Selective modification of apoB-100 in the oxidation of low density lipoproteins by myeloperoxidase in vitro

Chao-yuh Yang,[1,*,†] Zi-Wei Gu,[*] Manlan Yang,[†] Shen-Nan Lin,[**] Anthony J. Garcia-Prats,[§] Lynette K. Rogers,[§] Stephen E. Welty,[§] and Charles V. Smith[†,§]

Departments of Biochemistry,* Medicine† and Pediatrics,§ Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, and Analytical Chemistry Center,** University of Texas Medical School, 6431 Fannin Street, Houston, TX 77030

Abstract **Oxidative modification of LDL may be important in the initiation and/or progression of atherosclerosis, but the precise mechanisms through which low density lipoprotein (LDL) is oxidized are unknown. Recently, evidence for the existence of HOCl-oxidized LDL in human atherosclerotic lesions has been reported, and myeloperoxidase (MPO), which is thought to act through production of HOCl, has been identified in human atherosclerotic lesions. In the present report we describe the formation of 2,4-dinitrophenylhydrazine (DNPH)-reactive modifications in the apolipoprotein (apo) by exposure of LDL to myeloperoxidase in vitro. In contrast with the complex mixture of peptides from oxidation of LDL with reagent HOCl, oxidation with MPO in vitro produced a major tryptic peptide showing absorbance at 365 nm. This peptide was isolated and characterized as VELEVPQL(*C)SFILK . . . , corresponding to amino acid residues 53–66 . . . on apoB-100. Mass spectrometric analyses of two tryptic peptides from oxidation of LDL by HOCl indicated formation of the corresponding methionine sulfoxide (M=O), cysteinyl azo (*C), RS–N=N–DNP, derivatives of EEL(*C)T(M=O)FIR and LNDLNS VLV(M=O)PTFHVPFTDLQVPS(*C)K, which suggest oxidation to the corresponding sulfinic acids (RSO_2H) by HOCl. The present results demonstrate that DNPH-reactive modifications other than aldehydes and ketones can be formed in the oxidation of proteins and illustrate how characterization of specific products of protein oxidation can be useful in assessing the relative contributions of different and unexpected mechanisms to the oxidation of LDL and other target substrates. The data also suggest a direct interaction of the LDL particle with the active site on myeloperoxidase and indicate that effects of the protein microenvironment can greatly influence product formation and stability.**—Yang, C-y., Z-W. Gu, M. Yang, S-N. Lin, A. J. Garcia-Prats, L. K. Rogers, S. E. Welty, and C. V. Smith. **Selective modification of apoB-100 in the oxidation of low density lipoproteins by myeloperoxidase.** ***J. Lipid Res.*** **1999.** 40: **686–698.**

Supplementary key words myeloperoxidase • HOCl, hypochlorous acid • LDL, low density lipoproteins • oxidized LDL peptides • 2,4-dinitrophenylhydrazine • apoB-100

Oxidative modification of low density lipoprotein (LDL) may be important in the initiation and/or progression of atherosclerosis, but the relative contributions to initiation of atherogenesis and the precise mechanisms through which LDL oxidation and atherosclerosis progress in parallel remain controversial (1–4). Although many studies of the oxidation of LDL lipids have been published, far less is known about the oxidative modification of the apolipoprotein (apo). In addition, oxidative modifications of tissue proteins have been implicated in the pathogeneses of a number of other diseases and degenerative conditions (5, 6).

Myeloperoxidase, which catalyzes the oxidation of substrates by H_2O_2 in the presence of Cl^-, has been identified in human atherosclerotic lesions (7) and presumably arises from neutrophils and/or monocytes. Hazell et al. (8) have presented evidence for the existence of HOCl-oxidized LDL in human atherosclerotic lesions from studies using a monoclonal antibody specific for HOCl-treated LDL that does not cross-react with LDL oxidized or modified by other mechanisms. These investigators also reported that exposure of LDL to reagent or enzymatically generated HOCl resulted in oxidation of amino acid residues of apoB-100 and transformation of the LDL into a high-uptake form for macrophages (9). Modifications of lysine residues were the principle effects characterized, with conversion to the corresponding aldehydes, which can result in LDL aggregation by reaction of the aldehydes with unaltered primary amines to form Schiff base products (10). In other studies, Hazen and Heinecke (11) have characterized 3-chlorotyrosine as a biomarker of oxidation of LDL by MPO or HOCl in vitro, and have observed elevated levels of 3-chlorotyrosine in atherosclerotic tissues and in LDL isolated from atherosclerotic intima.

Recently, we have characterized a series of amino acid

Abbreviations: LDL, low density lipoproteins; MPO, myeloperoxidase; DNPH, 2,4-dinitrophenylhydrazine; GSSG, glutathione disulfide; GSOH, glutathione sulfenic acid; GSO_2H, glutathione sulfinic acid; GSO_3H, glutathione sulfonic acid; FAB, fast atom bombardment; MALDI, matrix-assisted laser desorption ionization; GSH, glutathione.

[1] To whom correspondence should be addressed.

residues on apoB-100 of LDL that are oxidized by reagent HOCl (12). In our studies, HOCl-mediated modification of the apoprotein in LDL was observed principally at Cys, Trp, Met, and Lys residues on the external surface of the LDL particle, with a preferential oxidation of the Cys residues. Nonetheless, oxidation of LDL by reagent HOCl in vitro would not necessarily be identical to that expressed in vivo. As a first step in testing that hypothesis, we investigated whether the products of the oxidative modification of apoB-100 of LDL by MPO are distinguishable from those obtained from oxidation with reagent HOCl by characterizing DNPH-reactive tryptic peptides from human LDL treated with MPO in vitro. The results of the present studies indicate that oxidation of the apoprotein in LDL by MPO *in vitro* is markedly different than that observed with HOCl. Additional studies indicate that the DNPH-reactive intermediates formed at cysteine residues by oxidation with HOCl appear to include the corresponding sulfenyl and sulfonyl chlorides and sulfinic acids.

MATERIALS AND METHODS

Materials

Myeloperoxidase (MPO, human polymorphonuclear leukocytes) was purchased from Calbiochem (San Diego, CA), hydrogen peroxide (30%) from Fisher Scientific (Pittsburgh, PA), 2,4-dinitrophenylhydrazine (DNPH) from Serva (Germany) or from Sigma, and N-tosyl-l-phenylalanine chloromethyl ketone (TPCK)-treated trypsin from Worthington Biochemicals (Freehold, NJ). All other reagents were purchased from Sigma Chemical Company (St. Louis, MO).

LDL preparation

LDL was isolated from plasma of fasting healthy donors by sequential ultracentrifugation in KBr solution at densities between 1.030 and 1.063 g/ml, as we have described previously (12). Briefly, aprotinin (0.056 unit/ml plasma), sodium azide (0.06% w/v), and EDTA (0.06% w/v) were added to plasma immediately after collection. For additional purification, LDL was recentrifuged in solution with density of 1.09 g/ml. Purified LDL was dialyzed against 50 mm phosphate buffer containing 0.15 m NaCl, pH 7.4 (50 mm PBS).

Oxidation of LDL by MPO

Oxidation of LDL by MPO was carried out according to the method described by Hazell et al. (9) with some modification. LDL in 50 mm PBS at a final concentration of 0.8 μm apolipoprotein was preincubated at 37°C for 10 min before addition of MPO at a final concentration of 1.2 μg/ml. Oxidation was initiated by addition of H_2O_2 at 1.5-min intervals with brief mixing after each addition, to a final concentration of 10 μm. Under these conditions, H_2O_2 was consumed by MPO within 1 min. Samples were protected from light between additions of H_2O_2. The total incubation time was 15 min and the sample was placed in ice for further processing. Replacement of the 50 mm PBS with a 50 mm phosphate buffer, without Cl^-, also was used in control studies of the oxidation of LDL by MPO/H_2O_2.

Reaction of LDL with 2,4-dinitrophenylhydrazine

The reaction of LDL with DNPH was performed as described (12). An equal volume of DNPH (5 mm in 2 N HCl) was added to the LDL. After incubation for 30 min at 4°C, 1 volume of 20% TCA was added. The precipitate was collected by centrifugation and washed with EtOH–EtOAc 1:1 (v/v) 3 times and dried with nitrogen.

Protein primary structure analysis

Delipidation and tryptic cleavage were conducted as described previously (12). (a) Enzymatic hydrolysis: Proteins were digested with TPCK-treated trypsin (enzyme:substrate ratio = 1:50) in 0.1 m ammonium bicarbonate, pH 8.0, at room temperature for up to 24 h. (b) HPLC for peptide separations and sequencing: A Vydac C_{18} column (250 × 4.6 mm) and trifluoroacetic acid (TFA)–acetonitrile gradient elution were used for primary peptide separations. The major peaks detected at 365 nm were collected manually and purified by sequential chromatography with phosphate buffer and ammonium acetate–acetonitrile gradient mobile phases (12). The sequences of the peptides thus obtained were determined with a gas phase automated sequencer (Applied Biosystems) with an online 120A PTH Analyzer.

