Articles

Biosynthesis of Conjugated Triene-Containing Fatty Acids by a Novel Isomerase from the Red Marine Alga *Ptilota filicina*†

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ABSTRACT: The biosynthesis of conjugated triene-containing fatty acids by the red alga *Ptilota filicina* is catalyzed by a novel enzyme, polyenoic fatty acid isomerase. The enzyme has been highly purified and is described here for the first time. Matrix-assisted laser-induced desorption mass spectrometry was used to determine that the major protein in the purified enzyme is composed of similar or identical subunits of M_r 58 119 Da. The native enzyme emerges with an apparent M_r of 174 000 Da from a gel permeation chromatography column. While this enzyme catalyzes the formation of conjugated trienes from a variety of polyunsaturated fatty acid precursors [arachidonate ((5*Z*,8*Z*,11*Z*,14*Z*)-eicosatetraenoate) is converted to (5*Z*,7*E*,9*E*,14*Z*)-eicosatetraenoate; γ-linolenate ((6*Z*,9*Z*,12*Z*)-octadecatrienoate) is converted to 6*Z*,8*E*,-10*E*-octadecatrienoate], this occurs most rapidly with eicosapentaenoate [(5*Z*,7*E*,9*E*,14*Z*,17*Z*)-eicosapentaenoate], which is likely the native substrate. Through a series of experiments utilizing γ-linolenates stereospecifically labeled with deuterium, we have determined that the enzyme intramolecularly transfers the bis-allylic *pro-S* hydrogen from the C11 position to the C13 position. Furthermore, the bis-allylic *pro-R* hydrogen at C8 in γ-linolenate is lost to the solvent. Using arachidonate as substrate, we demonstrated that the C11 olefinic position becomes protonated by a solvent-derived proton. There appears to be no requirement for molecular oxygen, and the transformation is catalyzed by this single enzyme.

There are numerous reports on the occurrence of conjugated dienoic, trienoic, and tetraenoic fatty acids in plants [e.g., Morris and Marshall (1966), Takagi and Itabashi (1981), Spitzer et al. (1991)], most of which are 18-carbon compounds originating from oleic acid [(9*Z*) -octadecenoic acid], linoleic acid [(9*Z*,12*Z*)-octadecadienoic acid], linolenic acid [(9*Z*,12*Z*,15*Z*)-octadecatrienoic acid], or stearidonic acid [(6*Z*,9*Z*,12*Z*,15*Z*)-octadecatetraenoic acid]. The biosynthesis of these conjugated polyenes from their fatty acid precursors, however, is still poorly understood. One of the more thorough investigations to date regarding the biosynthesis of conjugated polyenes in plants was by Crombie and Holloway (1985) who examined the conversion of oleic acid and linoleic acid to calendic acid (**1**) [(8*E*,10*E*,12*Z*)-octadecatrienoic acid] by cell-free homogenates of germinating marigold seeds. Using combinations of regiospecifically labeled oleic and linoleic acids, they were able to demonstrate that the conversion of linoleic acid occurred via removal of two hydrogens, one each from the C8 and C11 positions. There appeared to be no involvement of an oxygenated intermediate.

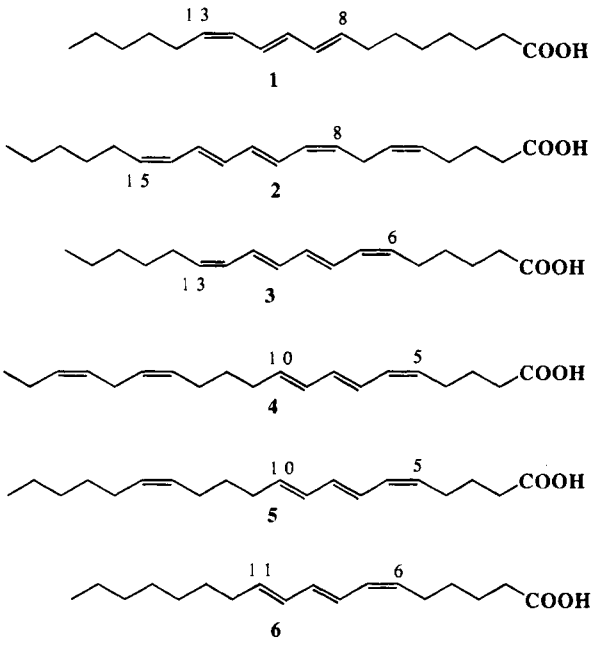

FIGURE 1: Structures of selected conjugated polyenoic fatty acid metabolites.

Burgess et al. (1991), studying arachidonic acid metabolism by cell-free preparations of the red marine alga *Bossiella orbigniana,* produced (5*Z*,8*Z*,10*E*,12*E*,14*Z*)-eicosapentaenoic acid (**2**) to which they assigned the trivial name bosseopentaenoic acid (BPA). They speculated that the biosynthetic pathway involves a 12-lipoxygenase or cytochrome P450

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catalyzed oxygenation because the cell-free biosynthesis of BPA from arachidonate required molecular oxygen. Gerwick et al. (1993), working with a related red marine alga, *Lithothamnion corallioides*, found this same conjugated tetraene to be produced in cell-free homogenates incubated with arachidonic acid. Subsequently, Hamberg (1992), using C18 polyunsaturated fatty acid precursors, showed that *L. corallioides* possesses a unique oxidase-type activity which forms the conjugated tetraene functionality without production of oxygenated intermediates. The enzyme does, however, require molecular oxygen for its activity. Using stereospecifically deuterated γ-linolenic acid [(6*Z*,9*Z*,12*Z*)-octadecatrienoic acid], it was shown that the enzyme oxidatively removes the *pro-S* and *pro-R* hydrogens at C8 and C11, respectively, resulting in the formation of (6*Z*,8*E*,-10*E*,12*Z*)-octadecatetraenoic acid (**3**).

Our interest in this area of metabolism stems from our findings of the past several years that many marine algae metabolize PUFAs[1] to a wide range of unusual and structurally unique oxylipins[2] (Gerwick et al., 1993a), as well as known eicosanoids such as 12*S*-HETE (Gerwick & Bernart, 1993) and hepoxilin B_3 (Moghaddam et al., 1990). In addition to oxidized fatty acid products containing oxygen derived from molecular oxygen, several metabolites with conjugated polyene systems have also been isolated. For example, during the course of our investigations of the natural product chemistry of *Ptilota filicina*, a temperate red alga from the Oregon coast, we discovered a previously undescribed conjugated eicosanoid, (5*Z*,7*E*,9*E*,14*Z*,17*Z*)-eicosapentaenoic acid (**4**) (Lopez & Gerwick, 1987). We were interested in examining the mechanisms of the isomerization reactions leading to its formation from a proposed EPA precursor. This conversion of EPA involves, in essence, a double isomerization in that the C8−C9 double bond is transferred to the C7−C8 position and the C11−C12 double bond migrates two carbons to form the C9−C10 double bond (Scheme 1). We have successfully isolated the enzyme responsible for this novel isomerization and in this paper provide evidence for the regio- and stereochemistry of the proton transfers and describe some of the characteristics of the protein.

