Purification of Arachidonate 5-Lipoxygenase from Porcine Leukocytes and Its Reactivity with Hydroperoxyeicosatetraenoic Acids*

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Arachidonate 5-lipoxygenase was purified to near homogeneity from the 105,000 × g supernatant of porcine leukocyte homogenate by immunoaffinity chromatography using a monoclonal anti-5-lipoxygenase antibody. Reaction of the purified enzyme with arachidonic acid produced predominantly 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid with concomitant formation of several more polar compounds in smaller amounts. These minor products were identified as the degradation products of leukotriene A4, namely, 6-trans-leukotriene B4 (epimeric at C-12) and an epimeric mixture of 5,6-dihydroxy-7,9,11,14-eicosatetraenoic acids. These compounds were also produced by reaction of the enzyme with 5-hydroperoxy-eicosatetraenoic acid. Association of the 5-lipoxygenase and leukotriene A synthase activities was demonstrated by several experiments: heat inactivation of enzyme, effect of selective 5-lipoxygenase inhibitors, requirements of calcium ion and ATP, and self-catalyzed inactivation of enzyme. The enzyme was also active with 12- and 15-hydroperoxy-eicosatetraenoic acids producing (5S,12S)- and (5S,15S)-dihydroperoxy-5,12-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acids, respectively. Maximal velocities of the reactions with these hydroperoxy acids as compared with that of arachidonic acid (100%, 0.6 μmol/3 min/mg of protein) were as follows: 5-hydroperoxy acid, 3.5%, 12-hydroperoxy acid, 22%, and 15-hydroperoxy acid, 30%.

The physiological role of arachidonate 5-lipoxygenase to initiate the biosynthesis of leukotrienes has been well established (1). Attempts have been reported to purify and characterize the enzyme from various animal species: rat basophilic leukemia cells (2-5), guinea pig (6), and human (7) polymorphonuclear leukocytes. The requirement of cofactors such as calcium ion (2) and ATP (6) was also described. According to a strategy to raise monoclonal anti-lipoxygenase antibodies with a partially purified enzyme as an antigen and to apply them to purification of lipoxygenases by immunoaffinity chromatography, we have been successful in the purification of 12-lipoxygenase from porcine leukocytes (8). This paper reports the application of the same method to the purification of 5-lipoxygenase from porcine leukocytes and also describes the reactivity of the purified enzyme with various hydroperoxy-eicosatetraenoic acids with special reference to the LTA4 synthase activity.

EXPERIMENTAL PROCEDURES

Materials—[1-14C]Arachidonic acid (59.6 mCi/mmol) was purchased from Amersham International (Amersham) and arachidonic acid from Nu-Chek Prep (Elysian, Minn.). 6-trans-LTB4, 12-0-methyl-5,12-diHETEs prepared from LTA4 methyl ester were kindly provided by Dr. S. Terao of Takeda Research Laboratories, and 5-HETE (racemic mixture) by Dr. M. Hayashi of Ono Research Institute. ATP, N,O-bis(trimethylallyl)trifluoracetamide, trimethylchlorosilane, and sodium borohydride were obtained from Wako Pure Chemical Industries (Osaka). Cooamassie Brilliant Blue R-250 and sodium deoxycholate from Nakarai Chemicals (Kyoto), 3,3'-diaminobenzidine-4-HCl from Dojin (Kumamoto), high molecular weight standard mixture for SDS-polyacrylamide gel electrophoresis and soybean lipoxidase (type I) from Sigma, dithiothreitol from Seikagaku Kogyo (Tokyo), and horse biotinylated anti-mouse IgG and Vectastain ABC kit from Vector Laboratories (Burlingame, CA). Cisilipol was provided by Dr. T. Horie of our university and AA861 by Drs. S. Terao and Y. Maki of Takeda Research Laboratories. Protein A-bearing Cowan I strain of Salmonella typhimurium (DCTK 6530) was a gift from Dr. W. L. Smith of Michigan State University. The bacterial cells were fixed in 1.5% formaldehyde and then heat-inactivated at 80 °C for 5 min (9). Myeloma cell line (SP2/0-Ag41) was provided by Salk Institute (San Diego). Precoated Silica Gel 60 F254 glass plates for TLC were purchased from Merck (Darmstadt), silicic acid (100 mesh) for column chromatography from Mallinkrodt Chemical Works, Affi-Gel 10 from Bio-Rad, and protein A-Sepharose CL-4B from Pharmacia.

