An Active Site Substitution, F87V, Converts Cytochrome P450 BM-3 into a Regio- and Stereoselective (14S,15R)-Arachidonic Acid Epoxynagenase*

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Cytochrome P450 BM-3 catalyzes the high turnover regio- and stereoselective metabolism of arachidonic and eicosapentaenoic acids. To map structural determinants of productive active site fatty acid binding, we mutated two amino acid residues, arginine 47 and phenylalanine 87, which flank the surface and heme ends of the enzyme's substrate access channel, respectively. Replacement of arginine 47 with alanine yielded a protein with reduced substrate binding affinity and arachidonate sp\(^3\) carbon hydroxylation activity (72% of control wild type). On the other hand, arachidonic and eicosapentaenoic acid epoxidation was significantly enhanced (154 and 137%, of control wild type, respectively). As with wild type, the alanine 47 mutant generated (15R)-hydroxyeicosatetraenoic, (14S,15R)-epoxyeicosatrienoic, and (17S,18R)-epoxyeicosatetraenoic acids nearly enantiomerically pure.

Replacement of phenylalanine 87 with valine converted cytochrome P450 BM-3 into a regio- and stereoselective arachidonic acid epoxynase ((14S,15R)-epoxyeicosatrienoic acid, 99% of total products). Conversely, metabolism of eicosapentaenoic acid by the valine 87 mutant yielded a mixture of (14S,15R)- and (17S,18R)-epoxyeicosatetraenoic acids (26 and 69% of total, 94 and 96% optical purity, respectively). Finally, replacement of phenylalanine 87 with tyrosine yielded an inactive protein.

We propose that: (a) fatty acid oxidation by P450 BM-3 is incompatible with the presence of residues with negatively charged side chains at the surface opening of the substrate access channel or a polar aromatic side chain in the vicinity of the heme iron; (b) the high turnover regio- and stereoselective metabolism of arachidonic and eicosapentaenoic acids involves charge-dependent anchoring of the fatty acids at the mouth of the access channel by arginine 47, as well as steric gating of the heme-bound oxidant by phenylalanine 87; and (c) substrate binding coordinates, as opposed to oxygen chemistries, are the determining factors responsible for reaction rates, product chemistry, and, thus, catalytic outcome.

Recombinant DNA techniques have greatly facilitated the molecular characterization of highly homologous P450 isoforms by providing access to sufficient quantities of recombinant proteins and have expedite the task of purifying and characterizing, as single molecular entities, otherwise nearly unresolvable protein isomers. Nevertheless, a three-dimensional structural description of a single mammalian, membrane-bound P450 is lacking, despite the availability of several highly purified eukaryotic P450 isoforms. Atomic structures are currently available for four water-soluble hemoproteins of bacterial origin (1–4). Among these, the hemoprotein domain of P450 BM-3, a fatty acid hydroxylase isolated from Bacillus megaterium, has been crystallized and its three-dimensional structure determined at 2-A resolution (2). Of all the structurally characterized P450s, the hemoprotein domain of P450 BM-3 shows the highest degree of functional and sequence homology to the mammalian enzymes and, in particular, to members of the CYP 4A gene subfamily of microsomal fatty acid hydroxylases (5–7). Native P450 BM-3 is a single polypeptide (119 kDa) containing a cytochrome P450 domain fused to a cytochrome P450 reductase domain with a 1:1:1 stoichiometric ratio between heme, FAD, and FMN (5–7). The recombinant P450 BM-3 holoenzyme has been shown to catalyze the NADPH-dependent hydroxylation of medium and long chain saturated fatty acids with optimal lengths of 14–16 carbons (8). While the regiochemistry of saturated fatty acid hydroxylation by P450 BM-3 is more or less carbon chain length-dependent, i.e. as chain length increases, regioselectivity shifts from the ω-1 to the ω-2 or ω-3 fatty acid carbon (7–9), the enzyme appears unable to catalyze fatty acid ω-oxidation, a reaction common to several mammalian P450 4A isoforms (6). Purified forms of P450 BM-3 were shown to catalyze the hydroxylation and the epoxidation of monounsaturated fatty acids such as palmitoleic acid (10). Furthermore, studies with palmitoleic acid indicated that the partition ratio between ω-2 hydroxylation and olefin epoxidation was pH-dependent and subject to differential inhibition by antibodies raised against purified P450 BM-3 (10, 11).

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1 The abbreviations used are: P450, cytochrome P450; AA, arachidonic acid; EPA, eicosapentaenoic acid; EET, cis-epoxyeicosatetraenoic acid; EETA, cis-epoxyeicosatetraenoic acid; DHET, cis-dihydroxyeicosatetraenoic acid; OH-AA, hydroxyeicosatetraenoic acid; FFB, pentafluorobenzyl; HPLC, high pressure liquid chromatography; RP-HPLC, reversed phase HPLC; NCI/GC/MS, electron capture, negative ion, gas liquid chromatography/mass spectrometry; MOPS, 4-morpholinepropanesulfonic acid.
Since, in those studies, a single protein was shown to be responsible for fatty acid sp³ carbon hydroxylation and olefin epoxidation, it was then concluded that P450 BM-3 existed in two or more pH-dependent substrate binding conformations and that antigen-antibody interactions could alter the equilibrium between these conformations (10, 11).

