions eluted within 10% buffer B in the TFA buffer system, whereas the remaining 14 eluted between 25% and 40% buffer B. The fractions were purified subsequently by rechromatography (*17*). Nine peptides containing Trp in the unoxidized protein were isolated from fractions 5, 6, 8, 13, 14, 16, and 17 and were sequenced by using an automatic peptide sequencer. Although the other 10 fractions had exhibited absorption at 365 nm, we isolated no peptides from them when they were rechromatographed. Of the nine peptides we did isolate, two pairs from neighboring fractions were indistinguishable by gas-phase sequencing. The peptide sequences, the locations of these peptides on apo B-100, and the retention times of each peptide that eluted from the buffer systems are listed in Table 1. The locations of the modified Trp residues in apo B-100 are illustrated in Figure 3.

The oxidized peptide fragments obtained from tryptic digestion of oxidized apo B-100 without treatment with DNPH are classified as the "A" series peptides, and the peptides isolated from the tryptic digests of DNPH-treated oxidized apo B-100 are classified as the "D" series peptides.

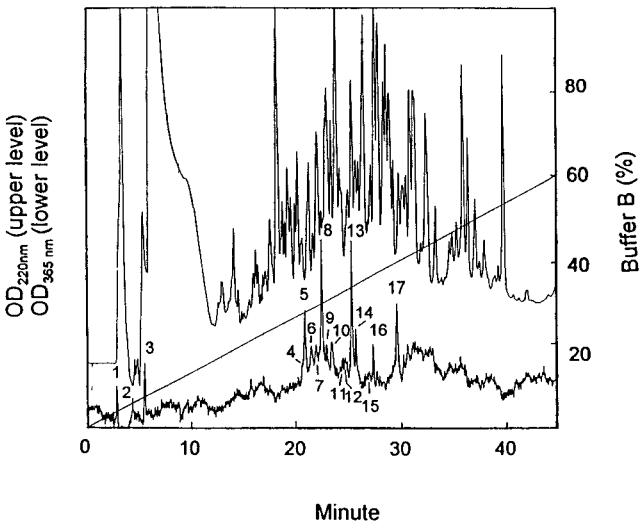

FIGURE 2: HPLC of Cu^{2+}-oxidized LDL not treated with DNPH. Tryptic digests of ox-LDL processed as in Figure 1, except without exposure to DNPH, were separated on a Vydac C18 column by using TFA buffer with an acetonitrile gradient from 0% buffer B to 60% buffer B in 45 min. The labeled peaks were collected and characterized. The absorbance units full scale (AUFS) at 220 and 365 nm were 1.0 and 0.03, respectively.

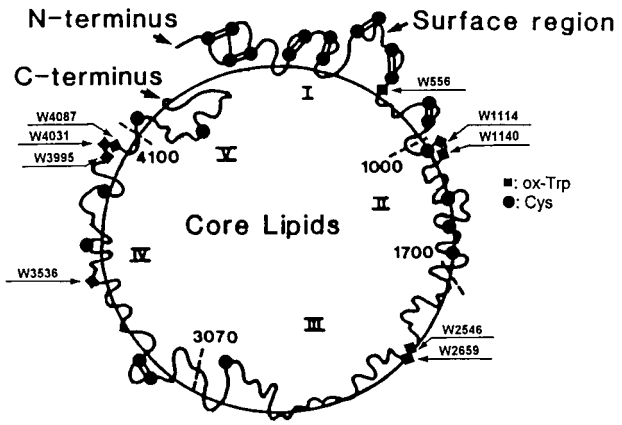

FIGURE 3: Relative location of the modified Trp residues on apo B-100. For simplicity, the trypsin-releasable regions are shown outside the core of the LDL particle and the trypsin-nonreleasable regions shown inside the lipid core of the LDL particle. The relative lengths of the peptides are not drawn to scale, and the surface−core orientation is presented to allow easy visualization of trypsin releasability. The scheme does not imply that the different parts of apo B-100 are physically on the surface or buried inside the lipoprotein particle. The five hypothetical domains are separated by dashed lines. Cysteine residues are indicated by solid circles, and disulfide bridges are indicated by double lines. The locations of the oxidized Trp residues on LDL are indicated by solid rectangles and are marked. (Modified from reference *17*, with permission.)

Peptides (D)10-1-3 and (D)12-1-10 were isolated from the tryptic peptide mixtures of DNPH-treated oxidized apo B-100, but not from treated tryptic peptides from LDL not treated with DNPH.