Preparation of Antibodies—Mice of C3H/He strain were given intraperitoneally a partially purified preparation of 5-lipoxygenase from porcine leukocytes (10). Spleen cells of the immunized mice were fused with myeloma cells (SP 2/0-Ag14) with the aid of polyethylene glycol according to the method of Godin (11). The fusion mixture was subjected to the "HAT selection" (11), and the hybridoma cells producing anti-5-lipoxygenase antibody were cloned in soft agar by the method of Kennet (12). A clone producing a species of monoclonal antibody, raised against a 5-lipoxygenase purified from porcine leukocytes, was selected for its ability to recognize and to bind with the enzyme from porcine leukocytes and to a lesser extent with the enzyme from guinea pig polymorphonuclear leukocytes.

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1 The abbreviations used are: 5-(H)(P)ETE, (5S)-hydroxy- or hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid; 12-(H)PETO, (12S)-hydroperoxy-5,8-cis-10-trans-14-cis-eicosatetraenoic acid; LT, leukotriene; LTA4, (5S,12S)-dihydroxy- or dihydroperoxy-6-trans-8-cis-10-trans-14-cis-eicosatetraenoic acid; LTB4, (5S,15S)-dihydroxy- or hydroperoxy-6-trans-8-cis-10-trans-14-cis-eicosatetraenoic acid; 5,6-diHETEs prepared from LTA4 methyl ester were kindly provided by Dr. S. Terao of Takeda Research Laboratories, and 5-HETE (racemic mixture) by Dr. M. Hayashi of Ono Research Institute. ATP, N,O-bis(trimethylallyl)trifluoracetamide, trimethylchlorosilane, and sodium borohydride were obtained from Wako Pure Chemical Industries (Osaka). Cooamassie Brilliant Blue R-250 and sodium deoxycholate from Nakarai Chemicals (Kyoto), 3,3'-diaminobenzidine-4-HCl from Dojin (Kumamoto), high molecular weight standard mixture for SDS-polyacrylamide gel electrophoresis and soybean lipoxidase (type I) from Sigma, dithiothreitol from Seikagaku Kogyo (Tokyo), and horse biotinylated anti-mouse IgG and Vectastain ABC kit from Vector Laboratories (Burlingame, CA). Cisilipol was provided by Dr. T. Horie of our university and AA861 by Drs. S. Terao and Y. Maki of Takeda Research Laboratories. Protein A-bearing Cowan I strain of Salmonella typhimurium (DCTK 6530) was a gift from Dr. W. L. Smith of Michigan State University. The bacterial cells were fixed in 1.5% formaldehyde and then heat-inactivated at 80 °C for 5 min (9). Myeloma cell line (SP2/0-Ag41) was provided by Salk Institute (San Diego). Precoated Silica Gel 60 F254 glass plates for TLC were purchased from Merck (Darmstadt), silicic acid (100 mesh) for column chromatography from Mallinkrodt Chemical Works, Affi-Gel 10 from Bio-Rad, and protein A-Sepharose CL-4B from Pharmacia. Preparation of Antibodies—Mice of C3H/He strain were given intraperitoneally a partially purified preparation of 5-lipoxygenase from porcine leukocytes (10). Spleen cells of the immunized mice were fused with myeloma cells (SP 2/0-Ag14) with the aid of polyethylene glycol according to the method of Godin (11). The fusion mixture was subjected to the "HAT selection" (11), and the hybridoma cells producing anti-5-lipoxygenase antibody were cloned in soft agar by the method of Kennet (12). A clone producing a species...
of antibody referred to as 5Lox-6, 5Lox-1, or 5Lox-9 was grown in the peritoneal cavity of mice, and the antibody in the ascites fluid was precipitated with ammonium sulfate and purified by the use of a protein A-Sepharose column (13). The details of the preparation of the monoclonal anti-5-lipoxygenase will be reported elsewhere. A monoclonal antibody against 12-lipoxygenase of porcine leukocytes [5LOX-6] was prepared as described above.