Microsomal P450 catalyzes the NADPH-dependent oxidation of AA to regiosomeric monohydroxylated and epoxidized metabolites (12–16). Depending on the P450 isoform composition of the enzyme preparation under study, arachidonic acid metabolism by P450 can proceed by one or more of the following pathways or reaction types: 1) allylic oxidation, 2) sp³ carbon hydroxylation at C₁₆ through C₂₀, and 3) olefin epoxidation. While the physiological significance of P450-dependent allylic oxidation remains obscure, the products of the latter two reactions display a variety of potent biological activities and have been implicated in processes such as hormonal signaling (12–16), the control of cell ion permeability and vascular tone (12–16), and the pathophysiology of experimental hypertension (12–16). The demonstration of in vivo chiral EET formation by human, rat, and rabbit organs established P450 as a member of the endogenous AA metabolic cascade and suggested new and important roles for the hemoprotein in the biosynthesis of lipid derived mediators of cell and organ function (9, 13). As with most eicosanoids, the regio- and stereochemical features of the P450-derived metabolites of AA define biological activity and/or potency and thus, functional significance (9). Studies using purified and/or recombinant mammalian P450 isoforms confirmed that P450 controlled, in an isoform-specific fashion, the regio- and stereoselectivity of oxygen insertion into the AA molecular template (9, 11, 13).

To initiate the delineation of protein and substrate determinants involved in productive substrate binding and metabolism, we recently characterized the metabolism of AA and several AA analogs by P450 BM-3 (17). These studies demonstrated that AA was metabolized by P450 BM-3 at the fastest rates ever reported for an NADPH-dependent P450 monoxygenase (3.2 μmol of product/nmol of P450/min at 30 °C), and that AA metabolism by P450 BM-3 regio- and stereoselectively generated (18R)-OH-AA and (14S,15R)-EET in a 5:1 molar ratio and with 96 and 99% optical purities, respectively (17). Similarly, EPA was metabolized by P450 BM-3 to (17S,18R)-EETA (97% optical purity) as the sole reaction product (17). Based on these results and similar experiments performed with the AA analogs EPA and eicosatrienoic acid, we developed a working model of the AA-bound active site of P450 BM-3 from which one concludes that during AA metabolism: 1) a single oxidant species accounts for fatty acid sp³ carbon hydroxylation and olefin epoxidation; 2) that catalytic outcome is critically dependent on active site spatial coordinates responsible for productive substrate binding and orientation between the heme-bound active oxygen and the acceptor carbon bond(s); and 3) the active site spatial coordinates that control AA binding restricts the degrees of freedom of substrate C=C bond rotation while, at the same time, allowing the molecule a moderate degree of lateral mobility (17). The above mentioned high catalytic rate, as well as the regio- and stereoselectivity displayed by P450 BM-3 during AA metabolism are indicative of a high degree of structural evolutionary specialization and, in particular, of those spatial coordinates that define protein substrate binding and orientation. We have utilized site-specific mutagenesis to change the properties of two amino acid residues flanking either the surface end or the heme end of the P450 BM-3 substrate access channel (2). Here we report that single amino acid replacements in the substrate access channel can either markedly alter the enzyme’s regioselectivity or result in inactive proteins. Furthermore, a valine for phenylalanine replacement at position 87 converts P450 BM-3 into an active and stereoselective (14S,15R)-AA epoxygenase.

**Materials and Methods**

**cDNA Manipulations and Protein Purification**—The original plasmid containing the cDNA coding for P450 BM-3 was a gift of Dr. Armand Fulco (Department of Biochemistry, UCLA, Los Angeles, CA). The cDNA was subcloned into the pIBI expression vector and expressed in DH5α Escherichia coli exactly as described (5). Site-specific mutations were introduced into the P450 domain using the M13 two-primer method of Kunkel et al. (18). Mutants were screened with the radiolabeled mutagenic oligomers and the incorporated changes confirmed by DNA sequencing. Mutated cDNAs were subcloned into the pIBI vector and expressed as above.

The recombinant P450 BM-3 proteins were purified as described in Ref. 5. Briefly, P450 BM-3 proteins were collected from cell lysates by precipitation with ammonium sulfate (60% saturation). After centrifugation and dialysis versus 50 mM MOPS buffer (pH 7.4) containing 50 mM KCl, the sample was applied to a DEAE-cellulose column equilibrated with dialysis buffer and the proteins were eluted using a linear salt gradient from 50 to 250 mM KCl. Fractions with a 416 nm to 280 nm absorbance ratio greater than 0.9 were pooled, concentrated, applied to a Sephacryl 200 hr column and eluted with 50 mM KCl and MOPS buffer (pH 7.4). Fractions with 416 nm to 280 nm absorbance ratios greater than 1.2 were pooled and utilized as such. P450 concentrations were determined from the difference absorbance spectrum of the CO-complex of the ferrous form of the enzyme using an extinction coefficient of 91 m⁻¹ cm⁻¹ for the wavelength pair of 450 versus 490 nm (19). The substrate binding spectra used for the determination of Kᵣ, the spectral binding constant, were obtained after the addition of increasing concentration of substrate at 25 °C. Difference spectra were recorded with a Hewlett Packard diode array spectrophotometer equipped with a Peltier temperature control device. Absorbance data for substrate binding were analyzed with the program Microsoft Excel®.