The peptide EELCTMFIR was prepared with an ABI 430A Synthesizer by the solid phase method as developed by Barany and Merrifield (13). The synthetic peptide was purified using the trifluoroacetic acid mobile phase and a Vydac C_{18} column. The purified peptide (0.33 mm in 8 ml 50 mm phosphate buffer, pH 7.4) was reacted with NaOCl (13.2 mm in 2 ml buffer) at 0°C for 15 min. The reaction mixture either was subjected to HPLC separation directly or was treated with DNPH. For DNPH derivatization, 10 μl of 2 mm DNPH in 2 m HCl was added to the reaction mixture. After 30 min at room temperature, the material was extracted 3 times with diethyl ether (10 μl each), the organic phase was discarded, and the aqueous phase was dried and subjected to HPLC purification.

Mass spectrometry

Mass spectra were obtained with a TSQ 70 (upgraded to TSQ 700) triple quadrupole mass spectrometer (Finnegan-Mat, San Jose, CA). An electrospray source (PerSeptive Biosystems, Vestec Products, Houston, TX) was used and modified to replace the metal spray needle with a fused silica glass capillary tube (360 mm o.d. and 50 mm i.d.). Infusion flow rate was 0.6 to 0.8 ml/min. Samples were introduced by a valve-loop system. Electrospray voltage was 3.5 kV. Lyophilized samples were dissolved in 50% methanol, 25% acetic acid, to approximately 2 pmol/μl, and 5 μl aliquots were injected. Low energy collision-induced dissociation used xenon as collision gas at 0.8 to 1 mtorr with collision energy at 6–10 eV. MALDI-TOF mass spectra were obtained on a Voyager Elite (PerSeptive Biosystem, Vestec Products) time-of-flight mass spectrometer equipped with a nitrogen laser (337 nm, 3 ns pulse). A positive linear mode was used, with an accelerating voltage of 25 kV and a pulse delay of 150 ns. Each spectrum was produced by accumulating data from approximately 50 laser shots. Fast Atom Bombardment (FAB) spectra were acquired on a VG ZAB-SEQ hybrid tandem mass spectrometer equipped with an 11-250J data system. Two- to 3-microliter aliquots of reaction mixture were applied to the sample probe with a liquid matrix of dithiodiethanol–thioglycerol 1:1 saturated with oxalic acid. Samples were ionized by bombardment with a primary neutral beam of xenon at 8 eV. Argon sufficient to attenuate the main beam by approximately 50% was admitted to the third field-free region and the instrument was scanned to detect ions losing neutral fragments of 129 Da, characteristic of the γ-glutamyl group.

HPLC of GSH and oxidation products

Similar oxidations of GSH and GSSG were investigated as model small molecule thiols and disulfides that are also physio-

logically relevant. HPLC analyses of GSH and oxidation products were achieved by a variation of the method described by Jayatilleke and Shaw (14). A Zorbax SB-C_{18} column was used with elution by mobile phases: A, 25 mm $NaHPO_4$, 5 mm tetrabutylammonium bisulfate, pH 4.5, and B, 80% mobile phase A with 20% MeOH, using a solvent program of 6 min at 100% A followed by a linear gradient to 85%A, which was held for 4 min. Detection was by absorbance at 210 nm, and concentrations were calculated from peak areas compared with experimentally derived standard curves. With this method, separation of GSH, GSSG, GSO_2H, and GSO_3H was sufficient for the purposes of the present studies.

GSO_2H was synthesized by the method of Calam and Waley (15). GSSG (2 mmol) was mixed with 3 mmol of $AgNO_3$ in 0.2 m NaOAc, pH 6.1, in the dark overnight at room temperature. The mixture was centrifuged, 6 mmol of NaCl was added to the supernatant, mixed, and the material was centrifuged. The resulting supernatant contained GSH and GSO_2H, as indicated by HPLC and mass spectrometry, and was used without further purification.

RESULTS

Oxidation of LDL by MPO and HOCl

HPLC analyses of the tryptic digests of DNPH-treated apoB-100, with detection at 220 nm (top trace, for detection of unmodified peptides) and at 365 nm (bottom trace, characteristic of the dinitrophenyl group) showed no distinct peaks with significant absorbance at 365 nm in freshly isolated human LDL (**Fig. 1A**). Exposure of LDL to myeloperoxidase, Cl^-, and H_2O_2, followed by DNPH treatment and trypsin digestion, produced several peaks detected at 365 nm, with a dominant single peak, designated 12 in chromatograms 1B and 1C. In chromatogram 1C, the trace produced by detection at 220 nm is deleted, so the peaks of absorbance at 365 nm can be seen more clearly. A similar analysis of LDL oxidized by reagent HOCl, derivatized with DNPH, and digested with trypsin, Fig. 1D, shows a much different pattern of products detected at 365 nm, most notably in the absence of a single major peak as observed with MPO oxidation and shown in Fig. 1B and 1C. Omission of MPO, H_2O_2, or even Cl^- from the reaction mixtures yielded chromatographic product profiles that were not markedly different from that obtained from native LDL (data not shown). The oxidations evidenced in Fig. 1B and 1C result from addition of 12.5 mole of H_2O_2 per mole of apoB-100. In contrast, the results shown in Fig. 1D arise from 2000 mole of HOCl per mole of apoprotein. In our previous studies, we found apoprotein modifications caused by HOCl/apoprotein mole ratios of 250/1 to be barely detectable by the methods used in these studies (12). Subsequent experiments using doses of HOCl identical to the doses of H_2O_2 used in the present studies with MPO were conducted and the results confirmed that no unexpected bimodal appearance and disappearance of products were observed with HOCl (data not shown), as would be expected if HOCl and MPO oxidized LDL through common

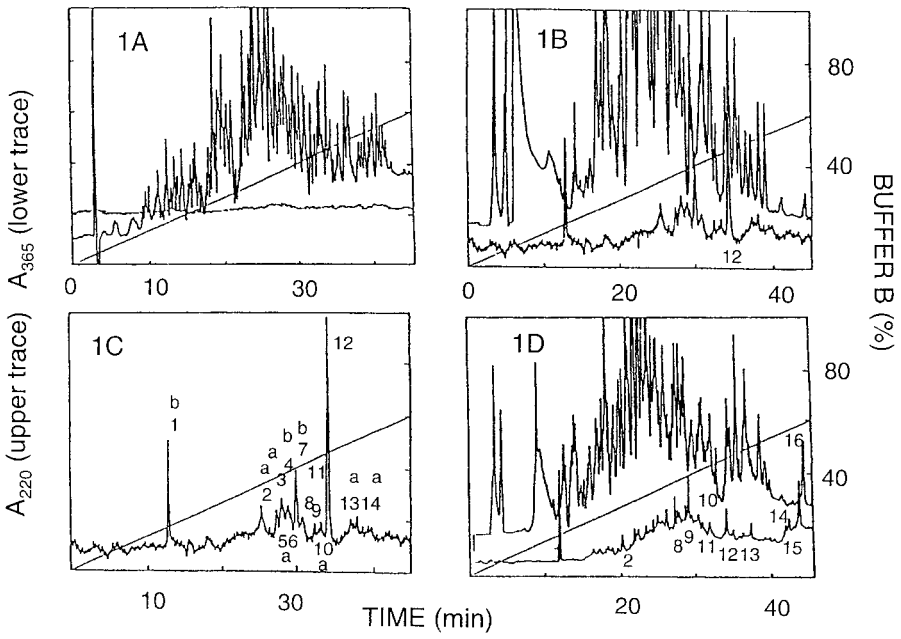

Fig. 1. HPLC of DNPH-treated apoB-100 tryptic peptides separated by reverse phase chromatography using the TFA-acetonitrile gradient: (A) native LDL treated with DNPH, the protein precipitated with acid, delipidated, washed, and digested with trypsin, and the tryptic peptides separated by reverse phase HPLC, showing absorbance detection at 220 (upper trace) and 365 nm (lower trace); (B) LDL exposed to MPO + H_2O_2 + Cl^-, then DNPH, and processed as in (A); (C) LDL exposed to MPO as in (B), but showing eluent absorption only at 365 nm; (D) LDL exposed to HOCl and processed as in (A). Fractions containing peptides exhibiting absorption at 365 nm were collected for further purification. Peaks in 1C marked with (a) showed no characteristic absorbance at 365 nm after rechromatography; peaks marked (b) showed absorbance at 365 nm after rechromatography, but no positive N-terminal sequence was observed by N-terminal sequence analysis.

TABLE 1. Oxidation of GSH and GSSG

	GSH	GSO_3H	GSO_2H	GSSG
		nmol		
GSH + H_2O_2	368	N.D.	N.D.	295
GSH + HOCl	548	45	248	174
GSH + MPO + H_2O_2	391	N.D.	N.D.	295
GSSG + H_2O_2	N.D.	N.D.	N.D.	1026
GSSG + HOCl	N.D.	N.D.	133	763
GSSG + MPO + H_2O_2	N.D.	101	N.D.	908

GSH or GSSG (1.0 μmol), in phosphate buffer, pH 7.4, at room temperature, was reacted with 2 μmol HOCl, 0.5 μmol H_2O_2, or 1.2 mg MPO + 10 μmol H_2O_2. After 15 min, the reaction mixtures were analyzed by HPLC. Product identities were assigned by coelution with standards, but it should be noted that the formation of other products cannot be precluded. Contents were calculated from peak areas using experimentally derived standard curves (GSH peak area was used to calculate GSO_2H). Data are presented in nmol and are means of at least three determinations. N.D., not detected (limit of detection is 5 nmol).

intermediates and mechanisms. Under otherwise identical reaction conditions with Cl^- and H_2O_2, separation of LDL from MPO by enclosure of the LDL in a dialysis bag inhibited formation of the major tryptic peptide absorbing at 365 nm to levels that were below our limits of detection (data not shown).

