Arachidonate 12-Lipoxygenase Purified from Porcine Leukocytes by Immunoaffinity Chromatography and Its Reactivity with Hydroperoxyeicosatetraenoic Acids*

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Chieko Yokoyama, Fukiko Shinjo, Tanihiro Yoshimoto, Shozo Yamamoto, John A. Oates, and Alan R. Brash

From the Department of Biochemistry, Tokushima University School of Medicine, Tokushima 770, Japan and the Departments of Medicine and Pharmacology and Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Arachidonate 12-lipoxygenase was purified to near homogeneity from the cytosol fraction of porcine leukocytes by ammonium sulfate fractionation, DEAE-cellulose chromatography, and immunoaffinity chromatography using a monoclonal antibody against the enzyme. The purified enzyme was stable (half-life of about 4 h at 4 °C) but was markedly protected from the inactivation by storage in the presence of ferrous ion or in the absence of air. The lag phase which was observed before the start of the enzyme reaction was abolished by the presence of 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid. An apparent substrate inhibition was observed with arachidonic acid and other active substrates; however, the substrate concentration curve was normalized by the presence of 0.03% Tween 20. Arachidonic acid was transformed to the ω-9 oxygenation product 12-hydroperoxy-5Z,8Z,10E,14Z-eicosatetraenoic acid. C-12 oxygenation also occurred with 5-hydroxy- and 5-hydroperoxyeicosatetraenoic acids; the respective maximal velocities of the rate with arachidonic acid. The dihydroperoxy and dihydroxy products were identified by their UV-absorption spectra, high performance liquid chromatography, and immunoaffinity chromatography using a monoclonal antibody against the enzyme.

Among these products, (8S,15S)-dihydroxy-5Z,9E,11E,13E-eicosatetraenoic acid, (5S,12S)-diHPETE, (14R,15S)-erythro-dihydroperoxy-5Z,8Z,10E,12E-eicosatetraenoic acid, and (14S,15S)-threo-dihydroxy-5Z,8Z,10E,12E-eicosatetraenoic acid were produced in larger amounts than the ω-9 oxygenation products. Furthermore, formation of 14,15-leukotriene A₄ was inferred from the characteristic pattern of its hydrolysis products comprised of equal amounts of (8R,15S)- and (8S,15S)-dihydroxy-5Z,9E,11E,13E-eicosatetraenoic acids together with smaller amounts of (14R,15S)-erythro- and (14S,15S)-threo-dihydroxy-5Z,8Z,10E,12E-eicosatetraenoic acids. These both lipoxygenase and leukotriene synthase activities were demonstrated with the homogeneous preparation of porcine leukocyte 12-lipoxygenase.

The first lipoxygenase enzyme to be discovered in animal tissues was the 12-lipoxygenase of human platelets (1). Subsequently, 12-lipoxygenase activity has been detected in many other tissues (2, 3), with one of the most abundant sources being the cytosol of porcine leukocytes (4). Studies in whole cells or with crude preparations of 12-lipoxygenase have indicated that these enzymes catalyze the formation of 12-HPETE from arachidonic acid (2, 4), and there is also evidence that the hydroperoxy analog 15-HPETE is further transformed to a mixture of more complex eicosanoids (5). It is also known that the 12-lipoxygenases from different tissues exhibit distinct characteristics. For example, the enzyme of porcine leukocytes catalyzes (12S)-oxygenation of 5-HETE whereas the platelet enzyme 5-HETE is an almost inactive substrate (4). The 12-lipoxygenase of rat basophilic leukemia cells is unique in having a dependence on the calcium ion for activity (6). Although these reactions of 12-lipoxygenases have been extensively investigated, the role of the 12-lipoxygenase pathway has not been elucidated. Unlike the cyclooxygenase and 5-lipoxygenase pathways there does not appear to be synthesis of potent biological mediators. Furthermore, the use of 12-lipoxygenase inhibitors in intact cells has generally yielded negative results (7).