**Enzyme Activity Determinations**—Oxygen and NADPH consumption were measured with a Clark-type oxygen electrode (YSI Instruments, Yellow Springs, OH) and a Hewlett Packard spectrophotometer, respectively, at room temperature and using 20–50 mM solutions of the enzyme in 50 mM MOPS buffer (pH 7.4) containing 50–100 μM fatty acid. After a 5-min preincubation, reactions were started by the addition of NADPH. NADPH utilization was measured at 340 nm and concentrations calculated using an extinction coefficient of 6.22 m⁻¹ cm⁻¹. For product identification and structural characterization, incubations were performed at 30 °C under atmospheric air and with vigorous mixing. Reaction mixtures in 50 mM Tris·Cl buffer (pH 7.4) containing 10 mM MgCl₂, 150 mM KCl, 8 μM sodium isocitrate, isocitrate dehydrogenase (1.0 IU/ml), dilaurelylphosphatidylcholine (0.05 μg/ml), and P450 BM-3 (2–10 μM, final concentration) were incubated 2.5 min prior to the addition of the sodium salt of either AA or EPA (25 mM each in 0.05 mM Tris·Cl buffer (pH 8.0) to final concentrations of 50–100 μM, each. After 1 min, reactions were started by the addition of NADPH (1 mM, final concentration). At different time points, aliquots of the incubates were withdrawn and the organic soluble products extracted thrice with equal volumes of ethyl ether containing HOAc (0.05%, v/v). After solvent evaporation under an stream of N₂, the products were resolved by RP-HPLC on a 5-μm Dynamax Microsorb C₁₈ column (4.6 × 250 mm, Rainin Instruments Co., Woburn, MA) using a linear gradient from 49.95% CH₃CN, 49.95% H₂O, 0.1% HOAc to 99.9% CH₃CN, 0.1% HOAc over 40 min at 1 ml/min (20). Products were quantified by on-line liquid scintillation using a Radiomatic Flo-one β-Detector (Radiomatic Instruments, Tampa, FL).

**Structural Characterizations**—The identification of 18-OH-AA, 14,15-EET, and of 17,18-EET was done using published methodology (20–22) and confirmed with synthetic standards. For the characterization of 14,15-EET, the organic soluble material extracted from incubates containing [1-¹⁴C]EPA (100 μM final concentration, 0.1 μCi/μmol), 5 nm P450 BM-3, and 1 mM NADPH was resolved by RP-HPLC as described above. The radioactive fraction eluting from the HPLC column with a retention time of 19.7 min was collected batchwise and submitted to catalytic hydrogenation under PtO₂. For structural characterization, the NICI/GCMS and chromatographic properties of the metabolite were compared to those of authentic 14,15-epoxyeicosanoic acid. PPB esters were prepared by reaction with pentafluorobenzyl bromide in triethylamine as described (21, 22). RP-HPLC was done on a 5-μm Dynamax Microsorb C₁₈ column (4.6 × 250 mm, Rainin Instruments Co.) using a linear gradient solvent from 49.95% CH₃CN, 49.95%
The atomic coordinates for "molecule A" of P450 BM-3 (2) were used to model the conformation of arachidonic acid in the substrate access channel. The fatty acid was docked into the access channel using the program InsightII (Biosym Corp., San Diego, CA). Inappropriate van der Waals contacts were minimized manually. The arachidonic acid carboxylate was positioned within electrostatic interaction distances of the Arg47 guanidino group with its $\omega$-end inserted into a pre-existing cavity above the heme group. The $\beta$-sheet strands on either side of Arg47 are colored in blue. The carbon atoms in Arg47 and Phe87 are yellow and the nitrogens blue. The heme group is in dark gray with its nitrogens blue, oxygens red and iron orange. The $\alpha$-carbon backbone of the $\alpha$-helix is green and the fatty acid carbon chain pink.

