Identification of a Specific Methionine in Mammalian 15-Lipoxygenase Which Is Oxygenated by the Enzyme Product 13-HPODE: Dissociation of Sulfoxide Formation from Self-Inactivation†

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ABSTRACT: Mammalian 15-lipoxygenases undergo a characteristic self-inactivation. The oxygenation of a single methionine to methionine sulfoxide, by 13(*S*)-hydroperoxyoctadecadienoic acid (13-HPODE), was previously suggested as the cause of the inactivation of rabbit reticulocyte lipoxygenase. The site of oxygenation is potentially near the enzyme's active site; however, the specific location of the modified amino acid residue has not been identified. To determine which of the methionine residues is oxygenated, we inactivated both human and rabbit 15-lipoxygenases with 13-HPODE and sequentially denatured, reduced, carboxymethylated, and digested the enzymes with trypsin. The digested mixtures were analyzed by reverse-phase HPLC chromatography. Mass spectrometric analysis of each of the methionine-containing fractions enabled us to locate the peptide segments containing the oxidized methionine in both enzymes separately. Tandem electrospray mass spectrometry identified the oxidized methionine residues to be amino acid 590 in the human enzyme and 591 in the rabbit enzyme. To investigate the significance of this oxygenation, Met^{590} in human 15-lipoxygenase was substituted with leucine by site-directed mutagenesis. The mutant protein was inactivated by 13-HPODE, yet no oxygenated peptide or other modified peptide could be identified by HPLC-MS analysis. We also found that human 15-lipoxygenase was inactivated during arachidonate oxidation and by the reaction product 15(*S*)-hydroperoxyeicosatetraenoic acid (15-HPETE), and no modified peptide was detected. Thus, methionine oxygenation is not essential for the inactivation of human 15-lipoxygenase. We suggest, however, that Met^{590} is an amino acid in the substrate binding pocket of human 15-lipoxygenase and interacts with the enzyme product 13-HPODE.

Lipoxygenases are a family of dioxygenases which catalyze the oxygenation of fatty acids specifically containing 1,4-*cis,cis*-pentadiene moieties. The lipoxygenase reaction results in a stereo- and regiospecific formation of a fatty acid hydroperoxide. Mammalian lipoxygenases metabolize arachidonic acid into a series of bioactive compounds, such as leukotrienes and lipoxins, which are implicated in inflammation and hypersensitivity (Hamburg & Samuelsson, 1974; Borgeat & Samuelsson, 1979; Samuelsson et al., 1987). In addition, reticulocyte 15-lipoxygenase appears to play a role in the maturation of red blood cells by triggering the peroxidation and degradation of mitochondrial membranes (Rapoport et al., 1979). It has also been found that 15-lipoxygenase is induced in inflammatory cells of human atherosclerotic lesions (Yla-Herttuala et al., 1991) and is capable of oxidizing low-density lipoprotein to its atherogenic form (Parthasarathy et al., 1989; Steinberg et al., 1989; Sigal et al., 1994). Thus, this particular lipoxygenase may be important in the early stages of atherosclerosis.

Mammalian lipoxygenases display a temperature-dependent, irreversible self-inactivation (Rapoport et al., 1979). The basis for this inactivation is unknown. Previous studies with rabbit reticulocyte 15-lipoxygenase showed that nearly stoichiometric amounts of enzyme product, 13(*S*)-hydroperoxyoctadecadienoic acid (13-HPODE),[1] were sufficient to inactivate the enzyme (Härtel et al., 1982). Using two-dimensional thin layer chromatography and cyanogen bromide cleavage, it was later found that the oxygenation of a single methionine to methionine sulfoxide correlated with enzyme inactivation by this hydroperoxide (Rapoport et al., 1984). It was proposed that this single methionine is a part of the enzyme's active site and that oxygenation of this methionine is responsible for the inactivation of the enzyme. The rabbit reticulocyte 15-lipoxygenase contains 16 methionines, and previous techniques failed to identify which methionine was oxygenated. Furthermore, a causal relationship between methionine sulfoxide formation and the self-inactivation has not been clearly established. For these reasons, we initiated studies to generate tryptic maps of human and rabbit 15-lipoxygenases and to identify the methionine that is oxygenated during incubation with 13-HPODE using HPLC-MS analysis. Using site-directed mutagenesis of the human 15-lipoxygenase, we have generated a methionine-substituted human 15-lipoxygenase and investigated the inactivation of this mutant enzyme.

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[1] Abbreviations: 13-HPODE, 13(*S*)-hydroperoxyoctadecadienoic acid; 15-HPETE, 15(*S*)-hydroperoxyeicosatetraenoic acid; ETYA, 5,8,-11,14-eicosatetraynoic acid; HPLC-MS, high-pressure liquid chromatography−mass spectrometry.

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MATERIALS AND METHODS

All chemicals and reagents were obtained from commercial sources in the highest purity available.

Purification of Human and Rabbit 15-Lipoxygenases. Human recombinant 15-lipoxygenase was purified to 98% purity from a baculovirus/insect cell expression system using anion-exchange chromatography on a Mono Q column as previously described (Kühn et al., 1993b). Native rabbit 15-lipoxygenase was purified to 98% purity from a 55% ammonium sulfate precipitate of rabbit reticulocyte lysate (purchased from Analytical Biological Services, Wilmington, DE) using a modification of the previously published procedures (Sloane et al., 1990). The ammonium sulfate precipitate was dissolved in distilled, deionized water and was dialyzed for 16 h against 100 volumes of argon-saturated buffer KHP-1 (20 mM KPO_4, pH 7.0, 20 μM ferrous ammonium sulfate, 7.5% saturated ammonium sulfate). After centrifugation and filtration through a 0.2-μm syringe filter (Gelman Sciences, Ann Arbor, MI), the ammonium sulfate sample was applied at 5 mL/min to a 150- × 21.5-mm hydrophobic interaction column (Bio-Gel TSK Phenyl-5-PW, Bio-Rad, Richmond, CA) equilibrated in buffer KHP-1. The column was developed at 5 mL/min on a Waters 650E chromatography system by a 60 min linearly decreasing ammonium sulfate gradient until the ammonium sulfate concentration was zero. The active fractions were pooled and concentrated to 5 mL by ultrafiltration (YM-30 membrane, Amicon, Danver, MA). The concentrate was desalted using a PD-10 column (Sephadex G-25 M, Pharmacia, Sweden) and eluted with buffer MOPS-1 (10 mM MOPS buffer, pH 6.8, 20 μM ferrous ammonium sulfate). The protein sample was then applied at 1 mL/min to a 10- × 100-mm Waters Protein Pak Q 8HR anion exchange column (Millipore, Milford, MA) equilibrated with buffer MOPS-1. The column was first developed with buffer MOPS-1 for 15 min, and then the NaCl concentration was increased to 20 mM over 5 min and the column was further developed with this salt concentration for an additional 1 h. The fractions containing lipoxygenase activity were pooled and concentrated to about 30 mg/mL by ultrafiltration. All purification steps were carried out at 4 °C or on ice. Each of the purified proteins was characterized by lipoxygenase activity and SDS gel electrophoresis using SDS−glycine 8−16% gradient gels (Novex, San Diego, CA). The method of Bradford (1976) was used for determining protein concentration.

Enzyme Incubation and Tryptic Digestion. Approximately 5 μL of highly purified human or rabbit 15-lipoxygenase (150 μg, 2 nmol) was treated with a 3-fold molar excess of 13(*S*)-hydroperoxyoctadecadienoic acid (13-HPODE) (Biomol, Plymouth Meeting, PA) and incubated anaerobically at 37 °C for 30 min. Solvent ethanol in the 13-HPODE solution was evaporated with N_2 gas before the protein solution was added to it. The incubated sample was diluted with 50 μL of phosphate-buffered saline (0.017 M KH_2PO_4, 0.05 M Na_2HPO_4, 0.15 M NaCl, pH 7.4) and denatured with 60 μL of freshly prepared denaturing buffer (0.4 M $NaHCO_3$, 8 M urea, pH 8). Dithiothreitol (45 mM, 8 μL) was added, and the sample was heated at 65 °C for 15 min. After cooling to room temperature, iodoacetamide (100 mM, 8 μL) was added and incubated at room temperature for 15 min. Here the cysteine residues of the proteins were reduced and alkylated to form *S*-carboxyamidomethylcysteines. The alkylated sample was finally diluted to 240 μL with water. Tryptic digestion was initiated by the addition of 5 μL (1 μg/μL) of trypsin-TPCK (Worthington, Freehold, NJ), and the mixture (approximately 245 μL) was incubated at 37 °C for 16 h.

