Limited Proteolysis and Active-Site Labeling Studies of Soybean Lipoxygenase 1†

Sampath Ramachandran,‡ Richard T. Carroll,‡ William R. Dunham,§ and Max O. Funk, Jr.*,‡

Departments of Chemistry and Medicinal and Biological Chemistry, University of Toledo, Toledo, Ohio 43606, and Biophysics Research Division, Institute for Science and Technology, University of Michigan, Ann Arbor, Michigan 48109

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ABSTRACT: Soybean lipoxygenase 1 was studied using limited proteolysis and active-site labeling to begin the structural characterization of the enzyme in solution. The serine proteases trypsin and chymotrypsin cleaved the large monomeric protein (95 kDa) into two large polypeptides, a C-terminal fragment of about 30 kDa and an N-terminal fragment of about 60 kDa. Under conditions that led to complete cleavage of the protein as judged by SDS–polyacrylamide gel electrophoresis, the catalytic activity of the protein was either reduced slightly (chymotrypsin) or enhanced (trypsin). The characteristics of the cleaved enzymes were the same as for native lipoxygenase 1 in all aspects examined: insensitivity to cyanide, fluoride, and EDTA, regiochemical and stereochemical consequences of catalysis, and EPR spectroscopy upon oxidation by product. The two fragments apparently were tightly associated as they could not be resolved under conditions which preserved the catalytic activity. Both native and protease-cleaved lipoxygenase 1 formed covalent adducts when treated with either ^{14}C-phenylhydrazine or 4-nitrophenylhydrazine. The label was found only in the 60-kDa fragment and following complete trypsin digestion was associated with a peptide beginning after Lys-482 in the primary sequence. Therefore labeling occurred in the vicinity of the conserved histidine cluster which has been postulated as the iron-binding site. From these observations it appears that lipoxygenase 1 exists as a pair of tightly associated domains with the iron-binding site located in the larger of the two.

Lipoxygenase is one of the central enzymes in the metabolism of polyunsaturated fatty acids in both plants and animals. For example, it catalyzes the inaugural step in the biosynthesis of leukotrienes from arachidonic acid in a wide variety of mammalian cells (Samuelsson et al., 1987). The hydroperoxide products of lipoxygenase catalysis as well as the leukotrienes are potent mediators of an array of biochemical events (Cathcart et al., 1991; Samuelsson & Funk, 1989). Because many of the biological activities of these compounds have pathophysiological consequences in humans (e.g., bronchoconstriction, smooth muscle contraction), the search for compounds which modulate lipoxygenase activity has been extensive. The design of such compounds has gone on in the absence of any knowledge of the three-dimensional structure of the enzyme. While the existence of mammalian lipoxygenases has been recognized since about 1974 (Hamberg & Samuelsson, 1974), the primary structures for these enzymes have only recently been elucidated (Matsumoto et al., 1988; Dixon et al., 1988), and only preliminary data from X-ray crystallography have been reported (Sloane et al., 1990). The catalytic activities of the mammalian lipoxygenases are influenced by a variety of factors (Hada et al., 1991). For example, the human 5-lipoxygenase is stimulated by a specific activating protein (Miller et al., 1990). The molecular events responsible for the effect of different conditions on catalysis have not been characterized. Like their plant counterparts, the mammalian lipoxygenases appear to be non-heme iron dioxygenases (Kroneck et al., 1991).

The physiological role of lipoxygenase catalysis in plants is less clear, although the activity of the enzyme has been implicated in the biosynthesis of compounds with growth regulatory and pest resistance properties (Siedow, 1991). The enzyme has been available in a highly purified form from soybeans since at least 1947 (Theorell et al., 1947). Most of the characterization of the catalytic mechanism of lipoxygenase has revolved around the interesting non-heme iron cofactor in the soybean enzyme. While the iron environment has been probed in a number of spectroscopic investigations, the protein ligands have not been identified (Feiters et al., 1990). The primary structures of several soybean lipoxygenases have become available from the sequence determination of the corresponding cDNAs (Shibata et al., 1987, 1988; Yenofsky et al., 1988). The soybean isoenzymes are identical in roughly 80% of the residues and share significant similarity with the mammalian enzymes. It has been suggested that a cluster of histidine residues conserved in all lipoxygenase amino acid sequences provides the ligands for the non-heme iron binding site (Shibata et al., 1988). Despite the early availability of highly purified lipoxygenase, only preliminary crystallization and X-ray diffraction data are presently available with respect to the determination of the three-dimensional structure of the protein (Stallings et al., 1990; Steczko et al., 1990; Boyington et al., 1990).

