Requirement for ω and (ω−1)-hydroxylations of fatty acids by human cytochromes P450 2E1 and 4A11

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Abstract Human liver microsomes and recombinant human P450 have been used as enzyme source in order to better understand the requirement for the optimal rate of ω and (ω−1)-hydroxylations of fatty acids by cytochromes P450 2E1 and 4A. Three parameters were studied: alkyl chain length, presence and configuration of double bond(s) in the alkyl chain, and involvement of carboxylic function in the fatty acid binding inside the access channel of P450 active site. The total rate of metabolite formation decreased when increasing the alkyl chain length of saturated fatty acids (from C12 to C16), while no hydroxylated metabolite was detected when liver microsomes were incubated with stearic acid. However, unsaturated fatty acids, such as oleic, elaidic and linoleic acids, were ω and (ω−1)-hydroxylated with an efficiency at least similar to palmitic acid. The (ω−1)/ω ratio decreased from 2.8 to 1 with lauric, myristic and palmitic acids as substrates, while the reverse was observed for unsaturated C18 fatty acids which are mainly ω-hydroxylated, except for elaidic acid showing a metabolic profile quite similar to those of saturated fatty acids. The double bond configuration did not significantly modify the ability of hydroxylation of fatty acid, while the negatively charged carboxylic group allowed a configuration energetically favourable for ω and (ω−1)-hydroxylation inside the access channel of active site.—Adas, F., J.P. Salaün, F. Berthou, D. Picart, B. Simon, and Y. Amet. **Requirement for ω and (ω−1)-hydroxylations of fatty acids by human cytochromes P450 2E1 and 4A11.** ***J. Lipid Res.*** **1999.** 40: **1990–1997.**

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The ubiquitous cytochrome P450 (P450) enzymes comprise a superfamily of monooxygenases present in both eukaryote and procaryote organisms (1). Cytochromes P450 from mammals are involved in the oxidation of a large number of exogenous and endogenous compounds including fatty acids (2). The physiological role of cytochromes P450 catalyzing the hydroxylation of fatty acids remains to be clarified. The CYP4A family appears to be mainly involved in oxidation of fatty acids and derivatives. The physiological role of certain bioactive metabolites generated from eicosanoids by this P450 family is now increasingly documented, and several data show their involvement in various cell functions. In addition, a significant regulatory role of CYP4A induction in the overall balance of fatty acid degradation by β-oxidation system is becoming increasingly evident (3). The peroxisomal β-oxidation system is particularly well suited for β-oxidation of fatty acids which are poor substrates for mitochondrial β-oxidation system (i.e., long-chain fatty acids). Unbalance for fatty acid degradation between a very efficient peroxisomal β-oxidation and the mitochondrial system should result in a rapid accumulation of toxic short- and medium-chain free fatty acids (i.e., lauric acid) generated by peroxisomes. Consequently, induction of members of CYP2E and CYP4A families should be associated with a detoxification process needed to reduce accumulation by cells of free fatty acids with the goal to maintain the membrane integrity. Study of the substrate specificity and the structure of metabolites generated by the major catalysts of fatty acid oxidation that belong to the CYP2E and CYP4A families are essential to demonstrate the individual role of P450s in the catabolic process. Several P450 isozymes are effective catalysts of hydroxylation of medium- and long-chain saturated and unsaturated fatty acids, although they show very different substrate selectivity and regiospecificity of the oxygene attack. The ethanol-inducible CYP2E1 isoform catalyzes not only the bioactivation of a large number of lipophilic compounds with low molecular weight, including aromatic and halogenated hydrocarbons, alcohols, ketones and nitrosamines (4–6), but also the hydroxylation of fatty acids. Recently, it was demonstrated that CYP2E1 from rat (7, 8), rabbit (9) and human (8, 10) livers was involved in the microsomal (ω−1)-hydroxy-

Abbreviations: P450, cytochrome P450 (E.C. 1.14.14.1) or CYP; RP-HPLC, reverse phase-high performance liquid chromatography; APCI, atmospheric pressure chemical ionization; LC–MS, liquid chromatography–mass spectrometry; MNNG, 1-methyl-3-nitro-1-nitrosoganidine; PPAR, peroxisome proliferator-activated receptor.