Scheme 1: PFI-Catalyzed Conversion of EPA to the Conjugated Triene (5*Z*,7*E*,9*E*,14*Z*,17*Z*)-Eicosapentaenoic Acid

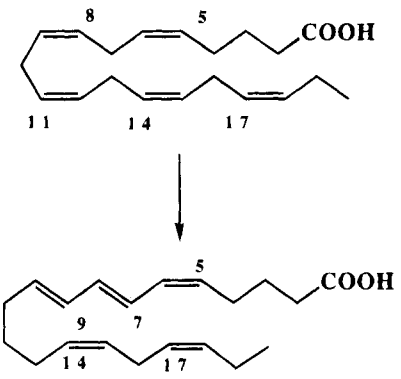


Table 1: Summary of a Typical Purification of PFI from Approximately 55 g (Wet Weight) of Frozen Tissue[a]

purif. step	total act. (μmol min^{-1})	sp act. (μmol min^{-1} mg^{-1})	unit yield (%)	total protein (mg)	purif. factor
S-100	1.15	0.010	100	110	1
55−90% $(NH_3)_2SO_4$	0.54	0.035	47	15.6	3.3
S-300 gel filtration	0.41	0.31	44	1.3	30
IEF	0.15	1.06	13	0.14	102

[a] The high-speed supernatant (S-100) fraction is taken as the initial purification step (the crude homogenate is not amenable to spectrophotometric analysis due to an excess of UV-absorbing material).

MATERIALS AND METHODS

Enzyme Isolation. Ptilota filicina was collected during low tide at Devil's Punchbowl on the Oregon coast on 26 Aug 1991. The freshly harvested plants were immediately frozen on dry ice and transported back to the laboratory, where they were stored at −70 °C. To prepare the enzyme, the frozen tissue, approximately 50 g, was ground in a stainless steel Waring blender with liquid nitrogen. The powdered tissue was slurried with approximately 2 vol of extraction buffer (100 mM NaH_2PO_4, 5 mM EGTA, 5 mM DTT, and 5 mM $MgCl_2$, pH 6.5) and homogenized with an Ultraturrex followed by a Potter-Elvehjem tissue homogenizer. The homogenate was then filtered through Miracloth (Calbiochem) and centrifuged at 12000*g* for 20 min. The supernatant was centrifuged at 100000*g* for 60 min. This high-speed supernatant was then precipitated with ammonium sulfate, and the material precipitating between 50% and 90% saturation was resuspended in a minimal volume of 100 mM NaH_2PO_4, pH 6.5 (approximately 5 mL). This material was passed down a 91 × 2.5 cm Pharmacia Sephacryl S-300 size-exclusion column and eluted with 20 mM NaH_2PO_4, pH 7.2. The fractions showing PFI activity were pooled and concentrated to approximately 2.0 mL using an Amicon Centriprep concentrator with a 30K molecular weight cutoff. The concentrated protein from size-exclusion chromatography was then subjected to preparative isoelectric focusing (IEF) using a Rainin RF-3 protein fractionator and Pharmacia Pharmalyte ampholytes, pH 4.0−6.5 (1% ampholytes in H_2O with 10% glycerol). The active fractions from IEF (pH 4.4−4.8) were pooled, and the buffer was exchanged with 20 mM NaH_2PO_4, pH 7.2, by three cycles of concentration and dilution with ice-cold buffer using an Amicon Centriprep 30K concentrator (see Table 1).

Enzyme Assay. Activity was assayed spectrophotometrically on a Hewlett-Packard 8452A diode array spectrophotometer by a modification of the method for lipoxygenase (Ben-Aziz et al., 1970). To a mixture of 100 mM NaH_2PO_4, pH 7.2, with 0.02% Tween 20 (reaction buffer) was added an appropriate amount of enzyme solution (5−100

[1] Abbreviations: BSA, bovine serum albumin; BPA, bosseopentaenoic acid; COSY; correlation spectroscopy; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EPA, eicosapentaenoic acid; EtOH, ethanol; FT-IR, Fourier transform infrared; GC/MS, gas chromatography/mass spectrometry; HETE, hydroxyeicosatetraenoic fatty acid; HPLC, high-performance liquid chromatography; IEF, isoelectric focusing; KIE, kinetic isotope effect; MALDI/MS, matrix-assisted laser-induced desorption/ionization mass spectrometry; MeOH, methanol; NMR, nuclear magnetic resonance; PFI, polyenoic fatty acid isomerase; PUFA, polyunsaturated fatty acids; RT, room temperature; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TMS, tetramethylsilane; UV/vis, ultraviolet/visible.

[2] Oxylipins are metabolites of unsaturated fatty acids of any chain length which undergo at least one O_2-dependent oxidation during the course of their biosynthesis (Gerwick, 1993; Gerwick et al., 1991).

μL), and the spectrophotometer was blanked. Reaction was initiated by adding 40 μg of arachidonic acid in 10 μL of ethanol to make a final volume of 1.0 mL with 131 μM arachidonate. Product formation was measured by monitoring absorbance at 278 nm. A unit of activity is defined as the amount of enzyme catalyzing the formation of 1 μmol of triene product per minute, using extinction coefficient ϵ = 57 000 L mol^{-1} cm^{-1} (Lopez & Gerwick, 1987). With appropriate conditions the reaction is essentially linear for several minutes. Protein determinations were done using the Bradford method (Bradford, 1976). Lipid-free BSA fraction V was used as the standard.

Molecular Mass Determinations. The mass of the native enzyme was determined by size-exclusion chromatography using Sephacryl S-300 (Pharmacia) on a 1.5 × 33 cm column. The following molecular mass standards were employed: apoferritin (443 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa) and cytochrome *c* (12.4 kDa) (Sigma). Elution buffer was 100 mM NaH_2PO_4, pH 7.2, with and without 100 mM NaCl (both gave the same results). The PFI preparation used had been subjected to ammonium sulfate precipitation, S-300 gel filtration, and concentration with a Centriprep 30K concentrator. Subunit mass of the IEF-purified material was determined by two methods, SDS−PAGE and matrix-assisted laser desorption/ionization mass spectrometry (MALD/IMS). Discontinuous gel electrophoresis was performed by the method of Laemmli (1970) on a 10% running gel and a 3% stacking gel with 0.6 M Tris in the running gel to reduce the spread of high-salt fractions. Sigma mark VIIL molecular mass standards were used. The gel was silver stained (Wray et al., 1981). MALD/IMS with time of flight detection was performed using sinapinic acid as the matrix (Karas & Hillenkamp, 1988; Jenson et al., 1993). Bovine serum albumin (M_r = 66.4 kDa) served as an internal standard.

Reaction under Anaerobic Conditions. A 5.5-mL volume of the reaction buffer plus 5% glucose was placed in a 15-mL three-neck flask with 1.4 × 10^{-3} unit of PFI (in 20 μL of NaH_2PO_4 buffer) and capped with a serum bottle septum. The flask was then subjected to three 15-min cycles of vacuum and purging with dry N_2. Under a positive pressure of dry N_2, 0.5 unit of glucose oxidase (Sigma G6125) was injected with a syringe through the septum for an additional 30 min to remove any residual O_2. The reaction of PFI was initiated by adding 0.2 mg of arachidonate in 50 μL of EtOH, again using a syringe and keeping the reaction vessel under pressure with N_2. At timed intervals 0.5-mL aliquots of the solution were removed, the reaction was quenched with 1.0 mL of MeOH, and the absorbance at 272 nm was measured (the triene λ_{max} in MeOH). A control reaction was performed in an identical manner but without sealing the vessel. Both reaction mixtures were stirred constantly at 23 °C.

Lipid Extraction. In experiments where reaction products were examined by NMR or GC/MS the lipid material was extracted in the following manner. The reaction was quenched with 5 vol of MeOH; then sufficient H_2O was added to make a final solution of MeOH/H_2O (50:50). This MeOH/H_2O solution was extracted with an equal volume of diethyl ether; the remaining aqueous phase was acidified to approximately pH 4.0 (determined with pH paper) by dropwise addition of 10% HCl. The acidified aqueous methanol solution was extracted three more times with diethyl ether. The ether extracts were pooled, washed twice with approximately 0.1 vol of H_2O, and reduced under vacuum using 100% ethanol as an azeotrope as necessary. The extracted lipids were resuspended in a small (1−3 mL) volume of MeOH and methylated with ethereal diazomethane.