**Preparation and Assay of Enzyme—**Porcine leukocytes were collected by the method described for the preparation of 12-lipoxygenase (14). About 65 g (wet weight) of leukocytes were obtained from 10 liters of whole blood, and the cells were suspended in 7 times the volume of 20 mM Tris-HCl buffer, pH 7.4, containing 0.5 mM dithiothreitol and 0.5 mM EDTA. The cell suspension was subjected to sonic disruption at 20 kHz for 15 s by the use of a Branson sonifier model 185D. The sonicate was centrifuged at 10,000 × g for 10 min, and the supernatant was further centrifuged at 105,000 × g for 60 min. The high-speed supernatant (350 ml, 7.6 mg of protein/ml) was referred to as the cytosol fraction and could be stored at −20°C for at least 1 month without an appreciable loss of 5-lipoxygenase activity.

For immunofluorescence chromatography of the enzyme, the anti-5-lipoxygenase antibody 5Lox-6 was conjugated to Affi-Gel 10 according to the manufacturer’s instruction. The immunofluorescence coefficients were calculated as described previously (6).

**Preparation of HPETEs—**[1-14C]-5-HPETE was prepared by incubation of the purified 5-lipoxygenase (0.15 mg of protein) with 25 pM [1-14C]-arachidonic acid in a 2 ml reaction mixture with the same composition described for the standard assay. The dried material was repurified by the same procedure. The purity of [1-14C]-5-HPETE thus prepared was examined by TLC (more than 92%). For the preparation of [1-14C]-12-HPETE, 12-lipoxygenase (0.50 mg of protein) was incubated with 40 μM [1-14C]-arachidonic acid in the standard assay mixture (14), which was scaled up to 2 ml as described for the preparation of 5-HPETE. The HPETE eluted from the affinity column was performed with a solvent mixture of petroleum ether/ethyl ether (7:3). The solvent was evaporated, and the residue was treated with diazomethane and then with a (4:1) mixture of ethyl ether/petroleum ether (75:25). UV spectra of the products were recorded as a solution in the above solvent system.

**RESULTS AND DISCUSSION**

**Enzyme Purification by Immunoaffinity Chromatography—**When the cytosol fraction of porcine leukocytes was applied to a column of Affi-Gel 10 to which monoclonal anti-5-lipoxygenase (5Lox-6) was linked, a bulk of protein passed through the column (Fig. 1). The 12-lipoxygenase was found in this fraction, which did not contain 5-lipoxygenase as
examined after removal of 12-lipoxygenase by the use of anti-12-lipoxygenase antibody. Then, the column was washed with sodium carbonate buffer at pH 10.0 containing 0.2% sodium deoxycholate. The 5-lipoxygenase activity was detected in the eluate, but the 12-lipoxygenase activity was not detectable. Elution was performed at 28°C rather than in the cold room at 4°C since proteins came out in sharper peaks. The column could be used for at least 1 year without an appreciable loss of its binding capacity.

This enzyme preparation was allowed to react with [1-14C]arachidonic acid, and the products were analyzed by silica gel TLC as presented in Fig. 2 (lane A). In addition to the major product in band 1, several minor products were found in bands 2–5. When sodium borohydride was added at the end of reaction (lane B), the major product (band 1 in lane A) was converted to a more polar compound (band 2 in lane B), which cochromatographed with authentic 5-HETE. Identity of the reduced product with 5-HETE was confirmed by gas chromatography-mass spectrometry as described below. Thus, the immediate reaction product (band 1 in lane A) was identified as 5-HPETE.