The peaks numbered in Fig. 1C were collected for further purification and analyses. The peaks labeled with an 'a' did not show peaks with significant absorbance at 365 nm with subsequent chromatography. The peaks labeled 'b' in this figure showed absorbance at 365 nm, but gave no amino acid content data on N-terminal sequence analyses. Peak 1C-12, after additional purifications with the phosphate buffer mobile phase and with the ammonium acetate-based mobile phase gave 1C-12-1-1. The other peaks isolated in these two HPLC purification steps gave no clear peptide sequence data. We characterized the material in 1C-12-1-1 by N-terminal sequence analysis to be VELEVPQL(*C)SFILK, which corresponds to amino acids 53–66 in the human apoprotein (16). Note that in this presentation the amino acid residues that have been identified by N-terminal sequence analyses are underlined, whereas the amino acids not underlined are implied from the published amino acid sequence data and the anticipated tryptic digestion sites. The N-terminal sequence of 1-C-12-1-1 is identical to that of a peptide characterized from the oxidation of LDL with reagent HOCl, indicated as peak 13 in Fig. 1D (12). However, this peptide was never more than a very minor product in the oxidation of LDL by HOCl, and modest, but characteristic differences in HPLC retention times of the two species indicate that 1-D-13 and 1-C-12-1-1 are different products sharing common N-terminal sequences. The N-terminal sequence analyses of 1-C-12-1-1 show no modifications up to the first K, with trace signals of K in cycle 14, T in cycle 15, and Q in cycle 17. Our sequencing analyses do not distinguish a modified C from an unmodified residue, and we have no direct evidence that C^{61} is oxidized by MPO to give rise to the DNPH-reactive site indicated by the DNPH-dependent absorbance at 365 nm. Previous studies have indicated that Cys^{61} does not exist in the thiol form, but appears to be disulfide-linked (17, 18). The facile oxidation of GSSG

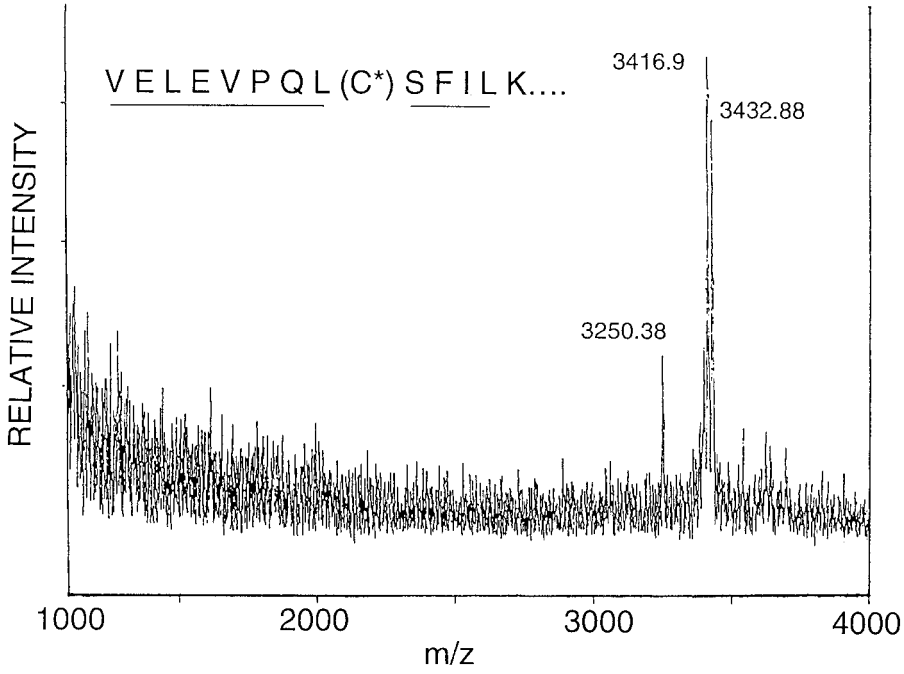

Fig. 2. MALDI-TOF mass spectrum of peptide 1C-12-1-1 from MPO oxidation of LDL. The peptide isolated by sequential HPLC purification was examined by MALDI-TOF. No ions are observed in the region of the anticipated molecular mass of the parent peptide, 1811.9. The identity of this modified peptide is not known at this time.

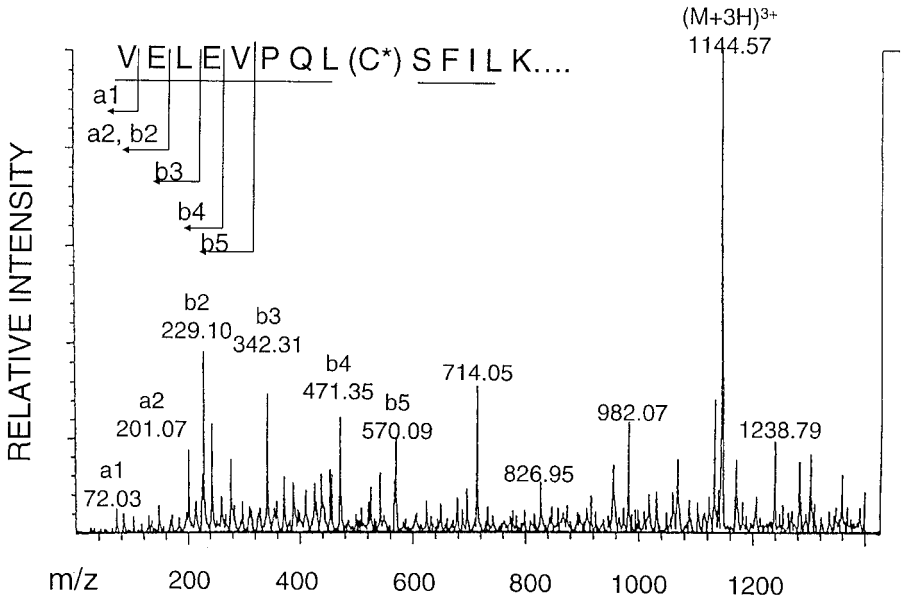

Fig. 3. ES-MS of peptide 1C-12-1-1 from MPO oxidation of LDL. The peptide isolated by sequential HPLC purification was examined by ES-MS. The ions labeled are assigned the respective N-terminal fragments consistent with the sequence obtained by gas phase sequencing. The ion at m/z 1144.57 may correspond to the y8" ion anticipated for the cysteinyl azo DNPH derivative (calculated mass 1145.5), but other y'' series fragments are not observed.

by HOCl (**Table 1**) suggests that similar oxidations of disulfides in proteins are not unreasonable.

Examination of the peptide 1-C-12-1-1 by MALDI-TOF yielded the spectrum shown in **Fig. 2**, which provides no indication of ions in the region of the mass expected, but indicates a molecule of much higher mass. Electrospray mass spectral characterization of the peptide 1C-12-1-1 did not show an apparent molecular ion in the range expected for the tryptic peptide predicted from the N-terminal sequence analysis and tryptic hydrolysis at K^{66} (m/z = 1619 for the unmodified peptide, or 1813 for [m + H + 194], for modification of the C^{61} to be de-

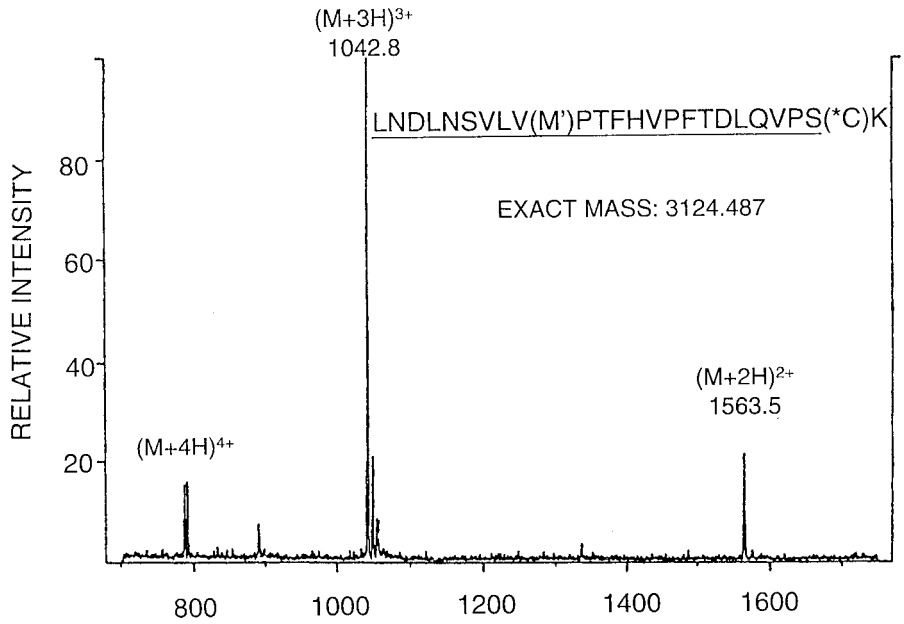

Fig. 4. Mass spectrum of LNDLNSVLV(M′)PTFHVPFTTDLQVPS(C*)K. The peptide isolated by HPLC was identified by N-terminal sequencing. The mass spectrum indicates modification of the parent sequence to the corresponding methionine sulfoxide (M + 16), indicated as (M′), and conversion of the C to the corresponding cysteinyl azo DNP derivative (M + 194).