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Definition of the role of an enzyme in cellular function requires a detailed examination of the properties of the purified protein. With the 12-lipoxygenase of bovine platelets the purification has proved difficult due to problems with instability of the enzyme after fractionation of the crude cytosol (8). In our experience, the 12-lipoxygenase of porcine leukocytes is also unstable during routine separation procedures. However, this particular enzyme is present in very high specific activity in the leukocyte cytosol. Therefore, we considered that isolation of the pure protein might be feasible using an alternative strategy for the chromatographic resolution. In the present paper we describe the application of immunoaffinity chromatography to achieve the first successful purification to homogeneity of a 12-lipoxygenase enzyme. Using this enzyme preparation we have characterized the reaction kinetics, substrate specificity and the structure of the products formed from octadecenoic acids and hydroxy- and hydroperoxy-eicosaenoic acids, especially 15-HETE.

EXPERIMENTAL PROCEDURES

RESULTS

Enzyme Purification—The 105,000 × g supernatant of sonicated porcine leukocytes (30 g wet weight) was used as the starting material. Earlier studies demonstrated that most of the 12-lipoxygenase activity was found in the cytosol fraction of leukocytes (4, 9). Since the 12-lipoxygenase was hardly detectable in particulate platelets (4, 9), the enzyme found in the leukocyte cytosol was not attributed to the contaminating platelets. After ammonium sulfate fractionation and DEAE-cellulose chromatography, the partially purified enzyme (specific activity, about 0.4 μmol/min/mg of protein) was applied to a lox-2-Affi-Gel 10 column for immunoaffinity chromatography (Fig. 1). After the bulk of inactive proteins was washed out, the pH of the elution buffer was increased up to 10.4 concomitant with an increase in the concentration of sodium chloride and Triton X-105 to elute the 12-lipoxygenase from the column. The enzyme thus purified gave a specific activity of 2–3 μmol/min/mg of protein if assayed in reaction mixture A immediately after elution (Table 1). The preparation usually showed essentially a single band on polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate as visualized by silver staining (Fig. 2).

General Properties of the Enzyme—The purified enzyme gave a molecular weight of about 72,000 as examined on 7.5% polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (Fig. 2). On the basis of this molecular weight and a specific enzyme activity of 3 μmol/min/mg of protein, the turnover number of the enzyme was calculated to be about 200/min at 30 °C. The enzyme was most active around pH 7.5. The purified enzyme was unstable with a half-life of about 24 h when stored at 4 °C in the air (Fig. 3). However, anaerobic conditions protected the enzyme from inactivation. Moreover, the presence of ferrous ion at 20 μM markedly stabilized the enzyme. Under these conditions the enzyme could be kept for 1 month at −70 °C. The purified enzyme (1 mg/ml) appeared as a colorless solution, which exhibited no significant absorption in the visible region. The content of inorganic iron in the purified 12-lipoxygenase was determined by atomic absorption spectroscopy. The result indicated about 0.45 atom of inorganic iron/mol of enzyme. The validity of the determination was confirmed by the satisfactory recovery of iron added to the enzyme solution as an internal standard.

Reactivity of 12-Lipoxygenase with HETEs and HPETEs—The enzyme was earlier shown to oxygenate C-12 and C-15 of 5-HETE, with reaction at C-12 being the more prominent (4). This finding was confirmed with the purified enzyme as shown in Fig. 4. The products were analyzed as the hydroperoxides and also after sodium borohydride reduction. Reaction with 5-HPETE gave the corresponding hydroperoxides together with 5-HETE (Fig. 4, lane E). The latter was also formed in incubation with boiled enzyme. The borohydride reduced products were previously identified by GC-MS as (5S,12S)-dihydroxy-6E,8Z,10E,14Z-eicosatetraenoic acid (the more polar and more prominent product) and (5S,15S)-dihydroxy-6E,8Z,11Z,13E-eicosatetraenoic acid (4). 12-HETE and 12-HPETE were inactive substrates as compared with control runs of a heat-denatured enzyme.

The enzyme was almost inactive with 15-HETE (Fig. 5). In sharp contrast, a variety of products were observed by reaction of 15-HPETE with the active enzyme (Fig. 5, lane D). After treatment with sodium borohydride, the reduced products were extracted and then analyzed by straight-phase HPLC (Fig. 6B) and reverse-phase HPLC (Fig. 7) with UV detection at 270 nm. Six major products were observed, and these were

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"Experimental Procedures" and additional references are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-1543, cite the authors, and include a check or money order for $3.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
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**FIG. 2.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified 12-lipoxygenase from porcine leukocytes. Electrophoresis of the purified enzyme (2.7 µg) was carried out with marker proteins as described in the miniprint. The proteins were visualized by silver staining.

