Characterization of the Substrate Binding Site of Polyenoic Fatty Acid Isomerase, a Novel Enzyme from the Marine Alga *Ptilota filicina*†

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ABSTRACT: The substrate binding site of polyenoic fatty acid isomerase (PFI) has been investigated using a series of alternate substrates and by examination of the pH dependence on the kinetic parameters of PFI with selected substrates. The pH dependence profile of PFI with EPA [(5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)-eicosapentaenoic acid] shows the enzyme to be catalytically active over a wide pH range, with activity being optimal below pH 6.0. Analysis of the kinetic parameters of DHA [(4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*)-docosahexenoic acid]; adrenic acid [(7*Z*,10*Z*,13*Z*,16*Z*)-docosatetraenoic acid]; EPA; arachidonic acid [(5*Z*,8*Z*,11*Z*,14*Z*)-eicosatetraenoic acid]; anandamide (arachidonyl-*N*-ethanolamide); and eicosatrienoic acid [(5*Z*,8*Z*,11*Z*)-eicosatrienoic acid] demonstrates that substrates possessing ω-3 olefins (DHA and EPA) have the lowest K_m values (1.9 and 9.6 μM, respectively). EPA and arachidonic acid showed the highest V_{max} values (6.0 and 2.8 μmol min^{-1} mg^{-1}, respectively). The twenty carbon ω-9 fatty acid eicosatrienoic acid showed a relatively large K_m and had a V_{max} approximately 20-fold less than EPA. Anandamide, a substrate analog lacking an ionizable carboxylate, showed a K_m similar to the other ω-6 fatty acids (arachidonic acid and adrenic acid); however, the V_{max} was approximately 5-fold lower than arachidonic acid and 8-fold lower than EPA. Moreover, anandamide demonstrated no pH dependency on its kinetic parameters over a range where EPA showed a 27-fold decrease in V/K_m. NMR spectroscopy was used to determine the structure of the product from reaction of PFI with DHA. These data showed the compound to be (4*Z*,7*Z*,9*E*,11*E*,16*Z*,19*Z*)-docosahexenoic acid. Reaction of PFI with dihomo-γ-linolenic acid resulted in the development of two products, one with the characteristic chromophore of a conjugated triene, the other with a chromophore characteristic of a conjugated diene. Analysis of the products from these reactions of PFI, in conjunction with the kinetic parameters from the alternate substrates, provides compelling evidence that the enzyme preferentially orients the substrate in the catalytic site with respect to the methyl terminus.

Although long recognized as constituents of plant seed oils, aside from the work of Crombie and Holloway (1985) describing the biosynthesis of calendic acid [(8*E*,10*E*,12*Z*)-octadecatrienoic acid] by germinating marigold (*Calendula offinalis*) seeds, little has been reported on the biosynthesis of conjugated olefin containing fatty acids in terrestrial plants. A linolenic acid isomerase from the anaerobic rumen bacterium *Butyrivibrio fibrisolvens* has been described by Tove and his colleagues which catalyzes the isomerization of linoleic acid to the 9*Z*,11*E* isomer (Kepler & Tove, 1967; Kepler et al., 1970, 1971). This enzyme is oxygen insensitive, functions in a narrow pH range centered around pH 7.1, and is reversibly inactivated by metal chelating reagents; however, the mechanism for the catalyzed reaction is yet to be determined (Schwab & Henderson, 1990). More recently, a reductive pathway in mammals for the metabolism of unsaturated fatty acids with odd numbered double bonds has been demonstrated (Smeland et al., 1992). Formation of conjugated Δ^3,Δ^5 dienoyl CoA as well as Δ^2,Δ^4 dienoyl CoA derivatives of unsaturated fatty acid intermediates is involved. The Δ^2,Δ^4 dienoyl CoA results from the activity of a newly described Δ^3,Δ^5-*t*-2,*t*-4 dienoyl-CoA isomerase (Chen et al., 1994).

Secondary metabolism of fatty acids by the marine algae demonstrates a variety of structurally novel metabolites (Gerwick, 1994). Among these natural products are numerous polyunsaturated fatty acids containing conjugated olefin systems (Lopez & Gerwick, 1987; Burgess et al., 1991; Hamberg, 1992; Mikhailova et al., 1995). Owing to the prevalence of these compounds among the marine algae, much of the work on conjugated fatty acid biosynthesis has pertained to these organisms. Hamberg (1992), for example, has shown that homogenates of the coralline red alga *Lithothamnion corallioides* will convert γ-linolenic acid [(6*Z*,9*Z*,12*Z*)-octadecatrienoic acid] to (6*Z*,8*E*,10*E*,12*Z*)-octadecatetraenoic acid. This reaction was demonstrated to be an oxidative process involving the abstraction of the 11-*pro-R* and 8-*pro-S* hydrogens. A similar oxidative mechanism is likely responsible for the production of bosseopentaenoic acid [(5*Z*,8*Z*,10*E*,12*E*,14*Z*)-eicosapentaenoic acid] from arachidonic acid reported in the marine red alga, *Bossiella orbigniana* (Burgess et al., 1991).

Recently, we described a novel enzyme, polyenoic fatty acid isomerase (PFI), which converts arachidonic acid

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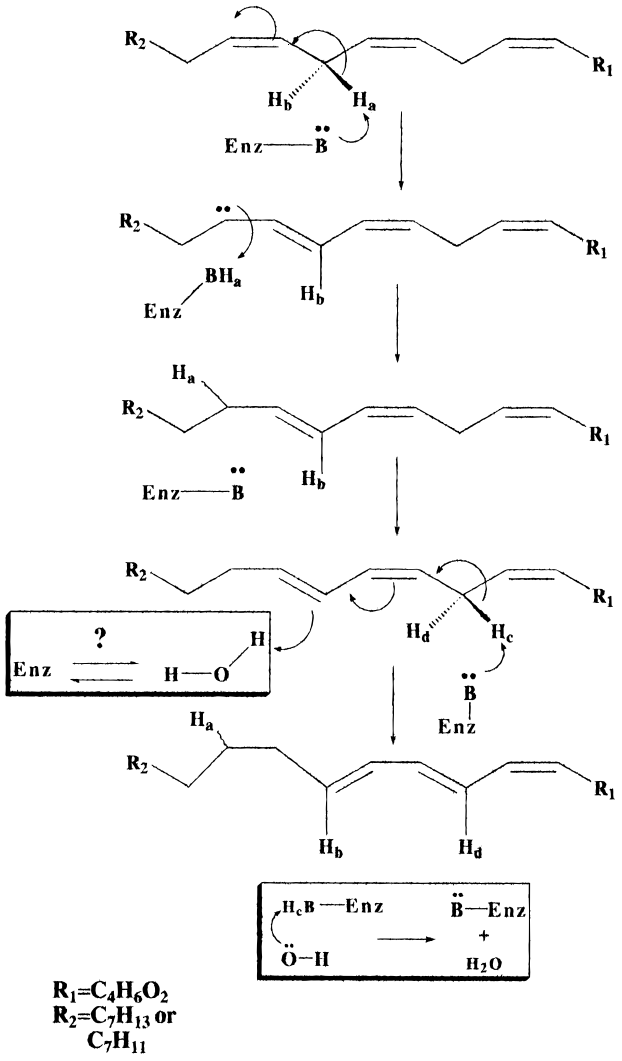
Scheme 1: Proposed Mechanism of Hydrogen Transfers in the PFI Catalyzed Isomerization of Polyunsaturated Fatty Acids