Lipoxygenase catalyzes the peroxidation of the 1,4-pentadiene unit in naturally occurring polyunsaturated fatty acids by molecular oxygen. In contrast to the uncatalyzed autoxidation of linoleic acid, lipoxygenase 1 catalysis is both regioselective for addition of oxygen at position 13 and stereospecific for formation of the *S* isomer (Van Os et al., 1979). The rate-limiting step is known from kinetic isotope studies to be the stereospecific removal of hydrogen from position 11 in linoleic acid (Egmond et al., 1973). The oxidation is probably a one-electron oxidation of the substrate producing a bound pentadienyl radical with concomitant

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‡ University of Toledo.

§ University of Michigan.

[1] Abbreviations: HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate, EPR, electron paramagnetic resonance; EDTA, ethylenediaminetetraacetic acid.

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reduction of the active iron(III) form of the enzyme to iron-(II) (Funk et al., 1990; Nelson et al., 1990). Combination with oxygen and reduction of the resulting peroxy radical with reoxidation of the non-heme iron would complete the catalytic cycle. The mechanism used by the enzyme to accomplish this chemistry is not known. For example, evidence that a basic group in the active site acts on the hydrogen at position 11 in linoleic acid has been presented (Corey et al., 1986). By weakening the C–H bond, the oxidation of the substrate would presumably be facilitated. The identity of the postulated basic group has not been revealed. Certain chemical modifications of lipoxygenase 1 have caused inactivation of the enzyme, and these reactions may have resulted from alteration of active-site residues. For example, the enzyme has been known for some time to be sensitive to oxidation by hydrogen peroxide (Mitsuda et al., 1967), but the chemical basis for this activation has not been positively identified. Inactivation of lipoxygenase 1 by 5,8,11,14-eicosatetraynoic acid was accompanied by the oxidation of a single methionine residue in the protein, but the position of this amino acid has not been determined (Kühn et al., 1984).

In the experiments described in this paper, we have begun to explore the organization of lipoxygenase 1 at the molecular level to gain a detailed understanding of the mechanism of action. Reported here are the results of limited proteolysis and chemical labeling experiments that provide the first insights into the structural features of the enzyme.

EXPERIMENTAL PROCEDURES

Materials. Lipoxygenase 1 was obtained from soybeans cv. Provar by extraction, differential ammonium sulfate precipitation, dialysis, and chromatofocusing (Funk et al., 1986). TPCK-treated trypsin, TLCK-treated chymotrypsin, soybean trypsin inhibitor, phenylmethanesulfonyl fluoride (PMSF), and linoleic acid were obtained from Sigma. ^{14}C-Phenylhydrazine was obtained from New England Nuclear and 4-nitrophenylhydrazine was obtained from Aldrich. The product of lipoxygenase catalysis was obtained as previously described (Funk et al., 1976).

Methods. Lipoxygenase activity was measured in a kinetic assay in which the change in absorbance at 234 nm of a linoleic acid solution (3.00 mL, 72 μM, pH 9.0, 0.1 M borate) was monitored after temperature equilibration (25 °C) and addition of an aliquot of lipoxygenase-containing solution (0.020 mL). The concentration of the enzyme was determined spectrophotometrically from the absorbance at 280 nm using a molar extinction coefficient of 120 000 L mol^{-1} cm^{-1} (Draheim et al., 1989).

Chymotrypsin digestion was carried out by treating lipoxygenase 1 (0.3 mL, 2.1 mg, 0.1 M Tris-HCl, pH 8.0) with α-chymotrypsin (0.030 mL, 0.21 mg, 0.1 M Tris-HCl, pH 8.0) at 25 °C for the specified period of time. The reactions were terminated either by combining the incubation solution with a solution of PMSF for gel electrophoresis or by dilution with buffer for kinetics. For kinetic assays, aliquots of the solution (0.010 mL) were combined with Tris-HCl (5.00 mL, 0.1 M, pH 7). For gel electrophoresis or HPLC, the solution (0.030 mL) was combined with PMSF in methanol (0.001 mL, 0.010 M). Trypsin digestions were carried out by treating lipoxygenase 1 (0.1 mL, 0.7 mg, 0.2 M Tris-HCl, pH 8.0) with trypsin (0.010 mL, 0.070 mg, 0.20 M Tris-HCl, pH 8.0) at 25 °C for the specified time period. The reactions were stopped by combining an aliquot of the digestion solution (0.010 mL) with a solution of soybean trypsin inhibitor (0.003 mL, 0.021 mg, 0.2 M Tris-HCl, pH 8.0).