The enzyme reaction as followed by the formation of 5-HPETE did not proceed in a linear fashion. As soon as the reaction started, it slowed down gradually and ceased after about 5 min even though the enzyme was still saturated with the substrate. Thus, a precise determination of the initial velocity was difficult. In routine assays the enzyme activity was determined by a 3-min reaction rather than a prolonged incubation, and expressed in terms of μmol of product/3 min/mg of protein. The specific activity of the purified enzyme determined by 3-min reaction was about 0.6 μmol/3 min/mg of protein and that determined by 1-min reaction was about 0.3 μmol/min/mg of protein. The characteristic time course of suicide-type was also described for other preparations of 5-lipoxygenase (4–7). The final specific activity of the previously purified enzymes was not described in view of such a peculiar time course. Ref. 5 reported a value of 350 ± 100 ng of 5-HETE (5-HPETE)/20 min/μg of protein at 37°C, which was equivalent to about 1.1 μmol/20 min/mg of protein. When the maximal reaction rate at the initial phase is read from the time course presented in Fig. 6 of Ref. 5, the enzyme activity per minute is about 0.3 μmol/min/mg of protein. Considering the difference in the incubation temperature (37 and 24°C), this reported value of the purified enzyme of rat basophilic leukemia cells may be lower than the final specific activity of our enzyme preparation. The other paper (3) also reporting the purification of the enzyme from rat basophilic leukemia cells did not provide us with sufficient parameters which were necessary for the calculation of the specific enzyme activity in terms of μmol/min/mg of protein.

As examined by SDS-polyacrylamide gel electrophoresis (Fig. 3), the enzyme after the first immunochromatography showed a protein band with molecular weight of about 72,000 and several other bands (lane B). Rechromatography of the enzyme under the same conditions brought about a preparation of near homogeneity exhibiting a major protein band with a molecular weight of 72,000 and several band in trace amounts (lane A). The presence of a high activity of 12-lipoxygenase did not allow the determination of the 5-lipoxygenase activity in the cytosol fraction. Therefore, the cytosol fraction was treated with anti-12-lipoxygenase antibody to remove 12-lipoxygenase. By the first immunoafinity chromatography of the 12-lipoxygenase-free cytosol fraction, the enzyme activity was recovered in 50% yield and the total protein in 0.49% yield. This step increased the specific enzyme activity from 6.4 nmol/3 min/mg of protein (the 12-lipoxygenase-free cytosol fraction) to 0.66 μmol/3 min/mg of protein. At the second chromatography the yield of the enzyme activity was 27% and that of protein was 28%, and the specific enzyme activity remained unchanged. Thus, the immunoafinity chromatography was an efficient method to purify the enzyme protein, but rechromatography did not bring about further increase of the specific enzyme activity due to the instability of the purified enzyme as described below. The enzyme purified by the first immunochromatography was used in most of the present work, and the important findings were
FIG. 3. SDS-polyacrylamide gel electrophoresis of 5-lipoxygenase. The 5-lipoxygenase preparations (A) after the second immunochromatography (15 μg) and (B) after the first immunochromatography (45 μg) were concentrated to 50 μl by lyophilization. The pass-through fraction (C) after the first immunochromatography (150 μg) and (D) a molecular weight standard mixture containing myosin from rabbit muscle (molecular weight, 205,000), β-galactosidase from Escherichia coli (116,000), phosphorylase b from rabbit muscle (97,400), bovine albumin (66,000), and egg albumin (45,000). Electrophoresis was carried out as described under "Experimental Procedures," and protein bands were stained with Coomassie Brilliant Blue R-250.

confirmed with the preparation obtained by the second immunochromatography.

The final preparation of the enzyme showed a band corresponding to a molecular weight of 72,000 ± 3,000 (n = 7) upon SDS-polyacrylamide gel electrophoresis with marker proteins. This value was close to the molecular weight of the purified 5-lipoxygenase of rat basophilic leukemia cells reported by one research group (73,000) (5) but somewhat different from the value described by the other group (90,000 as a monomeric enzyme) (3). The protein band was transferred from the polyacrylamide gel to a nitrocellulose membrane, and the blot was stained by the avidin-biotin complex method described under "Experimental Procedures." As compared with the control run without anti-5-lipoxygenase antibody, a colored band was observed selectively at the position corresponding to the purified enzyme with a molecular weight of 72,000 in the presence of any of the three monoclonal anti-5-lipoxygenase antibodies (5Lox-6, 5Lox-1, and 5Lox-9).