Characterization by N-Terminal Sequence Analyses of the Products of Trp Oxidation in LDL. In these peptides isolated from the oxidized LDL, the PTH derivatives of the residues corresponding to Trp in the unoxidized sequences eluted with HPLC retention times identical to the retention time observed for the PTH derivative of authentic kynurenine (not shown). The kynurenine derivative eluted between diphenylthiourea (DPTU) and PTH-Trp in the system used.

Mass Spectrometric Analysis of the Modified Trp Residues. The isolated peptides were examined by mass spectrometry. The analyses showed ions at M+4 Da in the peptides indicated by gas-phase sequencing analyses to contain kynurenine residues from oxidation of Trp, as illustrated for peptide (D)18-1-6 depicted in Figure 4. The +4 increase in *m*/*z* from the expected mass is localized to the Trp residue in the native sequence and corresponds to the increase in mass arising from conversion to kynurenine. Gas-phase automatic sequence analysis confirmed that the structure of this peptide had the amino acid sequence expected of this tryptic peptide from apo B-100, except that a residue that coeluted with the PTH derivative of kynurenine was observed in place of the Trp residue observed in the unoxidized apoprotein.

DISCUSSION

The working hypothesis of these studies was that the DNPH-reactive carbonyl groups that result from Cu^{2+} oxidation would form the corresponding hydrazones, which could be detected by absorbance at 365 nm. However, the results of the present studies indicate significant absorption at 365 nm by peptides due to formation of kynurenines from oxidation of the corresponding Trp residues. Giessauf et al. (*14*, *19*) postulated that Cu^{2+} catalyzes protein oxidation through close binding to Trp residues, with a site-specific redox reaction that yields a Trp• radical and Cu^{+}. The oxidation of Trp probably proceeds via the Trp-hydroperoxide, which is converted to NFK and kynurenine. NFK and kynurenine have been found in solutions of free Trp irradiated by UV-B light (*20*) and in several proteins exposed to free radicals (*12*, *21*, *22*) or to ozone (*13*, *23*). NFK and related compounds can be detected at 365 nm without any further modification.

Giessauf et al. (*14*) found that 8 or 9 of the 37 Trp residues in apo B-100 appear to be accessible to chelation with Cu^{2+} added to the aqueous phase, which led to a rapid-phase quenching of fluorescence, an effect that was reversible with denaturation and therefore more consistent with chelation than with oxidation of the Trp residues. One interpretation of their data would be that eight or nine Trp residues are located in environments accessible to the aqueous exterior of the LDL particle, and Giessauf et al. note that eight of the Trp residues in apo B-100 have surface probabilities greater than 1.0 and hydropathies less than −1.0 (*14*). The dominant blue shift of the fluorescence of Trp residues in LDL is also consistent with localization of the remaining 28−29 Trp residues existing in hydrophobic environments, such as the lipid core of the particles. The differences in reactivities of Trp or other residues toward Cu^{2+}-catalyzed oxidation or other transformations are likely to exhibit effects from structural features other than simple lipophilic/hydrophilic domain partitions, but characterizations of the specific products are needed. It is important to note that the level of specificity required for these assessments, and the meaning of the term "specific" we intend in our discussions, is that of individual amino acid residues, unique by position in primary sequence, in addition to the chemical natures of the products formed.