The same conditions were used in the treatment of human 15-lipoxygenase with 15(*S*)-hydroperoxyeicosatetraenoic acid (15-HPETE) (Oxford Biochemical Research, Oxford, MI) as well as the treatment of purified mutant lipoxygenase with 13-HPODE. To study the reaction of 5,8,11,14-eicosatetraynoic acid (ETYA) (Biomol) with human 15-lipoxygenase, the same amount of enzyme (approximately 5 μL, 0.5 mM) was treated with a 3-fold excess of ETYA. The enzyme/ETYA mixture was diluted with 50 mL of PBS buffer (final ETYA concentration was about 150 μM) and first incubated at room temperature for 1 h in the presence of air and then at 37 °C for 30 min, followed by the same treatment as above. A sample treated in absence of hydroperoxide products or ETYA was always carried out parallel to the reaction samples.

HPLC-MS Peptide Analysis. Approximately 500 pmol of the tryptic digest (60 μL) was directly analyzed by reverse-phase HPLC. Chromatography was carried out using a Vydac C4 column (2.1- × 150-mm, 5 μm particle size). The column was developed at 100 μL/min with a gradient consisting of solvents A (distilled, deionized water containing 0.1% trifluoroacetic acid) and B (80:20 acetonitrile−water containing 0.1% trifluoroacetic acid), as follows: 0−10 min, 0% B; 10−120 min, linear gradient to 100% B. The gradient was formed using an ABI model 140B microsyringe pumping system equipped with a static mixer (3 μL internal volume). The column effluent was passed through a flow splitter, where ca. 1.8 μL/min was directed to the mass spectrometer through a 100 μm i.d. fused silica transfer line. This transfer line was attached to the inlet needle of a TSQ700 triple stage quadrupole mass spectrometer (Finnigan-MAT, San Jose, CA) equipped with an electrospray ion source (Analytica, CT). A sheathing flow of 2-methoxyethanol (2.0 μL/min) was used in conjunction with a nebulizing nitrogen gas flow to assist the formation of microdroplets via electrospray. Data were acquired in centroid positive ion mode using a scan range of *m/z* 300−1900 in 3 s. Typical operating conditions for the ESI source were as follows: electrode/capillary entrance voltage, −3000 V; capillary exit, +40 V; tube lens, +180 V; drying gas temperature, 255 °C.

The main portion of the column effluent was directed to an absorbance detector (ABI model 783A) equipped with a 1.8 μL flow cell and then to a fraction collector. Absorbance was monitored at 214 nm. Fractions were collected in 1.5 mL polypropylene centrifuge tubes using 1 min intervals. In the subsequent tandem MS experiments, individual HPLC fractions were infused directly into the electrospray ion source using a syringe pump (Harvard model 22) at a flow rate 1 μL/min together with a sheathing flow of 1 μL/min of 2-methoxyethanol. Collision-assisted dissociation (CAD) spectra were then acquired on the +3 charged ion at a collision pressure of *ca.* 2.5 mtorr argon and energies (E_{lab}) of between −15 and −17 eV. Data were acquired in profile mode using signal averaging and then converted to centroid data.

Mutagenesis. Site-directed mutagenesis of human 15-lipoxygenase (Sigal et al., 1988) was performed by using

mismatched synthetic oligonucleotides by the Kunkel method (Kunkel, 1985) as previously modified (Evnin & Craik, 1988). Oligonucleotide was synthesized on an Applied Biosystems 308B DNA synthesizer at the Biomolecular Resource Center, University of California, San Francisco. To replace Met^{590} with leucine and generate the mutant protein M590L, a 29-mer with the sequence 5′-GCCAAGT-GATAGACAGCTGGAGAGAAGCC-3′ was used (mismatches are underlined). The mismatch contained in this oligonucleotide, G, converted the Met^{590} codon (ATG) to a leucine codon (CTG). Another mismatch, A, removed a *Van*91 I restriction site which was used to screen transformants. Uracil-laden plasmid pSS15LO, used as a template, was prepared as described (Sloane et al., 1995). This plasmid contains a *lac* promotor to express the 15-lipoxygenase and a bacteriophage f1 origin of replication to generate single strands. After the mutagenesis reaction, transformants were screened by digestion with restriction enzyme *Van*91 I. Positive mutant was verified by DNA sequencing (Sanger et al., 1977).

Purification of Human 15-Lipoxygenase and Mutant Protein M590L from Escherichia coli Expression System. Mutant protein M590L and the wild-type human 15-lipoxygenase expressed in pSS15LO were purified from *E. coli* by the following modification of published procedures (Kühn et al., 1993b). Bacteria was grown in 2 L of Terrific Broth (2.4% tryptone, 1.2% yeast extract, 0.017 M KH_2PO_4, 0.072 M K_2HPO_4, 0.04% glycerol) with 80 mg/L ampicillin at 37 °C overnight. After being harvested by centrifugation (10000*g*, 15 min) and washed with 10 mM Bis-Tris buffer (pH 7.5), the cells were resuspended in 100 mL of 10 mM Bis-Tris buffer (pH 7.5) containing 0.2 M NaCl and sonicated for 4 × 20 s with a Branson 450 Sonifier at output control 6. The cell debris was spun down (15000*g*, 15 min), and the supernatant was treated with 0.5% polyethylenimine to remove nucleic acid. Following removal of the polyethylenimine−nucleic acid precipitate by centrifugation (10000*g*, 15 min), the supernatant was precipitated with $(NH_4)_2SO_4$ at 60% saturation. The precipitate was redissolved in 10 mL of 10 mM Bis-Tris buffer (pH 6.8) and dialyzed against the same buffer at 4 °C overnight. After the insoluble material was removed by centrifugation, the supernatant was applied to a DEAE Sepharose (Pharmacia Biotech, Sweden) column (1.5- × 8.5-cm). The column was developed at 1.5 mL/min with a gradient consisting of solvents A (10 mM Bis-Tris, pH 6.8) and B (10 mM Bis-Tris, 0.2 M NaCl, pH 6.8), as follows: 0−20 min, 100% A; 20−25 min, linear gradient to 85% A; 25−75 min, 85% A; 75−85 min, linear gradient to 50% A; 85−155 min, 50% A. The column was further washed with 100% B, followed by 0.5 M NaCl, until ultraviolet absorbance (280 nm) returned to baseline. The fraction containing lipoxygenase activity was eluted with solvent containing 0.1M NaCl and concentrated to 2.5 mL by ultrafiltration and desalted on a PD-10 column. After filtration through a 0.2-μm syringe filter, the sample was applied at 1 mL/min to a 5- × 50-mm Mono S cation exchange column (HR 5/5, Pharmacia, Piscataway, NJ) equilibrated with 10 mM Bis-Tris (pH 6.0). The column was developed with a gradient consisting of solvents A (10 mM Bis-Tris, pH 6.0) and B (10 mM Bis-Tris, 0.5 M NaCl, pH 6.0), as follows: 0−10 min, 100% A; 10−15 min, linear gradient to 70% A; 15−42 min, 70% A; 42−45 min, linear gradient to 60% A; 45−60 min, 60% A; 60−90 min, 100% B on a Waters 650E chromatography system. The fraction containing lipoxygenase activity eluted at approximately 200 mM NaCl and was concentrated by ultrafiltration. The above purification steps led to a protein with about 85% purity. In order to get highly purified protein (>95% purity) for select HPLC-MS analyses, the protein sample was at times repurified on a Mono S column using the same solvent gradient as above. All purification steps were carried out at 4 °C or on ice. Each of the purified proteins was characterized by lipoxygenase activity and SDS gel (8−16%) electrophoresis. The method of Bradford was used for determining protein concentration.

Lipoxygenase Assay and Kinetics. The lipoxygenase activity of the purified proteins was assayed spectrophotometrically measuring the increase in absorbance at 234 nm. The standard assay consisted of 1 mL of PBS buffer (0.017 M KH_2PO_4, 0.05 M Na_2HPO_4, 0.15 M NaCl, pH 7.4) with 0.2% sodium cholate, 2 μM 13-HPODE, and 100 μM linoleate (Biomol) as substrate. The assay substrate solution was prepared as described (Kühn et al., 1993b).