The enzyme purified by the first immunoaffinity chromatography was most active around pH 7.5-8.0. When stored at 24 °C, the enzyme was inactivated rapidly. Half of the enzyme activity was lost for about 1 h, and the enzyme was almost inactive after 3 h. The enzyme inactivation was slower at 2 °C, and about 50% of the enzyme activity was retained after 24 h. No appreciable inactivation was observed by storage of the enzyme at −70 °C for 1 week.

Identification of Reaction Products—As shown in lane A of Fig. 2, the production of 5-HPETE (band I) from [1-14C] arachidonic acid was accompanied by several more polar products in small amounts (bands 2–5). Borohydride reduction did not change the positions of bands 3–5 (lane B). Subsequently, the reaction products were reduced and analyzed by reverse-phase HPLC (Fig. 4). As monitored by absorption at 270 nm and then at 240 nm, a doublet peak (Ia and b) appeared with a retention time of 11.9 min and 12.8 min, respectively, followed by two small peaks (IIa and b, 24.1 and 26.2 min). A peak detected at 240 nm (IV) was eluted at a retention time of 47.6 min. The radioactive materials in these peaks were subjected to TLC. Materials from peaks Ia and Ib appeared in the position corresponding to band 4 of the experiment of Fig. 2, and those from peaks IIIa and b migrated to the height of band 5. Peak IV corresponded to band 2. On the other hand, bands 4, 5, and 2 on the TLC plate (Fig. 2, lane A) were scraped, and the ethyl acetate extract from each band was analyzed by HPLC. The extract from bands 4, 5, and 2 gave the same elution profile as described above for peaks Ia and b, IIIa and b, and IV.

Compounds from HPLC peaks Ia and b exhibited UV spectra with an absorption maximum at 269 nm and shoulders at 258 and 280 nm. These spectra were identical with those of authentic 6-trans-LTB4 and its 12-epimer prepared from LTA4. HPLC of authentic 6-trans-LTB4 and its 12-epimer gave peaks at the position of Ia and b. Ref. 19 described separation of 6-trans-LTB4, and its 12-epimer by reverse-phase HPLC on which the former compound was eluted earlier than the other. The mass spectra of compounds Ia and b (trimethylsilyl ether derivative of the methyl ester) showed ion peaks at m/e 494 (M), 479 (M − 15), 404 (M − 90), 383 (M − 111), -CH2-CH=CH-(CH2)-CH3, 293 (M − (111 + 90)), 267, 229, 217, 203, 191, 169, and 129 (base peak), which were described for 6-trans-LTB4, and 12-epi-6-trans-LTB4 (19).

A mixture of compounds IIIa and IIIb exhibited a UV spectrum with a maximum at 272 nm and shoulders at 264 and 283 nm. The spectrum was identical with that of authentic 5,6-diHETE (an epimeric mixture), which gave peaks IIIa and b upon HPLC. Gas chromatography-mass spectrometry of a mixture of compounds IIIa and b (trimethylsilyl ether of methyl ester derivative) gave ion peaks at m/e 494 (M), 479 (M − 15), 463 (M − 31), 404 (M − 90), 291 (loss of -CH(OiMe3)- (CH2)-COOCH3), 203 (base peak, MeSiO+-CH2-CH3), COOCH3), and 171. An identical mass spectrum was recorded with authentic compounds. These UV and mass spectra were previously described for 5,6-diHETE (19). The stereochemical structures of compounds IIIa and b were not determined.

Compound IV showed an absorption maximum at 235 nm.
The mass spectrum of the trimethylsilyl ether derivative of the methyl ester had peaks at m/e 406 (M), 391 (M - 15), 375 (M - 31), 316 (M - 90), 305 (M - 101, loss of -CH(CH)2-COOCH3), 255 (M - 151, loss of -CH3-CH=CH-(CH2)3-COOCH3), 216, 215, and 203 (MeO-SiO2'-CH-(CH2)2-COOCH3). The UV and mass spectra were identical with those of authentic 5-HETE and consistent with those previously described (20).

