The Highly Stereoselective Oxidation of Polyunsaturated Fatty Acids by Cytochrome P450BM-3*

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Cytochrome P450BM-3 catalyzes NADPH-dependent metabolism of arachidonic acid to nearly enantiomerically pure 18(R)-hydroxyeicosatetraenoic acid and 14(S),15(R)-epoxyeicosatrienoic acid (80 and 20% of total products, respectively). P450BM-3 oxidizes arachidonic acid with a rate of 3.2 ± 0.4 μmol/min/nmol at 30 °C, the fastest ever reported for an NADPH-dependent, P450-catalyzed reaction. Fatty acid, oxygen, and NADPH are utilized in an approximately 1:1:1 molar ratio, demonstrating efficient coupling of electron transport to monooxygenation.

Eicosapentaenoic and eicosatrienoic acids, two arachidonic acid analogs that differ in the properties of the C-15–C-18 carbons, are also actively metabolized by P450BM-3 (1.4 ± 0.2 and 2.9 ± 0.1 μmol/min/nmol at 30 °C, respectively). While the 17,18-olefinic bond of eicosapentaenoic acid is epoxidized with nearly absolute regio- and stereoselectivity to 17(S),18(R)-epoxyeicosatetraenoic acid (99% of total products, 97% optical purity), P450BM-3 is only moderately regioselective during hydroxylation of the eicosatrienoic acid ω-1, ω-2, and ω-3 sp3 carbons, with 17-, 18-, and 19-hydroxyeicosatrienoic acid formed in a ratio of 2.4:2.2:1, respectively.

Based on the above and on a model of arachidonic acid-bound P450BM-3, we propose: 1) the formation by P450BM-3 of a single oxidant species capable of olefinic bond epoxidation and sp3 carbon hydroxylation and 2) that product chemistry and, thus, catalytic outcome are critically dependent on active site spatial coordinates responsible for substrate binding and productive orientation between heme-bound active oxygen and acceptor carbon bond(s).

Miura and Fulco (1, 2) originally reported by that extracts of Bacillus megaterium could monooxygenate fatty acids. The enzyme responsible for this reaction was isolated, purified, cloned, sequenced, and found to be a 120-kDa fusion protein of class II cytochrome P450 and NADPH:P450 reductase and was named cytochrome P450BM-3 (P450BM-3)3 (3, 4).

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1 The abbreviations used are: P450BM-3, CYP102, the soluble, bacterial P450 isolated from Bacillus megaterium; P450BM-P, the heme-protein domain of P450BM-3; EET, cis-epoxyeicosatrienoic acid; EPA, eicosapentaenoic acid; ETA, eicosatrienoic acid; 16-, 17-, 18-, 19-, and 20-OH-AA, 16-, 17-, 18-, 19-, or 20-hydroxyeicosatetraenoic acid; P450BM-3 contains heme, FAD, and FMN in a stoichiometry of 1:1:1, respectively (3). Both purified and recombinant P450BM-3 catalyze NADPH-dependent oxidation of medium and long chain saturated fatty acids, with optimum chain lengths of 14–16 carbons (3, 5). The regiochemistry of fatty acid hydroxylation by P450BM-3 is more or less chain length-dependent, i.e. as chain length increases, regioselectivity shifts from the ω-1 to the ω-2 carbon of the fatty acid (5). The turnover number of purified P450BM-3, in the monooxygenation of palmitic acid, is approximately 1.6 μmol/min/nmol at 25 °C (3, 5), which is similar to that of P450cam for camphor hydroxylation under similar conditions (6) and 100–1,000 times greater than that seen for most substrates with mammalian P450s. Monoxygenation of fatty acids by P450BM-3 is tightly coupled with a stoichiometry of NADPH, oxygen, and fatty acid consumed to hydroxylated fatty acid product formed of 1:1:1:1 (5). Under conditions of limiting substrate, P450BM-3 will further oxidize the initial metabolites to products that include diols and ketones (7).

In eukaryotes, AA serves both a structural role as a component of cellular membranes, and a critical functional role by participating in a variety of receptor/agonist-mediated signaling cascades (8). The latter role is a consequence of regio- and stereoselective oxygenations of AA that is utilized by mammalian cells to transduce the signal (8–13). The transduced chemical information is then decoded either by receptor mediated processes or, alternatively, by the direct effects of the oxygenated products on metabolic pathways (8–13). In addition to prostaglandin synthases and lipoxygenases, well recognized members of the AA cascade, the contribution of microsomal P450 to the metabolism of endogenous AA pools is now well established (9–13).

