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Synthesis of 8,9-leukotriene A_4 by murine 8-lipoxygenase ☆

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Abstract

Arachidonate 8-lipoxygenase was identified in phorbol ester induced mouse skin. We expressed the enzyme in an *Escherichia coli* system using pET-15b carrying an N-terminal histidine-tag sequence. The enzyme, purified by nickel–nitrilotriacetate affinity chromatography, showed specific activity of about 0.1 μmol/min/mg of protein with arachidonic acid as a substrate. When metabolites of arachidonic acid were reduced and analyzed by reverse-phase HPLC, 8-hydroxy derivative was a major product as measured by absorbance at 235 nm. In addition, three polar compounds (I, II, and III) were detected by measuring absorbance at 270 nm. These compounds were also produced when the enzyme was incubated with 8-hydroperoxyeicosa-5,9,11,14-tetraenoic acid. Neither heat-inactivated enzyme nor mutated enzyme produced these compounds, suggesting that they are enzymatically generated. Ultraviolet spectra of these compounds showed typical triplet peaks around 270 nm, indicating that they have a triene structure. Molecular weight of these compounds was determined to be 336 by liquid chromatography–mass spectrometry, indicating that they carry two hydroxyl groups. Compounds I and III were generated even under anaerobic condition, indicating that oxygenation reaction was not required for their generation from 8-hydroperoxyeicosa-5,9,11,14-tetraenoic acid. By analogy to the reactions of 5-lipoxygenase pathway where leukotriene A_4 is generated, it is suggested that 8-hydroperoxyeicosa-5,9,11,14-tetraenoic acid is converted by the 8-lipoxygenase to 8,9-epoxyeicosa-5,10,12,14-tetraenoic acid which degrades to compounds I and III by non-enzymatic reaction. In contrast, compound II was not generated under anaerobic condition, indicating that it was produced by oxygenation reaction. Taken together, 8-lipoxygenase catalyzes both dehydration reaction to yield 8,9-epoxy derivative and oxygenation reaction presumably at 15-position of 8-hydroperoxyeicosa-5,9,11,14-tetraenoic acid.

Keywords: Lipoxygenase; 8-Lipoxygenase; Arachidonic acid; Leukotriene; Expression

Lipoxygenases (LOXs) are a family of dioxygenases which catalyze the regio- and enantio-selective insertion of molecular oxygen into polyunsaturated fatty acids. Using arachidonic acid as a substrate, 5-, 8-, 12-, and 15-LOXs were identified in mammalian cells [1–3]. The 8-LOX was first found in mouse epidermis treated with phorbol ester [4,5]. The cDNA of the enzyme was cloned from phorbol ester-treated mouse skin [6,7]. The enzyme consists of 677 amino acids and shows 78% amino acid identity to human 15-LOX-2 which was cloned from human hair follicle [8]. The 8-LOX is also expressed in mouse brain as assessed by Northern blot analysis [6]. The enzyme was also found in *Aplysia californica* [9] and *Pseudoplexaura porosa* [10]. Amino acid determinants of the positional specificity of mouse 8-LOX and 15-LOX-2 were identified [11]. The reaction of 8-LOX was initiated by removal of the pro-10*R*-hydrogen from arachidonic acid [12], and

☆ *Abbreviations:* LOX, lipoxygenase; HPETE, hydroperoxyeicosatetraenoic acid; 8,9-LTA_4, 8,9-epoxyeicosa-5,10,12,14-tetraenoic acid; HPLC, high-performance liquid chromatography.

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the enzyme was shown to convert 5-hydroperoxyeicosatetraenoic acid (HPETE) to leukotriene A_4 by the identical hydrogen abstraction from 5S-HPETE [13]. In this paper, we showed that 8-LOX catalyzed dehydration of 8-HPETE to yield 8,9-epoxyeicosa-5,10,12,14-tetraenoic acid (8,9-LTA_4) as a putative intermediate which degraded to racemic dihydroxy compounds. We also demonstrated that the 8-LOX catalyzed 15-lipoxygenation reaction when 8-HPETE was used as a substrate.