[(5*Z*,8*Z*,11*Z*,14*Z*)-eicosatetraenoic acid] and EPA[1] [(5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)-eicosapentaenoic acid] to the (5*Z*,7*E*,9*E*,14*Z*)-eicosatetraenoic acid and (5*Z*,7*E*,9*E*,14*Z*,17*Z*)-eicosapentaenoic acid regioisomers, respectively (Wise et al., 1994). This enzyme, from the marine alga *Ptilota filicina*, is strictly an isomerase. The process, illustrated in Scheme 1, is proposed to involve two sequential isomerizations. The first, forming a putative diene intermediate, likely involves a 1,3 allylic shift followed by a second proton (or hydrogen) abstraction, migration of the π system, and subsequent protonation by a solvent derived proton. As is evident from this scheme, there is no net desaturation. Furthermore, there is no experimental evidence for an oxidized intermediate (Wise et al., 1994).

In this report we describe the results of the PFI[1] catalyzed isomerization of a broad range of alternative substrates. Based on detailed structural analysis of the product from reaction with docosahexaenoic acid (DHA), the development of a conjugated diene from reaction with dihomo-γ-linolenic acid, and analysis of the kinetic parameters of PFI with several other fatty acid substrates, we hypothesize that the enzyme orients the substrate in the catalytic pocket with respect to the methyl terminus and that it likely reacts, preferentially, with the protonated form of the substrate.

MATERIALS AND METHODS

Enzyme Isolation. The enzyme was purified as described previously from fresh-frozen tissue of the Oregon red marine alga *P. filicina* (Wise et al., 1994) with the modification of an additional hydrophobic interaction chromatography step. Briefly, purification entailed high speed centrifugation (100 000*g*) of the tissue lysate followed by ammonium sulfate precipitation (55−90% ammonium sulfate saturation), gel filtration on Sephacryl S-300, and then isoelectric focusing using a Rainen RF-3 preparative IEF fractionator. To effect final purification, the concentrated material from IEF was subject to hydrophobic interaction chromatography (HIC). The IEF material was brought to 30% ammonium sulfate saturation by adding an equal volume of a 60% ammonium sulfate saturated solution in 20 mM NaH_2PO_4 buffer, pH 7.2. This solution was loaded to a 0.7 × 5 cm column of phenyl Sepharose pre-equilibrated with 20 mM NaH_2PO_4 buffer, pH 7.2, at 30% ammonium sulfate saturation at 4 °C. The column was washed with equilibration buffer, then eluted with a 20% to 0% ammonium sulfate saturated phosphate buffer gradient and collected in 1.0 mL fractions. Active fractions, eluting at approximately 10% ammonium sulfate, were pooled and concentrated, and the buffer was exchanged with 20 mM NaH_2PO_4 buffer using a 30K Amicon Centriprep concentrator according to the manufacturer's instructions. This procedure consistently resulted in pure enzyme preparations as determined by SDS−PAGE (subunit molecular mass 61 000 Da; Wise et al., 1994). Enzyme activity [for EPA, $V_{max} = 6-14$ μmol min^{-1} mg^{-1}) remained stable for months stored at −20 °C.

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) as described previously (Wise et al., 1994). All assays were conducted in 100 mM NaH_2PO_4 with 0.02% Tween-20, pH 7.2 (buffer A), at 22 °C unless otherwise stated. A unit of activity is defined as the formation of 1 μmol of triene product/min, using an extinction coefficient $\epsilon = 57\,000$ L mol^{-1} cm^{-1} (Lopez & Gerwick, 1987). Protein determinations were performed using the Bradford method (Bradford, 1976); lipid free BSA fraction V (Sigma) was used as the standard.

Kinetic Analyses. Kinetic analyses were performed for the various substrates using purified enzyme unless otherwise stated. Duplicate independent experiments were performed using substrate concentrations ranging from (at least) 0.2- to 5.0-fold K_m. A minimum of five, and usually six, substrate concentrations were used for each analysis. The effect of pH on the kinetic parameters of PFI was evaluated from pH 5.0 to 10.0. A reaction buffer composed of 50 mM

[1] Abbreviations: BSA, bovine serum albumin; COSY, correlation spectroscopy; EPA, eicosapentaenoic acid; DEPC, diethyl pyrocarbonate; DHA, docosahexaenoic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; GLC/MS, gas liquid chromatography/mass spectrometry; HIC, hydrophobic interaction chromatography; HPLC, high pressure liquid chromatography; IEF, isoelectric focusing; IR, infrared; MES, 2-(*N*-morpholino)-ethanesulfonic acid; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; PFI, polyenoic fatty acid isomerase; PUFA, polyunsaturated fatty acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TMS, tetramethylsilane; TEA, triethanolamine; UV/vis, ultraviolet/visible.

NaH_2PO_4, 50 mM Na citrate, and 25 mM NaB_7O_3 with 0.3 M NaCl and 0.02% Tween-20 (buffer B) was used for these assays.

Kinetic parameters were determined either from analysis of double reciprocal plots using *Enzyme Kinetics* by Trinity Software or from nonlinear regression analysis using *Enzfitter* software by Biosoft, Cambridge, U.K. Double reciprocal analysis resulted in essentially linear plots with correlation coefficients >0.90. The pH dependence of V_{max} and V/K_m was analyzed by fits to the following equation, descriptive of a model where both protonated and unprotonated forms of the enzyme or substrate are active, using the curve fitting capability of *Sigma Plot* (Jandel Corp.):

$$Y = \frac{C_L(10^{(pK_a - pH)}) + C_H}{1 + 10^{(pK_a - pH)}} \quad (1)$$

In this equation, Y is the kinetic parameter being analyzed, C_L represents the limiting value of Y at low pH and C_H the limiting value at high pH. The points in the plots are experimental data, and the lines are fits to the above equation.