**FIG. 3.** Stability of the purified 12-lipoxygenase from porcine leukocytes. Immediately after elution of enzyme from the 2-Affi-Gel 10 column, the enzyme (120 µg/ml) was kept at 4 °C (○) or −70 °C (●) in the air. A Thunberg tube which contained the enzyme and was filled with nitrogen gas was kept at 4 °C (△). The enzyme was also kept at 4 °C in the presence of 20 µM ferrous ammonium sulfate either in the air (△) or in nitrogen gas (▲). A 10-µl aliquot was removed at intervals, and the 12-lipoxygenase activity was assayed with [1-14C]5-HETE as substrate in the standard assay mixture A. The enzyme protein was included in an amount of 2 µg (lanes B and D) or 7 µg (heat denatured in boiling water for 10 min, lanes A and C). The TLC plate was developed in the solvent system described previously (4) and exposed to an x-ray film for autoradiography. Migrations of 5-HETE and 5-HPETE are indicated by the arrows.

**FIG. 4.** Reaction of the purified 12-lipoxygenase with 5-HETE and 5-HPETE. The purified 12-lipoxygenase was allowed to react with either 50 µM [1-14C]5-HETE (100,000 cpm) (lanes A, B, and C) or 50 µM [1-14C]5-HPETE (100,000 cpm) (lanes D, E, and F) in the standard assay mixture A. The enzyme protein was incubated in an amount of 2 µg (lanes B, C, E, and F) or 7 µg (heat denatured in boiling water for 10 min, lanes A and D). After the reaction for 1 min sodium borohydride was added to the assay mixture (lanes C and F). TLC was performed twice in the same solvent system described previously (4). The TLC plate was exposed to an x-ray film for autoradiography. Migrations of 5-HETE and 5-HPETE are indicated by the arrows.

**FIG. 5.** Reaction of the purified 12-lipoxygenase with 15-HETE and 15-HPETE. The purified 12-lipoxygenase was allowed to react with 50 µM [1-14C]15-HETE (100,000 cpm) (lanes A and B) or [1-14C]15-HPETE (100,000 cpm) (lanes C and D) in the standard assay mixture A. The enzyme protein was included in an amount of 2 µg (lanes B and D) or 7 µg (heat denatured in boiling water for 10 min, lanes A and C). The TLC plate was developed in the solvent system described previously (4) and exposed to an x-ray film for autoradiography. Migrations of 15-HETE and 15-HPETE are indicated by the arrows.

diastereomer (IIb). We also noted quite consistently that when incubations were conducted on ice (as opposed to 30 °C), the prominence of the Ia and IIa products was greatly accentuated; this observation is currently under further study. Straight-phase HPLC analysis of incubations which were extracted with and without borohydride reduction indicated that Ia and IIa were formed as dihydroperoxides (Fig. 6, B and C). This type of analysis also revealed that the two major products IIIa and IIIb were indeed formed in the incubation as 8,15-dihydroxy (not hydroperoxy) derivatives. It was further noted that the abundance of these 8,15-diols, together with more minor 14,15-diols, was identical to the pattern of hydrolysis products of 14,15-leukotriene A₄. Our interpretation of these results is that the 12-lipoxygenase of porcine leukocytes converts 15(S)-HPETE to (14R,15S)-erythro-dihydroperoxide and (8S,15S)-dihydroperoxide and also it catalyzes the formation of 14,15-leukotriene A₄. As might be expected, the epoxide was formed when anaerobic conditions were employed whereas the oxygenation products were absent.
The purified enzyme (2 μg) was mixed with 50 μM 15-HPETE in the presence of 50 mM Tris-HCl buffer at pH 7.4 (total volume of 100 μl). Reaction was carried out at 30 °C for 1 min (A) or on ice for 10 min (B, C, and D). Reaction was performed under anaerobic conditions (D). Sodium borohydride was added to the reaction mixture, followed by extraction with ethyl ether (A and B). The reaction mixture was extracted with ethyl ether at acidic pH (C and D). The extract was analyzed by straight-phase HPLC (Alltech silica 5 μm, 4.6 × 250 mm) using solvent 3 (hexane, 2-propanol, acetic acid = 100:30:1) at a flow rate of 2 ml/min. Absorption at 270 nm was monitored.