Isolation of the triene methyl esters was accomplished by HPLC using two in-line Alltech Versapak 10 μM, 30 cm × 4 mm silica columns with a Waters M-6000 chromatography pump and a Model 480 LC spectrophotometer detector set at 272 nm. Elution was accomplished with 2% EtOAc in hexanes at a flow rate of 4.0 mL min^{-1} (3000 psi). The triene methyl ester eluted just after the arachidonate methyl ester in a volume of 40−45 mL.

GC/MS Analysis. All GC/MS analyses were performed on the methyl esters of the fatty acids. These were suspended in hexanes at a concentration of 20−50 ng μL^{-1}, and 1−2 μL was injected onto the column. Gas chromatography was performed on a Hewlett-Packard 5890 Series II gas chromatograph with a HP 5971A quadrupole mass selective detector interfaced with the Hewlett-Packard Chemstation using G1034B software for data analysis. An 11.5-m Ultra-1 open capillary column was used, with an initial temperature at 70 °C and a 20 °C min^{-1} ramp to a final temperature of 250 °C. Helium was used as the carrier gas.

Inhibitor Studies. The following lipoxygenase, prostaglandin H synthase, or cytochrome P-450 inhibitors were evaluated for their inhibitory activity with the enzyme at the concentration(s) indicated: acetylsalicylic acid, 330 μM; baicalein, 12 μM; dipyridamole, 10 and 100 μM; eicosatetraynoic acid (ETYA), 100 μM; esculetin, 50 μM; indomethacin, 56 μM; naproxen, 100 μM; NDGA, 15 μM; SKF 525A, 50 and 100 μM. Each inhibitor was made up in EtOH to allow introduction of the appropriate amount into a 1-mL reaction vessel in 5 μL (or less). The enzyme was preincubated with the inhibitor for at least 5 min before adding arachidonate (in 5 μL of EtOH; final concentration, 131 μM). The rate of change in absorbance at 278 nm was compared to a control assay using 5 μL of EtOH instead of inhibitor. Each assay was performed two or three times.

The effects of EDTA and *o*-phenanthroline were also evaluated. Aliquots of the partially purified enzyme were incubated for 2.5 h at RT in 100 mM NaH_2PO_4 (pH 7.2) with 5 mM EDTA. Comparisons of enzyme activity were by triplicate assay. For *o*-phenanthroline, the enzyme was incubated in a 1 mM solution of the chelator and 100 mM NaH_2PO_4 at RT and assayed at timed intervals for 17 h (single assay per time point). Control incubations were treated likewise, but without EDTA or *o*-phenanthroline. In addition the PFI was incubated with 5 mM EDTA at pH 4.2 in a 50 mM citrate/50 mM NaH_2PO_4 buffer and assayed at timed intervals (one assay at t = 0, 15, 30, 60, 120, and 240 min).

NMR, IR, and UV Analyses. All NMR experiments were performed on a Bruker ACP 300 instrument, and chemical shifts were assigned relative to an internal TMS standard. IR spectra were obtained on a Nicolet 510 FT-IR spectrometer. UV/vis spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer. The following data were obtained for the C_{20} methyl ester tetraene product **5**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.89 (3H, t, J = 6.8 Hz, H_3-20), 1.33 (6H, m, H_2-17,18,19), 1.45 (2H, tt, J = 7.4, 7.4 Hz, H_2-12), 1.73 (2H, tt, J = 7.4, 7.4 Hz, H_2-3), 2.01 (4H, m, H_2-13, 16), 2.11 (2H, m, H_2-11), 2.23 (2H, bdt, J = 7.5,

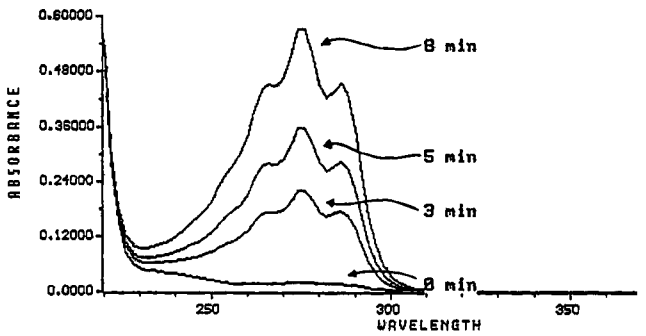


FIGURE 2: Development of the characteristic conjugated triene spectra of metabolite **5** at timed intervals during the incubation of 1.2×10^{-3} unit of partially purified PFI with 131 μM arachidonate.

7.5 Hz, H_2-4), 2.33 (2H, t, J = 7.5 Hz, H_2-2), 3.66 (3H, s, H_3-1′), 5.33 (3H, m, H-5,14,15), 5.71 (1H, dt, J = 14.1, 7.1 Hz, H-10), 6.04 (1H, bdd, J = 11.0, 11.0 Hz, H-6), 6.10 (1H, m, H-9), 6.16 (1H, m, H-8), 6.35 (1H, dd, J = 11.0, 13.7 Hz, H-7); IR (neat) υ_{max} 2953, 2926, 2856, 1741, 1457, 1436, 1243, 1199, 1170, 992, 966 cm^{-1}; UV (EtOH) 262, 272, 282 nm.[3]

The C_{18} methyl ester triene product **6** obtained from γ-linolenate gave the following data: ^1H NMR (300 MHz, $CDCl_3$) δ 0.89 (3H, t, J = 6.7 Hz, H_3-18), 1.24 (8H, m, H_2-14,15,16,17), 1.42 (4H, m, H_2-4,13), 1.66 (2H, tt, J = 7.4, 7.4 Hz, H_2-3), 2.09 (2H, dt, 7.2, 7.2 Hz, H_2-12), 2.20 (2H, dtd, 7.5, 7.5, 1.0 Hz, H_2-5), 2.32 (2H, t, J = 7.4 Hz, H_2-2), 3.66 (3H, s, H_3-1′), 5.37 (1H, dt, 10.9, 7.5 Hz, H-6), 5.71 (1H, dt, J = 14.4, 7.2 Hz, H-11), 6.00 (1H, bdd, J = 10.9, 10.9 Hz, H-7), 6.07 (1H, m, H-10), 6.16 (1H, m, H-9), 6.36 (1H, bdd, J = 10.9, 13.4 Hz, H-8).

Deuterated Substrates. The stereospecifically deuterated γ-linolenic acids [(11*R*)- and (11*S*)-γ-[11−^{2}H]linolenic acid and (8*R*)- and (8*S*)-γ-[8−^{2}H]linolenic acid) were synthesized as previously described (Fahlstadius & Hamberg, 1990; Hamberg & Samuelsson, 1967; Schroepfer & Bloch, 1965). As these syntheses involved a biological desaturation, the resulting deuterated species were diluted with endogenously produced γ-linolenic acid; hence the monodeuterated substrates ranged from 25 to 33% (see Table 2) (Hamberg, 1993).

RESULTS

Initial Enzyme Isolation and Characterization of the Enzyme Reaction. Frozen (-70 °C) tissue of *P. filicina* (50 g) was homogenized as described in Materials and Methods and subjected to high-speed centrifugation. The high-speed supernatant (S-100) fraction as well as the resuspended pellet from the high-speed centrifugation were assayed for activity with arachidonate. Incubation of arachidonate with the S-100 fraction resulted in the development of a chromophore indicative of a conjugated triene. Figure 2 illustrates the development of the conjugated triene spectrum. The microsomal fraction showed no such activity.