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lation of lauric acid and of several other fatty acids. In addition, the hydroxylation of two isomeric C18 unsaturated fatty acids, i.e., oleic (11) and elaidic (12) acids, was reported to be also catalyzed by the microsomal CYP2E1 from rat and human livers.

The mammalian CYP4A family encodes several cytochrome P450 isozymes which are involved in the oxidation of the terminal carbon, and to a lesser extent, of the (ω−1)-carbon of both saturated and unsaturated fatty acids (13). Interestingly, several members of the CYP4A subfamily also show the capability to catalyze mainly the ω-hydroxylation of potent chemotactic agents, such as prostaglandins and leukotrienes (14–16). CYP4A is induced in rat liver by hypolipidemic agents, such as clofibrate, and other peroxisomal proliferators (17). It has been recently demonstrated that CYP4A11 was the major lauric acid ω-hydroxylase in human liver (18), while the ethanol-inducible CYP2E1 was the major catalyst of (ω-1)-hydroxylauric acid in rat and human liver microsomes (7, 8, 10).

The role and significance of P450-dependent fatty acid hydroxylases are not well established. In rat and human, the induction of fatty acid hydroxylases by clofibrate and chemically related compounds involves transcriptional gene activation mediated by nuclear peroxisome proliferator-activated receptors (PPARs) (19, 20). It is noteworthy that in mammalian systems, clofibrate selectively induces fatty acid ω-hydroxylases from the CYP4A family (17), while ethanol enhances (ω−1)-hydroxylation of lauric and oleic (11) acids, mainly involving CYP2E1. The mechanism by which these P450 isoforms select the subterminal or the terminal carbon position for fatty acid hydroxylations remains unknown. It probably originates from substrate binding interactions with the active site of the enzyme. The CYP4A subfamilies are more selective for the terminal primary C–H bonds of the fatty acid, in preference to the more easily hydroxylated secondary C–H bonds at internal position such as (ω−1). Although P450-dependent fatty acid ω-hydroxylation in mammals was once thought to act as a catabolic pathway for eicosanoids, there are several lines of evidence that oxidized metabolites such as epoxy- and hydroxy-arachidonic acids are biologically active compounds (21–23).

In order to better understand the requirement for the optimal rate of ω and (ω−1)-hydroxylation of fatty acids by P450, three parameters were studied: *i*) the alkyl chain length, *ii*) the presence and configuration of double bond in the alkyl chain, and *iii*) the role of the carboxylic function. Human liver microsomes and recombinant human P450 cells were used as enzyme source.

MATERIALS AND METHODS

Chemicals

Lauric, myristic, palmitic, stearic, oleic, elaidic and linoleic acids were purchased from Fluka (Buchs, Switzerland), while radiolabeled [1-^{14}C]lauric (58 mCi/mmol), [1-^{14}C]myristic (50 mCi/mmol), [1-^{14}C]palmitic (55 mCi/mmol), [1-^{14}C]stearic (57 mCi/mmol), [1-^{14}C]oleic (55 mCi/mmol), and [1-^{14}C]linoleic acids (55 mCi/mmol) were from Amersham (Amersham, UK). NADPH and 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) were purchased from Fluka-Sigma (St. Quentin Fallavier). Modified cell microsomes containing human P450s were obtained from Gentest (Woburn, MA), while cytochrome b5 was from Oxford Biomedical Research (Oxford, MI). All chemicals and solvents were of the highest purity obtainable and were from Merck (Darmstadt, Germany) or Fluka-Sigma.

Preparation of human liver microsomes

Human liver samples were obtained from subjects who died after traffic accidents. In accordance with French law, local ethical committee (CHU, Brest, France) approval was obtained prior to this study. Human liver samples were frozen immediately after removal and the microsomal fraction was prepared according to a previously described method (24), and stored at −80°C until use. Microsomal protein content was determined using the Bio-Rad protein assay (Bio-Rad, Munich, Germany) based on the Bradford dye-binding procedure (25), using bovine serum albumin as standard. Human microsomal contents, in terms of specific P450 and various monooxygenase enzymatic activities, have been reported previously (26).