Lipid Extraction and Isolation. In experiments where reaction products were examined by NMR or GLC/MS, the lipid material was extracted and methylated as described previously (Wise et al., 1994). Isolation of the triene methyl esters from unreacted substrate 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. Isocratic elution with 2% EtOAc in hexanes at a flow rate of 4.0 mL/min (3000 psi) was used. Although baseline resolution was not achieved, the conjugated triene methyl esters eluted subsequent to the polyenoic fatty acid substrates on this system; hence it was possible to purify a portion of the reaction products from the unreacted substrate.

GLC/MS Analysis. All GLC/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, and 1−2 μL was injected onto the column. Gas chromatography was performed on a Hewlett Packard 5890 Series II GC 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, initial temperature at 70 °C with a 20 °C/min ramp to a final temperature of 250 °C. Helium was used as the carrier gas.

NMR, IR, and UV Analyses. All NMR experiments were performed on a Bruker ACP 300 instrument and chemical shifts assigned relative to an internal TMS standard or to the solvent signal (in benzene-d_6). UV/vis spectra were recorded on a Hewlett Packard 8452A diode array spectrophotometer. The following data were obtained for the C_{22} methyl ester of the product (**10a**) from DHA: ^{1}H NMR (300 MHz, $CDCl_3$) data: δ 0.97 (3H, t, J = 7.5 Hz, H_3-22), 1.47 (2H, tt, J = 7.5, 7.5 Hz, H_2-14), 2.09 (6H, m, H_2-13,15,21), 2.39 (4H, m, H_2-2,3), 2.77 (2H, bdd, J = 5.9, 5.9 Hz, H_2-18), 2.95 (2H, bdd, J = 6.6, 6.6 Hz, H_2-6), 3.67 (3H, s, H_3-1′), 5.27−5.43 (7H, m, H-4, 5, 7, 16, 17, 19, 20), 5.72 (1H, dt, J = 14.2, 7.2 Hz, H-12), 6.01 (1H, bdd, J = 10.9, 10.9 Hz, H-8), 6.11 (1H, bdd, J = 14.2, 10.0 Hz, H-11), 6.19 (1H, bdd, J = 13.1, 10.0 Hz, H-10), 6.40 (1H, bdd, J = 13.1, 10.9 Hz, H-9).

Protein Modification Reagents. To explore the role of specific amino acid residues on the catalytic function of the enzyme, several chemical modification reagents were employed. Inactivation of nucleophilic residues was attempted by incubating the enzyme in a 50 mM solution of iodoacetamide in 100 mM triethanolamine (TEA) buffer (pH 7.5) for 2 h (Coleman, 1968). Lysyl residues were modified by incubation of the enzyme in 1.0 mM pyridoxal in 100 mM NaH_2PO_4 buffer, pH 6.5, for 2 h (Price & Stevens, 1989).

EDC [1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide] in concert with glycine methyl ester was used to inactivate carboxylate residues (Hoare & Koshland, 1966). The enzyme was incubated in the presence of 5 mM EDC with 50 mM glycine methyl ester in 50 mM MES buffer, pH 5.0, for 60 min. Because the substrate has a carboxylate group, it was necessary to quench any unreacted EDC. Therefore, the reaction solution was brought to 10 mM glycine by adding an equal volume of a 20 mM glycine solution. After an additional 30 min incubation, duplicate assays of the EDC/glycine methyl ester treated enzyme were compared to a control treatment of enzyme in MES buffer with 10 mM glycine.

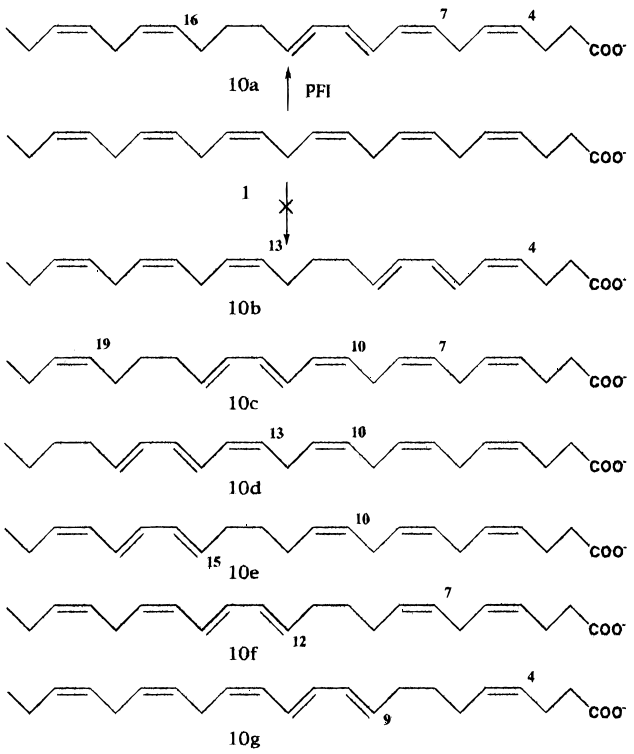
Diethyl pyrocarbonate (DEPC) was used to modify histidine residues (Miles, 1977). To evaluate this reagent, a 0.7 M solution of DEPC was made by diluting the stock reagent 1:10 in ethanol. A 1 μL aliquot of the DEPC solution was added to a 100 μL solution of NaH_2PO_4 buffer, pH 6.5, containing approximately 1×10^{-3} unit of PFI, making a final concentration of 6.9 mM DEPC. Control reactions were made by adding 1 μL of ethanol to an identical enzyme solution. Control and treatment solutions were assayed by diluting them with 900 μL of reaction buffer in a quartz cuvette and assaying in the standard manner.

Modification of cysteinyl residues was explored with Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)) (DTNB) (Coleman, 1969). A 250 μL preparation of PFI containing 16×10^{-3} unit in 100 mM TEA buffer, pH 8.0, was made up to 0.5 mM DTNB by adding an equal volume of a 1 mM DTNB solution in TEA. The control was diluted with an equal volume of TEA without DTNB. At timed intervals, over a period of 2 h, an aliquot of each solution was assayed for PFI activity using 131 μM arachidonic acid.