Eukaryotic P450s oxidize AA by one or more types of reactions: 1) allylic oxidation to generate six different regioisomeric hydroxyeicosatetraenoic acids containing a cis,trans-conjugated dienol, 2) hydroxylation at sp3 carbons near or at the methyl terminus to generate 16-, 17-, 18-, 19-, or 20-OH-AA, and 3) olefinic bond epoxidation to generate four regioisomeric EETs. While the physiological significance of allylic oxidation remains obscure, products of hydroxylation and olefinic bond epoxidation reactions display a variety of potent biological activities and have been implicated in processes ranging from hormonal signaling to the pathophysiology of hypertension (9–13).
As with other metabolites of the AA cascade, e.g. prostanoids and leukotrienes, regio- and stereochemical features define biological activity and/or potency. Thus, understanding active site topology and the structural determinants of asymmetric catalysis by P450s are prerequisites for modifying their mechanism of action, and ultimately, for rational pharmacological intervention. Studies using microsomal fractions, and/or solubilized and purified mammalian P450 isoforms have demonstrated that the hemoprotein controls the regio- and stereoselectivities of oxidation of AA in an isoform-specific fashion at three different levels: 1) the type of reaction catalyzed, i.e. epoxidation to form EETs by CYP2B and CYP2C isoforms (9, 11, 13); 2) positional selectivity, i.e. differentiation among four chemically equivalent olefinic bonds or between five sp<sup>2</sup> hybridized carbons (hydroxylations at C-16–C-20) (11, 13); and 3) absolute configuration. These processes are all the more remarkable since AA is an unbiased, acyclic molecule with high rotational freedom, and it is epoxidized by several mammalian P450 isoforms with unprecedented stereoselectivity (9, 11, 13).

As a guide to the protein structural determinants of polysaturated fatty acid metabolism by P450s, we describe herein the highly regio- and stereoselective metabolism of AA by recombinant P450BM-3, one of the four soluble P450s for which an atomic structure has been determined (16–19). Based on these results and similar analyses done with the AA cogeners EPA and ETA, we propose a model of the active site that accounts for the asymmetric catalysis by P450BM-3.

**EXPERIMENTAL PROCEDURES**

**Materials**—The fatty acids were obtained from NuCheck Prep, Inc. All other chemicals were obtained in the purest form available from Sigma.

**Methods**—The original plasmid, containing the gene encoding P450BM-3, was a gift from Dr. A. Fulco (Department of Biochemistry, UCLA, Los Angeles, CA). In preparation for our studies of the mechanism of P450BM-3, we constructed a new expression plasmid that contained only the gene encoding P450BM-3 and no extraneous *B. megaterium* DNA. We had previously constructed a plasmid for the expression of the heme domain of P450BM-3 and a construct from this project, pIBI-BMP2 (20), was linearized with SalI. Likewise, a vector had been constructed for the expression of the reductase domain of P450BM-3, pIBI-BMR (21). A 1.78-kilobase pair fragment from SalI-digested pIBI-BMR was recovered and ligated into the linearized pIBI-BMP2, giving pIBI-BM3. The sequence of the gene encoding P450BM-3 in this plasmid was confirmed. The plasmid was used to transform *Escherichia coli* strain DH5α. The protein was overexpressed and purified from these cells using published procedures (5). The concentration of the purified protein was estimated from the difference absorbance spectrum of the carbonyl complex of the ferrous form of P450BM-3, and the identification of the purified protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified material (2–5 mg/ml), final concentration, 0.2 μM, was used in catalytic hydrogenation and derivatization reactions.

**Enzyme Activity Studies**—The rates of P450BM-3-dependent oxygen and NADPH utilization were measured using 20–50 nM solutions of the enzyme in 50 mM sodium phosphate buffer, pH 7.4, containing 10 mM MgCl₂, 150 mM KCl, 8 mM sodium isocitrate, isocitrate dehydrogenase (1.0 IU/ml), dilauroyl phosphatidyl choline (0.05 μg/ml), and P450BM-3 (2–10 nM, final concentration) were incubated 2.5 min prior to the addition of the sodium salts of either AA, EPA, or ETA (25 mM each in 0.05 mM Tris-Cl buffer, pH 8.0) to final concentrations of 50–100 μM. Reactions were started by the addition of 1 mM NADPH (1 mM, final concentration). At different time points, aliquots were withdrawn, and the organic soluble products were extracted three times with equal volumes of ethyl ether containing HOAc (0.05%, v/v). After solvent evaporation under a stream of nitrogen, the products were resolved by RP-HPLC on a 5-μm Dynamax Microsorb Silica column (4.6 × 250 mm, Rainin Instruments Co., Woburn, MA) using a linear solvent gradient from 49.9% CH₃CN, 0.1% HOAc, 99.9% hexane at 2 ml/min (R<sub>t</sub> ~ 31.5, 33.1, and 36.5 min for 17-, 18-, and 16-OH-AA, respectively). Synthetic 19- and 20-OH-AAs were resolved by NP-HPLC as above using a solvent mixture of 1% 2-propanol, 0.1% HOAc, 99.9% hexane at 3 ml/min for 19- and 20-OH-AA, respectively. The enantiomers of methyl 14,15-epoxyeicosatrienoate were resolved by chiral phase HPLC as described previously (22).