The compound corresponding to band 3 (Fig. 2, lane A) remains unidentified. Migration of band 3 was not affected by borohydride reduction, ruling out a possibility that the compound in band 3 was a hydroperoxy product. A UV absorbing peak corresponding to (5S,12S)-diHETE was hardly detectable upon HPLC (position indicated by \( \text{II} \) in Fig. 4). This observation was in sharp contrast to the production of (5S,12S)-dihPETF in a larger amount by potato 5-lipoxygenase (21).

The production of 6-trans-LTB4 and its 12-epimer and 5,6-diHETEs suggested an intermediate formation of LTA4, and an attempt was made to trap LTA4 as 12-O-methyl-5,12-diHETE by the method of Borget and Samuelsson (22). After incubation of the enzyme with arachidonic acid, an excess amount of methanol was added to the reaction mixture. Upon reverse-phase HPLC a doublet peak cochromatographing with that of authentic 12-O-methyl-5,12-diHETE (an epimeric mixture) was detected by monitoring absorption at 270 nm. The compounds as a mixture exhibited an absorption spectrum with a maximum at 269 nm and shoulders at 260 and 279 nm. Essentially the same spectrum was described previously (22). The trimethylsilyl ether derivative of the methyl ester gave a mass spectrum with ion peaks at m/e 436 (M), 421 (M - 15), 404 (M - 32), 325 (M - 111, loss of -CH=CH2), and 293 (M - 32 + 111), 235 (M - 111 + 90), and 133. These ions were described previously for 12-O-methyl-5,12-diHETE (22).

Reactivity with 5-HETE—As described above, the reaction of the 5-lipoxygenase with arachidonic acid brought about an accumulation of 5-HETE and several degradation products of LTA4. Therefore, the purified enzyme was incubated with [1-14C]5-HETE, and the products were analyzed by TLC (Fig. 2, lanes D–F). Incubation with a heat-denatured enzyme (lane F) caused a nonenzymatic conversion of part of the added 5-HETE (band 1) to 5-HETE (band 2) and an unidentified compound (band 3). In contrast, 6-trans-LTB4 (a 12-epimeric mixture) and 5,6-diHETE (an epimeric mixture) were observed in bands 4 and 5 only in an enzymatic reaction (lane D). The product profile was unaffected by the addition of sodium borohydride (lane E). The results indicated that the purified preparation of 5-lipoxygenase also catalyzed the conversion of 5-HETE to LTA4 (LTA synthase reaction).

Since the association of the 5-lipoxygenase and LTA synthase activities was reported previously with a 5-lipoxygenase of potato tuber (21), these two enzyme activities were examined with our purified enzyme from porcine leukocytes under various experimental conditions. The LTA synthase reaction proceeded in a time course of suicide-type reaction described above for the 5-lipoxygenase reaction. When the enzyme was kept at various temperatures for 5 min, the 5-lipoxygenase and LTA synthase activities were lost almost in parallel as the temperature was raised (Fig. 5). We earlier reported two types of compounds, a benzoquinone derivative (AA861) (23) and a flavone derivative (circiliol) (24) as inhibitors of 5-lipoxygenase. These compounds at \( \mu \text{M} \) concentrations inhibited 5-lipoxygenase while inhibition of 12-lipoxygenase required much higher concentrations. Effect of these compounds was tested both by the 5-lipoxygenase assay and by the LTA synthase assay. As shown in Fig. 6, both AA861 and circiliol inhibited the LTA synthase reaction as well as the 5-lipoxygenase reaction. Several papers have reported a requirement of calcium ion for 5-lipoxygenase from various animal species (2–7). Calcium ion in the order of millimolar was necessary for 5-lipoxygenase from porcine leukocytes. The LTA synthase activity of the enzyme also required calcium ion. Barium and manganese ions showed about 60% of the effect of calcium ion on both the 5-lipoxygenase and LTA synthase reactions. We earlier reported that ATP and other nucleotides stimulated the calcium-dependent 5-lipoxygenase activity (4, 6). As illustrated in Fig. 7, the LTA synthase activity was also stimulated by the addition of ATP. For technical reasons the assays of both enzyme activities in the experiments described above (Figs. 5–7) were performed at a subsaturation level of substrate (2.5 \( \mu \text{M} \)). Effect of the concentration of arachidonic acid and 5-HETE on the 5-lipoxygenase and LTA synthase activities, respectively, was examined (Fig. 8). Reaction of 25 \( \mu \text{M} \) arachidonic acid or 5-HETE with a heat-denatured enzyme (60° C, 5 min) proceeded to a negligible extent. As compared at 40 \( \mu \text{M} \), the 5-lipoxygenase activity was about 25-fold higher than the LTA synthase activity. Such a big difference of the relative activities of the two enzyme reactions was consistent with an appreciable accumulation of 5-HETE when the enzyme was incubated with arachidonic acid (Fig. 2, lane A). It should be noted that the rate of LTA4 production was higher when the enzyme was incubated with arachidonic acid (crosses and
protein) was assayed for the 5-lipoxygenase activity with 2.5 μM [1-14C]arachidonic acid (closed circles) and the LTA synthase activity with 2.5 μM [1-14C]5-HPETE (open circles) in the presence of various concentrations of ATP. The enzyme activity determined in the presence of 10 mM ATP was expressed as 100%; 5-lipoxygenase, 0.30 nmol/3 min, and LTA synthase, 0.052 nmol/3 min.