RESULTS

PFI Activity with Alternative Substrates. To probe the nature of the substrate binding and catalytic site, a series of experiments was performed using the following substrates (see Figure 4): DHA (**1**) [(4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*)-docosahexaenoic acid]; adrenic acid (**2**) [(7*Z*,10*Z*,13*Z*,16*Z*)-docosatetraenoic acid]; EPA (**3**) [(5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)-eicosapentaenoic acid]; arachidonic acid (**4**) [(5*Z*,8*Z*,11*Z*,14*Z*)-eicosatetraenoic acid]; anandamide (**5**) (arachidonyl-*N*-ethanolamide); dihomo-γ-linolenic acid (**6**) [(8*Z*,11*Z*,14*Z*)-eicosatrienoic acid]; eicosatrienoic acid (**7**) [(5*Z*,8*Z*,11*Z*)-eicosatrienoic acid]; α-linolenic acid (**8**) [(9*Z*,12*Z*,15*Z*)-octadecatrienoic acid]; γ-linolenic acid (**9**) [(6*Z*,9*Z*,12*Z*)-octadecatrienoic acid]; and linoleic acid (**11**) [(9*Z*,12*Z*)-octadecadienoic acid]. Under standard assay conditions, all of these compounds proved to be substrates for PFI, as demonstrated by the development of a characteristic triene chromophore (tripeaked spectrum with λ_{max} = 276 nm; Lopez & Gerwick, 1987), with the exception of linoleic acid, which

Scheme 2: Selected Examples of Potential Conjugated Triene Containing Products from Reaction of PFI with DHA



showed the development of a chromophore with a single UV maximum at 238 nm, characteristic of a conjugated diene (Smith & Lands, 1972).

Characterization of the Reaction Product of DHA. Reaction of PFI with the 22 carbon fatty acid DHA (**1**) produced a triene chromophore as evidenced by UV spectroscopy; however, the regiochemistry of the conjugated triene system was not clear. It might be assumed that the product has the conjugated system starting at the first olefinic group (as is the case with the 18 and 20 carbon fatty acids examined (Wise et al., 1994)), resulting in (4*Z*,6*E*,8*E*,13*Z*,16*Z*,19*Z*)-docosahexaenoic acid (**10b**). However, other possibilities, including the 4*Z*,7*Z*,9*E*,11*E*,16*Z*,19*Z* (**10a**), 4*Z*,7*Z*,10*Z*,12*E*,14*E*,19*Z* (**10c**), 4*Z*,7*Z*,10*Z*,13*Z*,15*E*,17*E* (**10d**), 4*Z*,7*Z*,10*Z*,15*E*,17*E*,19*Z* (**10e**), 4*Z*,7*Z*,12*E*,14*E*,16*Z*,19*Z* (**10f**), or the 4*Z*,9*E*,11*E*,13*Z*,16*Z*,19*Z* (**10g**) isomers could not be discounted (Scheme 2).

To determine which isomer was produced, a large scale incubation of DHA (**1**) with PFI was performed using 10 mg of DHA in 50 mL of buffer A, pH 6.5 (608 μM DHA), and 18.5×10^{-3} unit of PFI at 22 °C. After 8 h, the increase in absorbance at 276 nm plateaued with 21% conversion of DHA to the triene product. The reaction was quenched with 5 volumes of MeOH and the lipids were extracted, methylated, and the methyl esters separated by HPLC. The isolated triene product (0.89 mg total) was analyzed by a combination of UV/vis spectroscopy, GLC/MS, ^{1}H NMR, ^{1}H−^{1}H COSY, and NOE difference experiments. In $CDCl_3$, signals at 2.77 and 2.95 ppm were observed, each exhibiting a broad doublet of doublet pattern characteristic of bisallylic protons. Both of these signals integrated for two protons, demonstrating the presence of two bisallylic methylenes. The H_2-2 and H_2-3 signals appeared as a broad multiplet at δ 2.39 integrating for four protons. The terminal methyl (H-22) protons appeared as a sharp triplet at 0.97 ppm. The ω-2 protons (H-21), characterized by a quadruplet at 2.11 ppm in DHA, were buried in the complex signal from the H-13 and H-15 methylenes.

To confirm the connectivity of the proton signals, a ^{1}H−^{1}H COSY experiment was conducted in benzene-d_6. In benzene the broad multiplet observed at δ 2.39 in $CDCl_3$ separated into distinct signals at δ 2.30 and δ 2.11 ppm. Also, the olefinic protons H-8, -9, -10, and -11 are shifted slightly downfield and H-12 shifted slightly upfield relative to the spectrum in $CDCl_3$. The bisallylic methylenes appear at δ 2.81 and δ 2.91. The ^{1}H−^{1}H COSY experiment showed coupling from the Δ-2 methylene at δ 2.30 through the methylene at δ 2.11 (H_2-3) to the olefinic protons at δ 5.28−5.50. The terminal methyl protons at δ 0.92 coupled to a cluster of methylene protons at δ 2.02 (integrating for six protons), and these coupled to a methylene at δ 1.39 as well as the olefinic protons at δ 5.28−5.50. This pattern of spin coupling results from the fact that the H_2-21 signal is buried in the peak attributable to the H_2-13 and H_2-15 methylenes. The bisallylic methylenes both showed crosspeaks only with the cluster of olefinic protons at δ 5.28−5.50. The low field olefinic spin system showed couplings from the cluster at δ 5.28−5.50 through the proton at δ 6.08 to the proton at δ 5.59 then to δ 6.13, δ 6.21, and finally to the signal at δ 6.51.

Proton decoupling experiments in $CDCl_3$ demonstrated a long range allylic coupling between the bisallylic group at δ 2.95 and the olefin at δ 6.01, thus establishing a ^4J coupling between one of the bisallylic groups and the conjugated olefin system. Irradiation at 2.39 ppm showed no effect on any of the low field signals, and irradiation of the bisallylic signal at 2.77 ppm only resulted in an ill-defined alteration of the signal at δ 5.17−5.43, indicating ^4J coupling to one (or both) of the two isolated olefins. In benzene-d_6, irradiation of the bisallylic signal at δ 2.91 resulted in a sharpening of the low field signal at δ 6.08. This spectrum could only result if two bisallylic groups are present and one is allylically coupled to the conjugated triene system. Hence the product of reaction of DHA with PFI is neither the **10b**, **10d**, nor the **10e** isomer.