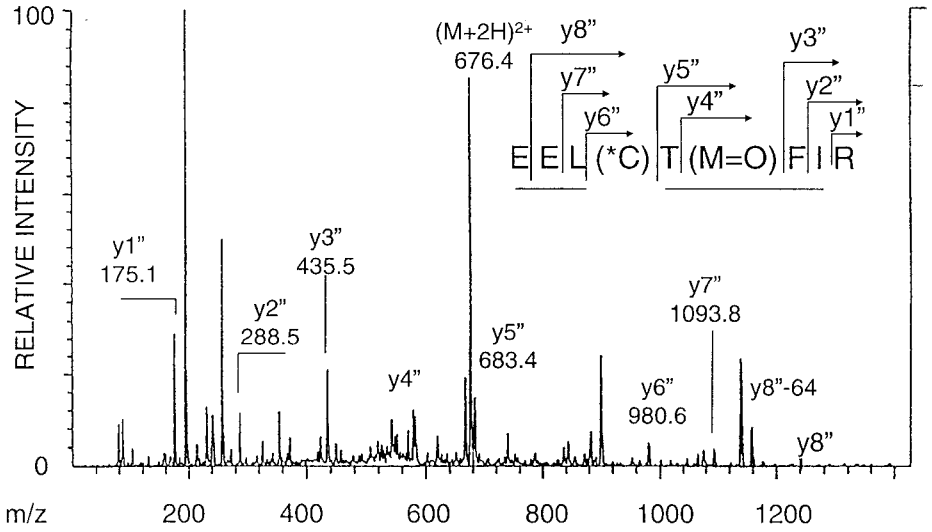

Fig. 5. Electrospray mass spectrum of EEL(C*)T(M′)FIR. The peptide isolated by HPLC and identified initially by N-terminal sequencing was analyzed by ES mass spectrometry. The parent mass and the y′′ series fragmentation ions indicate the formation of the methionine sulfoxide and the DNP-cysteinyl azo derivative, and further support the location of the latter modification on the cysteinyl residue.

scribed below). A series of N-terminal a and b series fragment ions were observed that are consistent with the peptide indicated by our gas phase N-terminal sequencing analyses, but the expected lower mass y′′ ions from the predicted C-terminal fragment ions are not observed (**Fig. 3**).

Peak 1D-12 was purified by HPLC and determined by N-terminal sequencing to be LNDLNSVLV(M=O)PTFHVPFTDQVPS(*C)K. Mass spectrometry indicated a mass of 210 Da greater than that expected for the parent unmodified tryptic peptide (**Fig. 4**). The oxidation of the methionine residue to the corresponding sulfoxide (M=O), and the absorbance at 365 nm, suggest that the additional 194 Da are most probably attributable to attachment of the DNP moiety (DNPH is 198 Da) to the cysteinyl residue, although an additional oxidation and loss of 2 Da are indicated. Electrospray mass spectrometry (ES-MS) of peak 1D-10-1, which was identified by N-terminal sequence analysis to be EEL(*C)T(M=O)FIR, gave a similar net increase in mass, and gave a full series of y′′ fragment ions that demonstrated the localization of the 194 Da increase in mass to the cysteine residue (**Fig. 5**). The products suggested by these data are illustrated in **Fig. 6**, which also outlines possible mechanisms for the transformations.

Oxidation of synthetic EELCTMFIR

To investigate the reaction schemes proposed in Fig. 6, we prepared EELCTMFIR by solid phase synthesis and purified the peptide by HPLC. Reaction of the peptide with HOCl without exposure of the material to DNPH gave a series of HPLC peaks detected at 220 nm, but showed no significant absorbance at 365 nm (**Fig. 7A**). Treatment of the initial reaction mixtures with DNPH gave products with absorbances at 365 nm (Fig. 7B). ES-MS of the material in peak 7A-2 (**Fig. 8**) indicated addition of 3 O atoms (at *m*/*z* 1189) and 4 O atoms (at 1205). Peak 7A-6 appears to be a dimer of the peptide containing 2 additional O atoms (*m*/*z* 2311.6, **Fig. 9**). The ion at *m*/*z* 2344 suggests a peptide dimer containing a total of 4 additional O atoms.

From the HOCl-oxidized EELCTMFIR treated subsequently with DNPH, we isolated a peptide whose ES-MS (**Fig. 10**) indicated an increase in mass of 244 Da, which is most readily explained as direct coupling with DNPH (+198–2) with the addition of 3 O atoms (+48). Fragment ions y_1'' through y_7'' indicate the methionine sulfoxide (not shown), sulfonhydrazide structure 3.4, presumably formed via the sulfonyl chloride (3.2) illustrated in **Fig. 11**. Hydrolysis of the sulfonyl chloride (3.2) would give the sulfonic acid (3.3), such as is observed for the ion at *m*/*z* 1205 in Fig. 8.

Oxidation of GSH

Oxidation of GSH by H_2O_2, reagent HOCl, and by MPO was studied in vitro, using a relatively simple HPLC analysis that enabled us to avoid limitations of methods that require derivatization. As is illustrated in **Fig. 12**, exposure of GSH to H_2O_2 alone gave GSSG, but with the conditions used no glutathione sulfinic (GSO_2H) or sulfonic acids (GSO_3H) were observed. However, at comparable extents of oxidation of GSH, HOCl produced readily detectable quantities of products that co-eluted with standards of the corresponding GSO_2H and GSO_3H. In contrast, oxidation of GSH by MPO (in the presence of Cl^- and H_2O_2) yielded only GSSG. Exposure of GSSG to H_2O_2 under similar conditions neither consumed GSSG measurably nor generated new product, as identified by HPLC (Table 1). HOCl did react with GSSG, giving GSO_2H as the principle product. Oxidation of GSSG

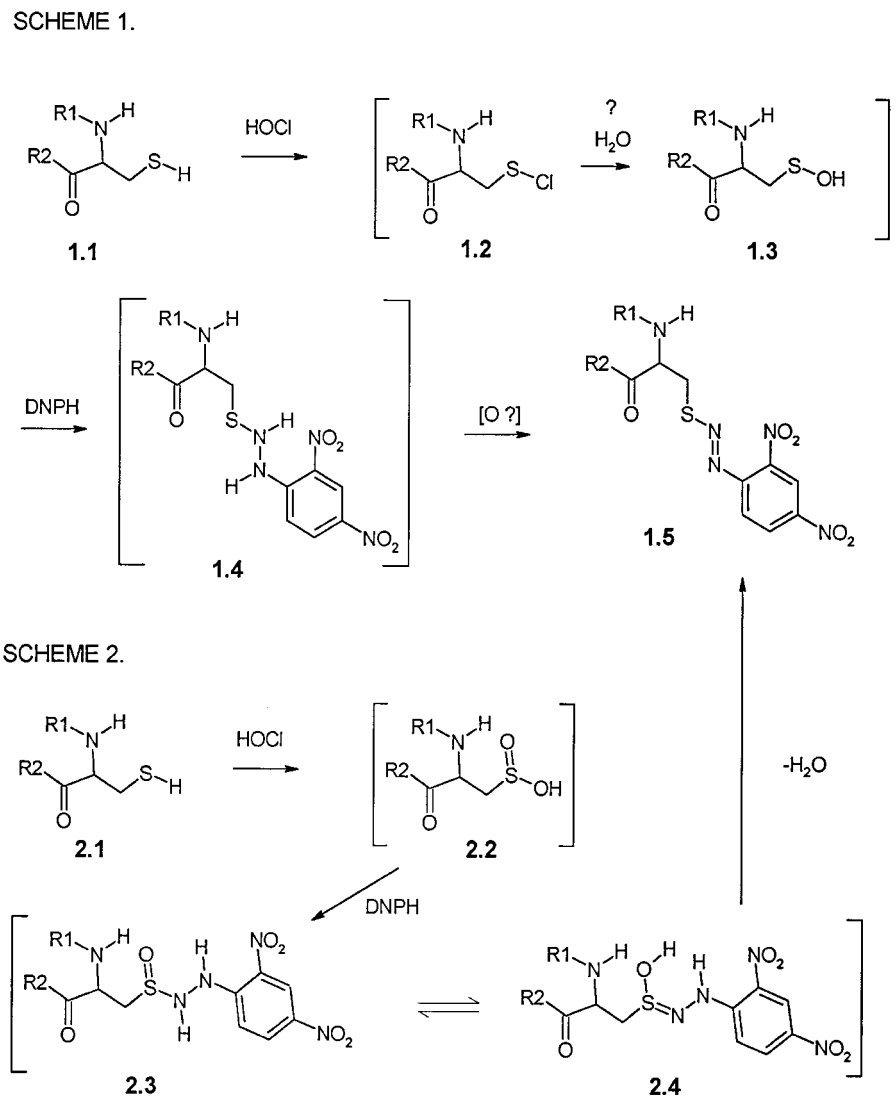

Fig. 6. Structure of proposed modification of peptides and potential mechanisms for product formation. The modifications of C residues in apoB-100 indicated by mass spectra in Figs. 4 and 5 are best explained by the corresponding DNP azo derivatives, 1.5. Possible mechanisms for these transformations include reaction of DNPH with the corresponding sufenyl chlorides (1.2) or sulfenic acids (1.3), with oxidation of the intermediate product, perhaps during workup. Alternatively, and in our view preferably, as indicated in Scheme 2, oxidation of the C groups to the sulfinic acids (2.2), condensation, tautomerization, and dehydration can account for the observed derivatives 1.5.

by MPO gave a material that co-eluted with GSO_3H, although we have not precluded the formation of some other product(s). Similar studies with GSO_3H indicated loss of starting material with exposure to HOCl, although new HPLC peaks were not observed, and no decreases in GSO_3H concentration were observed with the other oxidation conditions (data not shown). The data in Table 1 are based only on HPLC retention times for product identification, and coelution of other substances or failure of yet other products to elute under the conditions employed are not precluded.