broken line in Fig. 8) rather than 5-HPETE, indicating a better coupling of the two enzyme reactions.

Several lines of enzymological evidence support the concept that both the 5-lipoxygenase activity and the LTA synthase activity of the enzyme purified from porcine leukocytes can be attributed to the same single enzyme as proposed and discussed for the potato enzyme (21). Since the LTA synthase activity was much lower than the 5-lipoxygenase activity as described in Fig. 8, the production of 5-HPETE by the 5-lipoxygenase reaction was not efficiently coupled to the LTA synthase reaction. A question may be raised whether the enzyme was modified during the purification procedures resulting in an uncoupling of the two enzyme activities or a certain coupling factor was removed from the enzyme preparation. However, the overproduction of 5-HPETE (mostly detected as 5-HETE) from arachidonic acid was also described with the whole cells (25) and the cytosol (7) of human leukocytes.

Reactivity with 12-HPETE and 15-HPETE—The finding that 5-lipoxygenase of porcine leukocytes was also active with both 12-HPETE and 15-HPETE, prompted us to examine the activity with other HPETEs. The TLC autoradiogram presented in Fig. 9 indicated that the purified enzyme was also active with both 12-HPETE and 15-HPETE. When the enzyme was incubated with [1-14C]12-HPETE, there was a major product (band I in lane A), which was indistinguishable from the compound produced from 5-HPETE by 12-lipoxygenase of porcine leukocytes (lane B). The latter compound was (5S,12S)-dihPTE as preliminarily reported (8). The compound which was produced from 12-HPETE by 5-lipoxygenase and then reduced with sodium borohydride, cochromatographed with the enzymatically prepared (5S,12S)-dihPTE on reverse-phase HPLC. The borohydride-reduced product showed an absorption maximum at 269 nm with shoulders at 260 and 279 nm. The spectrum was previously described (14, 26). These observations together with the following mass spectrum of the borohydride-reduced product (trimethylsilyl ether and methyl ester) supported the identity of the 5-lipoxygenase product from 12-HPETE with (5S,12S)-dihPTE at m/e 479 (M − 15), 463 (M − 31), 404 (M − 90), 383 (M − 111, loss of −CH=CH−CH=CH2), 293 (M − 111 + 90), 267, 229, 217, 203 (Me3SiO−CH=CH2−COOCH3), 171, and 129 (base peak). which were described for (5S,12S)-dihPTE (14, 26). In addition to (5S,12S)-dihPTE thus identified, minor products with a little higher polarity were also observed in lanes A and B, which were presumably derived from (5S,12S)-dihPTE by nonenzymatic reduction. There was no conversion of 12-HPETE with heat-denatured 5-lipoxygenase (60 °C, 5 min).