For the characterization of EPA metabolites, the organic soluble material extracted from solutions containing [1-<sup>14</sup>C]EPA (100 μM, final concentration) and 0.1 μM P450BM-3 was resolved by RP-HPLC as described. The radioactive fraction eluting from the HPLC column with the retention time of authentic 17,18-epoxy-EPA (18.7 min) was collected batchwise and further characterized. To confirm the epoxide nature of the metabolite, an aliquot of the purified material (2–5 μg) was incubated, under an argon atmosphere and with constant mixing, with 0.25 ml of a mixture containing 20% EtOH, 40% H₂O, and 40% glacial HOAc. After 12 h at room temperature, the reaction mixture was diluted with 1 ml of 0.1 M KCl and extracted twice with equal volumes of ethyl ether. The resulting product co-eluted in RP-HPLC with synthetic vic-17,18-dihydroxy-5, 8, 11, 14-eicosatetraenoic acid (R<sub>t</sub> ~ 10 min) and, after derivatization to the corresponding TMS ether, PFB ester showed a NICI/GC/MS fragmentation pattern identical to that of an authentic standard (Fig. 1A). For regiochemical analysis, an aliquot of the hydrated epoxide (5 μg) was hydrogenated over PdO<sub>2</sub>, derivatized to the corresponding PFB ester (24), and purified by SiO<sub>2</sub> chromatography. The dry residue was dissolved in 200 μl of NaIO<sub>4</sub> (10 mg/ml in 70% CH<sub>3</sub>OH) and, after 2 h at 50 °C, the product was extracted into hexane and purified by RP-HPLC using a linear solvent gradient from 49.9% CH₃CN, 49.9% H₂O, 0.1% HOAc, to 99.9% CH₃CN, 0.1% HOAc over 40 min at 1 ml/min (R<sub>t</sub> ~ 43 min). The purified aldehyde, resulting from oxidative cleavage of the vic-diol precursor, was dried under a stream of N₂, mixed with 200 μl of 0.5% solution of methoxylamine hydrochloride in pyridine (Pierce), incubated 3 h at 30 °C, extracted into hexane, and then characterized by NICI/GCMS (Fig. 1B).

For structural analysis, the organic soluble products extracted from solutions containing [1-<sup>14</sup>C]ETA (100 μM, final concentration, 0.2 μCi/μmol), 2 mM P450BM-3, and 1 mM NADPH were purified by RP-HPLC as above. The radioactive material eluting from the RP-HPLC column between 18 and 20 min was collected batchwise and, after solvent evaporation, resolved into fractions a, b, and c (R<sub>t</sub> ~ 16.9, 23.1, and 37.1 min, respectively) co-eluted with the PFB esters of synthetic 17-, 18-, and 19-hydroxyeicosanoic acid, respectively. The PFB esters of hydroxylated a, b, and c were further characterized by NICI/GCMS.

**Chemical and Structural Characterizations—**The 18-Hydroxyeicosatetraenoic Acid.—To a room temperature mixture of synthetic methyl 18-hydroxyeicosatetraenoate (1.5 mg) (27), R<sup>−</sup>(+)-α-methoxy-α-trifluoromethylphenylethacetic acid (1.5 mg), and dimethylaminopropidine (0.2 mg) in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (1 ml) was added 1.3-dicyclohexylcarbodiimide (1.5 mg) in one portion with stirring. After 12 h, the solvent was removed in vacuo, and the residue was purified by PTLC (SiO<sub>2</sub>): 20% EtOAc/hexane, R<sub>t</sub> =...
A chiral standard of the 3,4-epoxy-hexan-1-yl benzoate was prepared as described (3Z)-hexan-1-ol as described previously (28) affording (3R,4S)-epox-hexan-1-ol, \( \text{[S]}^\text{1H} + 7.27^\circ (c 1.85, \text{CHCl}_3) \), as a colorless oil in 45% yield. The epoxy-alcohol (58 mg, 0.5 mmol) was dissolved in dry pyridine (1 ml) and cooled to 0 °C, and benzylo chloride (58 μl, 0.75 mmol, 1.5 equivalent) was added. After stirring at room temperature for 12 h, the reaction mixture was diluted with \( \text{CH}_2\text{Cl}_2 \) (2 ml), washed with saturated aqueous \( \text{CuSO}_4 \) solution (3 × 5 ml), brine (5 ml), dried over \( \text{Na}_2\text{SO}_4 \), and evaporated in vacuo.