To differentiate the remaining possibilities, a series of NOE difference experiments was performed. By irradiating the compound in $CDCl_3$ at 2.39 ppm (H_2-2, 3), a slight, though distinct enhancement of the signal at δ 2.95 (H_2-6) was observed. When the sample was irradiated at 2.95 ppm (H_2-6), an NOE was observed to signals at δ 2.39 (H_2-2, 3), δ 5.40, and δ 6.40 (H-9). Finally, irradiation at 2.77 ppm (H_2-18) resulted in an NOE at δ 0.97 (H_3-22), δ 2.09 (H_2-21), and δ 5.40. Taken together with the UV spectrum, the ^{1}H−^{1}H COSY, and the proton decoupled experiments, these results firmly establish the double bonds at the 4, 7, 9, 11, 16, and 19 positions. The relative stereochemistry of the conjugated system is based on coupling constant analysis. The olefins at the 4, 16, and 19 positions could not be unequivocally defined from this data; they are presumed to be unchanged from the substrate. With the caveat above, the product of the isomerization of DHA by PFI is determined to be (4*Z*,7*Z*,9*E*,11*E*,16*Z*,19*Z*)-docosahexaenoic acid (**10a**) (Scheme 2).

*Reaction of PFI with Dihomo-γ-linolenic Acid (**6**).* The reaction with dihomo-γ-linolenic acid (**6**) also provided insight into the binding characteristics of PFI. Although the reaction was relatively slow, the simultaneous development

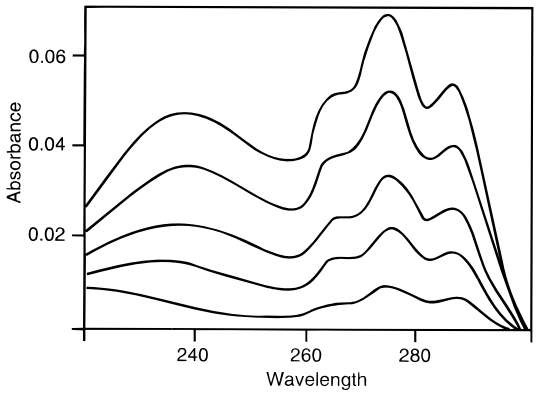


FIGURE 1: UV/vis spectra of the reaction of PFI with dihomo-γ-linolenic acid (**6**) showing the simultaneous development of a characteristic three-peaked conjugated triene chromophore (λ_{max} = 276 nm) and the putative conjugated diene chromophore (λ_{max} = 236 nm). Spectra were taken after 10, 20, 30, 45, and 60 min of reaction (from bottom to top).

of two chromophores, one with the characteristic triene absorption pattern and another with a single maximum at 238 nm, was clearly evident (Figure 1). This appeared to represent the production of both a conjugated diene and a conjugated triene, in concert. To further investigate these phenomena, a large scale incubation was performed using 33×10^{-3} unit of enzyme in 50 mL of buffer A, pH 6.5, with 5 mg of dihomo-γ-linolenic acid (**6**) (327 μM final substrate concentration) at 22 °C. The reaction was allowed to run at room temperature and monitored with periodic spectra. After 9 h, when the triene chromophore appeared to reach a maximum (28% conversion of substrate to the products below), the reaction was quenched with 5 volumes of MeOH and the lipids were extracted and methylated for GLC/MS analysis. Using $\epsilon = 24\,000\ M^{-1}\ cm^{-1}$ (Smith & Lands, 1972) and $\epsilon = 57\,000\ M^{-1}\ cm^{-1}$ (Lopez & Gerwick, 1987) as the extinction coefficients for the putative diene and the triene chromophores, respectively, the diene was calculated to be produced at a 2.6:1 ratio to the conjugated triene product throughout most of the reaction (just prior to quenching there was a perceptible increase in this ratio to 4.4:1; in earlier extended incubation studies with AA, we observed that the initial production of a conjugated triene is followed by a decrease in the triene chromophore and reciprocal increase in a diene chromophore—the observed decrease in triene was used to gauge the timing for termination of the incubation) (Wise, 1995). GLC/MS analysis of the methylated reaction products from this scaled-up incubation showed three distinct peaks on the chromatogram, all with a $M^+_{obs} = 320$, corresponding to the methyl esters of dihomo-γ-linolenic acid, the putative conjugated diene, and conjugated triene (Figure 2).

A similar reaction was performed with α-linolenic acid (**8**) using 16.5×10^{-3} unit of enzyme with 5 mg of α-linolenic acid in 50 mL of buffer A (360 μM), pH 6.5, and incubating overnight (23.5 h). The reaction was quenched with MeOH, and the lipids were extracted and methylated. Although the diene chromophore did not appear as rapidly, appearing only as a shoulder to the triene chromophore during the first 14 h of reaction, it was clearly present at termination of the reaction. The UV spectra showed a ratio of diene to triene of approximately 2:1 (using the same extinction coefficients as above). The GLC/MS analysis of the methyl esters again showed three distinct

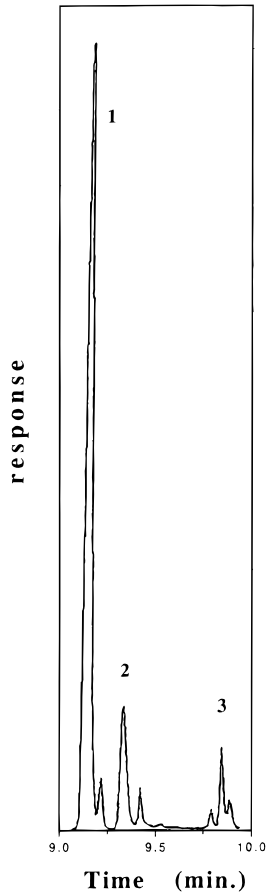


FIGURE 2: GLC chromatogram of the methyl esters of the reaction products of PFI with dihomo-γ-linolenic acid (**6**) (detector response represents the total ion count). Each of the major peaks showed an $M^+_{obs} = 320$ consistent with a molecular formula of $C_{21}H_{36}O_2$. Peak 1 has a t_R identical to authentic dihomo-γ-linolenate methyl ester, and hence, is the unreacted substrate. Peak 2 is likely the conjugated diene product and peak 3 the conjugated triene. The small peaks adjacent to the larger peaks all showed the same $M^+_{obs} = 320$ molecular ion and identical fragmentation patterns as the associated major peak and are likely thermal isomerization products generated on the GLC (a phenomenon commonly observed with conjugated PUFAs on GLC (Wolff & Miwa, 1965)).

peaks, all with $M^+_{obs} = 292$, consistent with a molecular formula $C_{19}H_{32}O_2$. The first peak was the methyl ester of the unreacted substrate, α-linolenic acid, the other two likely represent the conjugated diene and conjugated triene isomers.