50 μM of added 12-HPETE shortened the lag time, especially when a small amount of the enzyme was used. The functioning glutathione peroxidase (enzyme + glutathione) inhibited the 12-lipoxygenase reaction almost completely. These observations suggested that the same catalytic site of the enzyme was involved in the reactions with various substrates.

Normalization of Kinetic Properties of 12-Lipoxygenase — Before the enzyme reaction with arachidonic acid started, a lag phase up to about 30 s was noted, especially when a small amount of the enzyme was used. The functioning glutathione peroxidase (enzyme + glutathione) inhibited the 12-lipoxygenase reaction almost completely. These observations suggested an involvement of peroxide in the initiation of the 12-lipoxygenase reaction. The lag was abolished as the concentration of 12-HPETE in the assay mixture was raised and almost undetectable in the presence of 1 μM 12-HPETE. Preincubation of the enzyme with 0.1 μM 12-HPETE shortened the lag time depending on the preincubation time (10–60 s). The reaction rate after the lag phase was not affected by the concentration of added 12-HPETE. Hydrogen peroxide, cumene hydroperoxide, and t-butyl hydroperoxide did not abolish the lag phase.

As can be seen in Fig. 9A, the rate of 12-lipoxygenase reaction increased as the arachidonate concentration was raised up to 10 μM. A further increase in the substrate concentration reduced...
the reaction rate. Such an apparent substrate inhibition of 12-lipoxygenase was abolished by the addition of a low concentration of Tween 20, and an almost normal Michaelis-Menten curve was observed as shown by the closed circles in Fig. 9A. The optimal concentration of Tween 20 varied to some extent depending on the substrate concentration (Fig. 10A). Therefore, the standard mixture B for the spectrophotometric assay included 0.03% Tween 20 in addition to 1 μM 12-HPETE. Under these assay conditions the specific activity of the purified 12-lipoxygenase was about 5 μmol/min/mg of protein at 20 °C. This value was higher than the specific enzyme activity determined at 30 °C in the assay mixture (without Tween 20 and 12-HPETE as activators).

A lag phase was not observed in the reaction of the enzyme with 15-HPETE. However, the addition of 0.03% Tween 20 stimulated about 2-fold the reaction with 40 μM 15-HPETE. As shown in Fig. 5, the enzyme activity with 15-HPETE was hardly detectable. In the presence of 1 μM 12-HPETE and 0.03% Tween 20, the reaction of the enzyme with 50 μM 15-HPETE showed a slow increase in absorption at 270 nm (0.2% rate of 15-HPETE). The product obtained by incubation on ice for 10 min was tentatively identified as the 14-hydroperoxy derivative of 15-HPETE as determined by HPLC.

Reactions of 12-Lipoxygenase with Octadecenoic, Eicosaenoic, and Docosanoic Acids—In addition to reaction with arachidonic acid, the 12-lipoxygenase was active with other unsaturated fatty acids of various carbon chain lengths (Fig. 9, A–I). With all the unsaturated fatty acids tested, an apparent substrate inhibition was observed. The substrate saturation curve was normalized by the addition of a low concentration of Tween 20 (Fig. 10, B and C, for linoleic and γ-linolenic acids). It was noted that the Km for substrate was lower in the absence of Tween 20 although the maximal velocity was almost unaffected by addition of the detergent. When the maximal velocities were compared under the kinetically normalized conditions (in the presence of 1 μM 12-HPETE and 0.03% Tween 20), arachidonic acid and γ-linolenic acid were most active, followed by linoleic acid, α-linolenic acid, other eicosanoic acids, and docosanoic acid.

The reaction products from linoleic acid, α-linolenic, and γ-linolenic acids were reduced by treatment with triphenylphosphine in methanol and subsequently analyzed by straight-phase HPLC (Alltech silica, 5 μm, 4.6 × 250 mm, Alltech; solvent 2) with UV detection at 235 nm. The retention times were compared with standard mixtures of the pure cis/trans conjugated diene-containing hydroxy acids prepared by controlled autoxidation of the corresponding free fatty acids (10).