Materials and methods

Expression of 8-lipoxygenase. Mouse 8-LOX cDNA was prepared as described previously [13]. The cDNA was ligated into *Bam*HI site of pET-15b vector (Novagen) containing histidine-tag sequence at the N-terminal portion. *E. coli* strain BL21 (DE3) was transformed with the expression plasmid. The transformed *E. coli* was cultured in LB(−) until the absorbance 0.6–0.8 at 600 nm was attained, and expression of the enzyme was induced by adding 0.1 mM isopropyl-thio-β-D-galactopyranoside at 20 °C for 2 h. *E. coli* (3 L) were harvested by centrifugation and resuspended in 120 ml of 50 mM potassium phosphate buffer at pH 7.4 containing 0.3 M sodium chloride and 20 mM imidazole. The cells were disrupted by sonication three times each for 30 s using a Branson sonifier model 250. The sonicate was centrifuged at 100,000*g* for 60 min, and the supernatant solution was subjected to Ni–NTA column chromatography under manufacturer's instructions (Qiagen), except that the buffer for washing and elution contained 20% glycerol to stabilize the enzyme. SDS–polyacrylamide gel electrophoresis was performed by the method of Laemmli [14].

Site-directed mutagenesis of mouse 8-LOX cDNA was performed by polymerase chain reaction according to the method of Higuchi et al. [15]. His374 (CAT) was replaced with Leu (CTT) or His379 (CAT) with Leu (CTT). The nucleotide sequences of the mutants were confirmed by dideoxy sequencing [16].

Reaction of the enzyme with arachidonic acid and 8-HPETE. The purified enzyme was incubated with 25 μM arachidonic acid (Sigma) in a final volume of 1 ml containing 50 mM potassium phosphate buffer at pH 7.4% and 0.02% Tween 20. The production of 8-HPETE was monitored by measuring the absorbance at 235 nm due to a conjugated diene using a Beckman spectrophotometer DU-640. A molecular extinction coefficient of 27,000 was used to calculate the amount of 8-HPETE [17].

8-HPETE was prepared by the incubation of the purified 8-LOX and arachidonic acid. Extract of reaction mixture was subjected to thin-layer chromatography with a solvent mixture of diethyl ether/petroleum ether/acetic acid (85/15/0.1, by volume) at 4 °C. 8-HPETE was located by UV illumination, eluted from the silica gel with ethyl acetate, and dissolved in a small volume of ethanol.

Anaerobic reaction was carried out by continuous purging nitrogen gas. The purified 8-LOX (5 μg) was incubated with 25 μM 8-HPETE in a volume of 0.2 ml in 50 mM potassium phosphate buffer at pH 7.4 for 5 min at 37 °C. To reduce the metabolites, glutathione peroxidase (0.1 unit), and 5 mM glutathione were added to the mixture followed by incubation for another 10 min. PGB_2 (0.5 nmol) was added to the reaction mixture as an internal standard. The metabolites were extracted by Sep-Pak C18 and dissolved in ethanol.

High-performance liquid chromatography (HPLC). Reverse-phase HPLC was performed using a TSK ODS-120T column (5-μm particle, 4.6 × 250 mm) and a solvent mixture of methanol/water/acetic acid (68:32:0.01, by volume) at a flow rate of 1 ml/min. Absorption at 270 nm was monitored for 45 min and at 235 nm thereafter.

Liquid chromatography–mass spectrometry was performed by electrospray ionization, and molecular weights were determined by a Waters ZMD mass spectrometry. The ultraviolet spectrum of the metabolites of 8-HPETE by 8-LOX was measured using a Waters diode array detector model 996.