The residue was purified by PTLC (SiO\(_2\); 15% EtOAc, 85% hexane, 0.26) to give the epoxy-benzoate (98 mg, 100%) as a colorless oil. \( ^1\text{H} \) NMR (CDCl\(_3\), 250 MHz): \( \delta 1.06 \) (t, 3H, 6.6 Hz), 1.49–1.69 (m, 2H), 1.90–2.54 (m, 2H), 2.90–2.98 (m, 1H), 3.09–3.18 (m, 1H), 4.53 (t, 2H, 7.7 Hz), 7.46 (t, 2H, 7.7 Hz), 7.56 (apparent t, 1H, 7.7 Hz), 8.07 (d, 2H, 7.7 Hz). The (3S,4R)-enantiomer was obtained analogously. Comparisons using a Chiracel OC HPLC column (4.6 × 250 mm) eluted isocratically with 0.2% 2-ProOH, 0.1% EtOH, 99.7% hexane at 1.1 ml/min with UV monitoring showed the (3R,4S)-isomer had a retention time of 58 min, whereas the (3S,4R)-enantiomer and the biologically derived sample co-eluted with a retention time of 45 min.

Gas Chromatography-Mass Spectral Analysis—Samples were dissolved in dodecane and analyzed by NICI/GC/MS on a Nermag R1010C quadrupole instrument interfaced to a Varian Vista Gas chromatograph utilizing He and CH\(_4\) as carrier and reagent gases, respectively. Splitless injections were made onto a 30-m SPB-1 fused silica capillary column (0.25-mm inner diameter, 0.25-μm coating thickness, Supelco Inc. Bellefonte, PA). After 1 min at 100 °C, the oven temperature was raised to 300 °C at 10 °C/min.

RESULTS AND DISCUSSION
Rates of AA Oxidation by P450BM-3—The rate of fatty acid oxidation by P450BM-3 can be easily measured either polarographically using an oxygen electrode or spectrophotometrically monitoring absorbance changes at 340 nm. Seen in Fig. 2 is an oxygen electrode trace of O\(_2\) consumption during the NADPH-dependent metabolism of AA by P450BM-3. The order of addition of reactants is crucial, i.e. NADPH must be added last to avoid inactivating the reductase domain of P450BM-3. The order of addition of reactants is crucial, i.e. NADPH must be added last to avoid inactivating the reductase domain of P450BM-3.

Within the first 30 s of incubation, the rates of AA utilization and product formation began to decrease showing the lack of a clear linear relationship between product formation and incubation time. As the rate of AA oxidation decreased, the recovery of polyoxygenated products, derived from secondary oxygenations, increased concomitantly, and became predominant 2–3 min after initiation. When limiting amounts of AA were added, as shown in Fig. 2, the ratio of O\(_2\) consumed per mol of AA added was approximately 2, indicating that fatty acid polyoxy-
The spin of the heme iron from the low to the high spin state (30). Absorbance changes in the Soret region with substrate, and absorbance changes in the Soret region (5, 7) acid, extended incubation times (2–5 min) resulted in limited solubility of AA made impractical attempts to increase its concentration, and thus prolong enzyme-substrate saturation. The rates of product formation shown in Table I were obtained at 30 °C and after a 30 s incubation, and are approximations of the initial velocities. They are useful only for comparative purposes.

Among the fatty acids metabolized by P450BM-3, AA showed the highest oxidation rate, 3.2 μmol of product formed/min/nmol of P450BM-3 (Table I). To the best of our knowledge, the rates at which P450BM-3 catalyzes the redox coupled activation of molecular oxygen, the cleavage of the oxygen-oxygen bond, and the insertion of a reactive oxygen atom into the AA molecule are the highest ever reported for a mixed function oxidase, and in particular for a P450, NADPH-dependent, catalyzed reaction. It is of interest that these rates of metabolism (k cat ≈ 50 s 1) are similar to those of the electron transfer from NADPH to the FMN cofactor of the reductase domain of P450BM-3 (k ≈ 80 s 1) (29). More importantly, as shown below, under the reaction conditions employed, P450BM-3 displayed high regiochemical selectivity for the fatty acid ω-2 carbon and 14,15-olefinic bond, and generated 18-OH-AA and 14,15-epoxyeicosatrienoic acid (14,15-EET) as major reaction products (80% and 20% of the total products, respectively) (Table II). While limited solubility of AA made impractical attempts to increase the reaction rates shown in Table I, were obtained 1 min after incubation at 30°C, the relationship between the incubation time of 15.5 min (Fig. 4, fraction A), was conclusively identified as 18-OH-AA based on the following: 1) co-elution on reversed and normal phase HPLC with an authentic standard, 2) co-elution of its PFB-TMS derivative on capillary GC with a similarly derivatized standard, 3) NICI/GC/MS analysis of its PFB-TMS derivative showed the presence of an intense ion fragment at m/z 407 (loss of PFB, base peak) indicative of a monohydroxylated metabolite, 4) catalytic hydrogenation followed by conversion to the corresponding PFB-TMS derivative yielded a product that co-eluted on capillary GC with a similarly derivatized authentic standard, and 5) NICI/GC/MS analysis of its hydrogenated PFB-TMS derivative yielded a fragment ion at m/z 415 (loss of PFB, base peak), consistent with the presence of four double bonds in the parent molecule that had been reduced upon hydrogenation.