To estimate the enzyme kinetic parameters of mutant protein M590L and compare with that of the wild-type human lipoxygenase (both purified from the *E. coli* expression system), assay solutions in the same buffer with various substrate concentrations ranging from 1 to 100 μM were used. The reaction (1 mL) was started by addition of 1 μg of purified enzyme and performed in a quartz cuvette at 10 °C. The linear part of the reaction curves were used to calculate reaction rates using a molar extinction coefficient for conjugated dienes at 234 nm of 23 000 M cm^{-1} (Gibian & Vandenberg, 1987). Values for apparent K_M and V_{max} were estimated from Lineweaver−Burk and Hanes−Wolff analyses, using a best-fit linear regression by the Cricket Graph III program on a Macintosh computer. A Beckman DU650 spectrophotometer was used in all of the described assays.

In order to investigate the self-inactivation properties of both the mutant protein M590L and wild-type human 15-lipoxygenase, assays were carried out at both 22 and 37 °C monitoring the absorbance increase at 234 nm. The standard assay consisted of 1 mL of 10 mM potassium phosphate buffer (pH 7.0) with 0.2% sodium cholate and 100 μM linoleate or arachidonate (Biomol) as substrate.

Methionine Sulfoxide Determination. Protein samples treated with and without 13-HPODE were hydrolyzed under a N_2 atmosphere in sealed glass vials with 6 N HCl at 110 °C for 16 h. Amino acid analysis was performed on a Hewlett-Packard Amino Quant amino acid analyzer.

Determination of Iron Content. After inactivation with either 13-HPODE or 15-HPETE, the inactivated protein samples and the control samples were desalted with Bio-Spin 6 mini desalting columns (Bio-Rad) to remove any free iron existing in the samples. The iron content of the enzyme samples was determined by atomic absorption analysis with a Perkin-Elmer Model 2380 graphite furnace AA spectrometer.

RESULTS

Methionine Sulfoxide Determination. Previous studies suggested that 1 mol of methionine sulfoxide can be detected by amino acid analysis after inactivation of rabbit reticulocyte 15-lipoxygenase with 13-HPODE (Rapoport et al., 1984).

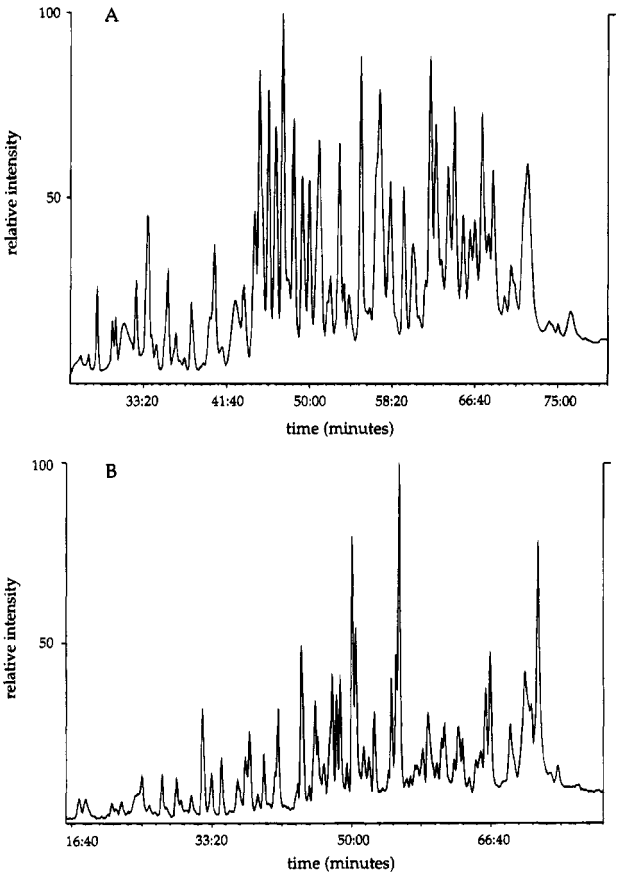

FIGURE 1: (A) Reverse−phase HPLC chromatogram of tryptic peptides derived from reduced and alkylated human 15-lipoxygenase. The column was developed as described under Materials and Methods at 2.5 μL/min with a gradient consisting of solvents A (distilled, deionized H_2O containing 0.1% trifluoroacetic acid) and B (80:20 acetonitrile−water containing 0.1% trifluoroacetic acid), as follows: 0−10 min, 0% B; 10−120 min, linear gradient to 100% B. The peptide segments contained in each fraction are listed in Table 1. (B) Reverse-phase HPLC chromatogram of tryptic peptides derived from reduced and alkylated rabbit 15-lipoxygenase. The column was developed under the above conditions, except 0−10 min, 2% B. The peptide segments contained in each fractions are listed in Table 2.

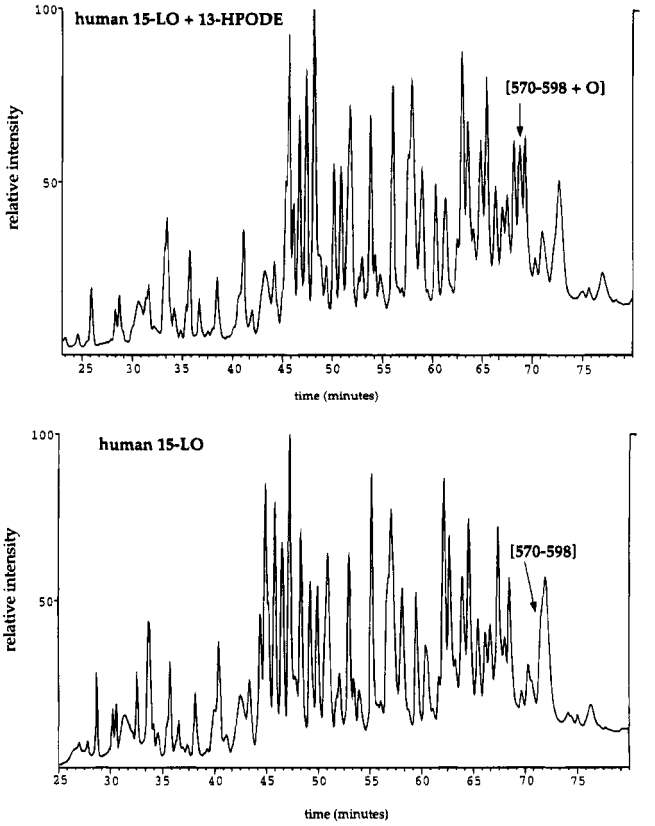

FIGURE 2: Comparison of the reverse phase HPLC chromatograms of tryptic peptides derived from human 15-lipoxygenase (15-LO) treated with or without 13-HPODE. Both of the samples were reduced and alkylated before digestion with trypsin. Fractions were separated from each other by RP-HPLC with conditions described in the legend to Figure 1. The fractions containing either peptide [570−598] or oxygenated peptide [570−598+O] are indicated by the arrows.

We confirmed these results with native rabbit 15-lipoxygenase and also with recombinant human 15-lipoxygenase purified from the bacterial expression system. The treatment of rabbit 15-lipoxygenase with 13-HPODE led to 1.17 mol more methionine sulfoxide formation per mole enzyme than the untreated sample. The value for the corresponding human enzyme experiment was 0.61 mol. The fact that an increase of approximately 1 mol of methionine sulfoxide could be detected in the experiments indicates the specificity of the reaction and motivated our further studies.

Peptide Mapping: Generation of Tryptic Digest Maps for Human and Rabbit 15-Lipoxygenases. To identify which amino acids were modified after the enzymes were inactivated with enzyme products (13-HPODE or 15-HPETE), we developed tryptic digest maps of both human and rabbit 15-lipoxygenases using HPLC and mass spectrometry. The chromatograms of tryptic digests for both human and rabbit enzymes are shown in Figure 1. The molecular weights of each component in a fraction were resolved by mass spectrometry. By comparison of the experimental mass data with the predicted molecular weights from the deduced primary structure (Sigal et al., 1988; Fleming et al., 1989), peptide fragments were assigned to each fraction. Each peak in the chromatograms contains one or more peptides which were uniquely identified by their molecular weights. The peptide fragments contained in each peak are shown in Tables 1 and Table 2 by the order of their retention times. These fragments account for approximately 95% of the predicted amino acid sequence of the proteins. All of the methionine-containing fragments are identified.