Results and discussion

Expression and purification of 8-lipoxygenase

An expression vector for mouse 8-LOX cDNA was constructed using pET-15b plasmid (Fig. 1A). As shown in Fig. 1B, the purified enzyme showed a single band with a molecular weight of approximately 75 kDa as analyzed by SDS–polyacrylamide gel electrophoresis. Fig. 1C shows enzyme activity as monitored by measuring the absorbance at 235 nm due to a conjugated diene. The specific activity of the purified enzyme was approximately 0.1 μmol/min/mg of protein. The 8-LOX reaction proceeded lineally only at the beginning, and the reaction rate decreased gradually. However, the reaction continued for approximately 20 min. This is a sharp contrast to the case of porcine 12-LOX, the reaction of which ceases within a few minutes by suicide inactivation [1,13]. When a larger amount of

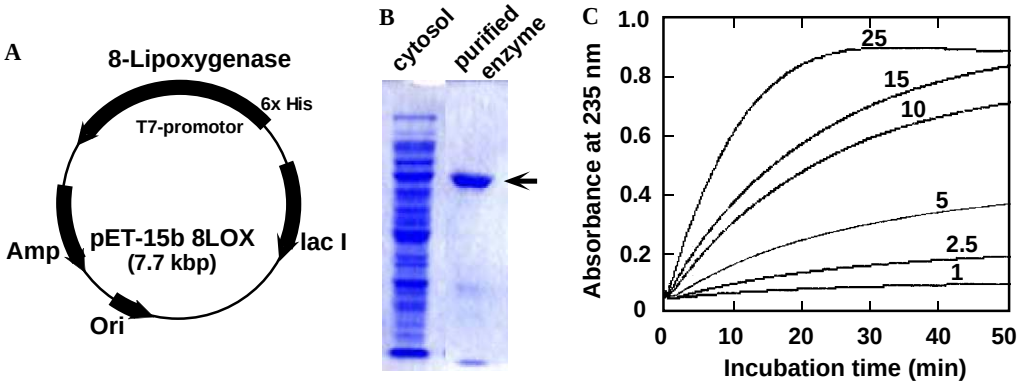

Fig. 1. Expression of mouse 8-LOX in *E. coli*. (A) An expression vector for mouse 8-LOX was constructed using pET-15b. (B) *E.coli* was transformed with the expression vector, and the high-speed supernatant was prepared after sonication. The enzyme was purified by Ni–NTA column chromatography and subjected to SDS–polyacrylamide gel electrophoresis; high-speed supernatant of *E. coli* sonicate (16 μg), the purified enzyme (1.3 μg). An arrow indicates the position of 75 kDa. (C) Various amounts (in μg) of purified 8-LOX were incubated with 25 μM arachidonic acid, and absorbance at 235 nm due to a conjugated diene of 8-HPETE was spectrophotometrically monitored.

the enzyme (25 μg) was used, the reaction leveled off around 30 min due to a complete consumption of 25 μM arachidonic acid, and the absorbance decreased thereafter.

Reaction of the enzyme with arachidonic acid and 8-HPETE

The purified 8-LOX was incubated with arachidonic acid, and the product was analyzed by reverse-phase HPLC as shown in the upper panel of Fig. 2. Three peaks (referred to as compounds I, II, and III) were detected between 16 and 21 min in addition to 8-HETE eluted around 85 min. The amount of compounds I and III was almost the same, and that of compound II was several fold greater than that of compound I or III. Total amount of these compounds was estimated to be approximately 10% of that of 8-HETE. The UV spectrum of these compounds showed triplet peaks around 270 nm as shown in the lower panel of Fig. 2, suggesting that these compounds have a conjugated triene. When molecular weight of these compounds was determined by liquid chromatography–mass spectrometry, it was calculated to be 336 for all of these compounds.

In order to examine the mechanism of biosynthesis of compounds I–III, several experiments were carried out. When the 8-LOX was incubated with 8-HPETE, compounds I–III were also produced as shown in Fig. 3A. The result indicates that the compounds I–III were produced from 8-HPETE, a primary product of arachidonic acid by 8-LOX. Neither heat-inactivated enzyme nor site-mutated enzyme (H374L) produced these compounds (Figs. 3B and D respectively). Essentially identical result was obtained with another mutated enzyme (H379L, data not shown). These results clearly indicated that compounds I–III were enzymatically generated from 8-HPETE. The UV spectrum and molecular weight of the compounds