Spatial geometries of these residues, it was proposed that, on the one hand, Arg47 controls entry to the substrate access channel and, therefore, fatty acid selectivity and, on the other hand, the aromatic side chain of Phe87 limits and/or controls substrate C–H acceptor bond access to the enzyme's heme iron and thus, the regioselectivity of oxygen attack (2). In the absence of atomic coordinates for substrate-bound P450 BM-3, we utilized a molecular modeling approach to visualize AA binding to P450 BM-3 (17). For these studies, the fatty acid carboxylate was placed within charge coupling distance of the Arg47 guanidinium group and the remainder of the molecule was built into the volume of the access channel defined by Arg47 at the surface and Phe87 above the heme (2, 17). To optimize occupancy, Phe87 was moved slightly back, thus allowing the $\omega$-end of AA to occupy an available active site cavity (2, 17). In this active site induced conformation, the fatty acid methyl end, i.e. the $\omega$ carbon, bends upward and away from the heme iron and the AA C18 pro-R hydrogen is positioned in close proximity to the enzyme's heme iron (17) (Fig. 1). Moreover, an additional small displacement of Phe87 places the AA 14,15-olefin in optimal proximity to the P450 BM-3 heme iron (17). Based on this model, we suggested that AA-bound P450 BM-3 oscillates between two alternate conformers characterized by the relative position of Phe87 with respect to the heme iron. These alternate conformations allow for a small degree of substrate lateral displacement, responsible for oxygen attack at C18 and the 14,15-olefin of AA (2, 17). To further define the roles of Arg47 and Phe87, as the outer and inner boundaries of the substrate access channel (2) (Fig. 1), we replaced them by site-specific mutagenesis. Mutated cDNAs were expressed, the corresponding recombinant proteins purified and their enzymatic properties studied as described under "Materials and Methods." A summary of the amino acid replacements introduced into the protein are shown in Table I. The positively charged side chain of Arg47 (Fig. 1) was either eliminated by replacement with alanine or replaced with a negatively charged glutamic acid (Table I). The aromatic ring of Phe87 (Fig. 1) was either removed by replacement with valine or, alternatively, its polarity was increased by substitution with tyrosine (Table I).

Substrate Access Channel: Arg47 Replacements—The poorly defined nature of Arg47 in the two crystallographically independent P450 BM-3 molecules analyzed indicated high struc-
The arginine residue at position 47 of the P450BM-3 polypeptide was replaced by alanine using site-specific mutagenesis. The resulting recombinant protein (2.5 nM, final concentration) was incubated with [1-14C]AA (1–5 μCi/μmol) and NADPH (100 μM and 1 mM, final concentrations, respectively) in the presence of an enzymatic NADPH regeneration system. After 30 s at 30°C, organic soluble products were extracted into acidified ethyl ether, resolved by RP-HPLC, and quantified by on-line liquid scintillation.

**TABLE II**

| Product distribution | Reaction rate a | % of wild type rate |
|----------------------|-----------------|---------------------|
| Total                | 100             | 2800 ± 96           | 82      |
| 18-OH-AA             | 67 ± 1          | 1,876 ± 28          | 72      |
| 14,15-EET            | 33 ± 1          | 924 ± 10            | 154     |

a Reaction rates are given in nanomoles of product/nmol of P450/min. Values are averages ± S.E., calculated from at least three different experiments.

Arachidonic acid epoxidaion by the P450 BM-3 R47A mutant

The arginine residue at position 47 of the P450 BM-3 polypeptide was replaced by alanine using site-specific mutagenesis. The resulting recombinant protein (2.5 nM, final concentration) was incubated with [1-14C]AA (1–5 μCi/μmol) and NADPH (100 μM and 1 mM, final concentrations, respectively) in the presence of an enzymatic NADPH regeneration system. After 30 s at 30°C, organic soluble products were extracted into acidified ethyl ether, resolved by RP-HPLC, and quantified by on-line liquid scintillation.

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Arachidonic acid epoxidation by Mutant P450 BM-3

The replacement of Arg47 (Fig. 1) by alanine (Table I) generated a mutant protein lacking a flexible and positively charged protruding side chain (2) and with potentially enhanced active site accessibility. Incubation of the R47A mutant P450 BM-3 with [1-14C]AA and NADPH (2.5 nM, 100 μM, and 1 mM final concentrations, respectively) resulted in the formation of two radioactive metabolites with HPLC retention times identical to those of authentic 18-OH-AA and 14,15-EET (Fig. 2). While the products formed are the same as those previously reported for wild type P450 BM-3 (17), the Arg for Ala replacement resulted in a reduced overall rate of AA oxidation (82% of wild type rates, Table II) (17). Furthermore, the amino acid replacement affected AA sp3 carbon hydroxylation and olefin epoxidation in opposite manners, i.e. an increased AA 14,15-olefin epoxide activity was accompanied by a concomitant reduction in AA ω-2 carbon hydroxylation (154 and 72% of wild type rates, respectively) (Fig. 2, Table II). An enhanced epoxide activity was also observed when the R47A mutant was incubated with EPA. As shown in Fig. 2 and Table III, the mutant enzyme metabolized EPA to 17,18-EETA with nearly absolute regioselectivity and at reaction rates higher than those reported for wild type P450 BM-3 (137% of wild type rates; Fig. 2, Table III) (17). Neither wild type P450 BM-3 nor the R47A mutant metabolized methyl-AA or methyl-EPA (data not shown). As with wild type enzyme, during AA metabolism by the R47A mutant, NADPH oxidation was coupled to oxygen reduction and substrate oxidation, as revealed by a 1:1 stoichiometry between NADPH and oxygen utilization (Table IV).

However, in contrast with wild type P450 BM-3, the R47A mutant enzyme diverts a small but significant portion of NADPH supplied electrons to H2O2 formation (Table IV). These results indicate that electron flow and oxygen chemistries are only marginally disturbed by the Arg47 for Ala replacement (Tables III and IV).