The Identification of an Oxygenated Methionine in Inactivated Enzymes. After treatment with 3-fold molar excess of 13-HPODE at 37 °C under N_2, the reaction mixture was cloudy and there was less than 5% lipoxygenase activity compared with the untreated sample. The 13-HPODE treated sample was then sequentially denatured, reduced, carboxymethylated and digested with trypsin. An HPLC-MS study was carried out on the digested protein to identify the oxygenated methionine residue. Figure 2 shows the reverse-phase HPLC chromatograms obtained from the tryptic digests of both the 13-HPODE treated and the untreated human 15-lipoxygenase. The shoulder containing peptide [570−598] disappeared in the treated sample, and a new peak was formed. This new peak contains a peptide segment which has a molecular mass 16 Da greater than that of peptide [570−598] as determined by mass spectrometry (Figure 3). This suggests that peptide [570−598] might have been oxygenated during 13-HPODE treatment. A search of the reconstructed ion chromatogram for all of the identified peptides and their theoretical corresponding oxygenated

Table 1: Results from the RP-HPLC-MS Analysis of Peptides Obtained from Trypsin Hydrolysis of Reduced and Alkylated Human 15-Lipoxygenase

retention time (min)	numerical sequence	amino acid sequence	retention time (min)	numerical sequence	amino acid sequence
27:03	214−217	LAER	51:43[a]	146−156	DGLILNMAGAK
28:38	305−310	LQPDGK	52:02	388−394	LIIPHLR
30:09	44−50	GKETELK	52:57	99−114	WVEGNGVLSLPEGTGR
30:09	485−490	TDVAVK	53:54	378−387	BLPSIHPIFK
30:09	621−625	AVLKK	55:08	189−198	DSLNVLTBWK
30:31	138−140	LYR	55:08	349−363	SSDFQLHELQSHLLR
31:22	563−569	LPPPTTK	56:42	431−450	QAGAFLTYSSFBPPDDLADR
33:36	244−250	SAHLPAR	56:42	626−640	FREELAALDKEIEIR
34:07	127−136	HREEELEERR	57:01	467−473	LWEIIYR
34:34	636−640	EIEIR	57:22	8−38	VSTGASLYAGSNNQVQLWLVGQHGEAALGKR
35:22	69−72	HLLK	58:08	8−37	VSTGASLYAGSNNQVQLWLVGQHGEAALGK
35:41	166−170	FLEDK	58:08	625−640	KFREELAALDKEIEIR
38:04	345−348	BWVR	59:27[a]	286−304	ANVILBSQQHLAAPLVMLK
39:54	94−98	FPBYR	62:08[a]	644−662	LDMPYEYLRPSVVENSVAI
40:21	199−205	DLDDFNR	62:39	69−93	HLLKDDAWFBNWISVQGPGAGDEVR
42:26	39−43	LWPAR	62:39	220−243	DSWKEDALFGYQFLNGANPVVLRR
43:18	38−43	RLWPAR	63:55	218−242	VRDSWKEDALFGYQFLNGANPVVLR
44:25	451−456	GLLGVK	63:55[a]	251−266	LVFPPGMEELQAQLEK
44:25	501−513	EITEIGLQGAQDR	64:31[a]	136−165	RKLYRWGNWKDGLILNMAGAKLYDLPVDER
44:53	141−145	WGNWK	64:31	220−242	DSWKEDALFGYQFLNGANPVVLR
45:10	457−466	SSFYAQDALR	64:31[a]	364−377	GHLMAEVIVVATMR
45:47	206−213	IFWBGQSK	65:26[a]	311−322	LLPMVIQLQLPR
46:30	115−126	TVGEDPQGLFQK	66:36	501−522	EITEIGLQGAQDRGFPVSLQAR
47:13	157−165	LYDLPVDER	67:22	523−562	DQVBHFVTMBIFTBTGQHASVHLGQLDWYSWV-PNAPBTMR
47:13	485−500	TDVAVKDDPELQTWBR			
47:50	514−522	GFPVSLQAR	67:22	51−64	VEVPEYLGPLLFVK
48:20	181−188	GLADLAIK	68:02	46−64	ETELKVEVPEYLGPLLFVK
49:11	171−180	RVDFEVSLAK	68:26[a]	405−430	TGLVSDMGIFDQIMSTGGGGHVQLLK
49:11	395−402	YTLEINVR	70:16	181−198	GLADLAIKDSLNVLTBWK
49:54	474−484	YVEGIVSLHYK	70:16	267−285	ELEGGTLFEADFSLLDGIK
50:52[a]	599−620	RQPVMVAVGQHEEEYFSGPEPK	71:26	570−598	DATLETVMATLPNFHQASLQMSITWQLGR
			71:56[a]	323−344	TGSPPPPLFLPTDPPMAWLLAK

[a] Denotes fragments that contain methionine. Letter B denotes acetamido-Cys.

Table 2: Results from the RP-HPLC-MS Analysis of Peptides Obtained from Trypsin Hydrolysis of Reduced and Alkylated Rabbit 15-Lipoxygenase

retention time (min)	numerical sequence	amino acid sequence	retention time (min)	numerical sequence	amino acid sequence
17:35	306−311	LQPDGK	52:40	190−199	DSLNVLAPWK
22:31	622−626	AVLEK	52:40[a]	475−485	YVQGIMGLYYK
24:27	564−570	LPPPTTK	52:40[a]	627−641	FREELAIMDKEIEVR
24:54	44−50	NKEEEFK	55:15	20−43	NKVELWLVGQHGEVELGSBLRPTR
27:20	128−136	HREQELEER	55:37	452−467	GLLGVESSFYAQDALR
29:01	167−171	FLEDK	55:37[a]	468−491	LWEIISRYVQGIMGLYYKTDEAVR
32:10	346−349	BWVR	58:27	56−64	YLGSLLFVR
33:14	245−251	SVQLPAR	59:04	100−127	WVVGDGVQSLPVGIGBTTVGDPQGLFQK
34:24	200−206	TLDDFNR	59:04	312−323	LMPMVIQLHLPK
36:20	44−55	NKEEEFKVNVSK	59:04	642−663	NEKLDIPYEYLRPSIVENSVAI
37:17	6−19	VBVSTGASIYAGSK	59:28[a]	365−378	GHLMAEVFTVATMR
37:48	158−166	LTDLPVDER	60:41	645−663	LDIPYEYLRPSIVENSVAI
39:28	502−513	EITEIGLQGAQK	61:06[a]	252−267	LVFPPGMEELQAQLEK
41:09	441−451	SFBPPDDLADR	62:46[a]	287−305	ANVILYBQQYLAAPLVMLK
43:56	207−212	IFWBGR	63:13	137−157	RKLYQWGSWKEGLILNVAGSK
45:34	147−157	EGLILNVAGSK	65:58	271−286	AGTLFEADFALLDNIK
45:55	389−395	LIVPHLR	66:32[a]	221−243	DSWQEDSLFGYQFLNGANPMLLR
45:55	396−403	YTLEINVR	68:58[a]	571−599	DATLETVMATLPNLHQSSLQMSIVWQLGR
45:55	492−501	DDLELQSWBR	70:41	69−99	HFLKEDAWFBNWISVQALGAAEDKYWFPBYR
46:38[a]	627−636	FREELAIMDK	70:41	200−217	TLDDFNRIFWBGRSKLAR
47:36	350−364	SSDFQVHELNSHLLR	70:41[a]	324−345	IGSSPPPLFLPTDPPMVWLAAK
48:09	379−388	BLPSIHPVFK	72:16	172−189	KIDFEASLAWGLAELALK
48:34	486−501	TDEAVRDDLELQSWBR	72:16[a]	406−440	NGLVSDFGIFDQIMSTGGGGHVQLLQQAGA-FLTYR
49:59	139−146	LYQWGSWK			
49:59	468−474	LWEIISR	72:16[a]	514−563	QGFPTSLQSVAQABHFVTMBIFTBTGQHSSIH-LGQLDWFTWVPNAPBTMR
50:26[a]	600−621	DQPIMVPLGQHQEEYFSGPEPR	74:36	173−189	IDFEASLAWGLAELALK

[a] Denotes fragments that contain methionine. Letter B denotes acetamido-Cys.