Kinetic Parameters with Alternate Substrates. Steady state kinetic analyses were performed using DHA (**1**), adrenic acid (**2**), EPA (**3**), arachidonic acid (**4**), eicosatrienoic acid (**7**), and anandamide (**5**) to quantify their kinetic parameters; the results are presented in Table 1.[2] This analysis shows a clear trend in K_m values, with the ω-3 fatty acids **1** and **3** having the lowest values. Eicosatrienoic acid (**7**), possessing an ω-9 olefin, had the highest K_m, and the ω-6 fatty acids **2** and **4**, as well as anandamide (**5**), showed intermediate K_m values. In terms of V_{max}, the C_{20} EPA (**3**) was highest, followed by arachidonic acid (**4**) (another C_{20} PUFA). The C_{22} substrates DHA (**1**) and adrenic acid (**2**) reacted somewhat more slowly,

[2] The kinetic parameters for EPA **3** and arachidonic acid **4** presented in Table 1 differ somewhat from those previously reported (Wise et al., 1994). The values in Table 1 reflect experimental conditions with a more appropriate range of substrate concentrations and hence are considered more accurate.

Table 1: Kinetic Parameters[a] of PFI with Various Substrates (±SE)[b]

	docosahexaenoate (**1**)	adrenate (**2**)	EPA (**3**)	arachidonate (**4**)	eicosatrienoate (**7**)	anandamide (**5**)
V_{max}^{app} (μmol min^{-1} mg^{-1})	1.84 ± 0.11	1.10 ± 0.035	6.0 ± 0.32	2.78 ± 0.16	0.31 ± 0.021	0.80 ± 0.09
K_m^{app} (μM)	1.9 ± 0.36	17.0 ± 1.5	9.61 ± 1.2	25.5 ± 3.9	52.8 ± 9.2	17.5 ± 5.0
V_{max}/K_m	0.97	0.065	0.62	0.11	0.006	0.045

[a] The V_{max} and K_m should be interpreted as apparent values because the reactions were not performed at the optimum pH and the effect of the Tween-20 on all substrates has not been evaluated. [b] Reactions were performed in buffer A at pH 7.0 except anandamide which was reacted in buffer B at pH 7.0.

followed by anandamide (**5**). The C_{20}, ω-9 fatty acid, eicosatrienoic acid (**7**), proved to be the poorest substrate in terms of V_{max} as well as K_m.

pH Profile of Kinetic Parameters. In initial experiments to evaluate the pH optimum of the enzyme, a number of buffers ranging from pH 2.0 to 8.0 were examined (Wise et al., 1994). Observation of a maximum velocity at pH 4.5 suggested the possibility that the enzyme prefers the protonated form of the substrate. It was subsequently discovered that PFI can utilize another nonionizable analog of arachidonic acid, anandamide (**5**), as a substrate (Wise et al., 1996). Again, the enzyme shows an apparent velocity maximum (under saturating conditions) at pH 4.5 with this compound; hence, it appears that an ionized carboxylate is not required for binding.

To investigate the identity of the residues involved in substrate binding and catalysis, the effect of pH on the reaction kinetics of PFI with EPA (**3**) as substrate was examined. The stability of the enzyme at the two extremes used in the pH dependence studies was evaluated by incubating the enzyme in buffer B at pH 4.0 and at pH 10.0. At intervals of 5, 10, and 15 min, 1 mL aliquots were removed, the pH was adjusted to 7.0 by dropwise addition of 5 N HCl or 5 N NaOH, and PFI activity was assayed. In three replicate experiments, after 15 min incubation, the enzyme showed >80% of the activity of the control at the lower pH and >90% of the control activity at the higher pH. Hence the enzyme is relatively stable at these pH extremes.

Figure 3 graphically illustrates the results of the pH dependence studies. The effect of pH on V/K_m shows a logarithmic decrease between pH 7.0 and 9.0, and the curve fit to eq 1 resulted in a pK_a = 7.1 ± 0.1. The V_{max} also displayed an upper and lower limit at low and high pH, with a calculated pK_a = 8.4 ± 0.1.

The fact that PFI is reactive with anandamide (**5**) provided an opportunity to explore the role of the carboxylate functionality in substrate binding and catalysis. Because anandamide (**5**) lacks the carboxylate functionality of fatty acid substrates, the pH effect on the kinetic parameters of this substrate might provide information about the mechanism of substrate binding. To evaluate this, steady state kinetic analyses were performed in buffer B at pH 7.0 and 9.0 using purified PFI (0.84 μg per assay, 1.9×10^{-3} unit). Table 2 shows the results of these experiments. These data demonstrate no significant pH dependence on the kinetic parameters of PFI with anandamide (**5**) as a substrate over a pH range where a 27-fold decrease in V/K_m was observed using EPA (**3**) as the substrate.

Protein Modification. After chemical modification, the enzyme was assayed under substrate saturating conditions. None of the protein modification agents used had any observable effects on enzyme activity. However, because of the possible role of a histidine residue in substrate binding (based on the pH dependency profile of V/K_m) we tried assessing the effect of DEPC under substrate limiting conditions at pH 6.5. Again, no discernible change in K_m or V_{max} was observed with the DEPC treated enzyme relative to the untreated control.