To establish the chemical nature of the substance formed (**5**), a larger scale incubation was performed. A preparation of enzyme from a Sephacryl S-300 size-exclusion column was diluted to 100 mL with reaction buffer to which 10 mg of arachidonate in 100 μL of EtOH (328 μM) was added. After approximately 40 min, the reaction was quenched with 5 vol of MeOH and the lipid material extracted. The methyl esters of the products and unreacted arachidonate were separated by HPLC. By a combination of ^1H NMR, ^1H−^1H COSY, GC/MS, and UV spectroscopy, the major product of arachidonate incubation was identified as a new conjugated triene metabolite [**5**, (5*Z*,7*E*,9*E*,14*Z*)-eicosatetraenoic acid].

Low-resolution mass spectral analysis of the methyl ester derivative of **5** indicated a molecular ion with a m/z = 318, in agreement with a molecular formula of $C_{21}H_{34}O_2$. ^1H−^1H COSY analysis readily demonstrated coupling from the C2-methylene at δ 2.33 through the aliphatic chain to the C5 olefinic proton at δ 5.33. Coupling from the C20 methyl at δ 0.89 through the aliphatic protons to the C15 olefinic proton at δ 5.33 was also clearly evident. The proton band at δ 5.33 was further coupled to a deshielded olefinic spin system descriptive of the conjugated triene. The UV spectrum of this product showed three maxima (Figure 2) characteristic of a conjugated triene with λ_{max}(EtOH) 262, 272, 282 nm. Additionally, the δ 5.33 ^1H NMR band showed coupling to one end of three sequentially coupled methylene groups (C11, C12, C13). Because the C2−C4, C16−C20, and C6−C13 spin systems all coupled to the signal at δ 5.33 (representing the protons at C5, C14, and C15), it was not clear whether this arachidonate metabolite was 5,7,9,14-eicosatetraenoic acid or 5,10,12,14-eicosatetraenoic acid. The question of the regiochemistry of the double bonds was resolved by a selective proton decoupling experiment. Irradiation of the C4 protons at δ 2.23 collapsed the broad triplet at δ 6.04 (H-6) to a sharp triplet, reflecting the loss of the long-range allylic coupling between these protons, thereby establishing the position of the conjugated double bonds to occur between C5 and C10. Coupling constants of $^3J_{5-6}$ = 11.0 Hz, $^3J_{7-8}$ = 13.7 Hz, and $^3J_{9-10}$ = 14.1 Hz defined the geometry of these three olefins as 5*Z*,7*E*,9*E*. We could not definitively determine the relative stereochemistry of the C14−C15 olefin in **5**; however, it is reasonable to expect this bond to be unchanged from the *cis* configuration of the substrate, as found in the naturally occurring EPA-derived conjugated triene metabolites (e.g., **4**; Lopez & Gerwick, 1987).

Enzyme Characterization. The SDS−PAGE of the isolated enzyme shows a single major band (Figure 3), likely to be the PFI, with an electrophoretic mobility corresponding to a molecular mass of 61 000 Da. By gel filtration on Sephacryl S-300 the native enzyme emerges in a volume corresponding to 174 000 Da. To better assess the mass of the subunit, the enzyme was subjected to MALD/IMS using a time of flight detector and BSA as an internal standard. By this technique the mass of the subunit was determined to be 58 119 Da with a standard deviation of 50 mass units (four replicate experiments). On preparative IEF the enzyme shows an isoelectric point at pH 4.5.

Reaction of PFI with Alternative Substrates. The substrate specificity of PFI with several alternative fatty acid substrates was evaluated by monitoring incubations under standard conditions by UV spectroscopy (see Materials and Methods). As shown in Figure 4, eicosapentaenoate is the best substrate (K_m^{app} = 32.8 ± 7.7 μM, and V_{max} = 14.0 μmol min^{-1}

[3] These maxima are different from those reported by Lopez and Gerwick (1987) for the natural product. The previously reported spectrum was obtained on an older, less reliable instrument. The data above are the correct values for this compound and the natural product. We thank Professor Alan Brash (Vanderbilt University) for pointing this out.

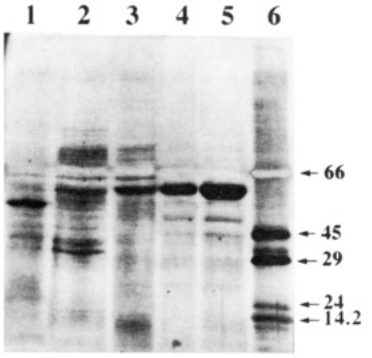


FIGURE 3: Silver-stained SDS−PAGE of the stages of purification of PFI. Lane 1, crude extract after high-speed centrifugation; lane 2, 55−90% ammonium sulfate cut; lane 3, active fraction from S-300 gel filtration; lane 4, material from IEF; lane 5, a 5-fold concentration of IEF-purified material; lane 6, molecular mass markers (values in kDa). The likely enzyme appears as a single prominent band after IEF (lanes 4 and 5).

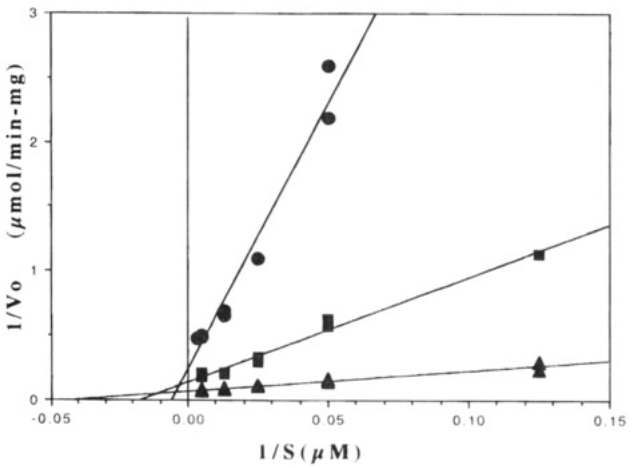


FIGURE 4: Lineweaver−Burk plot of PFI with eicosapentaenoate (▲), arachidonate (■), and γ-linolenate (●) as substrates. Assays were performed in 100 mM NaH_2PO_4 buffer at pH 7.2. Although this pH is not optimum for the enzyme, it was chosen because of the limited solubility of the substrates in acidic solutions. Each substrate concentration was assayed in triplicate (all data points are shown).

mg^{-1}),[4] followed by arachidonate ($K_m^{app} = 39.9 \pm 27$ μM, and V_{max} =7.0 μmol min^{-1} mg^{-1}) and γ-linolenate (K_m^{app} = 150 ± 35 μM, and V_{max} = 4.0 μmol min^{-1} mg^{-1}). α-Linolenate [(9Z,12Z,15Z)-octadecatrienic acid] also reacts with the enzyme, but approximately one-fifth as fast as γ-linolenate (data not shown). Linoleic acid does not serve as a substrate for triene production. These data are presented to demonstrate the relative affinities and velocities of EPA, arachidonate, and γ-linolenate as substrates for PFI. The values are given as apparent K_ms and V_{max}s because the assays were performed at a pH above the optimum (see below), the effect of Tween-20 on the enzyme and the critical micelle concentration for the substrates were not determined, and the enzyme was not demonstrably pure. An estimated k_{cat} for EPA at pH 7.2 is 41 s^{-1}, assuming one catalytic site per enzyme molecule.