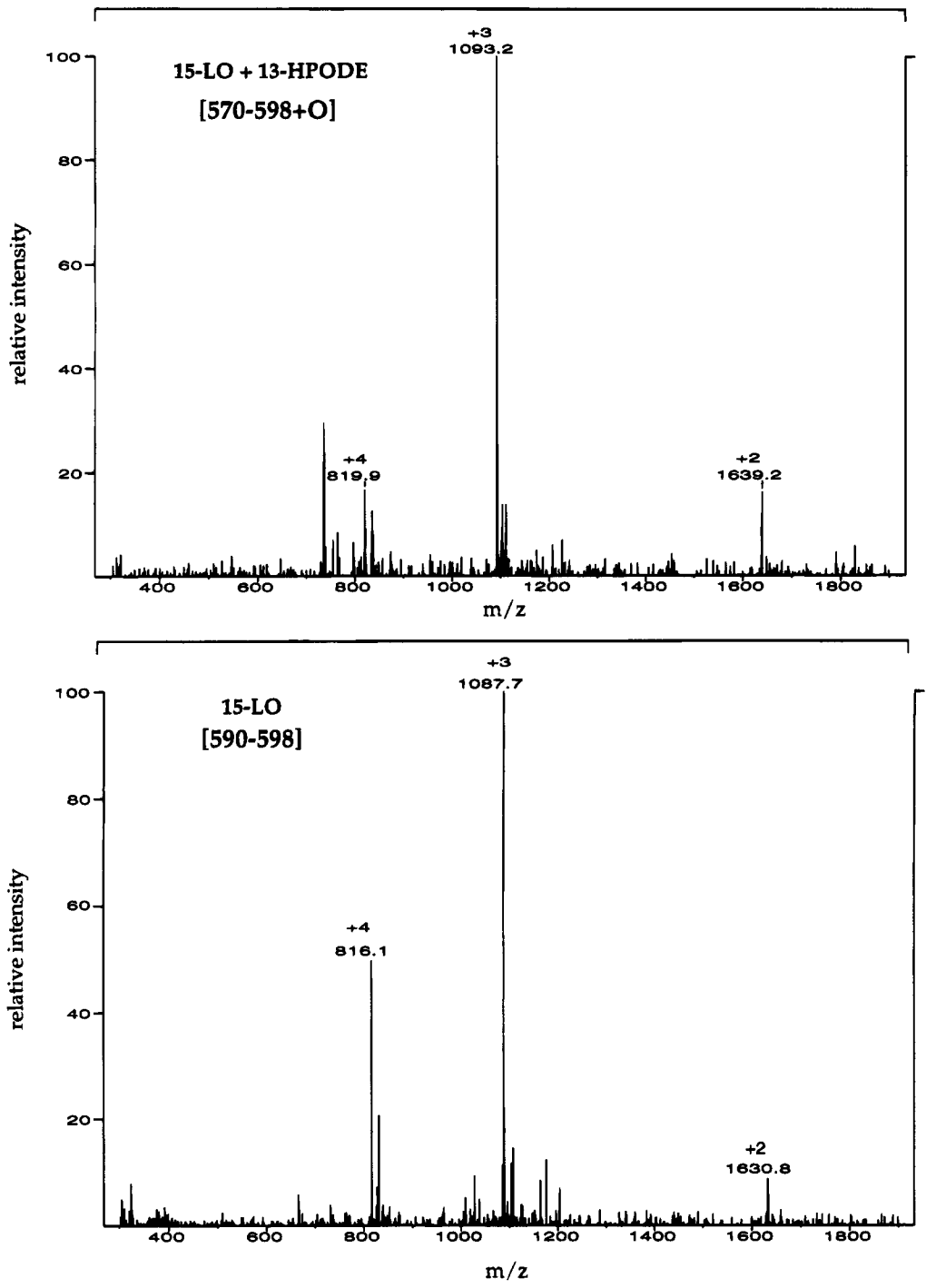

FIGURE 3: Mass spectra of peptides [570−598 + O] and [570−598]. (Upper panel) Mass spectrum of peptide [570−598+O]. The +2, +3, and +4 ions at *m/z* 1639.2, 1093.2, and 819.9, respectively, agree with the expected molecular mass (3276.8 Da). (Lower panel) Mass spectrum of peptide [570−598]. The +2, +3, and +4 ions at *m/z* 1630.8, 1087.7, and 816.1, respectively, agree with the expected molecular mass (3260.8 Da).

products was performed. The only peptide found to have a corresponding oxygenated product is peptide [570−598], which is composed of the following: Asp-Ala-Thr-Leu-Glu-Thr-Val-Met-Ala-Thr-Leu-Pro-Asn-Phe-His-Gln-Ala-Ser-Leu-Gln-Met-Ser-Ile-Thr-Trp-Gln-Leu-Gly-Arg. Analogous experiments were performed with rabbit 15-lipoxygenase, and HPLC-MS analysis revealed that the corresponding peptide [571−599] of rabbit lipoxygenase is also the only peptide that appears to be oxygenated during 13-HPODE treatment.

The peak at retention time 48.20 min (Figure 2) in the 13-HPODE treated human 15-lipoxygenase appears to have decreased when compared to the same peak in the untreated human 15-lipoxygenase. The investigation of this peak in the untreated human 15-lipoxygenase by mass spectrometry suggested that it contains the peptide [181−188] and an impurity. This impurity was not identified and could not be reproduced. In subsequent studies, this peak was smaller in intensity and the impurity was not found.

Extracted ion plots of the +3 ions, *m/z* 1087.7 and 1093.2 Da, which correspond to peptides [570−598] and [570−598+O] respectively, were performed on human 15-lipoxygenase treated with or without 13-HPODE (Figure 4). The absence of *m/z* 1093.2 in the untreated sample suggests that