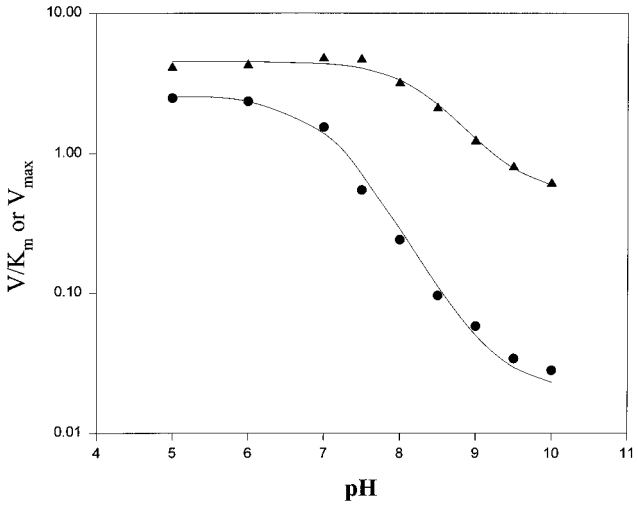


FIGURE 3: pH profiles of PFI with EPA as substrate: (▲) V_{max}^{app} and (●) V_{max}^{app}/K_m (V_{max} is expressed in terms of μmol min^{-1} mg^{-1} and K_m in terms of μM). The points represent experimental data; the solid line represents a fit to eq 1 (see Materials and Methods). A pK_a = 7.1 was determined from the V_{max}^{app}/K_m curve and pK_a = 8.4 from the V_{max}^{app} curve. The V_{max}^{app} values are approximated because the enzyme preparation used was not pure. The approximation is based on a correlation of the activity of the impure preparation with a known amout of the pure enzyme (0.17 μg); all experiments were performed with the same enzyme preparation.

Table 2: Effect of pH on Kinetic Parameters of PFI with Anandamide (**5**)[a]

	pH 7.0	pH 9.0
V_{max}^{app} (μmol min^{-1} mg^{-1})	0.80 ± 0.09	0.74 ± 0.09
K_m^{app} (μM)	17.5 ± 5.0	15.2 ± 4.5
V_{max}/K_m	0.046	0.049

[a] Reactions were performed in buffer B. Data represent the mean of two independent experiments ±SE.

DISCUSSION

Kinetic Parameters of Alternative Substrates. Comparison of the kinetic parameters using alternative substrates provides an intriguing insight into the nature and requirements of the binding site of PFI. From the relatively low K_m of docosahexaenoic acid (**1**) and EPA (**3**) (1.9 and 9.6 μM, respectively), it appears that an ω-3 olefin clearly increases the binding affinity of the substrate. The ω-6 olefinic groups

in adrenic acid (**2**) and arachidonic acid (**4**) give intermediate affinities (K_m = 17.0 and 25.5 μM, respectively), and eicosatrienoic acid (**7**) with an ω-9 olefinic group has a relatively high K_m (52.8 μM) despite its structural identity with EPA and arachidonic acid in the C1 to C13 region of the molecule. The enzyme was previously shown to convert γ-linolenic acid (**9**) [(6*Z*,9*Z*,12*Z*)-octadecatrienoic acid] to (6*Z*,8*Z*,10*Z*)-octadecatrienoic acid (Wise et al., 1994); in these experiments a K_m = 152 μM was determined.[3] The trend in these K_m values suggests that the enzyme recognizes an olefinic group proximal to the methyl end of the substrate. There is, however, a clear enhancement of catalytic efficiency associated with the presence of a carboxylate group. EPA (**3**), possessing both the carboxylate and the ω-3 olefin, has the highest V_{max} (6.0 μmol min^{-1} mg^{-1}) of any of the substrates tested, corresponding to a k_{cat} = 11.6 s^{-1}, assuming one catalytic site per molecule and a molecular weight of 116 kDa (Wise and Gerwick, work in progress). All of the fatty acids possessing either ω-3 or ω-6 olefins and a terminal carboxylate showed relatively high catalytic efficiencies. By comparison, eicosatrienoic acid (**7**) has a V/K_m value 2 orders of magnitude lower than EPA (**3**). Anandamide (**5**), lacking a carboxylate, has a V/K_m value nearly an order of magnitude lower than EPA (Table 1).

pH Dependency Profile of V/K_m and V_{max}. Interpretation of the pH dependency profile (Figure 3) is complicated by the fact that the substrate has an ionizable moiety. Although literature values for the carboxylate group of short chain (C_2−C_9) fatty acids are typically in the range of 4.8−5.0 (March, 1992), the pK_a of linoleic acid has recently been reported to range from 7.0 to 8.0 depending on concentration (Glickman & Klinman, 1995). It is therefore unclear whether the pK_a = 7.1 found in the V/K_m analysis represents an ionizable group on the free enzyme or the substrate. Likewise, the shift in the effect of pH on the V_{max} profile might reflect a change in the pK_a of either the substrate or the enzyme in the enzyme substrate complex.

The pH effect on the V/K_m profile does suggest that the enzyme prefers the protonated form of the substrate. If the carboxylate were the preferred form of the substrate, one would expect to see a downward curve of the V/K_m profile at the lower end of the pH range investigated. The fact that the opposite trend is observed indicates either that the enzyme preferentially binds the protonated form of the substrate or that the protonation state does not significantly effect binding. Moreover, the observation that the kinetic parameters of the nonionizing substrate anandamide showed no pH sensitivity is consistent with the hypothesis that it is the protonation state of the substrate which is responsible for this pH dependency. While comparison between anandamide and AA would have been desirable, the impractacality of using AA at higher pH's (its higher K_m would have required prohibitively high concentrations of AA at these pH's) made EPA the more reasonable choice. The differences between EPA and anandamide in these pH profiles are most rationally explained by the presence or absence of the carboxylate functionality rather than the ω-3 double bond. If a residue on the free enzyme were responsible for the pH effect, the calculated pK_a = 7.1 would suggest histidine; treatment of the enzyme with DEPC resulted in no alteration in V_{max} or V/K_m. Again, more direct evidence will be necessary to conclusively resolve this issue.

[3] A K_m this large undoubtedly reflects an effect of the low substrate solubility.