Fraction B (Fig. 4, retention time 22.6 min) was shown to contain 14,15-EET based on the following chromatographic and mass spectral evidence: 1) co-elution in reversed and normal phase HPLC with authentic 14,15-EET (23, 24); 2) co-elution in capillary GC of its PFB ester derivative with authentic 14,15-EET-PFB (23, 24); 3) the NICI mass spectrum of the PFB ester of fraction B was identical to that of synthetic 14,15-EET-PFB with major ion fragments at m/z: 319 (base peak, loss of PFB), 303 (20% of base peak, loss of PFB and oxygen) and 301 (12% of base peak, loss of PFB and water) (24); and 4) the NICI mass spectrum of the PFB ester of hydrogenated B was nearly identical to that of the PFB ester of authentic 14,15-epoxyeicosanoic acid with major ion fragments at m/z: 325 (base peak, loss of PFB), 309 (10% of base peak, loss of PFB and oxygen) and 307 (8% of base peak, loss of PFB and water) (24, 26).

Hydroxylation of saturated fatty acids (e.g. palmitic acid) by P450BM-3 occurs preferentially at the ω-2 carbon atom, with ω-1 and ω-3 hydroxylated products accounting for a substantial portion of the total metabolism (3, 5). In contrast, P450BM-3 hydroxylates the AA C18 carbon in a nearly exclusive fashion, i.e. 18-OH-AA accounts for better than 99% of the overall sp3 carbon hydroxylation. Hydroxylation at the fatty acid ω-3 carbon accounted for less than 1% of the total products and no metabolism was observed at the ω-1 or ω carbons (Fig. 5). Even though amino acid sequence analysis indicates a 25–30% sequence similarity between P450BM-3 and CYP4A gene subfamily isomers (a group of mammalian microsomal fatty acid ω-hydroxylases) (4), none of the CYP4A isomers catalyzes fatty acid or AA ω-2 oxidation or epoxidation (11, 13). However, the formation of 18-OH-AA and of 18(R)-OH-AA by rat hepatic and monkey seminal vesicle microsomal fractions, respectively, has been reported (31). Recent studies have also shown that hydroxylation at the C-18 position of AA was catalyzed by CYP1A1, CYP1A2, and CYP2E1 (11, 13, 25). Hydroxylation at

**Table I**

| Substrate | Reaction rate a | Spectral binding constant, Ks μM |
|-----------|-----------------|----------------------------------|
| AA        | 3.2 ± 0.4       | 1.2 ± 0.1                        |
| EPA       | 1.4 ± 0.2       | 1.6 ± 0.5                        |
| ETA       | 2.9 ± 0.1       | ND b                            |

a Reaction rates in μmol of product formed/min/nmol of P450BM-3. 
b Not determined.
the sp² carbons near or at the fatty acid methyl end require the delivery by the protein catalyst of a reactive, heme-bound oxygen species to a ground state sp² carbon atom. It is therefore likely that for all these reactions the oxygen chemistries and the mechanism(s) are similar yet independent of the fatty acid chain length and/or degree of saturation. Nevertheless, compared to saturated fatty acids, the AA molecule imposes additional steric requirements on the active site of the enzyme. During AA C18 hydroxylation, the P450 binding/active site must position the acceptor carbon atom not only in optimal proximity to the heme-bound active oxygen, but also with complete segregation of the fatty acid reactive bis-allylic methylene carbons at C-7, C-9, and C-13 and the 5,6-, 8,9-, 11,12-, and 14,15-olefinic bonds.