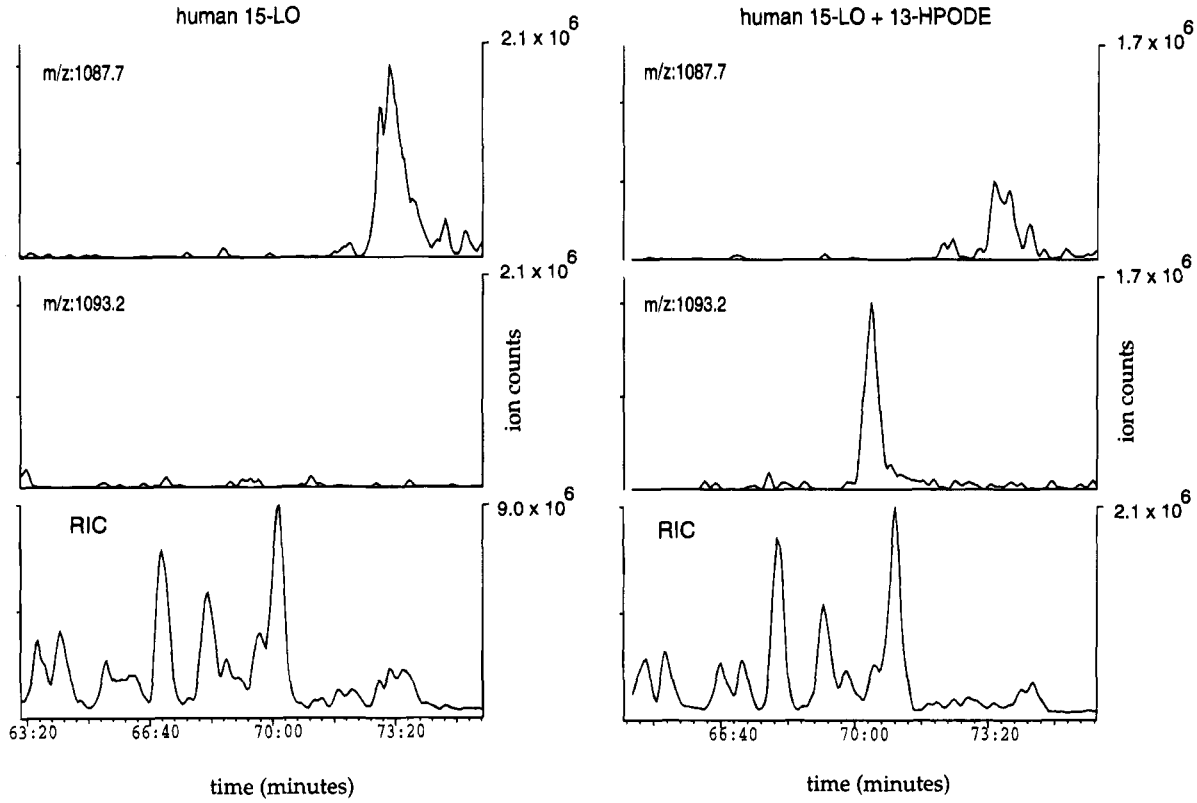

FIGURE 4: (Left) Normalized extracted ion chromatograms of the +3 ions of peptides [570−598] (top) and [570−598+O] (middle) in control human 15-lipoxygenase (15-LO). (Right) Normalized extracted ion chromatograms of the +3 ions of peptides [570−598] (top) and [570−598+O] (middle) in 13-HPODE treated human 15-lipoxygenase. The bottom two figures are the expanded potion of the reconstructed ion chromatogram (RIC) obtained from the reversed-phase HPLC chromatograms containing peptides [570−598] and [570−598+O] respectively.

the oxygenated peptide [570−598+O] was not related to sample handling but resulted from the treatment of human 15-lipoxygenase with 13-HPODE. In the 13-HPODE treated sample, there is only a very small peak corresponding to *m/z* 1087.7. Therefore this oxygenation reaction is a specific and high yield reaction (>70%).

Peptide [570−598] contains two methionines, Met^{577} and Met^{590}. To determine which one of these two methionines was oxygenated during 13-HPODE treatment, tandem mass spectrometry with electrospray ionization was carried out on the +3 ions of both the 13-HPODE treated and untreated samples. The resulting product ion spectra contained b_n- and y''_n-type ions (Biemann, 1990) (Figure 5). Met^{577} is surrounded by a set of doubly charged *y*-type ions, including y_{18}^{2+} through y_{25}^{2+}. Comparing the data obtained for both peptide [570−598] and [570−598+O], if Met^{577} had undergone oxygenation to methionine sulfoxide, an increase corresponding to 16 Da (*m/z* = 8 for +2 charge) for y_{22}^{2+} through y_{25}^{2+} in the 13-HPODE treated sample would be expected, while y_{21}^{2+} through y_{18}^{2+} would remain the same as that of the untreated sample. However, inspection of Figure 5 reveals that all of the doubly charged ions (y_{18}^{2+} through y_{25}^{2+}) are *m/z* = 8 bigger than that of the control sample. Thus Met^{577} was unchanged. This result agrees with the analyses of the *b* ions and the rest of the *y* ions for both samples.

The tandem mass spectrometry results suggest that the site of oxygenation must lie within residues 581 and 590, which contains the sequence PNFHQASLQM. The lack of a full set of *y*- or *b*-type sequence ions in this region of the spectrum made it difficult to unambiguously assign the site of oxygenation. However, the thioether substituent of Met^{590} is the most reactive moiety within this 10 amino acid subsequence. Therefore, Met^{590} is the most likely candidate for oxygenation by the reaction with 13-HPODE.

Using the same tandem mass spectrometric approach, data were generated for rabbit 15-lipoxygenase. A single site of oxygenation was narrowed to within residues 582−593 (PNLHQSSLQMSI). Applying the same arguments as for human 15-lipoxygenase, we suggest that Met^{591} of rabbit 15-lipoxygenase (the homologue of human Met^{590}) is the amino acid residue that is oxygenated by 13-HPODE (data not shown).

Generation and Characterization of Mutant Human 15-Lipoxygenase. In order to study the correlation of the product-based self-inactivation of mammalian lipoxygenase with the formation of methionine sulfoxide, a Met^{590}-substituted human 15-lipoxygenase was generated. Because Met^{590} in human 15-lipoxygenase aligns with Leu^{748} in soybean lipoxygenase (Sigal et al., 1988) and because leucine is a hydrophobic amino acid residue with a side-chain volume similar to that of methionine, we generated a mutant human 15-lipoxygenase with a leucine residue substituted for Met^{590}. Both the mutant protein, M590L, and the wild-type human 15-lipoxygenase were expressed in *E. coli*. The expression of each was confirmed by the formation of specific lipoxygenase products as identified by HPLC during the incubation of transformed bacteria with arachidonic acid as previously described (Kühn et al., 1993a).

The results of a typical purification of both the wild-type and mutant 15-lipoxygenases are shown in Table 3. Approximately 380-fold purification was achieved with 40%

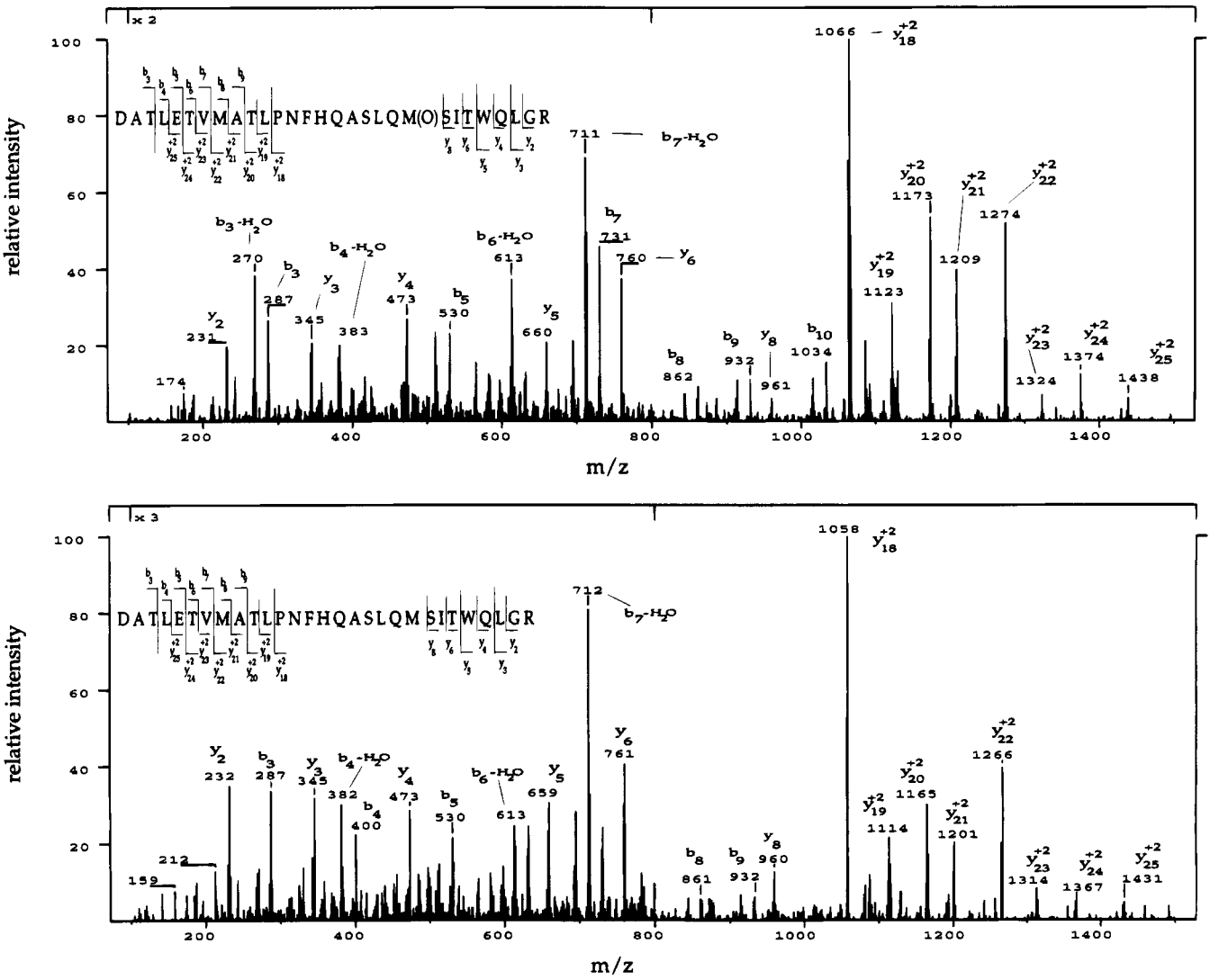

FIGURE 5: Tandem mass spectra of peptide [570−598] and [570−598+O]. (Upper panel) Tandem mass spectrum of the $(M+3H)^{3+}$ ion (*m/z* 1093.2) of [570−598+O]. (Lower panel) Tandem mass spectrum of the $(M+3H)^{3+}$ ion (*m/z* 1087.7) of [570−598]. The ion nomenclature is that proposed by Biemann (1990), where the *y*-type ions are numbered from the C-terminus and the *b*-type ions are numbered from the N-terminus.