SDS–polyacrylamide gel electrophoresis determinations were carried out with 12% polyacrylamide gels (Laemmli, 1970). The samples were prepared for electrophoresis by treatment in boiling water for 90 s in a 1:1 ratio with sample buffer prior to loading on the gels. Proteins were stained using Coomassie brilliant blue stain. The gradient gels were prepared as linear 8–20% gradients in acrylamide. Reverse-phase HPLC separation of fragments was carried out on a Vydac Protein C4 column (4.6 mm × 25 cm) using a flow rate of 1 mL min^{-1}. A linear gradient from 0 to 100% acetonitrile over 20 min, starting with 0.1% aqueous trifluoroacetic acid, was used to elute the proteins. Amino acid analysis of the small fragment from chymotrypsin digestion of lipoxygenase 1 was carried out after digestion with 6 N HCl at 110 °C for 24 h using HPLC with a postcolumn ninhydrin detection system (Pickering Laboratories). The reported values were averaged from determinations run in triplicate.

Resolution of the fragments without complete denaturation was attempted by treating the cleaved protein with 2, 4, and 6 M urea for 60 min at 4 °C followed by chromatography. Size-exclusion chromatography was carried out on Sephacryl S200 HR (Pharmacia) using 0.2 M Tris-HCl, pH 8.5, 0.3 M NaCl, and urea concentrations up to 6 M. Hydrophobic interaction chromatography was carried out on a Hydropore HIC column (Rainin, 4.6 × 100 mm) using 0.1 M potassium phosphate, pH 7, and 1.5 M ammonium sulfate. Linear gradients over 20 min were formed between the starting buffer and a buffer of the same composition without the ammonium sulfate. Ion-exchange chromatography was carried out on a Hydropore AX column (Rainin, 4.6 × 250 mm) using 0.010 M potassium phosphate, pH 7.0. A linear gradient over 25 min was formed between the starting buffer and 0.30 M potassium phosphate, pH 7.0, to elute the proteins.

Lipoxygenase 1 (4.0 mL, 2.0 mg, 0.1 M borate, pH 9.3) was treated with ^{14}C-phenylhydrazine (0.16 mL, 0.12 mg, 7.8 mCi mmol^{-1}, 0.1 M borate, pH 10) for 1 h at room temperature. The chymotrypsin-cleaved enzyme was treated in the same fashion at half the scale. Samples were analyzed by SDS–polyacrylamide gel electrophoresis. The gels were stained for protein with Coomassie brilliant blue or exposed to X-ray film for detection of the labeled proteins. Samples of either lipoxygenase 1 or trypsin-cleaved enzyme (2.5 mL, 2 mg, 0.1 M borate, pH 10.0) were treated with 4-nitrophenylhydrazine (0.25 mL, 0.08 mg, 0.1 M borate, pH 10.0) for 1 h at room temperature. Excess reagent was removed by passage through Sephacryl S200 HR (Pharmacia, 1.5 × 20 cm, 0.2 M Tris-HCl, pH 8). Samples treated in this way were used for either reverse-phase HPLC as before or complete trypsinization and sequence determination. For sequence determination the protein-containing fractions were pooled, concentrated (1 mL), and reduced and carboxymethylated (6 M guanidine hydrochloride, 10× SH content dithiothreitol, 37 °C, 2 h; 1.2× SH content iodoacetic acid, N_2 atmosphere, 90 min, room temperature, dark). The protein was dialyzed against Tris-HCl (0.1 M, pH 8.0, 2 M urea) and treated with trypsin (50 μg) at 37 °C for 16 h. The mixture of peptides was resolved on microbore HPLC using an Applied Biosystems 130A chromatograph with a C4 column and a linear gradient over 45 min from 0.1% aqueous trifluoroacetic acid to 0.1% trifluoroacetic acid in acetonitrile. Sequence determination was carried out on isolated peptides on an Applied Biosystems Model 477A protein sequencer.

The determination of iron was carried out by flame atomization atomic absorption spectroscopy of protein samples ranging in concentration from 1 to 5 mg mL^{-1} using an

Instrumentation Laboratories Model Video 11 spectrophotometer. Iron concentrations were assigned by interpolation with standard solutions ranging in concentration from 0.1 to 5.0 μg mL^{-1}. EPR spectroscopy was carried out at 9 GHz using a Varian Century Line spectrometer equipped with a homemade, gas-phase liquid helium transfer line and quartz dewar cavity insert. Temperature measurements were made using a 0.1-W Allen Bradley carbon composition resistor calibrated at liquid helium, liquid nitrogen, and ambient temperatures (Keeson & Pearlman, 1956). Frequency measurements were determined continuously using a Hewlett-Packard 5340A frequency counter. Field calibration (>100 mT) was accomplished using a Systron-Donner Model 3193 digital NMR gaussmeter. All spectra were obtained at 25 K using microwave powers that avoided modulation broadening (typically 5 mW power and 1 mT modulation amplitude). Scan time for each measurement was 4 min using a time constant of 0.128 s. The spectra were generated as difference spectra (sample minus buffer control) and were normalized to represent the same enzyme concentration. Oxidation with product was carried out at 100 μM enzyme followed by concentration (Centricon 30) to a final volume of 0.25 mL for each EPR sample. Mössbauer spectroscopy was obtained on samples of the enzyme enriched in iron-57 using a previously described tissue culture technique (Funk et al., 1986; Dunham et al., 1990). The distribution of regioisomers among the products was determined chromatographically (Chan & Levett, 1977). The stereochemistry of the products was determined after reduction and methyl esterification and conversion of the corresponding alcohols to the (+)-α-methoxy-α-trifluoromethylphenylacetic acid esters (Andre & Funk, 1986).