To further delineate the structural determinants responsible for AA active site binding and productive spatial orientation, the chiral properties of the metabolites formed by P450BM-3 were characterized. In previous studies it was shown that AA epoxidation and ω-2 carbon hydroxylation by mammalian P450 isoforms proceeded with a degree of enantiofacial selectivity unprecedented for P450 catalyzed oxidations of acyclic, unbranched molecules such as AA (9, 11, 13). As shown in Table II, EPA and ETA oxidation by P450BM-3 hydroxylates the ω-2 carbon atom of AA in a highly asymmetric mode generating 18(S)-OH-AA with 96% optical purity. Similarly, P450BM-3 catalyzed AA epoxidation yields 14(S),15(R)-EET with 99% optical purity. It thus appears that P450BM-3 evolved a highly structured and spatially rigid substrate binding site, capable of accommodating a polysaturated fatty acid such as AA in optimal orientation with regards to the heme-bound active oxygen. The unprecedented high enantiofacial selectivity of this protein, in conjunction with its moderate regioselectivity, indicates that during catalytically productive binding: 1) the AA carbon-carbon rotational freedom is substantially restricted, and 2) the active site spatial coordinates allow for a moderate degree of substrate lateral displacement. Finally, while 18(R)-OH-AA is the predominant enantiomer formed by mammalian microsomal enzymes (31), 14(S),15(R)-EET is the predominant enantiomer found in vivo in rat liver, plasma, and kidney (26, 32, 33).

**EPA and ETA Oxidation by P450BM-3—**The metabolism of EPA and ETA, two AA analogs (Fig. 5), was studied to probe the role that substrate structural features, in particular C–H bond acceptor chemistry, play as determinants of P450BM-3 catalytic outcome. We selected these AA analogs because: 1) radiolabeled EPA and limited amounts of radiolabeled ETA were commercially available, 2) all of the molecules are of the same carbon length, 3) between carbons C-1 and C-13, all three fatty acids (AA, EPA, and ETA) are structurally identical, 4) ETA, with its sp² C-14–C-15 carbons, was used to probe the role of the AA 14,15-olefinic bond in inducing oxygenation of...
The organic soluble products generated by solutions containing P450BM-3 (2.5 nm), [1-14C]EPA (100 μM) and NADPH (1 nm) were resolved by RP-HPLC as described under "Experimental Procedures." Shown is the radiochromatogram derived from a 2-min solution that contained a total of 1 pmol of P450BM-3. The retention time for fraction A is 18.7 min.

C–H bonds distal to the fatty acid ω-2 carbon, the substrate’s most metabolically active carbon atom (Table II), and EPA, with the extra olefinic bond at C-17–C-18 allowed for the analysis of C–H bond acceptor reactivity in the enzyme’s regioselectivity of oxygen insertion.

Incubation of EPA with P450BM-3 and NADPH resulted in the time-dependent formation of a radioactive product with a RP-HPLC retention time of 18.7 min (Fig. 6). Importantly, and at difference with AA, longer incubation times (>4 min) and/or higher enzyme concentrations (≥10 nm) did not result in substantial changes in the profile shown in Fig. 6, indicating that the EPA oxygenated metabolite is a poor substrate for P450BM-3. Structural analysis demonstrated that the sole product of EPA metabolism by P450BM-3 was 17,18-epoxyeicosatetraenoic acid (17,18-epoxy-EPA), i.e. no other product was generated during the first 3 min of incubation at 30°C; however, during prolonged incubations, the 17,18-epoxy-EPA underwent partial chemical hydration to 17,18-dihydroxyeicosatetraenoic acid. Additionally, as shown in Table I, EPA was metabolized at approximately half the rate estimated for AA. On the other hand, the EPA 17,18-olefinic bond is epoxidized at more than double the rate of the AA 14,15-olefinic bond (Tables II and III). Significantly, under the experimental conditions used, epoxidation of the EPA 14,15-olefinic bond was negligible (less than 1% of the total reaction products). The identification of the P450BM-3 metabolite as 17,18-epoxy-EPA was based on the following: 1) co-elution in reversed and normal phase HPLC with an authentic standard; 2) co-elution in capillary GC of its mass chromatogram derived from a 2-min solution that contained a total of 1 pmol of P450BM-3. The retention time for fraction A is 18.7 min.

For chiral analysis of the 17,18-epoxy-EPA, we initially utilized degradative ozonolysis followed by derivatization to the corresponding 3,4-epoxyhexan-1-yl benzoates. The Chiralcel OC HPLC properties of the synthetic standards were then compared to those of the biologically derived sample (see “Experimental Procedures” for further details). For routine nondestructive analysis, the optical antipodes of methyl 17,18-epoxyeicosatetraenoate were resolved with baseline separation by chiral phase HPLC on a Chiralcel OB column as shown in Fig. 7. Absolute configurations were assigned based on the results obtained by the above degradative ozonolysis procedure. Chiral analysis of the EPA epoxide metabolite demonstrated that its biosynthesis was highly asymmetric and generated 17(S),18(R)-epoxy-EPA with 97% optical purity (Table III). The high degree of regio- and stereochemical selectivity shown by P450BM-3 during the metabolism of EPA, illustrates the key role played by the C–H acceptor in directing catalytic outcome. Thus, assuming similar active site binding coordinates for AA and EPA, the π electron cloud associated with the EPA 17,18-olefinic bond is an efficient trap for the heme-bound oxygenating intermediate, more so than that associated with the AA ω-3 sp² carbon σ orbital. Therefore, C–H acceptor chemistry and active site binding coordinates, as opposed to reactive oxygen chemistry and/or heme-oxygen redox properties, provide a coherent explanation for product chemistry. It is of interest that for both the AA 14,15- and EPA 17,18-epoxyenases, oxygen was delivered to the olefinic bond’s si-re-face.