Table 3: Purification of Wild-Type Human 15-Lipoxygenase and Mutant Protein M590L from *E. coli* Cell Culture[a]

fraction	total protein (mg)	total activity[b] (units)	specific activity[b] (units/μg)	purification (fold)	yield (%)
lysate	1640 (1450)	4510 (3915)	0.0025 (0.0027)		100 (100)
$(NH_4)_2SO_4$	787 (608)	4014 (3709)	0.0051 (0.0061)	2.1 (2.3)	89.0 (94.7)
DEAE	310 (276)	3100 (2705)	0.010 (0.0098)	4.0 (3.63)	68.7 (69.1)
Mono S	1.64 (1.93)	1804 (1718)	1.10 (0.89)	440 (330)	40 (43.9)

[a] Data for the purification of wild-type human 15-lipoxygenase are shown inside the parentheses. [b] Units are expressed as nmole of conjugated diene formed per minute at 22 °C, based on ΔOD_{234}/min at the linear part of the reaction curve. An extinction coefficient of 23 000 M cm^{-1} was used (Gibian & Vandenberg, 1987).

yield. Various purification steps for both the mutant and wild-type enzymes were evaluated by total enzyme activity and SDS−PAGE gel electrophoresis as shown in Table 3 and Figure 6, respectively. Each purification achieved purity exceeding 85%.

The specific activity of the purified mutant protein M590L was comparable to that of the wild-type protein. In addition, both enzymes have relatively comparable apparent K_M and V_{max} as summarized in Table 4. The mutant protein M590L is still inactivated during its reaction with either linoleic acid or arachidonic acid at 22 °C (Figure 7). Higher temperature (37 °C) increased the inactivation rate as expected. Further addition of substrate did not lead to recovery of activity, confirming that the activity decrease was not due to substrate depletion (Figure 7). Control incubations of the enzyme without substrate at corresponding temperatures for same time span resulted in only slight decreases in activity (not shown). Hence, the fatty acid oxidation reaction appears to be the major cause of enzyme inactivation. These data demonstrate that the substitution of Met^{590} with leucine did not prevent the self-inactivation of human 15-lipoxygenase.

Amino Acid Analysis and HPLC-MS Analysis of Mutant M590L Treated with and without 13-HPODE. An HPLC-MS analysis of the mutant protein M590L was carried out. The mass of the mutant peptide [570−598] containing the M590L substitution, 3242.8 Da, was detected, while the mass

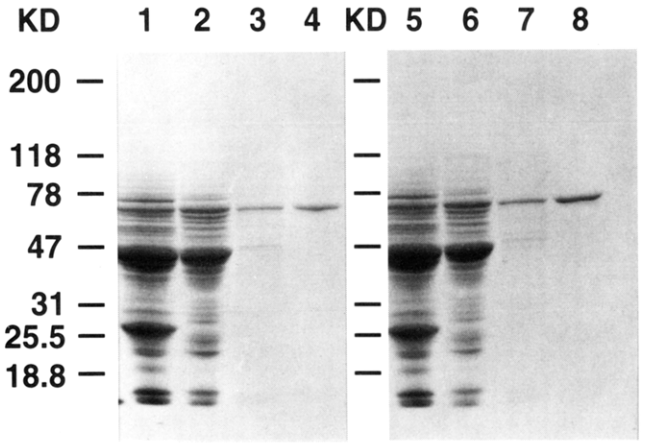

FIGURE 6: Purification of wild-type and mutant human 15-lipoxygenases. SDS−PAGE (8%-16%) stained with Coomassie Blue. (Lane 1) Ammonium sulfate fraction of wild-type human 15-lipoxygenase; (lane 2) the active fraction from the DEAE column for wild-type enzyme; (lane 3) the active fraction after Mono S column for wild type enzyme; (lane 4) human 15-lipoxygenase purified from baculovirus/insect cell expression system as a standard (Kühn 1993); (lane 5) ammonium sulfate fraction of mutant enzyme M590L; (lane 6) the active fraction from the DEAE column for mutant enzyme M590L; (lane 7) the active fraction after Mono S column for mutant enzyme M590L; (lane 8) human 15-lipoxygenase purified from baculovirus/insect expression system as a standard.

Table 4: Comparison of the Steady-State Kinetic Parameters of the Wild-Type Human 15-Lipoxygenase and Mutant Protein M590L[a]

	arachidonic acid		linoleic acid	
lipoxygenase	K_M (μM)	V_{max} (unit/μg)[b]	K_M (μM)	V_{max} (unit/μg)[b]
wild-type HLO	16.3 ± 7.2	1.22 ± 0.14	14.8 ± 9.3	1.72 ± 1.12
15 LOX M590L	22.7 ± 5.8	1.04 ± 0.21	10.8 ± 4.9	2.63 ± 1.24

[a] Values ± SEM. $n = 3$. [b] Unit = nmole of conjugated diene produced per minute.

of the corresponding wild-type peptide (3260.8 Da) was not detected. Mass spectrometric analysis of all of the tryptic digested peptide segments revealed that Met[590] was the only mutated site in the entire protein. After treatment with a 3-fold excess of 13-HPODE at 37 °C for 30 min, the mutant protein was 95% inactivated compared to the control sample. Amino acid analysis did not detect any increase of methionine sulfoxide when compared with the untreated sample. In addition, no new species was detected by the HPLC-MS analysis of the tryptic digest peptides, suggesting that no stable covalent modification on the protein resulted from this treatment. These data confirm that Met[590] is the site of oxygenation and also demonstrate that oxygenation of Met[590] is *not* necessary for the self-inactivation of mammalian 15-lipoxygenase.

Reaction of Human 15-Lipoxygenase with 15-HPETE and ETYA. As shown in Figure 7, the reaction of arachidonic acid also leads to enzyme inactivation with an even greater inactivation rate than with linoleic acid. The formation of methionine sulfoxide during the inactivation with arachidonic acid has never been reported. Human 15-lipoxygenase was incubated with a 3-fold molar excess of 15-HPETE at 37 °C under N_2 for 30 min. After this treatment, the protein was inactivated (~8% remaining lipoxygenase activity compared to the untreated sample). HPLC-MS analysis did not identify any oxygenated or modified peptide segment after reaction with 15-HPETE.

It has been reported that the treatment of soybean lipoxygenase with the inhibitor ETYA caused the oxygenation of a single methionine residue (Kühn et al., 1984). However, HPLC-MS analysis of the tryptic digest of human 15-lipoxygenase treated with ETYA showed no evidence for an oxygenated peptide.