To probe for changes in substrate binding affinity and/or spatial orientation resulting from the Arg47 for Ala substitution, we determined the spectral dissociation constants for AA and EPA as well as the chirality of the AA and EPA metabolites generated by the R47A mutant. Compared to wild type P450 BM-3, and as estimated by the values of the corresponding spectral dissociation constants shown in Table V, the mutated enzyme showed a substantially decreased binding affinity for AA and EPA. These results, in conjunction with the above mentioned changes in reaction rates, suggest that charge interactions between the Arg47 side chain and the fatty acid carboxylates may stabilize substrate binding in the spatial conformation that favors oxygenation at the substrate C18 C–H bond, i.e. AA sp3 ω-2 carbon hydroxylation and EPA 17,18-epoxidation. Similarly, the increased AA and EPA epoxide activity observed with the R47A mutant may reflect a decreased binding rigidity and greater freedom of substrate lateral displacement brought about by the removal of Arg47-asso-
**Table III**

Eicosapentaenic acid oxygenation by the R47A mutant isoform of P450 BM-3

| Product distribution | Reaction rate* | % of wild type rate | NADPH/O_2 ratio |
|----------------------|----------------|---------------------|-----------------|
| Total                | 1944 ± 110     | 137                 |                 |
| 17,18-EET            | 1916 ± 88      | 137                 |                 |
| 14,15-EET            | ≤1             | ND                  |                 |

*Reaction rates are given in nanomoles of product formed/μmol of P450/min. Values are averages ± S.E., calculated from at least three different experiments. ND, not determined.

**Table IV**

Stoichiometry of NADPH and oxygen utilization during the metabolism of arachidonic acid by different isoforms of P450

| P450 | NADPH | O_2 | H_2O_2 | NADPH/O_2 ratio |
|------|-------|-----|--------|-----------------|
| Wild type | 125 | 126 | ND     | 1.0             |
| R47A mutant | 125 | 140 | 5.8    | 0.9             |
| R47E mutant | 145 | ND  | ND     |                 |
| F87V mutant | 125 | 124 | 5.8    | 1.0             |
| F87V mutant | 189 | 190 | 8.6    | 1.0             |

**Table V**

Spectral dissociation constant for the binding of fatty acids to wild type and mutated isoforms of P450 BM-3

| Protein | AA         | EPA       |
|---------|------------|-----------|
| Wild type | 2.4 ± 0.7 | 1.6 ± 0.1 |
| R47A mutant | 11.0 ± 3.0 | 33.0 ± 1.0 |
| F87V mutant | 1.7 ± 0.5 | 1.3 ± 0.1 |

**Table VI**

Chiral properties of the metabolites generated by the R47A mutant isoform of P450 BM-3 during the metabolism of arachidonic and eicosapentaenic acids

| Substrate | Metabolite absolute configuration | Enantiomeric composition distribution |
|-----------|----------------------------------|--------------------------------------|
| AA        | (18S)-OH-AA                       | 96                                   |
| EPA       | (17S,18R)-EET                     | 96                                   |
of the volume available for substrate binding near the heme prosthetic group, it has little or no effect at the opposite end, the surface opening end of the access channel. Thus, compared to wild type P450 BM-3, the volume of the substrate binding region in F87V is more or less cylindrical instead of conical (Fig. 3, A and B). To study the functional role of Phe87, we replaced it with tyrosine or valine. With the Tyr for Phe87 replacement (Table I), we sought to increase the polarity of the phenyl ring and thus add a polarity component to its steric effects. On the other hand, in the valine mutant (F87V) (Table I), we sought to remove from the heme environment steric effects attributable to the aromatic ring.

The addition of a hydroxyl group to the Phe87 aromatic side chain, i.e. replacing this residue for tyrosine, generated a mutant (F87Y) protein catalytically inactive toward AA, EPA, and their corresponding methyl esters. Importantly, the Soret region absorbance spectra of the F87Y mutant were identical to those of the wild type P450 BM-3 (data not shown). Assuming that the tyrosine replacement does not introduce significant protein structural alterations, the metabolism of polyunsaturated fatty acids by P450 BM-3 appears to be incompatible with a heme environment containing the oxygen binding site in close proximity to a polar aromatic amino acid side chain. Significant restrictions in substrate heme accessibility are likely to be responsible for the aforementioned lack of catalytic turnover. In support of the latter, the addition of AA or EPA to a solution of F87Y P450 BM-3 failed to elicit typical spectral manifestations of substrate binding and heme iron spin change. While unable to metabolize AA, the F87Y mutant did support significant NADPH-dependent oxygen reduction (Table IV). Furthermore, the absence of measurable substrate oxidation and of H2O2 formation, as well as the observed 1.9–2.1 stoichiometric ratio between NADPH and oxygen utilization (Table IV), indicates that the F87Y mutant catalyzes a four-electron reduction
of molecular oxygen to H₂O. Control experiments demonstrated that: (a) the F87Y mutant protein lacked significant catalase-like activity, and (b) NADPH and oxygen utilization was partially fatty acid-independent (data not shown). The four-electron reduction of oxygen to water is characteristic of cytochrome oxidase(s), the structurally complex and multifunctional hemoproteins that serve as the terminal oxidases of the respiratory chain. Because of its importance for hemoprotein biochemistry and function, this unusual activity of the F87Y mutant is currently under investigation.