As mentioned, ETA lacks olefinic bonds in the vicinity of its ω-3 sp² carbon (i.e. C-17–C-18), as well as at C-14–C-15 (Fig. 5), and as a result the carbons at or near its methyl terminus are chemically similar to those of most saturated fatty acid substrates. Incubations of [1-14C]ETA (50–100 μM) with P450BM-3 resulted in the NADPH-dependent formation of radioactive metabolites with an average HPLC retention time of approximately 19.3 min (Fig. 8, fraction A). The broad, asymmetric nature of fraction A (Fig. 8) suggested the presence of more than one product. Fraction A was collected from the HPLC eluate and resolved by normal phase HPLC into three radioactive metabolites (Fig. 9). After HPLC purification, fractions a, b, and c (Fig. 9) were submitted to catalytic hydrogenation, derivatized to the corresponding PFB esters TMS ethers and their chromatographic and NICI/GC/MS properties were compared to those of the synthetic PFB esters of 16-, 17-, 18-, 19-, and 20-hydroxyeicosanoic acids. Under NICI conditions, the PFB ester, TMS ether derivatives of hydrogenated a, b, and
Chiral phase HPLC chromatographic properties of synthetic and enzymatically produced methyl 17,18-epoxyeicosatetraenoate. Racemic samples of synthetic methyl 17,18-epoxy-EPA (4 μg), enzymatically generated methyl 17,18-epoxy-[1-14C]EPA (6 μg, 4.0 μCi/μmol) of a mixture of the synthetic and biological product were resolved using a Chiralcel OB column (4.6 x 250 mm) and a solvent mixture of 0.5% 2-propanol, 99.5% hexane at 2 ml/min, with UV detection at 210 nm. For quantification, the enantiomers were collected individually and, after solvent evaporation, their radioactivity determined by liquid scintillation. Absolute configurations were assigned as described in the text.

![Retention time of pFB ester of authentic 17-, 18- and 19-hydroxyeicosanoic acids](image)

**Figure 7.** Chiral phase HPLC chromatographic properties of synthetic and enzymatically produced methyl 17,18-epoxyeicosatetraenoate. The organic soluble products generated by solutions containing P450BM-3 (5 nM), [1-14C]ETA (100 μM) and NADPH (1 mM), were resolved by RP-HPLC exactly as described in Fig. 4. Shown is the radiochromatogram derived from a 1-min solution that contained a total of 1 pmol of P450BM-3. The radioactive materials in fraction A eluted between 18.2 and 19.7 min.

![Chromatographic resolution of the products generated by P450BM-3 during the metabolism of ETA](image)

**Figure 8.** Chromatographic resolution of the products generated by P450BM-3 during the metabolism of ETA. The organic soluble products generated by solutions containing P450BM-3 (5 nM), [1-14C]ETA (100 μM) and NADPH (1 mM), were resolved by RP-HPLC exactly as described in Fig. 4. Shown is the radiochromatogram derived from a 1-min solution that contained a total of 1 pmol of P450BM-3. The radioactive materials in fraction A eluted between 18.2 and 19.7 min.

![Chromatographic resolution of the ETA metabolites generated by P450BM-3](image)

**Figure 9.** Chromatographic resolution of the ETA metabolites generated by P450BM-3. The [1-14C]-labeled metabolites (0.2 μCi/μmol) eluting from the RP-HPLC column between 18 and 20 min (Fig. 8) were collected batchwise and, after solvent evaporation, resolved by normal phase HPLC on a 250 mm Dynamax Microsorb silica column using an isocratic solvent mixture composed of 99.4% hexane, 0.5% 2-propanol, and 0.1% HOAc at 2 ml/min. The column eluent was monitored, on-line, for UV absorbance at 210 nm (top) and radioactivity as in Fig. 4 (bottom). The retention times for fractions a, b, and c were 16.9, 23.1, and 37.1 min, respectively.

| Product | Product distribution | Reaction rate (μmol/min) |
|---------|----------------------|-------------------------|
| 17-OH-ETA | 43 | 1.2 ± 0.04 |
| 18-OH-ETA | 39 | 1.1 ± 0.1 |
| 19-OH-ETA | 18 | 0.5 ± 0.06 |

*Reaction rates in micromoles of product formed/min/mmol of P450BM-3. Values are averages ± standard error of the mean calculated from at least three different experiments.