In initial experiments to evaluate the pH optimum of the enzyme, we examined a number of buffers at pHs ranging from 2.0 to 8.0. There was a 30% increase in rate of product formation from pH 8.0 to 6.5. From pH 6.5 to 4.5 the apparent velocity increased 2.4-fold, and below pH 4.0 activity slowly decreased. Observation of a maximum velocity at pH 4.5 might suggest that the enzyme recognizes the protonated form of the substrate. We have addressed this question by asssaying PFI with the methyl ester of arachidonate. Although the enzyme will utilize the methyl ester, the rate appears to be approximately 10-fold slower than that of the free acid at pH 7.2. Using the methyl ester, we again saw a 2.4-fold increase in velocity between pH 7.2 and 4.5. Due to the low solubility of the methyl ester in aqueous solution it is difficult to interpret these results unequivocally. We have also discovered that PFI will utilize another nonionizable analog of arachidonate, arachidonylethanolamide, as a substrate. The enzyme shows an apparent velocity maximum at pH 4.5 with this compound as well; hence, it appears that an ionized carboxylate is not required for binding and that the pH optimum at least partially reflects characteristics of the enzyme. The magnitude of the pH effect on K_m and V_{max} has not been assessed; experiments to explore these questions are in progress.

Inhibition by Lipoxygenase, Prostaglandin H Synthase, or Cytochrome P-450 Inhibitors. None of the nine inhibitors tested showed any significant inhibitory activity. Additionally, neither EDTA nor *o*-phenanthroline showed a significant effect on PFI activity.

Reaction in ^2H_2O. To determine whether either of the hydrogens involved in saturating the C11=C12 double bond of arachidonate originated from the solvent, the biosynthetic reaction was performed in deuterium oxide (99.8%) buffered with 100 mM NaH_2PO_4 at pH 7.2. The pH of the deuterium oxide was measured directly, and no correction was made for the deuterium effect (approximately 0.4 pH unit). Although the reaction rate is pH sensitive, in the range of pH 7.2−8.0 the effect is minor.

The reaction mixture consisted of 0.005 unit of partially purified enzyme, 5 mg of arachidonate (in 50 μL of EtOH), and 20 mL of buffer with 0.02% Tween-20. The reaction was monitored by observing the UV spectra of aliquots taken at intervals. After approximately 27 h, when no further increase in absorption at 278 nm was measured, the reaction was quenched with 5 vol of MeOH, and the lipids were extracted and methylated with CH_2N_2.

The absorbance (272 nm) of the conjugated triene (**5**) corresponded to 2.2 mg (out of 3.9 mg of total fatty acid methyl esters extracted). GC/MS showed two major peaks, representing the methyl ester derivative of unreacted arachidonate and the conjugated triene product. The molecular ion of the product triene methyl ester had a *m/z* of 319, demonstrating the incorporation of one deuterium atom from the solvent (*m/z* 318/319 ratio = 0.10). Unfortunately, the fragmentation pattern of the triene was not unequivocally diagnostic for the location of the incorporated deuterium atom, although this presumably occurred at either C11 or C12. ^1H NMR analysis was used to resolve this question.

The deuterium-containing triene product was separated from the arachidonate by HPLC of the methyl ester derivatives as described earlier, and a ^1H NMR spectrum was

[4] The K_ms for arachidonate and γ-linolenate represent an average of two experiments (±SD) using two different enzyme preparations. The K_m for EPA was calculated from duplicate experiments using the same enzyme preparation. The V_{max}s are calculated as described in Figure 4. This enzyme preparation had the highest specific activity we have achieved to date (standard assay conditions with arachidonate as substrate; 3.5 μmol $min^{-1}mg^{-1}$), and it therefore provides a better estimate of V_{max}.

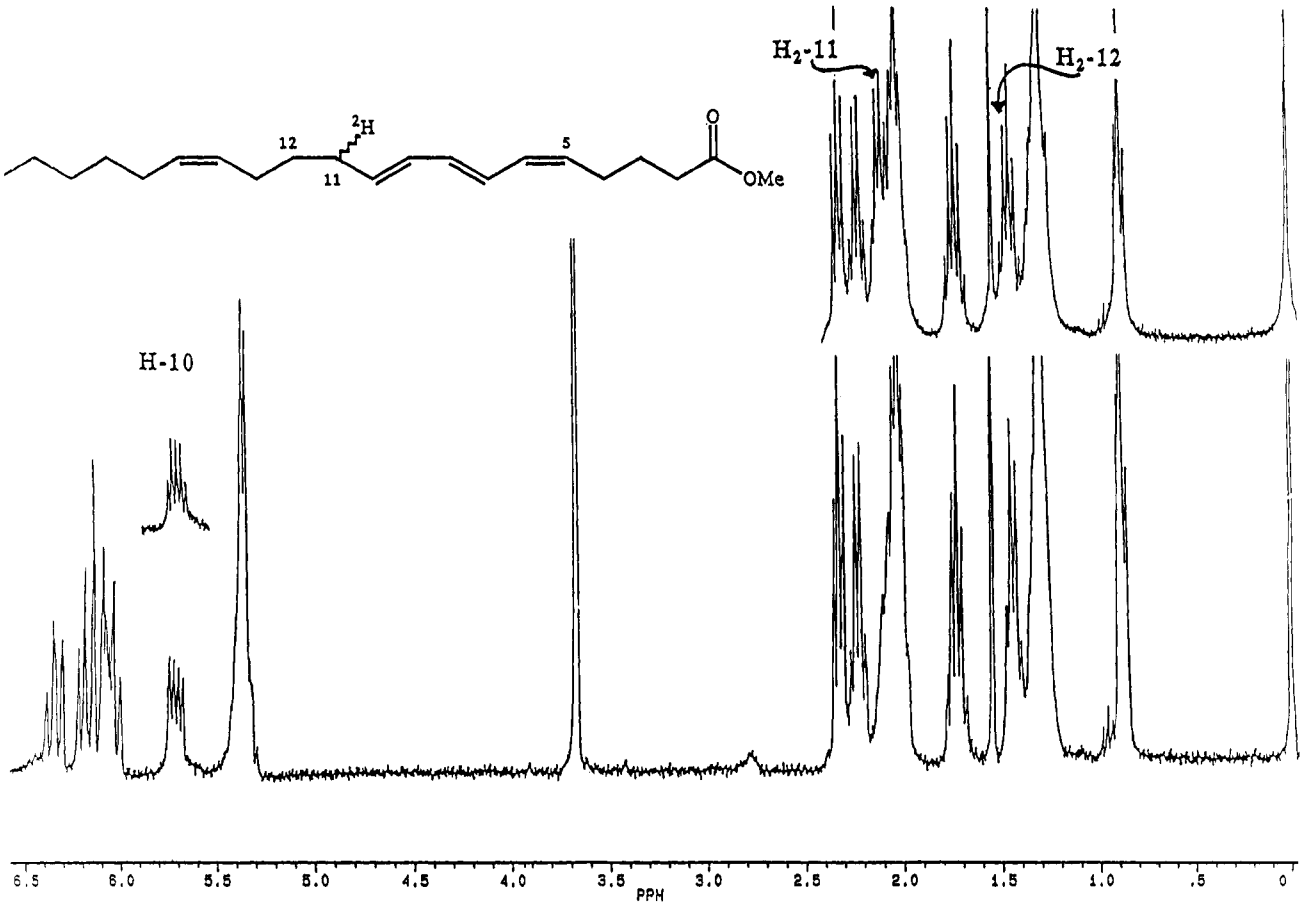


FIGURE 5: ^{1}H NMR spectrum of the methyl ester derivative of the triene product after incubation of PFI with arachidonate in deuterated water (bottom) and portions of the ^{1}H NMR spectrum of the unlabeled methyl ester derivative of the C_{20} conjugated triene (**5**) (top). The reduction of the H-10 and H_2-12 pentets in the unlabeled methyl ester derivative of **5** to quartets, as well as the diminution of the intensity of the H-11 signal, clearly demonstrates the incorporation of the solvent-derived deuterium at the C11 position.

obtained. Figure 5 shows the spectrum of the deuterated triene with salient features of the normal protonated species superimposed above it. Collapse of the pentet at 5.7 ppm and the multiplet at 1.45 ppm representing protons at C10 and C12, respectively, as well as diminution of the signal at 2.1 ppm in the deuterated product, unequivocally demonstrated incorporation of one deuterium atom at C11.