The nearly absolute degree of enantiofacial selectivity with which wild type P450 BM-3 catalyzed AA sp³ hydroxylation and 14,15-olefin epoxidation (17) indicated that: (a) AA binding to the active site of P450 BM-3 severely limits the molecule’s freedom of C–C bond rotation, (b) the active site binding coordinates allow for a limited degree of substrate displacement along the longitudinal axis of the access channel resulting in more than one catalytically productive active site-AA complex, and (c) Phe⁸⁷ provides a distinct steric barrier to active site-substrate configurations favoring oxygen delivery to the AA or EPA 14,15-olefin (17) (Figs. 2 and 3A). Incubation of [1⁴C]AA with the F87Y mutant P450 BM-3 and NADPH, followed by product extraction and HPLC analysis, revealed this single amino acid substitution caused important alterations in the regioselectivity of AA oxidation by P450 BM-3. Thus, as reported (17) and shown in Fig. 4, AA metabolism by wild type P450 BM-3 generated a 5:1 mixture of 18-OH-AA and 14,15-EET. Compared to wild type, the F87V mutant enzyme metabolized AA at a significantly reduced overall rate (approximately 38% of wild type rates, Table VII). This reduced catalytic rate could be accounted for by the loss of AA sp³ carbon hydroxylation activity since, under identical conditions, the mutant enzyme catalyzed 14,15-EET formation at a rate twice that of wild type P450 BM-3 (Table VII). It is of interest that, in addition to the above effects on reaction rates, the replacement of Phe⁸⁷ by Val resulted in the generation of small amounts of H₂O₂ due to non-productive cycles of O₂ reduction and inactivation, and acceptor C–H bond insertion. Assuming that the Phe⁸⁷ for Val replacement does not introduce major protein structural alterations and/or changes in the kinetics of product release, the documented catalytic dissociation between P450 BM-3 supported hydroxylation and epoxidation reactions is in agreement with the proposal that these reactions are mediated by distinct P-450 BM-3 active site-substrate conformations (Fig. 3, A and B) (2, 17). Studies of the metabolism of AA and of several AA analogs by P450 BM-3 indicated that steric factors, as opposed to the chemistry of the reactive oxygen intermediate, may play the predominant role in determining the nature of the reaction products (17). Furthermore, our current understanding of the biochemistry of oxygen activation by P450 enzymes supports the concept that sp³ carbon hydroxylation of molecular oxygen to H₂O. Control experiments demonstrated that: (a) the F87Y mutant protein lacked significant catalase-like activity, and (b) NADPH and oxygen utilization was partially fatty acid-independent (data not shown). The four-electron reduction of oxygen to water is characteristic of cytochrome oxidase(s), the structurally complex and multifunctional hemoproteins that serve as the terminal oxidases of the respiratory chain. Because of its importance for hemoprotein biochemistry and function, this unusual activity of the F87Y mutant is currently under investigation.

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Arachidonic Acid Epoxidation by Mutant P450 BM-3

and olefin epoxidation are mediated by a common oxidant. Inasmuch as olefin epoxidation is thermodynamically preferred over \( sp^2 \) carbon hydroxylation, the decreased rates of AA metabolism observed with the F87V mutant enzyme may reflect changes in electron transfer kinetics brought about by the removal of the Phe\(^{87} \) aromatic side chain from the environment of the protein redox active center. Alternatively, charge interactions between the side chain of Arg\(^{47} \) and the substrate carboxylic acid, the proposed substrate anchoring site, may limit heme-iron access to the AA and EPA 14,15-olefins by providing a significant energy barrier to the freedom of substrate longitudinal displacement.

Incubation of \([1-\text{\textsuperscript{14}C}]\)EPA with the F87V mutant protein resulted in the NADPH-dependent formation of two radioactive products with HPLC retention times identical to those of authentic 17,18-EETA and its hydration product, 17,18-DHETA (17) (Fig. 5). A third metabolite, eluting with an HPLC retention of 19.7 min was identified as 14,15-EETA based on the following criteria: (a) catalytic hydrogenation over PtO\(_2\) generated a product with normal and RP-HPLC chromatographic properties identical to those of authentic 14,15-epoxyeicosanoic acid (21 and 33.1 min for normal and RP-HPLC, respectively), (b) catalytic hydrogenation followed by derivatization to the corresponding PFB ester yielded a product with normal and RP-HPLC retention times identical to those of the synthetic 14,15-epoxyeicosanoic acid (12 and 46 min for normal and RP-HPLC, respectively), and (c) catalytic hydrogenation, followed by derivatization to the corresponding PFB ester of synthetic 14,15-epoxyeicosanoic acid (data not shown). For chiral characterization of enzymatically derived 14,15-EETA, the metabolite was reduced by catalytic hydrogenation, converted to the corresponding PFB ester and compared by chiral phase HPLC with racemic and/or enantiomerically pure synthetic standards. As shown in Fig. 6, Chiralcel OD chromatography resolved the optical antipodes of synthetic 14,15-epoxyeicosanoate-PFB with base-line resolution. Co-injection of a mixture of racemic (10 \( \mu \)g) and (14S,15R)-epoxyeicosanoate-PFB (15 \( \mu \)g), was resolved using a Chiralcel OD column with UV detection at 210 nm, exactly as described under Materials and Methods.