RESULTS

Limited Proteolysis. In a recent study of the isoenzymes of soybean seed lipoxygenase, peptide mapping experiments were carried out in order to compare the primary structures of the proteins. Despite the high degree of sequence identity of the isoenzymes, distinctive patterns of peptides were obtained from different isoenzymes, indicating significant differences in the occurrence of proteolysis sites in the areas of nonidentity within the structures (Draheim et al., 1989). Cleavage of lipoxygenase 1 by the serine proteases was subsequently found to be very sensitive to the conditions of the experiment. For example, inclusion of small amounts (<0.01%) of SDS in chymotrypsin digestions led to selective cleavages resulting in a limited number of fragments. Under these conditions, however, lipoxygenase activity was rapidly lost as digestion took place. In the absence of SDS and with relatively large protease to protein ratios (1:10 w/w), the digestion of lipoxygenase 1 occurred with only a modest loss of catalytic activity as illustrated in Figure 1 (top), in which the SDS–polyacrylamide gel electrophoresis of samples from a time course of the reaction is presented. While the protein was completely converted into fragments that could be separated by SDS–polyacrylamide gel electrophoresis, the samples retained about 80% of the activity of an untreated control. Similarly, when lipoxygenase 1 was treated with trypsin, the protein was converted into two large fragments as illustrated in Figure 1 (bottom). In this case complete digestion was accompanied by a significant increase in catalytic activity. The trypsin-cleaved enzyme consistently showed an increase in activity (30–60%) in comparison to samples of the enzyme identically incubated but with the protease omitted. One of the more highly activated samples was subjected to a kinetic analysis to see if the activating effect could be ascribed to either K_M

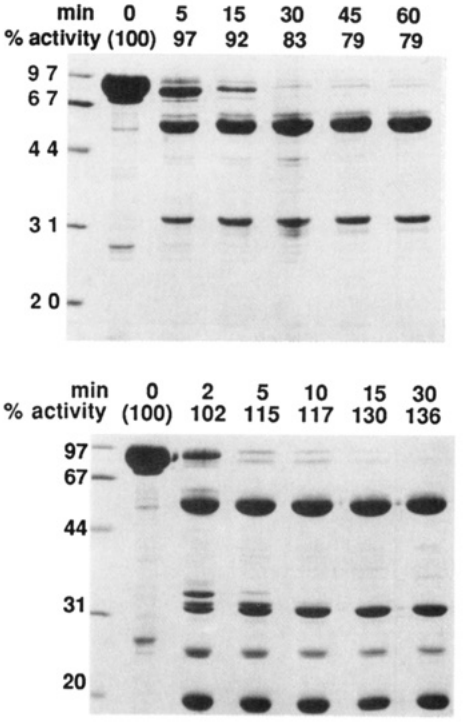

FIGURE 1: SDS–polyacrylamide gel electrophoresis determinations of the time course for the limited proteolysis of lipoxygenase 1 carried out in Tris-HCl, pH 8.0, 25 °C. Samples were incubated for the indicated time periods. Activity refers to the oxygenation of linoleic acid (72 μM) at pH 9.0. (Top) Chymotrypsin digestion. (Bottom) Trypsin digestion. The large band at the bottom of each lane representing a digestion time point is the soybean trypsin inhibitor used to stop the reaction.

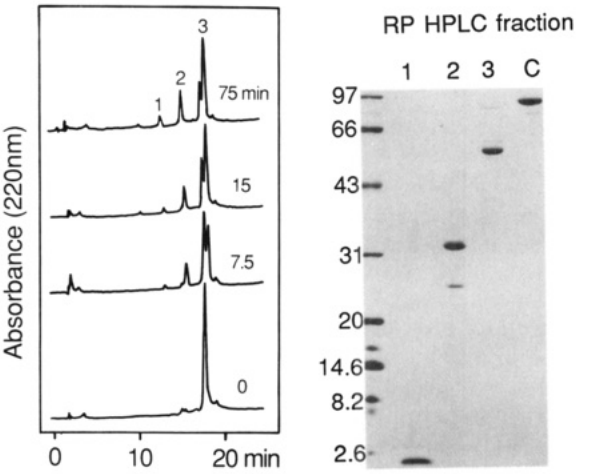

FIGURE 2: (Left) Reverse-phase HPLC determinations of the time course for chymotrypsin digestions. (Right) SDS–gradient polyacrylamide gel electrophoresis determinations of HPLC peak fractions from left panel, Coomassie brilliant blue stain. C represents lipoxygenase 1 not treated with chymotrypsin.

or k_{cat}. Values were obtained from Lineweaver–Burk plots for K_M and k_{cat}, respectively: lipoxygenase 1, 14 μM, 142 s^{-1}; digested enzyme, 11 μM, 189 s^{-1}. While both values were changed by the digestion, the increase in k_{cat} is probably the more significant.