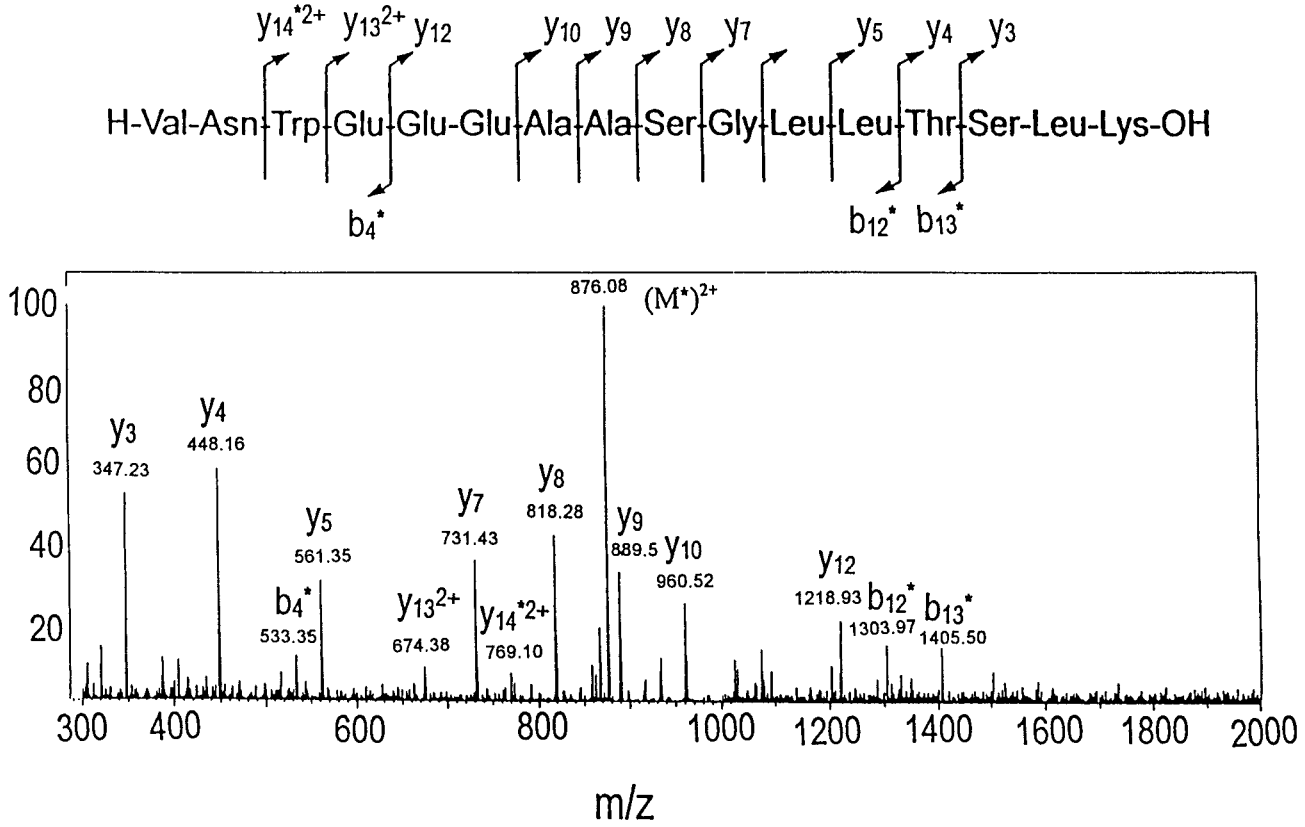

FIGURE 4: Electrospray and tandem MS analyses of oxidized peptides. The mass spectrum of product ions from the decomposition of *m*/*z* 876.09, $(M+4+2H)^{2+}$ from peptide (D)18-1-6 (Table 1), is shown. The peptide sequence ions are labeled according to the nomenclature proposed by Biemann (*26*). Superscripted stars indicate ions that are 4 Da greater than the *m*/*z* calculated for the respective unmodified peptides.

Characterization of the products of oxidation of bovine β-casein in HOCl by using similar methods of DNPH derivatization, trypsin digestion, and HPLC (*24*) indicated oxidation of tyrosine residues to the corresponding quinone methides, with subsequent conversion with DNPH to the corresponding hydrazones. With similar analyses, oxidation of LDL with HOCl indicated preferential oxidation of surface Cys residues, apparently to the corresponding sulfinic acids. Other amino acid residues, such as Trp, Met, and Lys, were also identified as possible modified sites, but no tryptic peptides exhibiting significant absorbance at 365 nm were observed in samples not treated with DNPH (*15*). The oxidation of Trp residues we observed in our earlier studies of the oxidation of LDL by reagent HOCl in vitro (*15*) did not produce the corresponding kynurenines, as evidenced by the absence in the tryptic peptides of detectable absorbances at 365 nm without reaction with DNPH, and N-terminal sequence analyses did not yield peaks comigrating with the (PTH) derivative of kynurenine.

In the Cu^{2+}-catalyzed oxidations in the present studies, we observed only those peptides in which the Trp residues had been converted to kynurenines or related compounds exhibiting detectable absorbance at 365 nm. The Trp-containing peptides from LDL oxidized by Cu^{2+} formed different PTH derivatives than did Trp residues from LDL oxidized by HOCl. The PTH-derivative of the Trp residues from Cu^{2+}-oxidized LDL revealed a peak between DPTU and PTH-Trp by PTH amino acid analysis, whereas the PTH-Trp of HOCl-oxidized peptides did not reveal a peak at this position, nor any other position within the chromatographic range of the standard Applied Biosystems for PTH analysis.