The reported regio- and stereoselective epoxidation of EPA to (17S,18R)-EETA by P450 BM-3 was attributed to efficient trapping of the heme-bound active oxygen by the fatty acid reactive 17,18-olefin \( \pi \) electron cloud (17). As illustrated by the HPLC chromatograms in Fig. 5, the F87V mutant P450 BM-3 catalyzed the epoxidation of EPA to 14,15-EETA and 17,18-EETA. Additionally, small and variable amounts of the 17,18-EETA hydration product 17,18-DHETA were recovered (28 and 72% of reaction rates are given in nanomoles of product/nmol of P450/min. Values are averages \( \pm \) S.E., calculated from at least three different experiments. To accommodate the findings, a revised scheme is proposed for the metabolism and reaction rates are given in nanomoles of product/nmol of P450/min. Values are averages \( \pm \) S.E., calculated from at least three different experiments. To accommodate the findings, a revised scheme is proposed for the metabolism of eicosapentaenoic acid by the F87V mutant isoform of P450 BM-3 (Table IX).

**Table IX**

| Reaction rate\(^a\) | Product distribution | % of the wild type rate |
|---------------------|----------------------|------------------------|
| Total               | 521 \( \pm \) 24     | 100                    |
| 14,15-EETA          | 146 \( \pm \) 5      | 28                     |
| 17,18-EETA          | 376 \( \pm \) 11     | 72                     |

\( ^a \) Reaction rates are given in nanomoles of product/nmol of P450/min. Values are averages \( \pm \) S.E., calculated from at least three different experiments.

**FIG. 5.** Chromatographic comparison of the eicosapentaenoic acid metabolites isolated from incubates containing wild type or the F87V mutant isofom of P450 BM-3. The wild type and the mutant isoform of P450 BM-3 (5 \( n \)m, final concentration) were incubated at 30°C, in the presence of NADPH and [1-\textsuperscript{14}C]EPA (1 and 0.1 mM, respectively). After extraction into acidified Et\(_2\)O, reaction products were resolved by RP-HPLC as described under "Materials and Methods." Shown are the radiochromatograms of products generated after 1 min of incubation at 30°C.

**FIG. 6.** Chiral phase HPLC resolution of the optical isomers of synthetic and enzymatically produced 14,15-epoxyeicosanoate PFB esters. Racemic samples of synthetic 14,15-epoxyeicosanoate-PFB (20 \( \mu \)g), (14S,15R)-epoxyeicosanoate-PFB (12 \( \mu \)g), and of a mixture of racemic (10 \( \mu \)g) and (14S,15R)-epoxyeicosanoate-PFB (15 \( \mu \)g), were resolved using a Chiralcel OD column with UV detection at 210 nm, exactly as described under "Materials and Methods."
of total products for 14,15- and 17,18-EETA + 17,18-DHETE, respectively) (Fig. 5, Table IX). As with AA, the removal of the Phe87 aromatic ring from the heme environment was associated with decreased rates of EPA oxidation (Table IX) and concomitant increases in substrate-dependent, NADPH oxidase (Table IV). Furthermore, as shown in Tables V and VIII, the Phe87 replacement by Val did not significantly change the affinity of the protein for EPA, nor did it affect the enantiofacial selectivity of oxygenation (Table VIII). Thus, while the removal of the aromatic ring in Phe87 allows further displacement of the EPA molecule along the longitudinal axis of the access channel (Fig. 3B), the overall EPA binding coordinates do not appear to be significantly altered by this replacement (Table VIII). It is of interest that regardless of the fatty acid, the $s_i, r_e$ faces of the 14,15- and 17,18-olefin are always selected for epoxidation (Fig. 7), indicating that regioselectivity results predominantly from freedom of longitudinal displacement within the confines of the substrate access channel and not from oxygen chemistries and/or unique binding configurations.

The presence in P450 BM-3 of an hydrophobic substrate binding site uniquely adapted to AA binding and metabolism not only indicates high evolutionary specialization, it also raises interesting questions with regards to the mechanism and/or driving force(s) responsible for the release of the oxygenated products. As reported (17), the products of AA oxidation are efficiently recycled through the enzyme system and undergo extensive secondary oxidations, even in the presence of high AA concentrations (17). Thus, (14S,15R)-EET and (18R)-OH-AA compete effectively with AA for binding to the enzyme active site. Hence, it is likely that during catalytic turnover, reversible changes in active site configuration may participate in facilitating oxygenated product release.