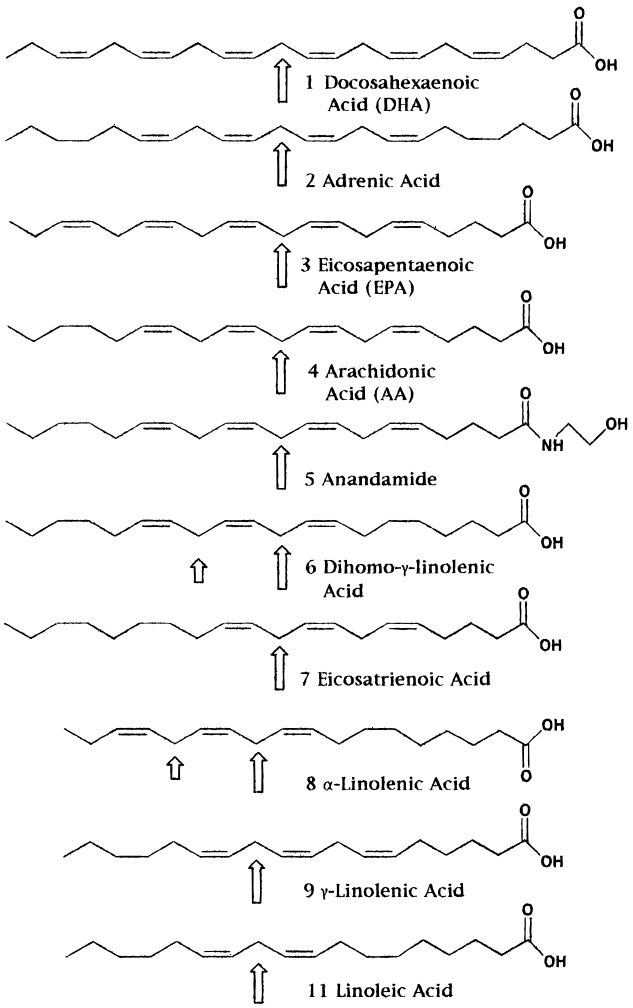


FIGURE 4: Polyenoic fatty acid substrates for PFI aligned with respect to the methyl termini (except for the C_{18} fatty acids which are aligned with respect to the carboxyl termini of the C_{20} substrates). The arrows point to the position of the initial proton (hydrogen) abstraction, assuming a conjugated diene intermediate in the formation of the conjugated triene product. The two arrows under dihomo-γ-linolenic acid (**6**) and α-linolenic acid (**8**) indicate that both a conjugated diene (large arrow) and a conjugated triene (small arrow) are formed from these substrates; the size of the arrow depicts the relative proportion of products formed. Only a conjugated diene product was formed from linoleic acid (**11**).

Substrate Orientation in the Binding Pocket. We suggest that the enzyme recognizes the olefin(s) proximal to the methyl terminus for binding and that the carboxylic acid group is used to position the substrate in the reaction site for the most efficient alignment of catalytic moieties. Figure 4 shows the substrates oriented with their methyl termini aligned. This figure illustrates the relative positions of the abstracted protons viewed in a linear fashion.

In the case of EPA (**3**), the combination of the ω-3 olefin, the carboxyl group, and the appropriate chain length allows for the most efficient positioning within the catalytic pocket. DHA (**1**) is also an excellent substrate in terms of binding, but, because of the additional two carbons, probably does not fit into the catalytic site as well as EPA. The fact that the only product of reaction of DHA with PFI is the 4*Z*,7*Z*,9*E*,11*E*,16*Z*,19*Z* isomer (**10a**) presents substantial evidence that the enzyme positions the substrate with respect

to the ω-terminus. Eicosatrienoic acid (**7**), by virtue of its 1,4,7 heptatriene functional group, is converted to the conjugated triene, but lacking an ω-3 or ω-6 olefin the binding efficiency is quite low ($K_m = 52.8\ \mu$M) and the V_{max} is more than 2 orders of magnitude lower than for EPA. Ostensibly, α-linolenic acid (**8**) and γ-linolenic acid (**9**) appear to defy this trend. Because the chain length of these two substrates may not allow for binding of the ω-terminus in a catalytically permissive orientation, the primary binding mechanism for these substrates may well be through the carboxylic acid group. From this orientation the protons at the C11 position in γ-linolenic acid (**9**) would occupy a space close to the protons in the C10 position of arachidonic acid, allowing for catalysis. This would explain the relatively high K_m ($150 \pm 35\ \mu$M) previously determined for γ-linolenic acid (**9**) (Wise et al., 1994). Reaction with α-linolenic acid (**8**), like dihomo-γ-linolenic acid (**6**), resulted in a preponderance of conjugated diene products (putative conjugated dienes from C9−C12 and/or C13−C16). Again, this is consistent with a proclivity of the enzyme to first abstract a proton from the C11 position of C_{18} substrates.

The evidence of a diene product, free in solution, from reaction of PFI with dihomo-γ-linolenic acid demonstrates that the overall reaction is likely not a concerted one. Although consistent with our model (invoking a conjugated diene intermediate), the conjugated diene product from reaction with dihomo-γ-linolenic acid should not be construed as incontrovertible evidence for this mechanism. Rather, these results simply demonstrate that this substrate probably aligns in the catalytic pocket in such a manner that a C10 methylene proton is transferred to the C12 position, resulting in the putative 8*Z*,10*E*,14*Z* stereoisomer. Because this product lacks another appropriately placed olefin, it cannot be further isomerized and is therefore released from the enzyme. However, it appears that there is some latitude in the catalytic site, allowing approximately 25% of the dihomo-γ-linolenic acid to be converted to a conjugated triene by an initial abstraction of a C13 methylene proton. This may result when the substrate occasionally aligns in a manner allowing initial abstraction of the C13 proton. This intermediate can then undergo subsequent isomerization, resulting in a conjugated triene product.

In our previous report on the regio- and stereochemistry of the hydrogen transfers catalyzed by PFI, we demonstrated a substantial kinetic isotope effect k_H/k_D using (8*R*)-[8-^{2}H]-γ-linolenic acid as a substrate. The isotope effect was only manifested by an enrichment of deuterium in the unreacted substrate. No deuterium enriched diene intermediate (or any other chemical species including product) was observed (Wise et al., 1994). The observation of a free diene product from reaction with dihomo-γ-linolenic acid (**6**) and α-linolenic acid (**8**) brings into question our previous conclusion that the diene intermediate is tightly bound. However, unlike the other substrates examined, neither dihomo-γ-linolenic acid (**6**) nor α-linolenic acid (**8**) has an olefin on the carboxyl side of the putative enzyme bound conjugated diene intermediate. This might be a factor in product release that could account for this apparent discrepancy.

The physiological role of conjugated fatty acids in marine algae is yet to be determined. However, from the broad phylogenetic and geographic distribution of these metabolites (Burgess et al., 1991; Lopez & Gerwick, 1987; Mikhailova et al., 1995) they undoubtedly serve an important biological function. We believe that the exploration of the biosynthetic machinery involved in the production of these compounds as well as other marine algal secondary metabolites will continue to unveil a variety of novel enzymes possessing unique catalytic features.

SUPPORTING INFORMATION AVAILABLE

Figures giving 300 MHz NMR, ^{1}H−^{1}H COSY, homonuclear ^{1}H-decoupling, and NOE difference spectra data for compounds in this study (11 pages). Ordering information is given on any current masthead page.

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