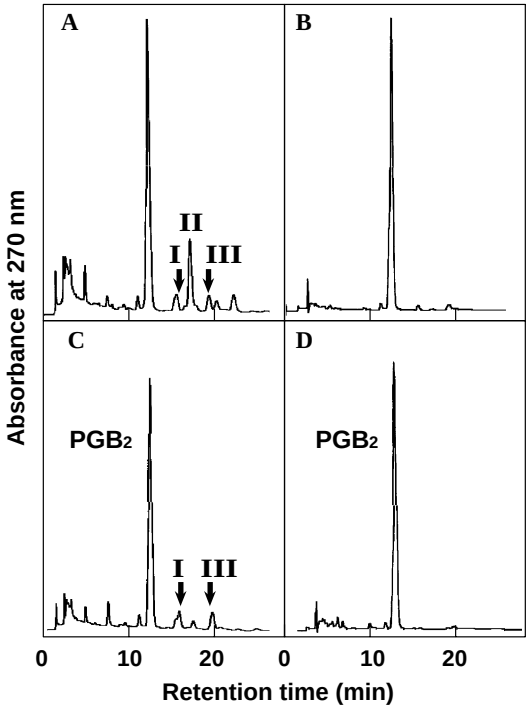

Fig. 3. Enzymatic transformation of 8-HPETE to the compounds I–III. (A) The purified enzyme was incubated with 8-HPETE. Reverse-phase HPLC was performed as in Fig. 2. (B) Heat-inactivated enzyme was used. (C) The reaction was carried out under anaerobic condition. (D) Used was the mutated (H374L) enzyme devoid of activity.

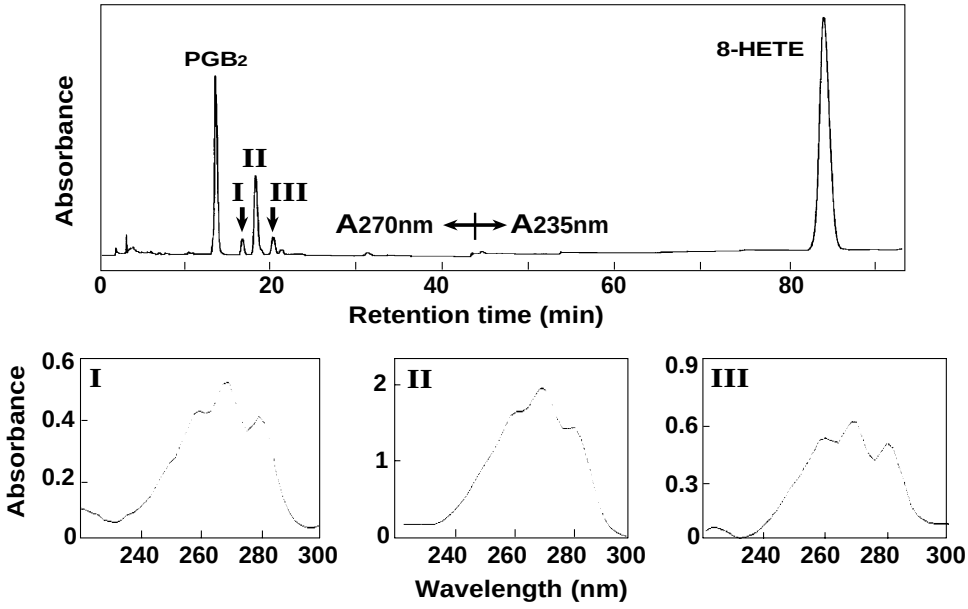

Fig. 2. Reverse-phase HPLC analysis of 8-LOX reaction products. Upper: the purified enzyme was incubated with arachidonic acid, and the reduced metabolites were subjected to reverse-phase HPLC. Absorbance at 270 nm was monitored for 45 min and at 235 nm thereafter. PGB_2 was included as an internal standard. Lower: UV spectrum of metabolites I–III. The compounds I, II, and III show typical triplet peaks around 270 nm, indicating that they have triene structure.