Determination of Iron Content of the 13-HPODE and 15-HPETE Inactivated Protein. Iron release could be one

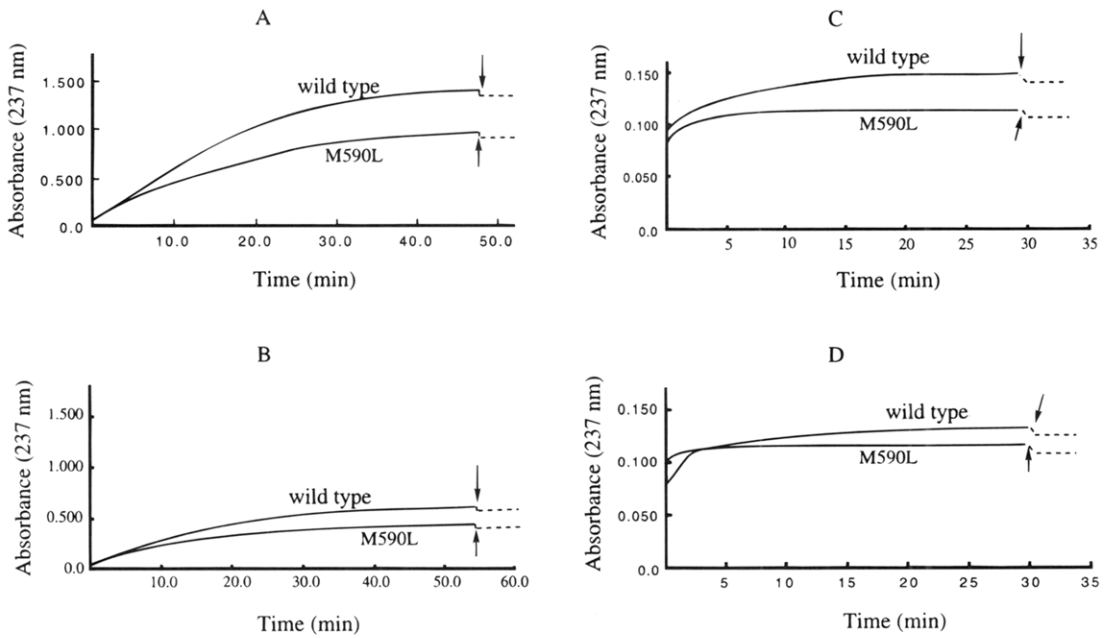

FIGURE 7: Suicidal inactivation of the wild-type human 15-lipoxygenase and mutant protein M590L at 22 and 37 °C. Enzymes purified from *E. coli* expression system (0.5 μg) were used for each reaction, and the increase in absorbance at 234 nm was recorded. As shown with dashed lines, the inactivation of the enzymes is not caused by product depletion or reversible product inhibition since further addition of substrate (indicated by arrows) failed to restore the activity. (Panel A) Reactions of enzymes with linoleate at 22 °C. (Panel B) Reactions of enzymes with linoleate at 37 °C. (Panel C) Reactions of enzymes with arachidonate at 22 °C. (Panel D) Reactions of enzymes with arachidonate at 37 °C. The assay solutions contain 100 μM substrate and 0.2% of sodium cholate in 10 mM potassium phosphate buffer (pH 7.0).

Table 5: Iron Content of Protein Samples after Various Incubations

sample	amount of hydroperoxy	incubation time (37 °C)	specific activity[a,b]	inactivation (%)[c]	Fe/enzyme (mol/mol)[d]	iron release (%)[c]
HLO	0	0	1.06 ± 0.08	0	0.632 ± 0.011	0
HLO	0	0.5 h	1.02 ± 0.03	3.8	0.589 ± 0.014	6.3
HLO + 13-HPODE	3 molar ratio	0.5 h	0.063 ± 0.004	94.1	0.326 ± 0.005	47.6
HLO + 15-HPETE	3 molar ratio	0.5 h	0.21 ± 0.01	80.2	0.244 ± 0.012	61.9

[a] Unit = nmole of conjugated diene produced per minute per μg of enzyme. [b] Value ± SEM. $n = 5$. [c] Mean values in the corresponding left column are used in the calculation. [d] Value ± SEM. $n = 3$.

possible explanation for the inactivation of lipoxygenases by hydroperoxy products since the iron center is essential for enzyme activity (Pistorius & Axelrod, 1974). After inactivation with either 13-HPODE or 15-HPETE, the iron content of the inactivated protein samples was determined by atomic absorbance (Table 5). Incubation of human 15-lipoxygenase with 13-HPODE or 15-HPETE led to 94% and 80.2% enzyme inactivation, respectively. The decrease of iron content resulting from these incubations was 47.6% and 61.9%, respectively. We suggest that iron release may contribute to, but does not completely account for, the inactivation.

DISCUSSION

Earlier studies utilizing amino acid analysis, cyanogen bromide cleavage, and two-dimensional chromatography advanced the hypothesis that near stoichiometric amounts of 13-HPODE were sufficient to inactivate rabbit 15-lipoxygenase and that the formation of a single methionine sulfoxide was responsible for this inactivation (Rapoport et al., 1984). These prior studies, however, were not able to elucidate which methionine was oxygenated or whether such oxygenation was causally responsible for product-based inactivation. Using HPLC and mass spectrometry, we have developed tryptic maps of both the rabbit and human 15-lipoxygenases which enable the detection of modifications of the enzymes. We have confirmed earlier results that methionine sulfoxide is formed during incubation with 13-HPODE and localized this modification for the first time to Met^{590} in the human enzyme and Met^{591} in the rabbit enzyme. No other covalent modifications were detected.

The generation, purification, and characterization of a mutant human 15-lipoxygenase with a leucine substituted for methionine at position 590 enabled us to probe the requirement of the methionine residue in the inactivation of the enzyme. Compared with wild-type enzyme, the mutant enzyme had comparable specific activity and apparent K_M and V_{max}. Importantly, it also had a similar inactivation profile during the enzyme reaction and was inactivated when incubated with 13-HPODE. It did not, however, form any additional methionine sulfoxide compared with untreated sample as detected by amino acid analysis, nor was there any covalent modification of the enzyme as detected by HPLC-MS analysis. These results clearly dissociate methionine sulfoxide formation from the product-based enzyme inactivation observed for mammalian enzymes. The inactivation of wild-type enzyme in the presence of 15-HPETE and the absence of any covalent modification further support the conclusion that methionine sulfoxide formation is not integral to the inactivation mechanism.

Other explanations for the inactivation need to be considered. Our results suggest that release of the catalytically active iron may contribute to, but cannot completely account for, the loss of activity. This is consistent with a previous report that when rabbit reticulocyte 15-lipoxygenase was treated with stoichiometric amounts of 13-HPODE at 10 °C, the ferric form of the enzyme was formed accompanied with a spectral change reflecting the transition of the iron atom of the enzyme from the ferrous to the ferric state. Subsequent heating of the same sample to 37 °C resulted in no further spectral change, but the enzyme lost its activity (Härtel et al., 1982). This result also implies that the change of iron coordination environment or iron release is not the cause of the enzyme inactivation.

The secondary reaction products and oxygen-containing radicals formed during the interaction of the hydroperoxide with the enzyme are other possible causes of the enzyme inactivation. They may lead to noncovalent modification of the enzyme or generate a protein radical which is stable only in the native protein environment. However, the denaturing step previous to tryptic digestion in our study may destroy these unstable modifications and may therefore not be detectable by HPLC-MS analysis. Furthermore, nonspecific protein modification due to free radical formation may escape detection by our technique.

The three-dimensional structure of soybean 15-lipoxygenase has recently been determined (Boyington et al., 1993; Minor et al., 1993). Because there is no substrate or inhibitor bound in these X-ray crystal structures, it is of interest to document specific residues that may be in contact with either substrate or product. Our results suggest a specific interaction between 13-HPODE and Met^{590} of the human 15-lipoxygenase. The homologue of Met^{590} in soybean 15-lipoxygenase appears to be Leu^{748} on the basis of sequence alignment. Interestingly, Leu^{748} is about 15 Å away from the iron center and is close to an open cavity in the enzyme (W. Minor and B. Axelrod, personal communication). This cavity is suggested to be the substrate binding pocket since it is also exposed to the catalytic iron atom (Boyington et al., 1993). The only other amino acid residues that have been implicated to be in contact with the substrate cavity are Ile^{417} and Met^{418} of human 15-lipoxygenase (Sloane et al., 1991). The homologues of these residues in soybean lipoxygenase, Thr^{556} and Phe^{557}, also surround the same cavity (Boyington et al., 1993). Finally, the fact that modification of Met^{590} does not occur during treatment with 15-HPETE indicates the increased rigidity one would expect from 15-HPETE as compared with 13-HPODE may contribute to the observed selectivity of methionine oxygenation. All of these findings need to be accounted for in any model of the enzyme's active site.

In conclusion, we have identified the methionine which is oxygenated during the inactivation of mammalian 15-lipoxygenases with 13-HPODE. The specificity of this

oxygenation suggests that Met^{590} is near the substrate binding pocket of human 15-lipoxygenase. Evaluation of a mutant enzyme that lacks this residue suggests that methionine sulfoxide formation is not causally related to enzyme inactivation. The application of tryptic mapping and HPLC-MS provides us with a means to further study the enzymatic mechanism and characteristics of the substrate binding pocket of mammalian lipoxygenases.

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