Reverse-phase HPLC and SDS–gradient polyacrylamide gel electrophoresis analysis of samples from the time course for digestion of lipoxygenase 1 by chymotrypsin revealed that the enzyme was cleaved into three polypeptides of approximate apparent molecular mass 60, 30, and 2 kDa (Figure 2). The peptides were subjected to N-terminal sequence determination through at least 13 cycles of Edman degradation. The

Table I: Amino Acid Analysis of the Small Fragment from Chymotrypsin Cleavage of Lipoxygenase 1

amino acid	found	275–317	amino acid	found	275–317
Asx	3.8	4	Met	0.0	0
Thr	1.7	2	Ile	5.6	6
Ser	2.0	3	Leu	5.2	5
Glx	3.9	4	Tyr	1.6	2
Pro	3.9	4	Phe	(2.0)	2
Gly	2.3	2	Lys	3.3	3
Ala	0.1	0	His	1.9	2
Cys	0.0	0	Arg	0.9	1
Val	2.5	3			

N-terminal sequence of the 30-kDa fragment matched exactly with the sequence reported (Shibata et al., 1987) for positions 3–16 in lipoxygenase 1 (position 2: Phe). The small fragment turned out to be an internal peptide, matching exactly the sequence reported for lipoxygenase 1 at positions 275–288 at its N-terminus. Finally, the N-terminal sequence of the largest peptide was found to match exactly positions 318–331 in the lipoxygenase 1 sequence. Therefore, chymotrypsin cleaved lipoxygenase 1 into a 30-kDa N-terminal fragment and a 60-kDa C-terminal fragment which were separated by at least a 2-kDa peptide in the native protein. Because the preceding analyses left in doubt the whereabouts of amino acids 289–317 in these experiments, the amino acid composition of the small fragment was determined. The amino acid ratios found for the peptide matched closely with the anticipated composition of the peptide extending from position 275 through position 317 (Table I). The fragments obtained from trypsin digestion were also isolated by reverse-phase HPLC and subjected to Edman degradation. In the case of trypsin digestion only fragments of approximately 60 and 30 kDa apparent molecular mass were obtained, and only samples of the 60-kDa polypeptide were susceptible to automated Edman analysis. The first 12 N-terminal amino acids from the fragment matched exactly with positions 319–330 in the reported sequence for lipoxygenase 1. It has been previously demonstrated that lipoxygenase 1 is N-terminally blocked (Shibata et al., 1987) which accounts for the failure to obtain sequence from the 30-kDa fragment. No small fragment comparable to the one found in chymotrypsin digestions was detected in either reverse-phase HPLC chromatograms or SDS–gradient polyacrylamide gel determinations.

Resolution and Labeling of the Fragments. The chymotrypsin-cleaved enzyme migrated as a single unresolved band through size-exclusion chromatography columns under strictly native conditions. A somewhat confounding feature of size-exclusion chromatography was the tendency for native lipoxygenase 1 to be retained significantly on the columns and consequently to run at an anomalously low apparent molecular weight. This made it necessary to discriminate carefully between activity from residual native enzyme and the cleaved and separated fragments. The effect of denaturing agents on the clipped enzyme was investigated in an attempt to disrupt domain interactions without eliminating the tertiary structure required for catalytic activity. Catalysis by the cleaved enzyme was stable for at least 60 min at room temperature in 6 M urea. Attempted resolution of the fragments using either size-exclusion, hydrophobic interaction, or ion-exchange chromatography was not successful. In each case partial resolution of the fragments was obtained. However, fractions containing only one of the large fragments displayed no catalytic activity and contained no iron. By contrast, fractions containing both fragments contained iron and had specific activities comparable to native lipoxygenase 1. Mixing the resolved fragments in no instance led to reconstitution of catalytic activity.