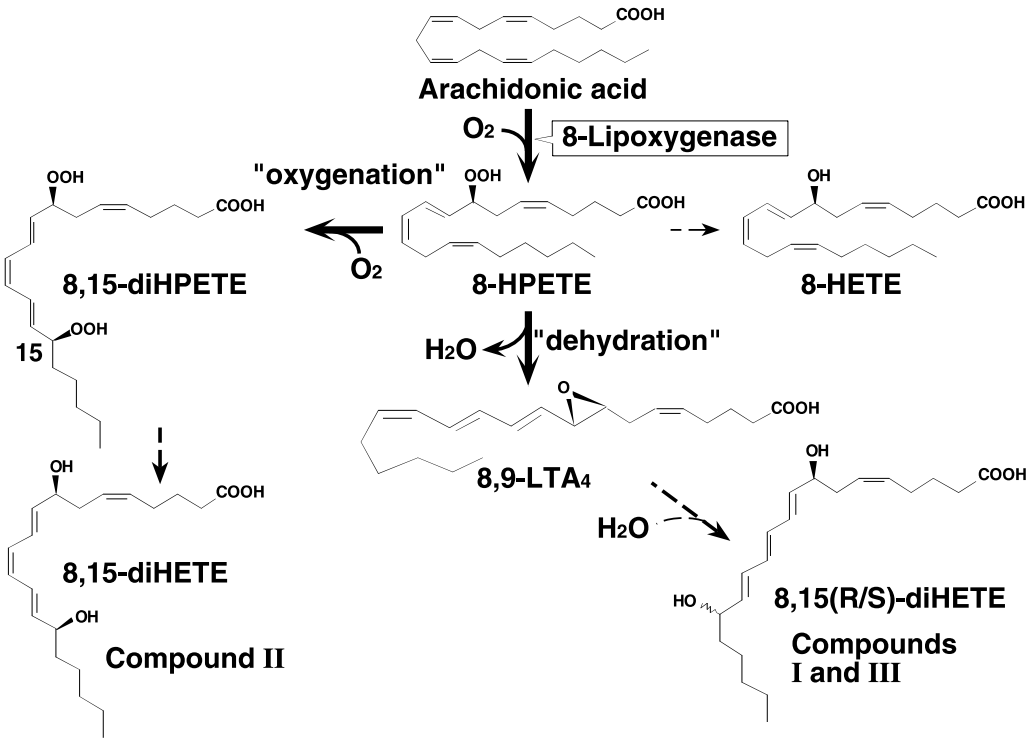

Fig. 4. Metabolism of arachidonic acid by 8-LOX. The enzyme catalyzes not only 8-LOX reaction but also dehydration of 8-HPETE producing 8,9-LTA_4 as a putative intermediate which degrades to a racemic mixture of dihydroxy compounds. The enzyme also catalyzes 15-lipoxygenation of 8-HPETE producing 8,15-diHPETE.

I–III suggest that they carry a conjugated triene with two hydroxyl groups. We then performed the reaction under anaerobic conditions to examine whether the oxygenase reaction is necessary for the production of these compounds. As shown in Fig. 3C, compound II was not produced under anaerobic condition, whereas compounds I and III were generated under both aerobic and anaerobic conditions (Figs. 3A and C). These results suggest that molecular oxygen is required for the production of compound II, but not for compounds I and III. As summarized in Fig. 4, our experiments suggest that the enzyme catalyzes not only 8-LOX reaction but also the conversion of 8-HPETE to 8,9-LTA_4 as an intermediate compound which is non-enzymatically hydrolyzed to dihydroxy racemic compounds I and III. The compound II appears to have a conjugated triene and two hydroxyl groups, and the generation of this compound from 8-HPETE required molecular oxygen (Fig. 3C). These results suggest that molecular oxygen is introduced into 8-HPETE generating diHPETE which is reduced by glutathione peroxidase being added after the reaction. It was previously reported that mouse 8-LOX expressed in *E. coli* converted arachidonic acid to 8S-HPETE and then to 8S,15S-diHPETE [18]. It is reasonable to assume that hydrogen abstraction at 13 position of 8-HPETE is initiated by the 8-LOX followed by the insertion of molecular oxygen into 15 position yielding 8,15-diHPETE. It should be mentioned that porcine leukocyte 12-LOX catalyzed 8-lipoxygenation in a similar fashion with 15-HPETE as a substrate yielding 8S,15S-diHPETE [19]. In conclusion, this study suggests that 8-LOX catalyzes both dehydration and 15-lipoxygenation of 8-HPETE in addition to 8-lipoxygenation of arachidonic acid.

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