Peptides (A)5-2-3 and (D)12-1-9 corresponded to the same tryptic peptide as peptide 5-1 (SEILAHWSPAK) isolated from LDL oxidized by HOCl. Electrospray mass spectrometric analysis of peptide 5-1 from HOCl oxidation and DNPH treatment indicated ions at *m*/*z* of M+14 and M+194, relative to the *m*/*z* calculated for the unmodified tryptic peptide, which suggest introduction of an oxygen atom, with loss of 2H, as one possibility for the M+14 ion, and covalent attachment of DNPH, which has a mass of 198 Da, with additional oxidation to account for the decreased mass, but the exact identities of these modifications are not known at this time.

Two pairs of the nine Cu^{2+}-oxidized peptides characterized in the present studies, (A)5-2-4 and (A)6-2-1, and (A)13-2-1 and (A)14-2-1, were isolated by HPLC from their neighboring fractions. The slight differences in the retention times of peptides (A)5-2-4 and (A)6-2-1 (Table 1) in the TFA and the phosphate chromatography systems suggest that minor differences exist in the modifications of the parent peptides, but the natures of these differences are not known at the present time.

Based on the studies of apo B-100 we described previously (*17*), all but one of the nine peptides identified in the present studies as being oxidized by Cu^{2+} are located either in the trypsin-releasable region or in the mixed region. The Trp residue in peptide NLTDFAEQYSIQD**W**AK is located in a trypsin-nonreleasable region of LDL, but the oxidation of the nearby Trp in peptide TIDQMLNSELQ**W**PVPDIYLR (Table 1 and Figure 4) might alter the conformation of apo B-100 sufficiently to affect access of the Trp in peptide NLTDFAEQYSIQD**W**AK to the aqueous phase surface of

the LDL. Although the trypsin-releasable peptides are not necessarily located on the surface of the LDL particle, that is the most reasonable working hypothesis, and our present results suggest that the Trp residues located on the surface of LDL are those most likely to be oxidized by means of Cu^{2+} catalysis.

Other amino acid residues that may be modified during Cu^{2+}-catalyzed oxidation of LDL may not be detected by our analytical procedures. Leeuwenburgh et al. measured increases in levels of *o*-tyrosine and dityrosine in apo B-100 with oxidation of LDL in vitro and in vascular lesions and concluded that the absence of an increase in *o*-tyrosine levels in lesion LDL or atherosclerotic tissue suggested that redox-active metal ions are unlikely to serve as catalysts for LDL oxidation in artery walls (*23*). However, the levels of copper ion they employed in their studies (100 μM) were much higher than the concentrations used in most studies, including our present investigations (5 μM). They did not observe increases in dityrosine levels with Cu^{2+}-catalyzed oxidation of LDL, whereas LDL oxidation by myeloperoxidase, H_2O_2, and tyrosine showed marked increases in dityrosine levels. Leeuwenburgh et al. also observed comparable increases in dityrosine levels with the oxidation of LDL by H_2O_2 in the presence of Cu^{2+}, suggesting that several parameters can exert detectable effects on product profiles, even as assessed without positional specificity.

More recently, Hazen and co-workers have described tyrosine nitration by myeloperoxidase + NO_2^- + H_2O_2 (*25*), suggesting a potentially significant mechanism for LDL modification and initiation of atherogenesis. Although their studies published to date have addressed principally nitrotyrosine formation and lipid peroxidation, the earliest effect they have reported was a rapid loss of about 20 of the 37 Trp residues in apo B-100, with a slower secondary phase in which nitrotyrosine accumulation and lipid peroxidation are increased.

In summary, the results of this study are consistent with our previous findings on the sites of HOCl- and myeloperoxidase-mediated oxidation of LDL (*15*, *18*), which indicated that sensitive amino acid residues on the surface of LDL are likely to be oxidized. Further, the specific products formed by oxidation in our studies are remarkably characteristic of the mechanisms of oxidation employed. The results to date support our working hypothesis that approaches such as those we describe can be adapted to distinguish candidate mechanisms of oxidation of cells and tissues in vivo, although substantial progress in analytical capabilities will be essential to the development of this line of investigation.

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