Intramolecular Hydrogen Transfer in Stereospecifically Labeled γ-Linolenate. From the results of the incubation of PFI with arachidonate in D_2O it was clear that protonation of C12 results from transfer of a substrate-derived proton (or hydrogen). This could occur either as an intermolecular transfer or an intramolecular transfer. We first examined the intramolecular mechanism. An allylic 1,3-proton shift from C10 was most easily envisioned; however, the possibility of transfer from C7 could not be ruled out. To test which of these two processes occurs, the enzyme was incubated in separate experiments with (11*R*)-, (11*S*)-, (8*R*)-, and (8*S*)-deuterio-γ-linolenic acids. As indicated earlier, γ-linolenic acid functions reasonably well as a substrate. With respect to the bis-allylic methylene groups, C7 and C10 of arachidonate correspond to C8 and C11 of γ-linolenate.

For these experiments the enzyme had been additionally purified by isoelectric focusing (Table 1, Figure 3). Because it was determined that the conversion of arachidonate to the conjugated triene was approximately 2−3 times faster at pH 4.5−5.0 than at pH 7.2, all reactions with ^{2}H-labeled γ-linolenate were performed at pH 5.0.

Table 2: Enrichment of Deuterium in Stereospecifically Labeled γ-Linolenic Acid and Its Conjugated Triene Product and Ratios of the *m/z* 207 and 208 Fragments from the Triene Products of the Reaction of PFI with the Two Stereoisomers of γ-[11-^{2}H]Linolenate[a]

	% ^{2}H enrichment			
substrate	substrate	unreacted substrate	product	product *m/z*, 208/(207 + 208)
(11*S*)-γ-[11-^{2}H]linolenate	32.2	74.5	19.4	0.30
(11*R*)-γ-[11-^{2}H]linolenate	25.6	23.9	24.1	0.36
(8*S*)-γ-[8-^{2}H]linolenate	32.9	34.6	31.3	
(8*R*)-γ-[8-^{2}H]linolenate	32.4	69.2	4.7	
γ-linolenate control				0.25

[a] The % ^{2}H enrichment is calculated as [(M + 1) − (^{13}C abundance)]/[M + [(M + 1) − (^{13}C abundance)].

As is clearly evident from Table 2, incubation of PFI with (11*S*)-γ-[11-^{2}H]linolenic acid resulted in retention of deuterium in the product (19.4%) and a pronounced isotopic enrichment of the unreacted substrate (74.5%). Reaction with (11*R*)-γ-[11-^{2}H]linolenic acid again showed retention of deuterium in the product (24.1%); however, no isotopic enrichment of the unreacted substrate was observed (23.9%). The ratios of the fragments at *m/z* 207 and 208 indicated the position of the ^{2}H label in the products (Table 2). Cleavage of the undeuterated and methylated triene product at the C12−C13 bond results in a methyl-C1 to C12 fragment with a nominal mass of 207 (Figure 6). Transfer of deuterium from C11 to C13 results in a normal isotopic ratio for this fragment. The product formed from (11*R*)-γ-[11-

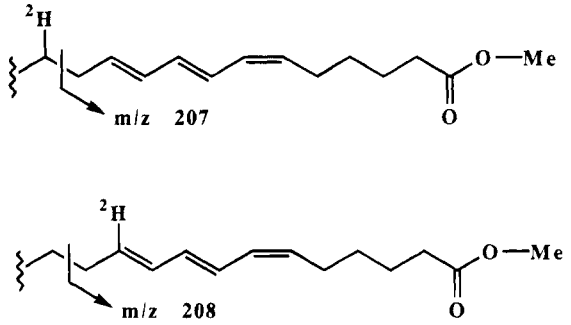


FIGURE 6: Fragmentation of the methylated triene product from γ-[11-^{2}H]linolenate to form *m/z* 207 or *m/z* 208 fragments. The relative abundance of these fragments indicates whether migration of the deuterium label from C11 to C13 has occurred during the formation of the conjugated triene.

^{2}H]linolenic acid gave a 208/(207 + 208) ratio of 0.36, while the methyl ester of the triene product formed from (11*S*)-γ-[11-^{2}H]linolenic acid showed a ratio of 0.30. The conjugated triene formed from unlabeled γ-linolenic acid gave a ratio of 0.25. Hence, the deuterium enrichment in the product derived from (11*S*)-[11−^{2}H]-γ-linolenic acid is due to a deuterium transfer to C13, while that derived from (11*R*)-γ-[11-^{2}H]linolenic acid is due to retention of deuterium at C11.

When the enzyme was incubated in the presence of (8*S*)-γ-[8-^{2}H]linolenic acid or (8*R*)-γ-[8−^{2}H]linolenic acid under conditions essentially identical to those described for the γ-[11-^{2}H]linolenic acids, a pronounced isotope effect was observed with the (8*R*)-γ-[8-^{2}H]linolenic acid resulting in enrichment of the unreacted deuterated substrate. The corresponding triene product showed only 4.7% deuterium [due to a small enantiomeric impurity in the substrate (Hamberg, 1993)]. The deuterium enrichment of the unreacted (8*S*)-γ-[8-^{2}H]linolenic acid was slightly increased over that of the starting material, and a small decrease in deuterium was observed in the product (see Table 2), again reflecting slight enantiomeric impurity of the substrate. These results clearly demonstrate that the *pro*-8-*R* hydrogen in γ-linolenic acid, and by analogy the *pro*-7-*R* hydrogen in arachidonate, is lost to the solvent during the isomerization.

Intermolecular Proton (Hydrogen) Transfer. To explore the possibility of an intermolecular transfer of hydrogen, unlabeled arachidonic acid and labeled (11*S*)-γ-[11-^{2}H]-linolenic acid were co-incubated with PFI to observe whether any of the deuterium label would be transferred to the C_{20} conjugated triene product. The (11*S*)-γ-[11-^{2}H]linolenic acid and unlabeled arachidonate were mixed in approximately 10:1 molar ratios (215 μM γ-linolenic acid with 21.5 μM arachidonate) to compensate for the higher affinity of the enzyme for arachidonate. The arachidonate was added 30 min after initiation of the reaction with γ-linolenate and PFI to allow for several turnovers of the enzyme (methylated compound **6** was observed by GC/MS of the final reaction mixture). After 3.75 h the reaction was quenched with MeOH and the lipids extracted. The C_{20}-derived conjugated triene product (**5**), isolated as its methyl ester, possessed a *m/z* 319/318 ratio of 0.28. This is identical to the (M + 1)/M ratio found in the methyl ester of arachidonate as well as the triene product of arachidonate produced in the absence of deuterated γ-linolenic acid.