We also applied direct mass spectral examination of a reaction mixture of GSH + HOCl. FAB MS of a crude reaction mixture, using scanning constant neutral loss of mass 129 to identify γ-glutamyl-containing species, gave the spectrum shown in **Fig. 13**. The FAB spectrum for the GSO_2H we synthesized is shown in **Fig. 14**. The ion at m/z 308 indicates GSH, as we observed also by HPLC, but the ion at m/z 340 is characteristic of the sulfinic acid. The spectrum of the GSO_3H purchased commercially shows no ions at m/z 340, little if any content of GSH or GSO_2H, and shows strong ions from exchange with up to three sodium ions. The ion at m/z 340 in the FAB spectrum of the crude reaction mixture of GSH + HOCl (Fig. 13) supports the HPLC evidence for formation of the sulfinic acid in the oxidation of GSH by HOCl, and the ions at 377.9, 399.9, and 422.1 support formation of the sulfonic acid, even though a parent ion $[M + H]^+$ containing no sodium ion (at 356) was not observed clearly.

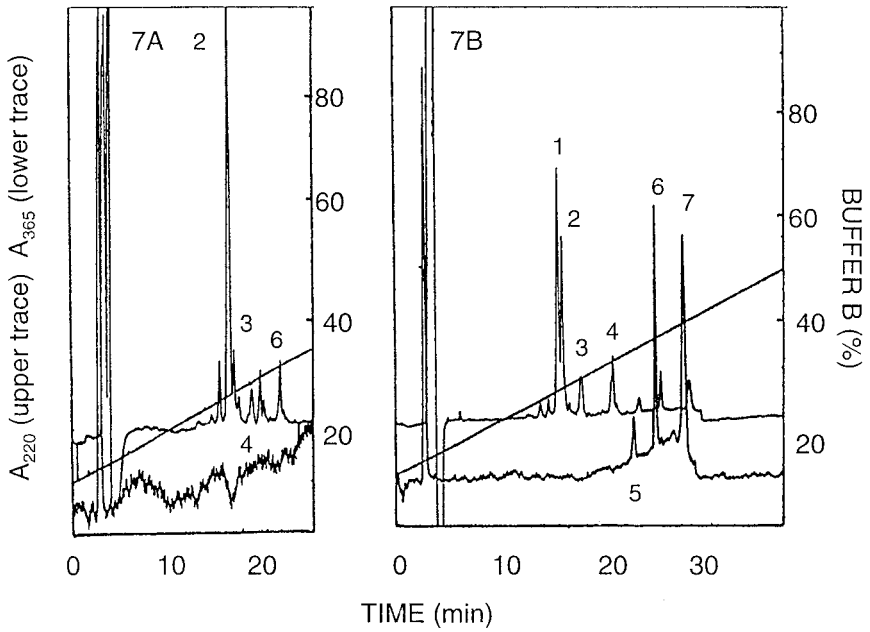

Fig. 7. HPLC of HOCl-treated peptide EELCTMFIR without (A) and with (B) treatment with DNPH after peptide oxidation. EELCTMFIR was synthesized and purified by HPLC. The purified peptide was reacted with HOCl in phosphate buffer at 0°C for 15 min, then subjected to HPLC analysis directly (A) or treated with DNPH prior to HPLC (B).

DISCUSSION

The HPLC analyses of tryptic peptides absorbing at 365 nm clearly indicate a marked difference in the oxidation of LDL by MPO and the effects of exposure to reagent HOCl (Fig. 1). This selectivity was not simply a function of extent of oxidation, as exposure of LDL to doses of reagent HOCl equal to the doses of H_2O_2 used in the MPO-catalyzed oxidations illustrated in Figs. 1B and 1C produced no products detectable by the methods used in the present studies (data not shown). The selectivity of the MPO-catalyzed oxidation of LDL is most readily attributed to the intermediacy of an enzyme–substrate complex. Marquez and Dunford (19) have reported kinetic evidence for an enzyme-bound intermediate in the MPO-catalyzed chlorination of taurine, and Libby et al. (20, 21) have observed similar effects in their studies of substrate oxidation by chloroperoxidase. However, large proteins, especially those ensconced in even more bulky lipoprotein particles, should have more difficulty in accessing the restrictive active site of MPO (22) than would the much smaller molecules studied by the other investigators. Nevertheless, the present HPLC data (Fig. 1) indicate that freely diffusible HOCl is not likely to be the oxidizing species responsible for the product pattern observed MPO-catalyzed oxidation of LDL.

The mass spectral analyses of the principle peptide isolated from MPO-oxidized LDL were not entirely consistent with the structure of the tryptic peptide predicted from the primary sequence of apoB-100 (16). However, the mass spectra are not indicative of an impure or unstable mixture. Although the MALDI spectrum of this peptide (Fig. 2) gives what appears to be a possible molecular ion (at $m/z = 3432.88$) that suggests a product other than the expected tryptic fragment ($m/z = 1619$ for the unmodified peptide), the spectrum also is consistent with a single, relatively pure compound, albeit of significantly greater molecular weight than was expected (Fig. 2). The HPLC analyses also indicate that the substance isolated is highly unlikely to be a heterogeneous mixture. The gas phase N-terminal sequence data and the N-terminal a and b fragment ions observed by ES-MS (Fig. 3) further support the purity of the peptide, and that VELEVPQ(*C) SFIL is a part of the structure.

The additional mass could arise from intermolecular crosslinking of apoproteins, but the remarkable selectivity with which the final product seems to be formed would make such a process quite surprising. An intermolecular bond formed between the apoB-100 peptide and a selected site on the MPO molecule is a possibility, but we have no indication that the MPO enzyme activity is diminished during incubation, and the amounts of MPO used in these studies would severely limit formation of stoichiometric products. Another explanation consistent with the data available at the present time is that the apoprotein is induced to form one or more intramolecular covalent crosslinks.

However, the transformations responsible for generation of peptide 1-C-12-1-1 do not generate a second N-terminal sequence. The greater mass of the peptide is most readily explained by alterations of one or more normally recognized sites for hydrolysis by trypsin. The DNPH-dependent absorbance at 365 nm indicates that MPO oxidation also must generate and retain a site reactive with DNPH. Al-

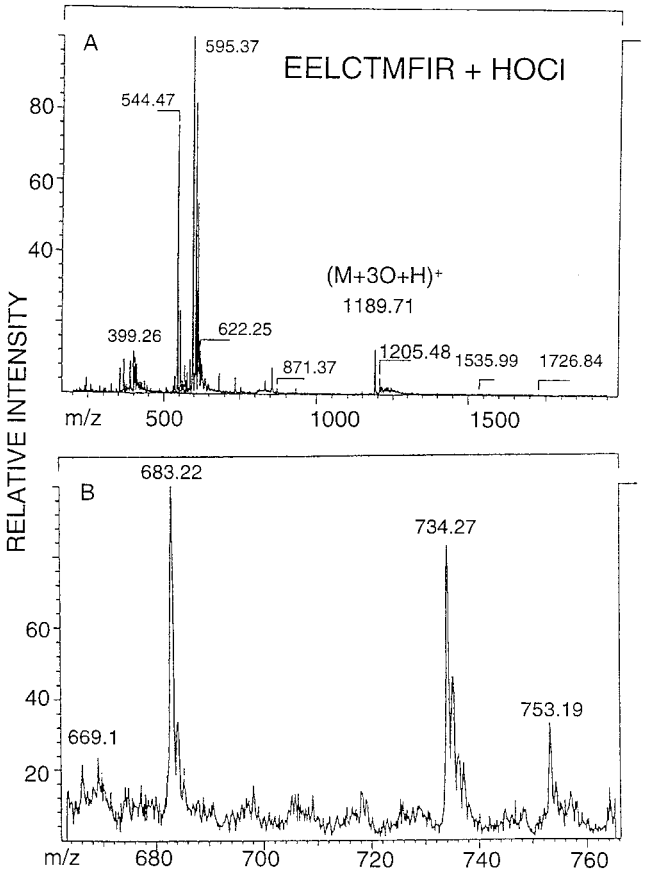

Fig. 8. Electrospray-MS of peptide 7A-2. The mass spectrum of HPLC peak 7A-2 indicates incorporation of 3 O atoms at *m/z* 1189.7, and 4 at 1205.5. The latter ion is probably the cysteic acid, methionine sulfoxide, but the former could be either the cysteic acid with intact methionine, or methionine sulfoxide cysteine sulfinic acid. The ion at 683.22 shown in panel B suggests the latter because this is the mass calculated for the y_5'' fragment ion containing the methionine sulfoxide, as opposed to the *m/z* 667 calculated for the unoxidized methionine.