Analysis of the 12-lipoxygenase reaction with linoleic acid revealed a single major product which co-chromatographed with 13-hydroxy-octadecadienoic acid (3% 9-hydroxy isomer). Subsequent GC-MS analysis of the methyl ester trimethylsilyl ether derivative of the major enzymatic product and the standard confirmed the structure. A prominent ion was recorded at m/z 382 (M+), about 20% relative abundance, followed by a series of weak ions at m/z 367, 351, 339, 335, and 325, and then prominent ions at m/z 311, 225, 143, 130, and 73. An identical pattern was observed in the sample and the 13-hydroxy-octadecadienoic acid standard.

On straight-phase HPLC the four hydroxy derivatives obtained via autoxidation of α-linolenic acid eluted in the order 15-, 12-, 16-, and 9-hydroxy acids. The enzyme-derived product chromatographed with the first eluting isomer. GC-MS confirmed the reduced product was the 13-hydroxy-octadecadienoic acid. A weak molecular ion was observed at m/z 380 (M+) with further series of weak ions at m/z 365, 348, and 333, followed by the particularly dominant signal of the base peak at m/z 311 (loss of the terminal pentene carbon radical).

Using the same approach, the triphenylphosphine-reduced enzymatic product from γ-linolenic acid was identified as the 10-hydroxy-octadecadienoic acid. It co-eluted with the second of the four hydroxy analogs obtained via autoxidation (the order of elution being 13-, 10-, 9-, and 6-hydroxy acids). A second product from the enzyme reaction (about 5% yield) co-chromatographed on HPLC with the 13-hydroxy standard, but this minor compound was not investigated further. GC-MS of the methyl ester trimethylsilyl ether derivative of the major product and of the 10-hydroxy standard showed essentially identical patterns with the M+ ion being absent, but fragment ions corresponding to M-15 and M-31 were recorded at m/z 365 and 349, respectively, with other diagnostic ions at m/z 269 (loss of the terminal octene carbon radical), 179, 147, 137, 129, 119 (base peak), 105, 91, and 73.

**DISCUSSION**

The monoclonal antibody technique was first applied to prostaglandin research by Smith and co-workers (11, 12). A cyclooxygenase-specific antibody was utilized for histological studies on the enzyme (11), and later a monoclonal antibody against prostacyclin synthase was applied to purification of the enzyme by immunoaffinity chromatography (12). Recently we prepared several species of monoclonal antibody specifically directed against 12-lipoxygenase (9) and 5-lipoxygenase (13) of porcine leukocytes. These monoclonal antibodies were used for successful purification by immunoaffinity chromatography of 5-lipoxygenase (14) and 12-lipoxygenase as described in this paper. The advantage of the monoclonal antibody technology is that a highly specific anti-lipoxygenase antibody can be prepared using a partially purified enzyme as antigen. Immunoaffinity chromatography can then be performed to isolate an essentially homogeneous sample of lipoxygenase from a crude preparation of the enzyme. We have found that this approach has been very successful with enzymes which were difficult to handle by conventional methods. A critical step in the immunoaffinity technique is the recovery of enzyme after it is bound to the antibody on the column. The most commonly used conditions for the elution of protein include an alkaline pH, but this may be associated...
with loss of enzymatic activity. This problem occurred with our 12-lipoxygenase, and it proved to be the most difficult step to resolve. Many trials were made before we were able to identify suitable conditions (addition of a non-ionic detergent and other reagents) for the recovery of catalytically active enzyme from the immunoaffinity column.

The oxygenation reactions of cyclooxygenase (15) and lipoxygenases (16, 17) are known to be preceded by a lag phase which can be abolished by addition of the hydroperoxide product. We observed the same phenomenon with the purified 12-lipoxygenase. The effect was particularly marked with small amounts of enzyme. Addition of 1 μM 12-HPETE eliminated the lag phase and allowed a more precise monitoring of the initial velocity of reaction with different substrates. By standardizing the reaction conditions in this manner and by using a continuous spectrophotometric assay, we were able to find a marked substrate inhibition of the enzyme. This substrate saturation effect was eliminated by addition of a low concentration of Tween 20 to the reaction mixture. It seems likely that the action of the detergent is to facilitate a more uniform dispersion of the substrate. However, the leukocyte 12-lipoxygenase is somewhat hydrophobic in nature, and, therefore, another possibility is that the Tween 20 helps maintain a more catalytically active conformation of the enzyme.