For ETA, as was the case for AA and palmitic acid, extended incubation times (≥2 min) resulted in further metabolism of 17-, 18-, and 19-hydroxy-ETA. Among the secondary oxidation products, 18-oxo-ETA was identified using GC/MS. As shown in Table I, P450BM-3 metabolized ETA at rates comparable to those obtained with AA. Under conditions favoring primary metabolism, the enzyme generated 17-, 18-, and 19-hydroxy-ETA in a 2.4:2.2:1 molar ratio, respectively (Table IV). Importantly, the moderate degree of regioselectivity of the enzyme for the ETA ω-1, ω-2 or ω-3 sp3 carbon atoms is similar to that previously obtained with several saturated fatty acid substrates (5, 34). Thus, for all the polyunsaturated fatty acids tested here, the preferred sites for the P450BM-3 catalyzed oxygen insertion were the ω-2 and ω-3 carbon atoms. P450BM-3 metabolized the ω-2 or the ω-3 carbons of ETA at rates comparable to those of EPA epoxidation (in micromoles of product/min/mmol of P450: 1.2 and 1.1 for 17- and 18-OH-ETA, and 1.4 for 17, 18-epoxy-EPA, respectively) (Tables III and IV). On the other hand, 19-OH-ETA and 14,15-EET are generated at lower rates (Ta-
bles II and IV). It is therefore likely that all three molecules occupy more or less similar spatial coordinates in the active site of P450BM-3.

Modeling AA Binding to the Active site of P450BM-P—Only the atomic coordinates of the substrate-free form of P450BM-P are known (19). Attempts to soak either AA or palmitic acid into preformed crystals of P450BM-P to obtain crystals of substrate bound enzyme has resulted in either low occupancy of the active site by the substrate fatty acid or disorder in the crystals.2 Thus, to enable us to visualize substrate binding in the active site of P450BM-3, we have utilized a molecular modeling approach in which AA was "docked" into the substrate access channel and active site. The fatty acid carboxylate was positioned within charge coupling distance of the guanidinium group of Arg47 at the mouth of the access channel (19). The remainder of the AA molecule was built into the volume of the substrate access channel defined by Arg47 at the surface and Phe87 above the heme, maintaining the appropriate bond angles and with reduced van der Waals contacts with amino acid side chains and backbone atoms. During energy minimization of AA in the substrate access channel and active site, Phe87 moved slightly allowing the ω-end of the AA molecule to occupy more readily an existing active site cavity (Fig. 10). In this model, the ω-end is bent upward and toward the fatty acid 14,15-olefinic bond. The pro-R hydrogen of C-18 of AA was positioned within charge coupling distance of the guanidinium group of Arg47 at the mouth of the access channel (19).

FIG. 10. Model of AA bound to P450BM-P.

In conclusion, while epoxidation and a combination of epoxidation and sp3 hydroxylation have been demonstrated in mammalian P450s of the CYP1 and CYP2 families (9, 11, 12, 25, 35, 36), with highly enantioselective epoxidations catalyzed by CYP2C11 and CYP2C23 (36, 37), none of the characterized eukaryotic AA epoxygenases appears capable of epoxidizing a single AA olefinic bond with complete exclusion of the other three. The present study has demonstrated that the common reactive oxygen intermediate that has been proposed for P450 monooxygenations (38) can catalyze epoxidation and sp3 carbon hydroxylation of AA to 18-OH-AA and 14,15-EET (80 and 20% of total products, respectively) in the reaction catalyzed by P450BM-3. In addition, by comparing the stereoselective epoxidation of EPA at the 17,18-olefinic bond, and by contrast, the less selective 17-, 18-, and 19-hydroxylation of ETA with that of AA, we find that in contradistinction to the nature and chemical properties of the reactive oxygen intermediate(s), the chemistry of the reaction products are critically dependent on: 1) the chemical properties of the acceptor C–H bonds and 2) the optimal orientation of the C–H acceptor with respect to the heme-bound reactive oxygen intermediate. We also can conclude from these stereoselective oxidations of AA and EPA that the active site geometry responsible for substrate binding and orientation must restrict the freedom of substrate C-C bond rotation while, at the same time, allowing some degree of substrate lateral mobility that, for P450BM-3 permits 14,15-EET formation. This substrate lateral mobility may be controlled by the positional relationship of the active site residue Phe87 to the heme iron. Thus, substrate chemistry and protein structural features, as opposed to oxidant chemistry, are the key determinants of catalytic outcome. Finally, in as much as the biological function(s) of P450BM-3 are yet to be determined, the high catalytic rates and unprecedented degree of regio- and stereochemical selectivity displayed by the enzyme during the metabolism of AA and EPA are indicative of evolutionary specialization and suggest a role for P450BM-3 in the metabolism of bacterial unsaturated fatty acids.

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