Assay of monooxygenase enzymatic activities

The hydroxylations at ω and (ω−1)-positions of a series of fatty acids were measured by adding microsomal protein (0.3 mg) to a reaction mixture containing substrate (0.1 mm; specific activity 2.5 mCi/mmol), 0.12 m potassium phosphate buffer, pH 7.4, and 5 mm $MgCl_2$. The enzymatic reaction was initiated by the addition of 1 mm NADPH and stopped after 10–30 min by 0.8 mL of a 10% H_2SO_4 aqueous solution. The metabolites and residual substrate were extracted twice with 5 mL of diethylether. The organic phase was dried under a stream of nitrogen, the residue was then dissolved in 100 μL acetonitrile, and 20 μL were injected for RP-HPLC analysis. Enzyme kinetic parameters were determined by adding a series of fatty acids to the reaction mixture in the range 12.5–200 μm.

Conditions of RP-HPLC analysis

The ω and (ω−1)-hydroxylated metabolites and residual substrates (lauric, myristic, palmitic, oleic, and linoleic acids) were separated by RP-HPLC using a 5-μm Ultrasphere C18 column 150 × 4.6 mm (Beckman, Gagny, France). The mobile phase (containing 0.2% acetic acid in a mixture of water–acetonitrile) program began isocratically for 30 min at a flow rate of 2 mL/min with mixtures of water–acetonitrile 75:25, 65:35, 60:40, 55:45, 60:40 (v/v) for C12, C14, C16, C18, C18:1, and C18:2, respectively. The isocratic phase was followed by a 5-min linear gradient of 95% acetonitrile in water for 15 min, in order to elute the residual substrate. Radioactivity of RP-HPLC effluents was monitored with a computerized on-line scintillation counter (Flo-One Beta radiometric detector, Packard, Meriden, CT). The rate of radiolabeled metabolites generated was calculated from peak surfaces and expressed as nmol/min per mg of protein.

HPLC/mass spectrometry

Hydroxylated metabolites were analyzed by HPLC/APCI–mass spectrometry on a Navigator LC/MS mass spectrometer (Thermo Quest, Manchester, UK), equipped with an ionization source at atmospheric pressure running on negative ion mode (11, 12). The RP-HPLC chromatographic conditions were the same as described above.

Incubation of human recombinant P450s

Human P450 isoforms CYP2E1 and CYP4A11 (Gentest) were obtained from human B-lymphoblastoid cell lines transfected separately with human P450 cDNAs. Cytochrome P450 2E1 (Gen-

test M106k) and 4A11 (Gentest M121a) preparations did not contain cytochrome b5. It was added to the incubation mixture in the molar ratio of 1:2 for P450/b5. The incubation method was the same as described above for human liver microsomes, except for the incubation time which was increased to 60 min.

Methylation of the carboxylic group of palmitic acid

In a first step, diazomethane was prepared in a diethylether phase, following the procedure described by Sigma from 1-methyl-3-nitro-1-nitrosoguanidine (MNNG). Esterification was performed by adding [^{14}C]palmitic acid (0.1 mm, specific activity 2.5 mCi/mmol) to 3 mL of ether containing diazomethane at room temperature for 2–3 h. The control of the complete esterification of the palmitate was checked by HPLC with radiometric detection. Methylated palmitic acid (0.1 mm) was then incubated with either human microsomes (FH3) at 37°C for 15 min, or human recombinant P450s (CYP2E1 and CYP4A11) at 37°C for 60 min. As methylpalmitate was not efficiently soluble in buffer, acetonitrile (1% final concentration) was added in the incubation mixture. Such a concentration of acetonitrile accompanied with a strong mixing allowed a total solubilization of the substrate in the buffer, and furthermore did not inhibit the catalytic activity of cytochromes P450.