A thorough characterization of the properties of the leukocyte 12-lipoxygenase may offer important clues as to the still unknown physiological role of the enzyme. The substrate specificity toward polyunsaturated fatty acids constitutes one such important property. Notably the porcine leukocyte enzyme has a quite distinct substrate preference compared to the 12-lipoxygenase of platelets. In addition to its reactivity with eicosapolyenoic acids, the leukocyte 12-lipoxygenase was found to oxygenate α-linolenic, γ-linolenic, and also linoleic acids, a finding included in our preliminary report and in a recent publication by Claeys et al. (18). The latter authors concluded that linoleic acid was the preferred substrate of the enzyme. In our experience the preferred substrate depends on the concentration of the fatty acid used in the test; at concentrations below 30 μM the fastest rate of reaction is observed with arachidonic acid, while in the 100 μM range the substrate inhibition (in the absence of detergent) results in linoleic acid giving the most rapid reaction. Claeys et al. (18) pointed out the possible relevance of the fact that porcine leukocytes contain twice as much linoleic acid as arachidonic acid in phospholipids.

As reported previously (5, 19), when a suspension of porcine leukocytes is incubated with arachidonic acid, a variety of dihydroxy derivatives is produced. One series of dihydroxy compounds is formed via the synthesis of 15-HPETE. The mechanism of biosynthesis of these derivatives was studied using incubations in H218O or under 18O2 and using stereoselectively tritiated arachidonic acid. These experiments with intact porcine leukocytes showed that 15-HPETE was further converted by both lipoxygenase and leukotriene A synthase pathways. From a body of circumstantial evidence it was suggested that the leukocyte 12-lipoxygenase participated in both these types of reaction (5). With the availability of the purified enzyme, we have now been able to verify this prediction.

As summarized in Fig. 11, the purified enzyme catalyzes both oxygenation of various substrates and leukotriene A, synthesis. First, we have been able to isolate the oxygenation products as the intact hydroperoxides rather than hydroxy compounds and thus obtain the first direct evidence for the syntheses of dihydroxy products by an oxygenase mechanism. 5-HPETE and 5-HETE were oxygenated at the C-12 position. In addition to such 12-lipoxygenase reactions, the same purified enzyme oxygenated C-8, C-9, and C-14 of 15-HPETE. The products were isolated as (8S)-hydroperoxy and (14R)-hydroperoxy derivatives of 15-HPETE. Also, the product analyses pointed to the synthesis of 14,15-leukotriene A, as deduced from the presence of the characteristic pattern of its dihydroxy hydrolysis products, 8,15-diHETEs and 14,15-diHETEs. As described in this paper, an inhibition of 15-HPETE metabolism by increasing concentrations of arachidonic acid, a parallel heat inactivation of the enzyme activities with 15-HPETE and arachidonic acid as substrates, and a characteristic stimulation by Tween 20 of the reactions with 15-HPETE as well as other substrates suggested that the 14,15-leukotriene A, synthesis from 15-HPETE was an integral catalytic activity of 12-lipoxygenase. The association of lipoxygenase and leukotriene synthase activities observed with the porcine leukocyte 12-lipoxygenase has also been reported for other purified lipoxygenases, and this can now be considered as a common property of mammalian lipoxygenases. The 5-lipoxygenase of potato tubers transformed 5-HPETE to leukotriene A, (5S,12S)-diHPETE was also produced in a considerable quantity (20). The purified 5-lipoxygenases of porcine leukocytes (14) and human leukocytes (21) were recently shown to catalyze leukotriene A, synthesis from
5-HPETE. Association of the two enzymatic activities is supported by another study; rabbit reticulocytes contain a 15-HPETE, which was purified and shown to transform 15-HPETE to 14,15-leukotriene A₄ (22).

Another facet of the enzymatic reaction is the positional specificity of the oxygenation. The 15-HPETE activity of our purified 12-HPETE was essentially the sole product (together with a small amount of 12-HETE and 10-hydroxy-11,12-epoxy-5,8,14-eicosatrienoic acid formed by nonenzymatic degradation of the 12-HPETE). However, as shown in Fig. 4, when 5-HPETE or 5-HPETE was the substrate, a small but significant portion of the substrate was oxygenated at C-15 in place of C-12. Presumably the 15-oxygenation of 5-HPETE and 5-HPETE is attributed to the 15-oxygenase activity of 12-HPETE rather than a separate 15-oxygenase. A similar observation was reported for the potato 5-HPETE (20). The rabbit reticulocyte 15-HPETE converts arachidonic acid to a mixture of 15-HPETE and 12-HPETE in a ratio of about 15:1 (22).