All the changes in catalytic outcome and/or reaction rates reported here are the consequence of single amino acid replacements in the vicinity of the heme prosthetic group. Furthermore, these substitution-dependent changes appear to take place in the absence of major protein and/or active site structural modifications, i.e. changes in enzyme regioselectivity occurred without alterations in stereoselectivity. The pioneering studies of Negishi and collaborators and others with microsomal P450 isoforms (23, 24), in conjunction with the results reported here, show that substrate selectivity and catalytic outcome can be critically dependent on the chemical nature of unique residues located in finite, more or less predictable, areas of the protein active site. Thus, instead of the chemical properties of the oxidant species, the chemistry of the reaction products may be controlled by active site binding coordinates and the resulting spatial orientation of acceptor bonds with respect to the heme-bound reactive oxygen. These as well as other published studies are beginning to provide a molecular description of active site determinants for regio- and stereoselective metabolism in different P450 isoforms (24). These studies also contribute to the delineation of the molecular basis responsible for the catalytic versatility of these functionally diverse but, otherwise, structurally homologous proteins.

Finally, while it is apparent that charge interactions associated with the side chain of Arg47 serve as a fatty acid anchoring point and thus may regulate substrate displacement along the longitudinal axis of the access channel, the Phe87 aromatic side chain controls heme access at the distal end of the access channel. Consequently, we propose that the high turnover regio- and stereoselective metabolism of fatty acids such as AA and EPA is controlled by substrate anchoring at the surface opening of the access channel and by steric gating of the heme-bound reactive oxygen. Furthermore, the experimental data also shows that substrate-active site binding coordinates, as opposed to oxygen chemistries, are the predominant factors responsible for reaction rates and the chemistry of the reaction products.

REFERENCES

1. Poulos, T. L., Finzel, B. C., Gunsalus, I. C., Wagner, G. C., and Kraut, J. (1985) J. Biol. Chem. 260, 16122–16130
2. Ravichandran, K. G., Boddu palli, S. S., Hasemann, C. A., Peterson, J. A., and Deisenhofer, J. (1992) Science 261, 721–736
3. Hasemann, C. A., Ravichandran, K. G., Peterson, J. A., and Deisenhofer, J. (1994) J. Mol. Biol. 236, 1169–1185
4. Cupp-Vickery, J., Li, H., and Poulos, T. L. (1995) Nat. Struct. Biol. 2, 144–153
5. Men. L. P., and Fulco, A. J. (1987) J. Biol. Chem. 262, 6676–6682
6. Fulco, A. J. (1991) Annu. Rev. Pharmacol. Toxicol. 31, 177–203, and references therein
7. Narhi, L. O., and Fulco, A. J. (1987) J. Biol. Chem. 262, 6683–6690
8. Miura, Y., and Fulco, A. J. (1975) Biochim. Biophys. Acta 398, 305–317
9. Boddu palli, S. S., Estabrook, R. W., and Peterson, J. A. (1990) J. Biol. Chem. 265, 4233–4239
10. Ruettinger, R. T., and Fulco, A. J. (1981) J. Biol. Chem. 256, 5728–5734
11. Narhi, L. O., and Fulco, A. J. (1986) J. Biol. Chem. 261, 7160–7169
12. Capdevila, J. H., Falck, J. R., and Estabrook, R. W. (1992) FASEB J. 6, 731–736, and references therein
13. McGiff, J. C. (1991) Annu. Rev. Pharmacol. Toxicol. 31, 339–369, and references therein
14. Olie, E. H. (1994) Prog. Lipid Res. 33, 329–354, and references therein
15. Harder, D. R., Campbell, W. B., and Roman, R. J. (1995) J. Vasc. Res. 32, 79–92, and references therein
16. Capdevila, J. H., Zeldin, D., Makita, K., Karara, A., and Falck, J. R. (1995) in Cytochrome P450: Structure, Mechanism, and Biochemistry (Ortiz de Montellano, P. R., ed/2nd Ed., pp. 443–471, Plenum Press, New York, and references therein
17. Capdevila, J. H., Wei, S., Helvig, C., Falck, J. R., Beolusldtsev, Y., Truan, G., Graham-Lorenze, S. E., and Peterson, J. A. (1996) J. Biol. Chem. 271, 22063–22071
18. Kunkel, T. A., Bemenek, K., and McClary, J. (1991) Methods Enzymol. 204, 125–139
19. Omura, T., and Sato, R. (1964) J. Biol. Chem. 239, 2370–2378
20. Capdevila, J. H., Falck, J. R., Dishman, E., and Karara, A. (1990) Methods Enzymol. 187, 385–394
21. Capdevila, J. H., Dishman, E., Karara, A., and Falck, J. R. (1991) Methods Enzymol. 206, 441–453
22. Falck, J. R., Lumin, S., Blair, I., Dishman, E., Martin, M. V., Waxman, D. J., Guengerich, F. P., and Capdevila, J. H. (1990) J. Biol. Chem. 265, 10244–10249
23. Linberg, R. L., and Negishi, M. (1989) Nature 339, 632–634
24. Negishi, M., Uno, T., Darden, T. A., Sueyoshi, T., and Pedersen, L. G. (1996) FASEB J 10, 683–689, and references therein
25. Conolly, M. L. (1983) J. Appl. Crystallogr. 16, 548–558