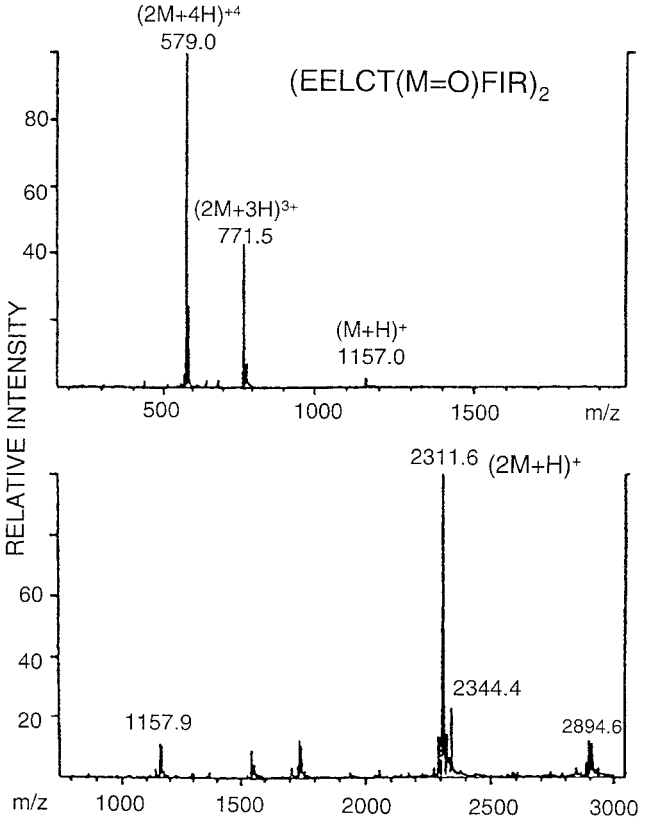

Fig. 9. Electrospray mass spectrum of peptide 7A-6. The ion at *m/z* 2311.6 indicates a dimer formed by oxidation of EELCTMFIR by HOCl, including the incorporation of 2 O atoms, such as through oxidation of both M to the corresponding methionine sulfoxides (M=O). The multiply charged ions $(M + 4H)^{4+}$ and $(M + 3H)^{3+}$ are indicated. The ion at *m/z* 1157 matches the parent monomeric peptide modified only by a single oxidation, presumably of M to (M=O). In the parent spectrum, the ions at *m/z* 2344 and 2377 suggest incorporation of 2 and 4 additional O atoms, respectively, possibly indicating oxidation of the cysteine S atoms to the corresponding sulfonyl derivatives. The nature of the bond(s) linking the dimer is(are) not known at the present time.

though no logical combination of modifications have been identified using the published primary structure of human apoB-100 (16), the loss of enzyme recognition of selected tryptic digestion sites remains the leading hypothesis for explaining the results presently available.

Another key finding from the present studies is that not all DNPH-derived increases in absorbance arising from oxidation of proteins are due to hydrazones of aldehydes or ketones. We initially rationalized the HOCl-mediated alterations of the cysteine residues of apoB-100 as proceeding through thioaldehydes or aldehydes, with subsequent formation of the corresponding DNPH-derived hydrazones. The mass spectral data are not consistent with these hypotheses, but suggest the reactions illustrated in Fig. 6. In addition to the increase of 16 Da by oxidation of the methionine to the sulfoxide (not shown in Fig. 6), the additional increase of 194 Da is best explained by product 1.5. Other studies have demonstrated that products other than aldehydes and ketones are formed by oxidation of proteins (5, 6, 23–26), but the present data are best explained by the formation of DNPH-reactive sites on cysteine moieties suggestive of sulfenyl chlorides or the sulfenic or sulfinic acids, or even the sulfonyl chlorides (Figs. 6 and 11).

The modification of biological macromolecules by chemically reactive metabolites and the contributions of these alterations to drug toxicities and disease processes have been recognized for many years and have been studied extensively (27–29). The present observations suggest that biological macromolecules may themselves be converted to chemically reactive intermediates capable of altering other macromolecules or other sites on the same molecule. Unraveling the products and thereby the mechanisms of such processes will be difficult, but the evidence associating protein oligomerization with hyperoxic lung injury (30, 31) and possibly with other diseases, such as Alzheimer's (32), indicates that these questions are likely to be important and merit careful investigation.

Clearly a product of GSH oxidation, GSSG frequently is

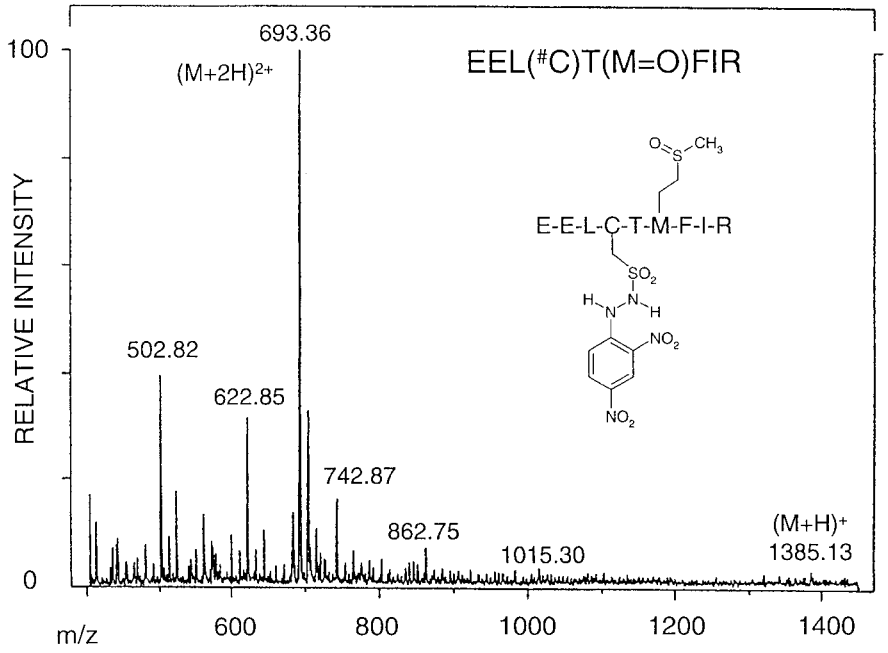

Fig. 10. Electrospray-MS of peptide 7B-6. Spectra indicate oxidation of M to methionine sulfoxide (M′) and conversion of the C to the corresponding DNPH-derived sulfonamide (C#), presumably through the sulfonyl chloride (RSO_2Cl). The y′′ series ions from y_1′′ through y_7′′ were observed (not shown), further supporting the assigned structure.

called 'oxidized glutathione'; however, products other than GSSG can be produced by oxidation of GSH, and products other than disulfides can be formed through the oxidation of cysteine sulfhydryls in proteins. The ability of GSSG to be reduced by the NADPH-dependent glutathione reductase system to sustain supplies of GSH distinguishes GSSG from many of the other products of oxidation of GSH. For this reason, the reactions of HOCl with GSH may have markedly different effects on biological defense functions and cell viability than would equivalent quantities of hydrogen peroxide or lipid hydroperoxides. Although one molecule of GSH is capable of reducing four molecules of HOCl, the formation of products that are not reduced by the glutathione reductase-NADPH system and the potential formation of protein modifications that are not similarly repairable by reduction of disulfide bonds in proteins, through thiol-disulfide exchange reactions, are likely to exert physiological effects that are equally irreversible (33–35).

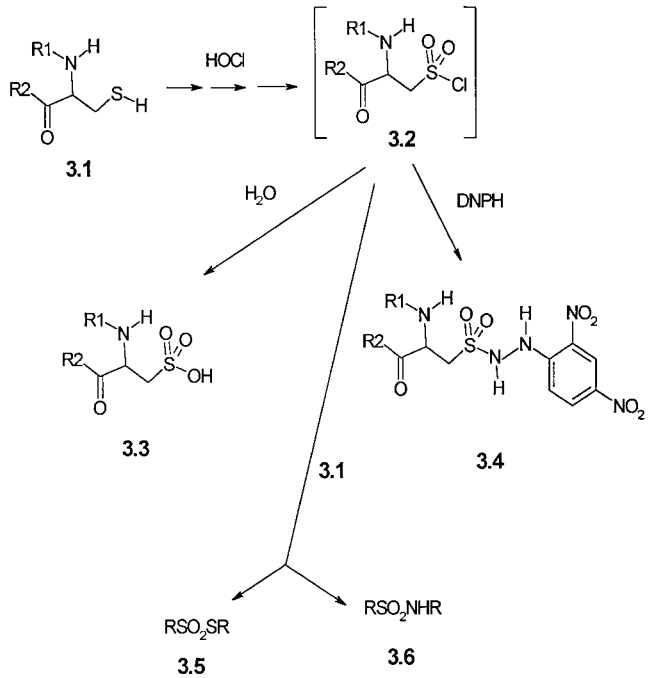

Fig. 11. Proposed structures and mechanisms of formation of cysteic acid (3.3) and DNP-sulfonhydrazide (3.4) in the reaction of EELCTMFIR with HOCl. The oxidation of the methionine group is not addressed in this scheme, nor are the potential mechanisms through which peptide dimerization might be effected.