In conclusion, the 12-HPETE of porcine leukocytes catalyzes a variety of reactions. Eicosapolyenoic acids, octadecapolyenoic acids, and docosapolyenoic acids are acceptable substrates for the primary oxygenation. The oxygenation can occur in the ω-6 or the ω-9 positions, depending on the particular substrate. Reaction with hydroperoxy acids can lead to the syntheses of several different dihydroperoxy derivatives and also epoxide of the leukotriene type. It should occur in the substrates for the primary oxygenation. The oxygenation catalyzes a variety of reactions. Eicosapolyenoic acids, octadecapolyenoic acids, and docosapolyenoic acids are acceptable substrates for the primary oxygenation. The oxygenation can occur in the ω-6 or the ω-9 positions, depending on the particular substrate. Reaction with hydroperoxy acids can lead to the syntheses of several different dihydroperoxy derivatives and also epoxide of the leukotriene type. It should be noted that these are by no means minor reactions. The reactions occur at a considerable rate, and they give these derivatives as the quantitatively major products.

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Additional references are found on p. 16721.
Non-radioactive 15-HPETE was also prepared by the use of soybean lipoygenase and purified by straight-phase RPC (Alltech Silica 5 μ. 3 ml) and silica gel column chromatography. Non-radioactive 13-hydroxy-9,11,15-eicosatrienoic acid, 10-hydroxy-8,11,13-
eicosatrienoic acid, 15-hydroxy-9,11,15-eicosatrienoic acid and 15-hydroxy-8,10,14-eicosatrienoic acid were prepared by oxidation of α- linoleic acid, ω-6-linolenic acid, linoleic acid and arachidonic acid, respectively, in presence of α-tocoopherol (6). For the preparation of a mixture of 15,12-epoxy- and 12,15-epoxy-15-
eicosatrienoic acid, and 12,15-epoxy- and 15,12-epoxy-13-
eicosatrienoic acid, a controlled autoxidation of 15-HPETE methyl ester was carried out in the presence of 5% (w/v) α-tocoopherol (dry flake) under an O₂ atmosphere for 15 h at room temperature (about 50°C). After the autoxidation reactions, hydroxy-products were reduced to hydroxy-
compounds by the addition of triphenylphosphine. 12,15-epoxy-15-
eicosatrienoic acid, and 13,12-epoxy- and 15,12-epoxy-13-
eicosatrienoic acid were prepared in mg quantities via chemical synthesis of 14,15-inositol hexaacetate 16. Identification of these compounds was confirmed by GC-MS. 12,15-epoxy-15-
eicosatrienoic acid was prepared by the double oxygenation reaction of the soybean lipoygenase (16).

Purification and Assay of 12-Lipoxygenase of Porcine leucocytes
Leucocytes were collected from porcine blood by the method described previously (3). About 30 g wet weight of leucocytes were obtained from 1 l of whole blood, and the cells were dispersed to 3 × 10⁷ cells on 3 ml volume of 20 ⽥ potassium phosphate buffer, pH 7.4. The cell suspension was subjected to sonic oscillation at 0.2 kHz twice for 30 s by the use of a Branson sonifier model UCD-100. The sonicate was centrifuged at 10,000 × g for 30 min and the supernatant was collected. The supernatant (200 ml) was mixed with one ninth the volume of 10% solution of streptomycin sulphate, and after 30 min the mixture was centrifuged at 30,000 × g for 30 min. Solid ammonium sulphate was added to the supernatant solution, and the fraction obtained at 30-50% saturation was passed through a Sephadex G-50 column (3.5 × 25 cm) equilibrated with 20 ml potassium phosphate buffer (pH 7.4) containing 0.05 M Tris HCl. Active fractions were collected (60 ml) and they were applied to a DEAE-cellulose column (5 × 40 cm) equilibrated with the buffer described above. 12-Lipoxygenase was eluted with the same buffer in which the concentration of potassium chloride was increased to 0.15 M. Active fractions were pooled (20 ml) and applied to a DEAD-Sepharose column (2 × 15 cm) equilibrated with 50 ml of 50 mM diethanolamine buffer at pH 7.8 containing 30% glycerol, 0.1 M sodium chloride and 0.05 M Tris HCl. The fraction was washed with 50 ml of the same medium at room temperature and the protein in the eluate was eluted by 0.5 M diethanolamine buffer at pH 7.8 containing 30% glycerol, 0.6 M sodium chloride, 0.25 Tris HCl and 2 M uric acid. 50-ml fractions were collected in tubes each containing 1 ml of 20% sucrose. Immediately after the activity of each fraction was assayed. The active fractions were combined (8-12 ml) and a solution of ferric ammonium sulphate was added to the final concentration of 0.15 M. The solution was dialyzed against the addition of 4 times the volume of 20 ml of phosphate buffer at pH 7.5 containing 20 mM ferric ammonium sulphate, and concentrated to the original volume by the use of a Diaflo membrane PM-10 (30 ml). The concentrated solution was dialyzed again as described above, and the enzyme solution was concentrated to 1-2 ml with the aid of a Diaflo membrane. The enzyme solution was stored at 0°C in the presence of nitrogen gas.