O_2 Requirement. Results from the anaerobic reaction of PFI demonstrate that the initial velocity of the reaction is

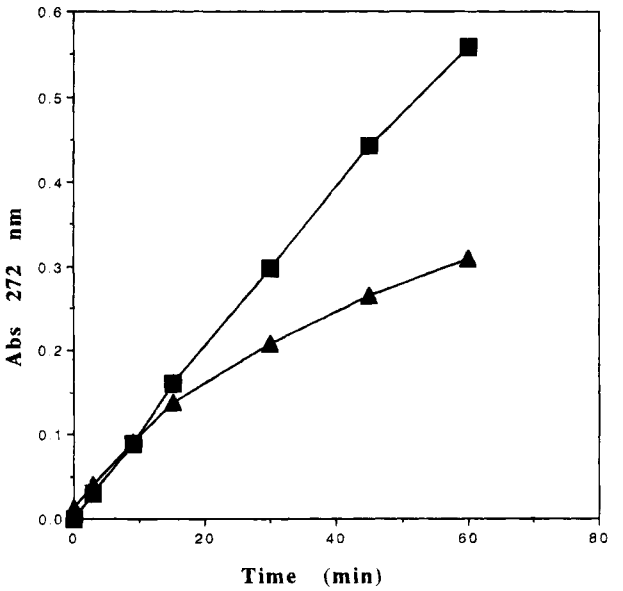


FIGURE 7: Comparison of the velocity of triene formation with (■) and without (▲) oxygen. PFI was incubated in a sealed, evacuated vessel purged with N_2 and preincubated with glucose and glucose oxidase to remove the last traces of molecular oxygen. After the addition of arachidonate, aliquots were removed at 0, 3, 9, 15, 30, 45, and 60 min and quenched with MeOH. The absorbance at 272 nm (the λ_{max} in MeOH) was determined as a measure of triene formation. A control reaction was performed in an open vessel. Both were vigorously stirred.

unaffected by the lack of O_2 (Figure 7). It would appear that the enzyme does not require molecular O_2, certainly not in stoichiometric amounts. Essentially identical results were obtained whether the reaction was run in the presence or absence of glucose and glucose oxidase. As a further check that anaerobic conditions were obtained by this procedure, a similar reaction was performed using *Gracilariopsis lemaneiformis* lipoxygenase (Moghaddam & Gerwick, 1990) and arachidonate. No hydroperoxide formation was observed (as determined from a spectrophotometric assay of diene formation, i.e., increased absorption at 238 nm). It is of interest that the rate of triene formation under anaerobic conditions appears to slow (relative to the aerobic control) after a prolonged incubation. This phenomenon was observed in three replications of this experiment but has not been further evaluated.

DISCUSSION

One of the first hypotheses on the biogenesis of conjugated trienes in terrestrial plants (Gunstone, 1965) suggested that they result from the oxidation of the bis-allylic methylene group of linoleic acid to form the (9*Z*,12*Z*)-11-hydroxyoctadecadienoic acid derivative followed by 1,4-dehydration to form either 8,10,12- or 9,11,13-octadecatrienoic acid. Morris (Badami & Morris, 1965) agreed that the fatty acid precursor for these conjugated trienes was linoleic acid; however, he proposed (10*E*,12*Z*)-9-hydroxyoctadecadienoic acid and (9*Z*,11*E*)-13-hydroxyoctadecadienoic acid as intermediates to these two conjugated trienes. These latter two proposed intermediates are likely products of lipoxygenase metabolism. Crombie and Holloway (1985) found experimentally that linoleic acid is the immediate precursor of calendic acid (**1**), a conjugated triene-containing fatty acid from marigold seeds (*Calendula officinalis*). These experiments showed that regiospecific abstraction of the C8 and C11 hydrogens from linolenic acid results in the formation

of the conjugated triene product. No oxygenated intermediates appeared to be involved in the reaction; however, a requirement for molecular oxygen was not assessed. This biosynthetic pathway may, in fact, be analogous to the system in *L. corallioides* (Hamberg, 1992). Although this transformation in *C. officinalis* was presented as a radical mechanism, there was no direct experimental evidence to support this proposal.

It is interesting to note that, in the investigation by Gerwick et al. (1993) of oxylipin metabolism by *L. corallioides*, an enzyme-catalyzed conversion of arachidonate to both (5*Z*,8*Z*10*E*,12*E*,14*Z*)-eicosapentaenoic acid (**2**) and (13*R*)-hydroxyeicosatetraenoic acid was seen in which the latter product was similar to the intermediate proposed by Gunstone (1965). While the *L. corallioides* enzyme requires molecular oxygen, ^{18}O-labeling studies clearly revealed that the hydroxyl oxygen originates from water (Gerwick et al., 1993). Hamberg's (1992) continuing studies of the *L. corallioides* system have shown that although this alga can introduce a bis-allylic hydroxyl group in a variety of polyunsaturated fatty acid precursors, this metabolic capacity is unrelated to its ability to oxidatively form conjugated tetraene products (e.g., metabolite **3**).

Another enzyme known to isomerize linoleic acid to a conjugated product, (9*Z*,11*E*)-octadecadienoic acid, has been isolated from the anaerobic bacterium *Butyrivibrio fibrisolvins* (Kepler & Tove, 1967). Detailed experiments with the isolated enzyme, linoleic acid Δ^{12}-*cis*-Δ^{11}-*trans*-isomerase, showed that the transformation of (9*Z*,12*Z*) -octadecadienoic acid into (9*Z*,11*E*)-octadecadienoic acid occurred through abstraction of a proton from the C11 methylene and stereospecific *pro-R* protonation at the C13 position from a solvent-derived proton (Kepler et al., 1971).

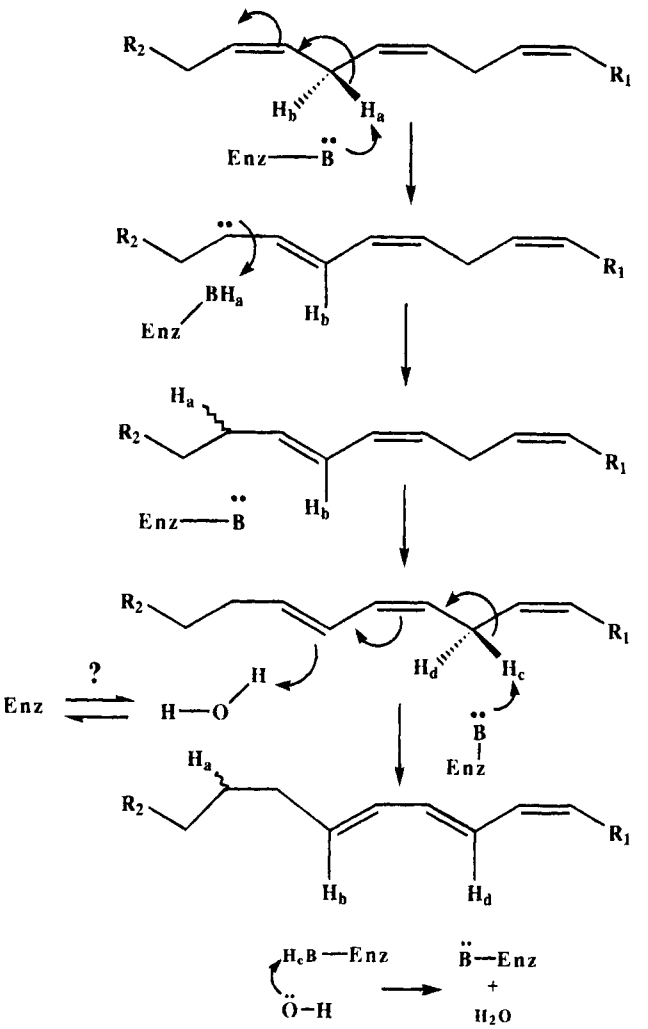
Polyenoic fatty acid isomerase represents the first example, to our knowledge, of a single enzymatic isomerization of two double bonds, one allylically and one homoallylically (Schwab & Henderson, 1990). The sequence of these steps is not clear at present, although we suggest that it occurs first with the formation of a conjugated diene followed by a second isomerization to form the conjugated triene. Hence, the enzyme performs a rather complex series of proton (or hydrogen) abstractions and transfers to accomplish this "double" isomerization, with apparently distinct mechanisms.