The results of the present studies indicate that mechanisms other than S-thiolations need to be considered in the covalent aggregation of proteins. Protein aggregates should affect biological systems adversely, both through loss of normal functions of the constituent proteins and through the physical properties of the aggregates. These covalent clumps, if formed largely through the formation of disulfide bonds and denaturation of tertiary structure, might be resolved through reduction of the disulfide bonds through glutaredoxin, thioredoxin, or related systems, or at least through lysosomal hydrolysis and re-synthesis of the altered proteins from the amino acids thus recovered. However, more complex covalent modifications, such as the ones implicated in the HOCl-mediated and/or the MPO-catalyzed oxidation of apoB-100, might compromise even the process of lysosomal

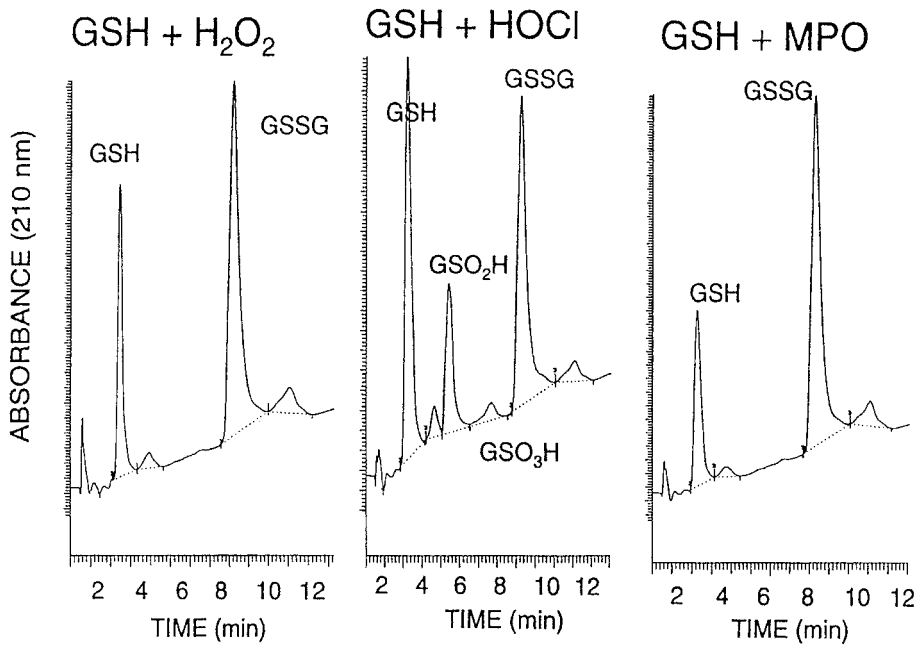

Fig. 12. HPLC separation of GSH and products of its oxidation in vitro. GSH (1.0 μmol), in phosphate buffer, pH 7.4, at room temperature, was reacted with 0.5 μmol H_2O_2, 2 μmol HOCl, or 1.2 μg MPO + 10 μmol H_2O_2. After 15 min, the reaction mixtures were analyzed by HPLC. Product identities were assigned by coelution with standards, purchased or in the case of GSO_2H synthesized as described in Methods. The identities of the other peaks are unknown at this time.

digestion and re-synthesis of proteins (36, 37). We have observed by western analyses two DNPH-reactive proteins of about 40 and 120 kDa in the BAL fluids of rats exposed to hyperoxia (30). Isolation of the reactive bands and N-terminal sequence analyses of the proteins showed both to be 100% identical over 16 amino acids to rat β-casein, although rat β-casein is about 25 kDa. The chemical structures of the DNPH-reactive products and the

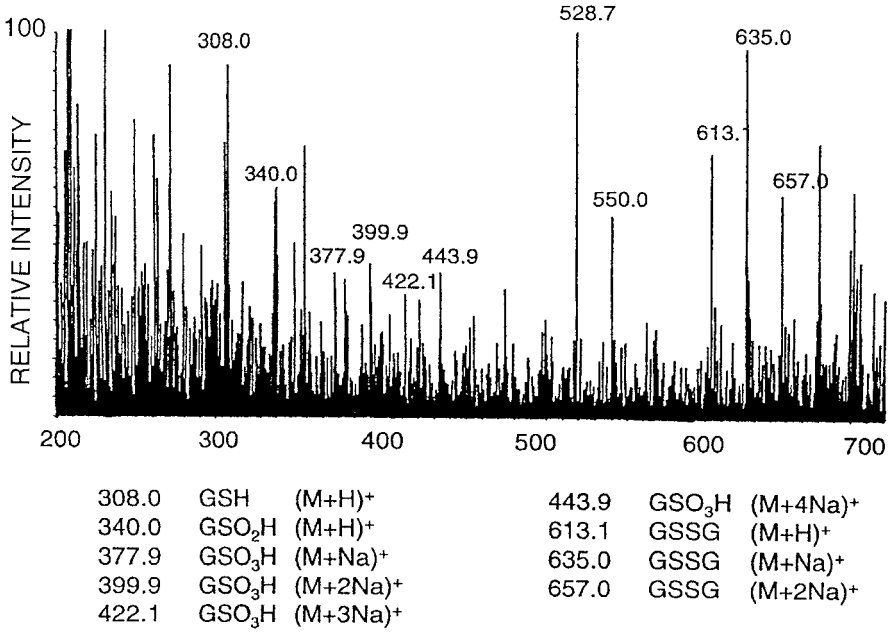

Fig. 13. FAB mass spectrum of GSH oxidation by HOCl in vitro. GSH and HOCl (1:2) were reacted at room temperature for 15 min, and the reaction mixture was analyzed directly by fast atom bombardment. Spectra were acquired with constant neutral loss of m/z of 129, for loss of the glutamyl moiety. Structural assignment of ions are indicated. The production of GSO_2H, GSO_3H, and GSSG are indicated by the respective ions, but no indications of halogenated derivatives are observed.

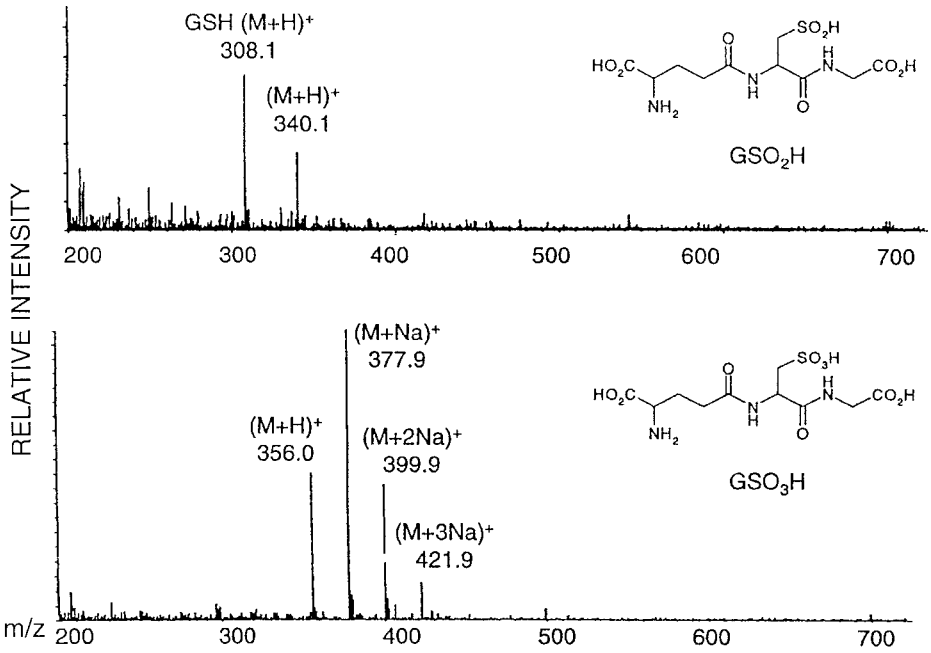

Fig. 14. FAB mass spectrometry of glutathione sulfinic (GSO_2H) and sulfonic (GSO_3H) acids. GSH produced in the synthesis of GSO_2H from GSSG was not removed prior to analysis. The ion at m/z 340 is assigned to the protonated molecular ion of GSO_2H, and is not observed in GSH, GSSG (not shown) or in GSO_3H (B).

mechanisms responsible for the oxidative alterations are not known at this time, but the implications of oxidative protein oligomerization appear to be not unlike those indicated by the present studies. In other investigations, we noted intraalveolar accumulation of proteinaceous material in rats exposed to hyperoxia, and a modest but statistically significant attenuation of parameters of lung injury that correlated with attenuation of the extent of accumulation of these proteinaceous exudates in rats treated with the 21-aminosteroid U74389 (31).

In conclusion, our data indicate that freely diffusible HOCl does not contribute significantly to MPO-catalyzed oxidation of LDL in vitro. The generation of DNPH-reactive sites in apoB-100 by oxidation of HOCl appears to involve generation of sulfinic acids from cyst(e)ine residues in the apoprotein. The metabolic activation of biological molecules, including macromolecules, to form chemically reactive intermediates capable of contributing to initiation and/or progression of atherosclerosis, hyperoxic lung injury, or related toxicities and diseases is likely to be a formidable challenge for research, but recent studies suggest that these processes may be of sufficient importance to justify the effort.