The standard 12-lipoxygenase assay mixture (350 μl) contained 50 mM Tris HCl buffer at pH 7.4, 100 μM (50,000 cpm) [3H]arachidonic acid and 200 μM (10 μCi) arachidonic acid. The reaction was started by the addition of enzyme, and the increase in absorbance at 234 nm was followed at 37°C. A molecular absorbance coefficient (27,000) was applied to the hydroxy acids.

Proteins was determined by the method of Bradford (18) with bovine serum albumin as a standard. The enzyme activity was expressed in terms of μmol of products/min per mg of protein at 30°C in assay A and at 20°C in assay B. Purity of enzyme preparations was examined by 7.5% polyacrylamide gel electrophoresis in the presence of 0.1% SDS according to the method of Laemmli (19). For molecular weight determination the standard mixture of the following marker proteins (Sigma) was also run: cytochrome c from horse erythrocytes, 28,000; sodium, 45,000; bovine plasma albumin, 66,000; phosphorylase b from rabbit muscle, 97,000; trypsin inhibitor from soybean, 61,000; myosin from rabbit muscle, 200,000.

High Performance Liquid Chromatography — A Waters pump model 600A was equipped with an injector, a Waters model 510 pump, a Waters 8450 UV detector, and a Waters 996 photodiode array detector. RP-HPLC was performed with a 4.6 × 100 mm Nova-Pak C18 column (10 μm) at a flow rate of 1 ml/min. The mobile phase was a mixture of water, acetonitrile (90:10), (90:10) and (80:20) (v/v). The column was maintained at 40°C.

GC Chromatography — Mass Spectrometry — The instrument was a Varian 8200-59 GC quadrupole with a 15 m × 0.26 mm fused silica capillary column. Samples in dot matrix were injected at 150°C and after 1 min the oven temperature was programmed to 200°C. Mass spectra were recorded in the electron impact mode at an electron energy of 70 eV. The position of the hydroxy group on the hydroxy isomers of polyunsaturated fatty acids were confirmed by the mass spectra of the hydroperoxide analogs analyzed by the methyl ester trimethylsilyl ether derivatives. Hydroperoxide M value to simplify the mass spectra, with fragmentation dominated by cleavage from the trimethylsilyl ether, leaving an unassigned side to the positional hydroxy group.

Analysis of free lipids in 12-Lipoxygenase — Immediately after elution of enzyme from immobilized affinity chromatography columns using an iron-free buffer (treated with Celite 545), the enzyme activity was determined. Enzyme solution was concentrated by Diaflo membrane PM-10. After dialysis against the addition of 4 times the volume of 20 ml of phosphate buffer at pH 7.5 containing 20 mM ferric ammonium sulphate, and concentrated to the original volume by the use of a Diaflo membrane PM-10. The concentrated solution was dialyzed again as described above, and the enzyme solution was concentrated to 1-2 ml with the aid of a Diaflo membrane. The enzyme solution was stored at 0°C in the presence of nitrogen gas.

The standard 12-lipoxygenase assay mixture (350 μl) contained 50 mM Tris HCl buffer at pH 7.4, 100 μM (50,000 cpm) [3H]arachidonic acid and 200 μM (10 μCi) arachidonic acid. The reaction was started by the addition of enzyme, and the increase in absorbance at 234 nm was followed at 37°C. A molecular absorbance coefficient (27,000) was applied to the hydroxy acids.

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