The enzyme was also active with 15-HPETE as shown in lane C in Fig. 9. Heat-denatured enzyme (60 °C, 5 min) was totally inactive. The reaction product (band II in lane C) comigrated with (5S,15S)-dihPTE prepared from 15-HPETE by soybean lipoxygenase (band II in lane D). Reverse-phase HPLC showed the same retention time for the 5-lipoxygenase product from 15-HPETE and the 15-lipoxygenase product from 5-HPETE, both of which were reduced with sodium borohydride to more polar compounds with the same polarity. Identification of both of the reduced compounds as (5S,15S)-dihPTE was confirmed by their spectra with ab-
sorption maxima at 244 nm (18) and mass spectra (trimethylsilyl ether and methyl ester) (18) as follows; at m/e 494 (M), 479 (M – 15), 463 (M – 31), 404 (M – 90), 394, 393 (M – 101, loss of -(CH2)7-COOCH3), 332 (M – 90 and 71), 314 (M – 2 x 90), 225 (-(CH=CH)-2CHOSiMe2)-(CH2)-CH3), 203 (Me3SiO’=CH-(CH2)3-COOCH3) and 173 (base peak, Me3SiO’=CH-(CH2)3-CH3). In addition to (5S,15S)-dihydroperoxyesters (HPETEs) as a major product, there were minor products a little below band II, which were presumably produced by nonenzymatic reduction of hydroperoxy groups. Moreover, trace amounts of unidentified products were observed midway between band II and the origin.

Relative activities of the 5-lipoxygenation with arachidonic acid, 12-HPETE, and 15-HPETE are shown in Fig. 8. Both 12-HPETE and 15-HPETE were oxygenated by the purified 5-lipoxygenase from porcine leukocytes with 22 and 30% maximal velocity of the arachidonate 5-lipoxygenation, respectively.

Although the enzyme protein was highly purified by immunoaffinity chromatography, instability of the purified enzyme hindered a reasonable increase in the specific enzyme activity, for example, phosphatidylcholine as reported by Goetze et al. (5). Results of our investigations with such a purified enzyme demonstrated reactivity of 5-lipoxygenase with various HPETEs. The reaction with 5-HPETE showed the LTA synthase activity, and association of the LTA synthase activity with the 5-lipoxygenase activity was supported by several experiments. It should be carefully considered whether or not the LTA synthase activity associated with the purified 5-lipoxygenase can account for most of the activity in the cytosol fraction. Although a precise determination of the LTA synthase activity in the cytosol fraction was difficult due to the presence of a certain peroxidase activity which decomposed 5-HPETE added as such or generated from arachidonic acid and the incubation of arachidonic acid with the 12-lipoxygenase-free cytosol fraction accumulated 5-HPETE (part of which was reduced to 5-HETE), LTA4 as judged by its degradation products was produced only in a small amount as in the reaction with the purified 5-lipoxygenase. The pass-through fraction at the immunoaffinity chromatography step had a hardly detectable activity of LTA synthase after removal of 12-lipoxygenase by the use of its antibody. These observations may rule out the presence of a separate LTA synthase in the cytosol fraction. The enzyme activity of 12-lipoxygenase to synthesize 14,15-epoxy compound of LTG type was predicted as in the reaction with the purified 5-lipoxygenase. The pass-through fraction at the immunochromatography step had a characteristic requirement of the LTA synthase activity and a characteristic requirement of the enzyme from such a casual activity of contaminating hemoprotein. The enzyme was also active with 12-HPETE and 15-HPETE. The versatile reactivity with various HPETEs is also the case of other lipoxygenases, i.e. rabbit reticulocyte 15-lipoxygenase (29), porcine leukocyte 12-lipoxygenase (8, 27), and soybean lipoxygenase (30). Experiments which allow a precise chemical insight into the reaction mechanism of the enzymes, for example, the stereoselective hydrogen abstraction from the substrates, are required to understand the apparently versatile function of these lipoxygenases by a simplified principle.

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REFERENCES


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