RESULTS

Characterization of fatty acid metabolites

The RP-HPLC profiles of fatty acids incubated with human liver microsomes showed the presence of two major metabolites with retention times corresponding to (ω–1) and ω-hydroxylated metabolites. Furthermore, negative ion APCI–mass spectrometry analysis of these metabolites confirmed their elemental composition. They were characterized by selected ion monitoring as deprotonated molecule $[M-H]^+$, with ions at m/z 215, 243, 271, 297, and 295 for the hydroxylated metabolites generated from lauric, myristic, palmitic, oleic or elaidic, and linoleic acids, respectively. These results were consistent with the expected mass fragmentation pattern of subterminal and terminal hydroxylated fatty acids. Mass spectra analysis of metabolites also showed that epoxide derivatives from oleic and elaidic acids were not generated to a detectable level by microsomes. Metabolites from linoleic acid were resolved by HPLC/APCI–MS analysis as a complex mixture containing epoxides, diols, and hydroxylated derivatives. Only the 17-hydroxy and 18-hydroxy-octadecadienoic derivatives characterized by their retention times and GC–MS (11) were studied in this report.

Incubation of saturated and unsaturated fatty acids with hepatic human microsomes

Human liver microsomal preparations hydroxylated fatty acids at the ω and (ω–1) positions (**Fig. 1**). The rate of total metabolites formation decreased when increasing the alkyl chain length from C12 to C16. The formation of both ω and (ω–1)-hydroxylated metabolites decreases from 2.7 ± 0.54 to 0.22 ± 0.04 and from 1.4 ± 0.27 nmol/min per mg to 0.24 ± 0.07 nmol/min per mg, respectively, when increasing the chain length from lauric to palmitic acid. Interestingly, no hydroxylated metabolite was detected when human liver microsomes were incubated with stearic acid. However, unsaturated analogs such as oleic (Z C18:1), elaidic (E C18:1), and linoleic (C18:2) acids were ω and (ω–1)-hydroxylated with an efficiency at least similar to palmitic acid. The (ω–1)-hydroxylation values were 0.20 ± 0.06, 0.60 ± 0.35, and 0.16 ± 0.09 nmol/min per mg, for oleic, elaidic, and linoleic acids, respectively, while the

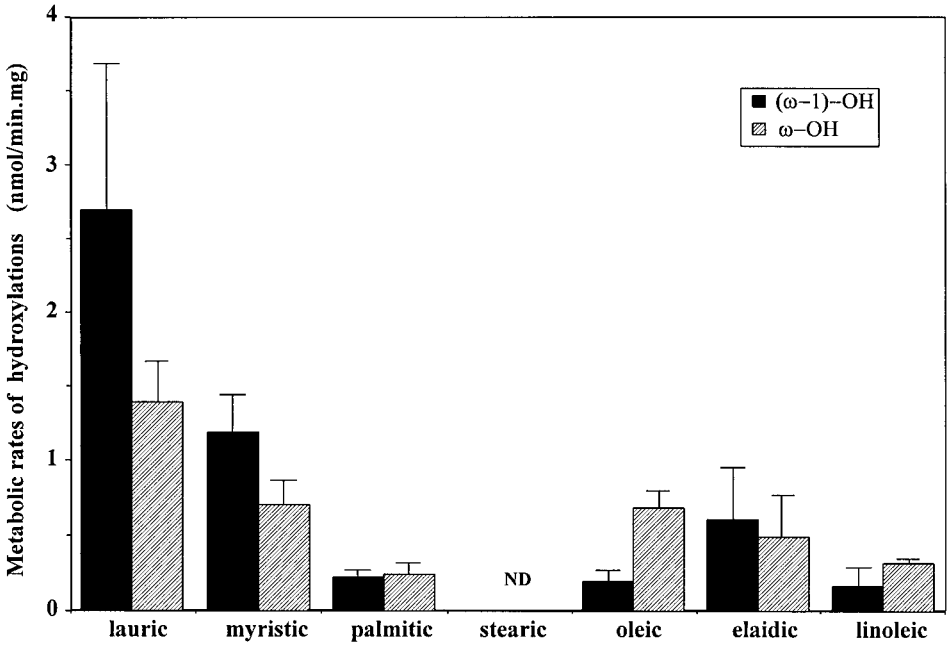

Fig. 1. Metabolism of various fatty acids (0.1 mm; specific activity 2.5 mCi/mmol) by human liver microsomes (0.3 mg; n = 5). Hydroxylated metabolites were analyzed by RP-HPLC coupled with radiometric detection, as described under Materials and Methods section; ND, not detectable.