Our rationale for the experiments using stereospecifically deuterated substrates was as follows. If a carbon−hydrogen bond were broken in a rate-limiting step, one would expect to see a kinetic isotope effect (KIE) using the deuterated substrates. In these experiments, the KIE would manifest itself as an enrichment of the unreacted deuterated substrate. An increase of the M + 1 molecular ion over the normal isotopic abundance attributable to ^{13}C in the product would result only upon deuterium retention. Observation of a KIE and retention of deuterium in the product would indicate bond breakage and transfer of deuterium within the substrate. This latter situation was observed when (11*S*)-γ-[11-^{2}H]linolenate was used as substrate. However, when (8R)-[8−^{2}H]-γ-linolenate was used as substrate, the reaction showed a pronounced KIE and the product triene was substantially depleted in deuterium. This can only be interpreted as a result of carbon−deuterium bond breakage in a rate-limiting step with subsequent loss of the deuterium.

The *m/z* 208/207 fragments formed in the mass spectrometer by fragmentation of the C12−C13 bond further substantiate the transfer of the C11 *pro-S* hydrogen to the C13 position (Table 2). Although the 208/(207 + 208) ratio obtained from the methyl ester of the product from unlabeled γ-linolenate (0.26) is higher than one would expect for a C_{13} compound, it is possible that some of the 208 peak intensity is due to a McLafferty rearrangement. The slightly higher 208/(207 + 208) ratio seen in the triene produced from (11*S*)-γ-[11-^{2}H]linolenate (0.30) is attributable to a 4.6% enantiomeric impurity in the substrate (Hamberg, 1993). Any (11*R*)-γ-[11-^{2}H]linolenate present in the (11*S*)-γ-[11-^{2}H]linolenate preparation would certainly be enriched in the product. The significantly higher 208/(207 + 208) ion ratio (0.36) from the methyl ester product of (11*R*)-γ-[11-^{2}H]linolenate incubation clearly indicates that the *pro-R* hydrogen at C11 remains at that position in the product [this substrate was only 1.7% enantiomerically impure (Hamberg, 1993)]; therefore, it must be the *pro-S* hydrogen which is transferred.

The transfer of protons or hydrogens during the isomerization of arachidonate or eicosapentaenoate to the corresponding 5*E*,7*Z*,9*Z* conjugated triene product is shown in Scheme 2. The *pro-S* hydrogen at C10 (H_a) is abstracted and transferred to C12 to form the proposed bound diene

Scheme 2: Regio- and Stereochemistry Involved in the Isomerization of a Polyenoic Fatty Acid to the Conjugated Triene Product



$R_1=C_4H_6O_2$
$R_2=C_7H_{13}$ or C_7H_{11}

intermediate. The *pro-R* hydrogen at C7 (H_c) is then removed with subsequent electron migration and protonation at the C11 position by a solvent-derived proton. We suggest that the transfer of the C10 *pro-S* hydrogen (or proton) to C12 occurs as a 1,3-allylic shift, likely catalyzed by a single catalytic functionality. Determination of the stereochemistry of the proton addition at C12 would provide some insight into the involvement of one or more catalytic residues in this process; experiments are underway to resolve this question.

By this scheme, the enzyme-bound diene intermediate (we have no evidence for a free diene in solution) is then subject to a second isomerization involving abstraction of the C7 *pro-R* hydrogen (or proton) and migration of the π system to form the conjugated triene. Protonation at C11 occurs with a solvent-exchangeable proton, the stereochemistry of which remains undetermined. The fact that only deuterium-enriched substrate and undeuterated triene product were observed upon reaction with (8*R*)-γ-[8-^{2}H]linolenate would suggest that if the reaction does proceed through a diene intermediate (Scheme 1) the first step must be reversible and the diene intermediate must be enzyme bound. If the diene intermediate were free to dissociate from the enzyme, then it is this species which would become enriched with deuterium following reaction with (8*R*)-γ-[8-^{2}H]linolenate.

While we have drawn this reaction (Scheme 2) as an anionic mechanism, we emphasize that we are uncertain about the nature of the electron transfers. Passage of the enzyme down either Sephadex G-25 or Sephacryl S-300 results in quantitative recovery of activity, indicating that the addition of cofactors, such as NAD or FMN, is not required. The lack of a requirement for any added redox cofactors does not preclude the possibility that they may be covalently or otherwise tightly bound to the enzyme. Similarly, because incubations of the enzyme with EDTA or *o*-phenanthroline did not reduce its catalytic capabilities, it is unlikely that there are loosely associated divalent metal cations. Again this cannot be taken as rigorous proof that metal ligands are not involved; it may simply indicate that the metal ligand is tightly bound or deeply embedded in the enzyme core.

The ability of the enzyme to abstract a proton from the bis-allylic C10 and C7 methylene groups poses some intriguing questions. The two best characterized classes of enzymes catalyzing this kind of reaction are the lipoxygenases and prostaglandin H synthase (Smith et al., 1991). In both of these, the bis-allylic hydrogens are removed in radical mechanisms with the resulting allylic radical being quenched by molecular oxygen to form a hydroperoxide. In the case of PFI, there is no hydroxy or hydroperoxide formed (reaction in the presence of glutathione and glutathione peroxidase has no effect) and experiments under anaerobic conditions (Figure 7) have shown that stoichiometric quantities of oxygen are not needed. The results of the anaerobic incubations are enigmatic; we hypothesize that there may be some change in the conformation and/or function of the enzyme under reducing conditions or possibly a side reaction involving a cofactor (still to be determined).

SDS−PAGE analysis of the partially purified enzyme indicates that it is composed of similar or identical subunits of 61000 Da (this conclusion has been confirmed in subsequent work with enzyme showing a single band on SDS−PAGE; M. L. Wise and W. H. Gerwick, in progress). The mass of the native enzyme determined by gel filtration (M_r = 174 000 Da) would indicate a trimer. Although not unknown, homotrimeric enzymes are somewhat unusual. Analytical centrifugation or mass spectrometry will be used to confirm the weight of the native enzyme (in progress).

Unlike the well-characterized aerobic reaction of lipoxygenases from plant and animal sources, or even the relatively new enzyme described by Hamberg (1992) which produces a conjugated tetraene, PFI appears not to have any requirement for molecular oxygen. Further, it is not inhibited to any extent by the conventional inhibitors of mono- and dioxygenases. The enzyme is strictly an isomerase; i.e., no net desaturation occurs as in the case of calendic acid (**1**) or BPA (**2**) biosynthesis.

With the multitude of structurally novel oxylipins found in the marine environment, it is not surprising that marine organisms are also rich sources of enzymes which catalyze unique biosynthetic reactions. It is our hope that further investigation into these pathways may simultaneously reveal unknown features of the physiology and ecology of the algae, extend the range of known enzymatic transformations of arachidonic acid and its congeners, and provide insight into the use of these enzymes as tools for the synthesis of novel bioactive compounds.

ACKNOWLEDGMENT

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