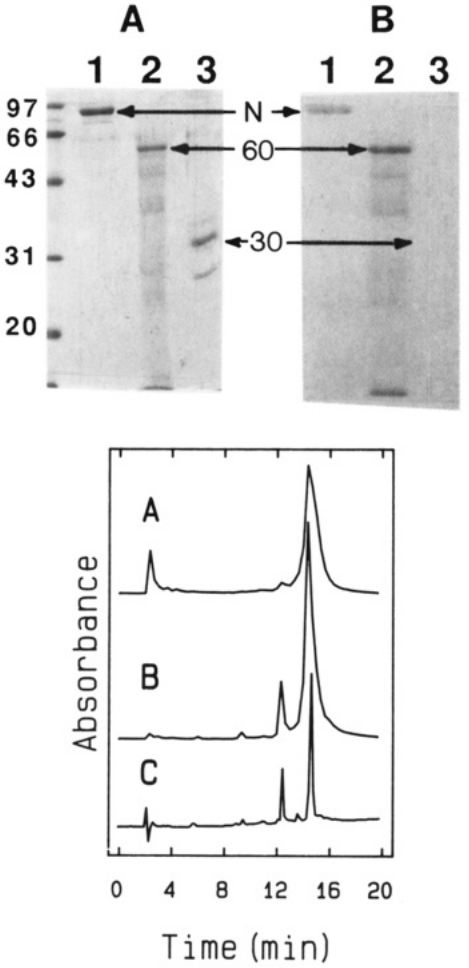

FIGURE 3: (Top) SDS–polyacrylamide gel electrophoresis determinations of lipoxygenase 1 and lipoxygenase 1 after treatment with chymotrypsin upon treatment with ^{14}C-phenylhydrazine. Panel A, Coomassie brilliant blue stain. Panel B, autoradiogram. (Bottom) Reverse-phase HPLC determinations of lipoxygenase 1 after treatment with trypsin upon treatment with 4-nitrophenylhydrazine. Trace A, detection at 340 nm. Trace B, detection at 280 nm. Trace C, lipoxygenase 1 treated with trypsin but not 4-nitrophenylhydrazine, detection at 220 nm.

Two labeling experiments were carried out to localize the active site of the enzyme to one of the large fragments. Samples of lipoxygenase 1 were treated with ^{14}C-phenylhydrazine. It has been demonstrated that the enzyme is rapidly inactivated by autoxidizing solutions of phenylhydrazine (Gibian & Singh, 1986) Radioactivity was detected in the intact protein after inactivation and determination by SDS–polyacrylamide gel electrophoresis using autoradiography (Figure 3, top). This result indicated that covalent labeling of the protein accompanied the inactivation process. When the enzyme was first treated with chymotrypsin and then inactivated by ^{14}C-phenylhydrazine treatment, radioactivity was detected in the 60-kDa fragment but not in the 30-kDa fragment. As a confirmation of the preceding result, the enzyme was similarly treated with 4-nitrophenylhydrazine both with and without prior limited trypsin digestion. Samples from these experiments were analyzed by reverse-phase HPLC (Figure 3, bottom). The detection wavelengths were 220, 280, and 340 nm in the three chromatograms presented. A sample of the clipped enzyme that was not treated with phenylhydrazine served as the control, and detection was carried out at 220 nm to reveal the positions of the proteins. The samples of the trypsin-clipped enzyme treated with 4-nitrophenylhydrazine were analyzed with detection at 280 nm to reveal the presence

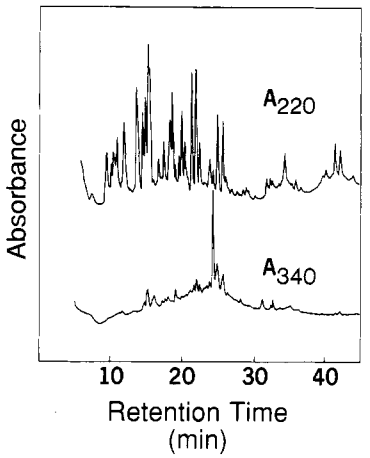

FIGURE 4: Reverse-phase HPLC determination of peptides obtained from lipoxygenase 1 treated with 4-nitrophenylhydrazine followed by reduction and carboxymethylation and complete trypsinization (2 M urea).

of the tryptophan residues in the fragments and at 340 nm to reveal the presence of the 4-nitrophenylhydrazine labeled species. In lipoxygenase 1 there are two tryptophan residues in the 30-kDa fragment and 12 in the 60-kDa fragment. The ratio of the integrated areas of the two peaks in Figure 3 (bottom), chromatogram B, was 89:11, in rough agreement with the expectation from the tryptophan content of the fragments, 86:14. At the 340-nm detection wavelength, a peak area ratio of 99:1 was found, corresponding to nearly exclusive labeling of the 60-kDa fragment. Neither fragment showed significant absorption at 340 nm from injection of the clipped enzyme not treated with 4-nitrophenylhydrazine.