This work was supported by National Institutes of Health grants HL-27341 and GM-44263, and by a grant-in-aid 97G-213 from the American Heart Association, Texas Affiliate, Inc.

Manuscript received 27 February 1998, in revised form 21 August 1998, in re-revised form 2 December 1998, and in re-re-revised form 10 December 1998.

REFERENCES

1. Steinberg, D., S. Parthasarathy, T. E. Carew, J. C. Khoo, and J. L. Witzum. 1989. Beyond cholesterol. Modifications of low density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320:** 915–924.
2. Steinbrecher, U. P., H. Zhang, and M. Lougheed. 1990. Role of oxidatively modified LDL in atherosclerosis. *Free Radical Biol. Med.* **9:** 155–168.
3. Stocker, R. 1994. Lipoprotein oxidation: mechanistic aspects, methodological approaches and clinical relevance. *Curr. Opin. Lipidol.* **5:** 422–433.
4. Berliner, J. A., M. Navab, A. M. Fogelman, J. S. Frank, L. L. Demer, P. A. Edwards, A. D. Watson, and A. J. Lusis. 1995. Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics. *Circulation*. **91:** 2488–2496.
5. Stadtman, E. R., and B. S. Berlett. 1997. Reactive oxygen-mediated protein oxidation in aging and disease. *Chem. Res. Toxicol.* **10:** 485–494.
6. Dean, R. T., S. Fu, R. Stocker, and M. J. Davies. 1997. Biochemistry and pathology of radical-mediated protein oxidation. *Biochem. J.* **324:** 1–18.
7. Daugherty, A., J. L. Dunn, D. L. Rateri, and J. W. Heinecke. 1994. Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions. *J. Clin. Invest.* **94:** 437–444.
8. Hazell, L. J., L. Arnold, D. Flowers, G. Waeg, E. Malle, and R. Stocker. 1996. Presence of hypochlorite-modified proteins in human atherosclerotic lesions. *J. Clin. Invest.* **97:** 1535–1544.
9. Hazell, L. J., and R. Stocker. 1993. Oxidation of low-density lipoprotein with hypochlorite causes transformation of the lipoprotein into a high-uptake form for macrophages. *Biochem. J.* **290:** 165–172.
10. Hazell, L. J., J. J. M. van den Berg, and R. Stocker. 1994. Oxidation of low-density lipoprotein by hypochlorite causes aggregation that is mediated by modification of lysine residues rather than lipid oxidation. *Biochem. J.* **302:** 297–304.
11. Hazen, S. L., and J. W. Heinecke. 1997. 3-Chlorotyrosine, a specific marker of myeloperoxidase-catalyzed oxidation, is markedly elevated in low density lipoprotein isolated from human atherosclerotic intima. *J. Clin. Invest.* **99:** 2075–2081.
12. Yang, C-Y., Z-W. Gu, H-X. Yang, A. M. Gotto, Jr., and C. V. Smith. 1997. Oxidative modifications of apoB-100 by exposure of low

density lipoproteins to HOCl in vitro. *Free Radical Biol. Med.* **23:** 82–89.
13. Barany, G., and R. B. Merrifield. 1980. Solid phase peptide synthesis. *In* The Peptides: Analysis, Synthesis, Biology. E. Gross and J. Meienhofer, editors. Academic Press, New York. 3–284.
14. Jayatilleke, E., and S. Shaw. 1993. A high-performance liquid chromatographic assay for reduced and oxidized glutathione in biological samples. *Anal. Biochem.* **214:** 452–457.
15. Calam, D. H., and S. G. Waley. 1962. Some derivatives of glutathione. *Biochem. J.* **85:** 417–419.
16. Yang, C-Y., Z-W. Gu, S-a. Weng, T.W. Kim, S-H. Chen, H. J. Pownall, P. M. Sharp, S-W. Liu, W-H. Li, A. M. Gotto, Jr., and L. Chan. 1989. Structure of apolipoprotein B-100 of human low density lipoproteins. *Arteriosclerosis.* **9:** 96–108.
17. Hori, H., R. E. Fenna, S. Kimura, and M. Ikeda-Saito. 1994. Aromatic substrate molecules bind at the distal heme pocket of myeloperoxidase. *J. Biol. Chem.* **269:** 8388–8392.
18. Yang, C. Y., T. W. Kim, S. A. Weng, B. Lee, M. Yang, and A. M. Gotto, Jr. 1990. Isolation and characterization of sulfhydryl and disulfide peptides of human apoB-100. *Proc. Natl. Acad. Sci. USA.* **87:** 5523–5527.
19. Marquez, L. A., and H. B. Dunford. 1994. Chlorination of taurine by myeloperoxidase. *J. Biol. Chem.* **269:** 7950–7956.
20. Libby, R. D., T. M. Beachy, and A. K. Phipps. 1996. Quantitating direct chlorine transfer from the enzyme to substrate in chloroperoxidase-catalyzed reactions. *J. Biol. Chem.* **271:** 21820–21827.
21. Libby, R. D., A. L. Shedd, A. K. Phipps, T. M. Beachy, and S. M. Gerstberger. 1992. Defining the involvement of HOCl or Cl_2 as enzyme-generated intermediates in chloroperoxidase-catalyzed reactions. *J. Biol. Chem.* **267:** 1769–1775.
22. Nathan, C. F., B. A. Arrick, H. W. Murray, N. M. DeSantis, and Z. A. Cohn. 1980. Tumor cell anti-oxidant defenses. Inhibition of the glutathione redox cycle enhances macrophage-mediated cytolysis. *J. Exp. Med.* **153:** 766–782.
23. Hazen, S. L., F. F. Su, D. M. Mueller, J. R. Crowley, and J. W. Heinecke. 1996. Human neutrophils employ chlorine gas as an oxidant during phagocytosis. *J. Clin. Invest.* **98:** 1283–1289.
24. Radi, R., J. S. Beckman, K. M. Bush, and B. A. Freeman. 1991. Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J. Biol. Chem.* **266:** 4244–4250.
25. DeMaster, E. G., B. J. Quast, B. Redfern, and H. T. Nagasawa. 1995. Reaction of nitric oxide with the free sulfhydryl group of human serum albumin yields a sulfenic acid and nitrous oxide. *Biochemistry.* **34:** 11494–11499.
26. Waldhauser, L., and J. Utrecht. 1991. Oxidation of propylthiouracil to reactive metabolites by activated neutrophils. Implications for agranulocytosis. *Drug Metab. Dispos.* **19:** 354–359.
27. Miller, E. C., and J. A. Miller. 1947. The presence and significance of bound aminoazo dyes in the livers of rats fed p-dimethylaminoazobenzene. *Cancer Res.* **7:** 468–480.
28. Smith C. V., B. H. Lauterburg, and J. R. Mitchell. 1985; Covalent binding and acute lethal injury in vivo: how has the hypothesis survived a decade of critical examination? *In* Drug Metabolism and Disposition: Considerations in Clinical Pharmacology. G. Wilkinson and M. D. Rawlins, editors. MTP Press, London. 161–181.
29. Nelson, S. D., and P. G. Pearson. 1990. Covalent and noncovalent interactions in acute lethal cell injury caused by chemicals. *Annu. Rev. Pharmacol. Toxicol.* **30:** 169–195.
30. Knight, S. A., C. V. Smith, and S. E. Welty. 1997. Iron and oxidized β-casein in the lavages of hyperoxic Fischer-344 rats. *Life Sci.* **62:** 165–176.
31. Awasthi, S., A. Gyurasics, S. A. Knight, S. E. Welty, and C. V. Smith. 1998. Protein oxidation biomarkers in hyperoxic lung injury in rats: effects of U-74389. *Toxicol. Lett.* In press.
32. Gotz, M. E., G. A. Kunig, P. Riederer, and M. B. H. Youdim. 1994. Oxidative stress: free radical production in neural degeneration. *Pharmacol. Ther.* **63:** 37–122.
33. Prutz, W. A. 1996. Hypochlorous acid interactions with thiols, nucleotides, DNA, and other biological substrates. *Arch. Biochem. Biophys.* **332:** 110–120.
34. Chesney, J. A., J. W. Eaton, and J. R. Mahoney, Jr. 1996. Bacterial glutathione: a sacrificial defense against chlorine compounds. *J. Bacteriol.* **178:** 2131–2135.
35. Winterbourn, C. C., and S. O. Brennan. 1997. Characterization of the oxidation products of the reaction between reduced glutathione and hypochlorous acid. *Biochem. J.* **326:** 87–92.
36. Grant, A. J., W. Jessup, and R. T. Dean. 1992. Accelerated endocytosis and incomplete catabolism of radical-damaged protein. *Biochim. Biophys. Acta.* **1134:** 203–209.
37. Gupta, S., L. K. Rogers, and C. V. Smith. 1994. Biliary excretion of lysosomal enzymes, iron, and oxidized protein in Fischer-344 and Sprague-Dawley rats and the effects of diquat and acetaminophen. *Toxicol. Appl. Pharmacol.* **125:** 42–50.