To localize the label found in the 60-kDa fragment further, lipoxygenase 1 was inactivated with 4-nitrophenylhydrazine, subsequently reduced and carboxymethylated, and subjected to complete digestion using trypsin in the presence of 2 M urea. The peptides obtained from the digestion were subjected to microbore reverse-phase HPLC. The effluent was measured at two wavelengths, 220 nm for peptides and 340 nm for label. As depicted in Figure 4, the label was found for the most part in one peptide. This peptide was isolated and subjected to automated Edman sequence determination. The sequence was found to match exactly with the first 16 amino acids following Lys-482 in the reported sequence for lipoxygenase 1: A-Y-V-I-V-N-D-S-C-Y-H-Q-L-M-S-H.

Properties of the Clipped Enzyme. Catalysis by the chymotrypsin-treated lipoxygenase had the same features as the native enzyme. Activity was maximal around pH 9 and was not sensitive to added sodium fluoride, sodium cyanide, or EDTA (1 mM). Also, the regiochemical and stereochemical consequences of catalysis by the clipped enzyme were not significantly different from those of native lipoxygenase. The EPR spectrum for the clipped enzyme upon treatment with 1 equiv of product hydroperoxide was obtained. The EPR spectrum of the chymotrypsin- and trypsin-cleaved enzymes had the same $g6$ features as observed for the native enzyme (Figure 5). The iron atom in the trypsin-cleaved enzyme also had Mössbauer parameters that were quite comparable to those for the native enzyme (Dunham et al., 1990): δ/Fe equal to 1.091 ± 0.003 mm s^{-1} and ΔE_q equal to 3.064 ± 0.011 mm s^{-1} versus δ/Fe equal to 1.08 ± 0.01 mm s^{-1} and ΔE_q equal to 3.00 ± 0.01 mm s^{-1}. There was a slight increase in the line width (full width at half-maximum) from 0.15 ± 0.02 to 0.23 ± 0.04 mm s^{-1} going from the native to the trypsin-cleaved enzyme, but the difference was too small to attribute

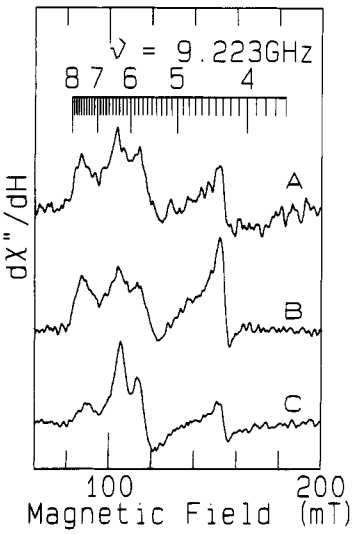

FIGURE 5: EPR spectra for lipoxygenase 1: (A) treated with chymotrypsin, (B) treated with trypsin, and (C) native upon subsequent treatment with 13-hydroperoxy-(9*Z*,11*E*)-9,11-octadecadienoic acid (1:1).

to a significant change in the coordination sphere of the iron atom.

DISCUSSION

From the limited proteolysis data presented here, it is evident that soybean lipoxygenase 1 consists of at least two domains. The possibility that additional subdomains exist was not excluded by these experiments. In the case of chymotrypsin cleavage, the enzyme was divided into two large fragments of approximately 60 and 30 kDa and a linker peptide of 43 amino acids. Interestingly, the folding pattern of the lipoxygenase polypeptide apparently brings two relatively hydrophobic chymotrypsin sites to surface-exposed positions. Because the two additional chymotrypsin substrates in the small fragment were not cleaved, it may be speculated that these Phe residues (283 and 286) were in a less accessible or buried location and that the small peptide was not released until the protein was denatured for SDS–polyacrylamide gel electrophoresis. The overall fragmentation pattern was confirmed by the results of limited trypsin digestion. As with chymotrypsin, two large fragments were obtained. Cleavage occurred at Arg-318, one position removed from the second chymotrypsin site, Tyr-317. While a trypsin site existed in lipoxygenase 1 at Lys-277, this was apparently not susceptible to proteolysis since no small fragment was detected in either reverse-phase HPLC chromatograms or SDS–gradient polyacrylamide gel determinations. This also indicated the likelihood that the amino acid chain turned away from the surface of the protein after residue 275, rendering Lys-277 inaccessible to the protease. It was also noted that while the second amino acid residue in lipoxygenase 1 was a substrate for chymotrypsin, positions 7 and 9 (both Lys) were not trypsin substrates. This may indicate that the enzyme does not contain a long N-terminal extension but that this portion of the protein is incorporated into a protease-inaccessible region.

Chymotrypsin digestion had only a small effect on the catalytic activity of lipoxygenase 1. The specific activity of the cleaved enzyme was reduced by approximately 20%. Otherwise, there were no significant differences between the digested and native enzymes in any of the features examined: regiochemical and stereochemical consequences, susceptibility to inhibition, iron content, and EPR spectroscopy of the product-oxidized form. These data indicated that chymo-

trypsin cleavage caused a minimal change in the native structure in a way that would affect catalysis. Under conditions where the activity of the cleaved enzyme was stable (1 h, 6 M urea), the fragments were only partially resolved using size-exclusion, hydrophobic interaction, or ion-exchange chromatography. No catalytic activity was detected in any completely resolved fragment, although chromatographic fractions containing both fragments also contained catalytic activity. Therefore the fragments could not be separated without complete denaturation, suggesting a structure consisting of two tightly associated domains. Reconstitution experiments with resolved fragments and iron salts did not result in recovery of catalytic activity. These observations indicated that while limited proteolysis clearly divided lipoxygenase 1 into large fragments, interactions between the two (or three) pieces were necessary for maintenance of the native three-dimensional structure. In contrast to chymotrypsin cleavage, limited digestion with trypsin caused a significant increase in specific activity for lipoxygenase 1. Apparently the cleavage of the polypeptide resulted in a change to a conformation for the protein characterized by a higher specific activity. This is the second observation indicating that conformational changes may result in enhanced catalytic activity for a soybean seed lipoxygenase. We have recently reported the characterization of monoclonal antibodies to the seed isoenzymes (Wheelock et al., 1991). In that study antibodies capable of stimulating catalytic activity (by up to 60% for one of the lipoxygenases) were discovered. It appears that lipoxygenase is capable of catalysis-sensitive conformational changes both intramolecularly and intermolecularly. These observations were interesting in relation to the recently discovered properties of the mammalian 5-lipoxygenase. This enzyme was found to be stimulated by a membrane-associated activating protein referred to as 5-lipoxygenase activating protein or FLAP (Miller et al., 1990). It is tempting to speculate about the results of the proteolysis experiments on the soybean enzyme in light of this feature of the mammalian enzyme. The large proteolytic fragment from the soybean enzyme was roughly equivalent in size to the mammalian enzyme and was also the location found for the greatest sequence homology between the proteins. It is possible that the soybean enzyme has a built-in activating protein, namely, the 30-kDa fragment, in comparison to the mammalian enzyme with its FLAP. There is, however, no apparent relationship between the FLAP primary sequence and that for the N-terminal 30-kDa fragment from proteolysis of the soybean enzyme.

Experiments with phenylhydrazine and 4-nitrophenylhydrazine demonstrated that only the largest fragment (60 kDa) from chymotrypsin-cleaved protein became labeled during inactivation of the enzyme. The position of the label within the large fragment was located by complete trypsinization, HPLC purification, and microsequence determination. The label was found associated with a peptide beginning after the trypsin site at Lys-482. The isolated peptide contained two of the histidine residues conserved among lipoxygenases and believed to be part of the non-heme iron binding site. This is the first experimental evidence that these residues are at or near the catalytic site of lipoxygenase 1. While phenylhydrazine reacts to form covalent adducts with numerous heme proteins, the chemical mechanism for this process has not been explicitly elucidated. The formation of aryl–iron adducts clearly takes place in, for example, the reaction of phenylhydrazine with myoglobin (Ringe et al., 1984). Upon denaturation, the aryl group can be transferred to a neighboring nitrogen atom of the porphyrin ring (Swanson & Ortiz de Montellano, 1991). It is likely that phenyldiazene and phenyl radicals are intermediates in the formation of these covalent adducts. The inactivation of lipoxygenase 1 by phenylhydrazine presumably follows a similar course, with a reactive intermediate (phenyl radical or aryl–iron) being formed which reacts (free radical addition or reductive elimination) to form the covalent adduct and inactivate the enzyme. Whatever the mechanism might be, intermediates of this reactivity would not be expected to act at a great distance. In the case of heme proteins, the intermediate never gets further than the porphyrin moiety. Therefore it is reasonable to propose that inactivation involves the iron atom and labeling takes place in the immediate vicinity of the iron-binding site.

In summary, the observations reported here have provided the first insights into the molecular organization of the enzyme lipoxygenase 1. The protein is most likely arranged as a set of two tightly associated subdomains of approximately 60 and 30 kDa molecular mass. The site susceptible to phenylhydrazine modification resides in the larger fragment and includes a portion of the conserved histidine cluster, the proposed non-heme iron binding site. The results are consistent with iron activation of phenylhydrazine to give a reactive intermediate capable of labeling the protein. These are the first experimental results indicating that this portion of the polypeptide may be involved in iron binding and catalysis. Digestion of lipoxygenase 1 by trypsin was found to increase the specific activity of the enzyme. This was interpreted as occurring as a result of a conformational change made possible by the cleavage.

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