TABLE 1. Kinetic parameters of fatty acid metabolism by human liver microsomes

Fatty Acid	(ω-1)-Hydroxylated Metabolite			ω-Hydroxlyated Metabolite			(ω–1)ω Ratio
	V_{max}	K_m	V_{max}/K_m	V_{max}	K_m	V_{max}/K_m	V_{max}
	nmol/min·mg	*μM*	*μL/min·mg*	*nmol/min·mg*	*μM*	*μL/min·mg*	
C12:0 (n = 5)	7.2 ± 4.1	84 ± 45	90 ± 30	2.1 ± 1.2	13 ± 6.6	230 ± 210	3.4 ± 0.2
C14:0 (n = 2)	1.17 ± 0.5	52.5 ± 17.7	20 ± 3	0.6 ± 0.3	82.5 ± 14.8	7 ± 3	2.8 ± 0.3
C16:0 (n = 2)	0.52 ± 0.3	102 ± 13.4	5 ± 1	0.51 ± 0.24	47 ± 9.9	8 ± 2	1 ± 0.2
C18:0 (n = 2)	ND	ND	—	ND	ND	—	—
C18:1 Z (n = 3)	0.93 ± 0.05	81 ± 39	10 ± 2	2.6 ± 1.7	46.6 ± 28	50 ± 6	0.35 ± 0.08
C18:1 E (n = 1)	1.4	28.5	49.1	0.48	53.5	9.0	2.9
C18:2 (n = 1)	0.2	48.5	4.1	0.55	52.6	10.5	0.36

The ω and (ω–1)-hydroxylase activities were measured using purified radiolabeled substrates, as described in Materials and Methods section. The ratio V_{max}/K_m expressed as μL/min·mg represent the intrinsic clearance; ND, not detectable.

ω-hydroxylation activities were 0.70 ± 0.12, 0.50 ± 0.28, and 0.33 ± 0.03 nmol/min per mg, respectively.

Kinetic parameters

Enzyme properties were determined following classic Michaelis-Menten kinetics and are reported in **Table 1**. Kinetic parameters of fatty acid ω and (ω–1)-hydroxylations in human liver microsomes indicated the involvement of a single enzyme in the range of substrate concentrations used. Accordingly, the low affinity K_m of lauric ω-hydroxylation higher than 500 μm (8) was not detected in this range of substrate concentrations. High apparent velocities for (ω–1)-hydroxylated fatty acids were observed for lauric, myristic, oleic, palmitic and linoleic acids. Based on apparent V_{max}, values of microsomal (ω–1)-oxidation were in decreasing order lauric > myristic > oleic > elaidic > palmitic > linoleic acids. Similarly, high apparent velocities for ω-hydroxylated fatty acids were observed for oleic, lauric, myristic, linoleic, and palmitic acids. Based on apparent V_{max} (ω) values, catalytic efficiency of human liver microsomes was highest for lauric > oleic > linoleic > myristic > palmitic acids. The apparent K_m values were in a same order of magnitude for all the fatty acids, but were 6 times higher for myristate when compared to laurate.

The intrinsic clearance V_{max}/K_m (μL/min·mg protein) allowing us to predict first-pass elimination in the liver showed a high ω-hydroxylation of lauric acid. Furthermore, the change of configuration of the double bond from *cis* (Z) (oleic acid) to *trans* (E) (elaidic acid) increased the intrinsic clearance of monounsaturated C18 fatty acid 5-fold. The V_{max} (ω–1)/ω ratio decreased from 3.4 to 1 with lauric (C12), myristic (C14), and palmitic (C16) acids as substrates. Interestingly, the reverse was observed for unsaturated C18 fatty acids which are mainly ω-hydroxylated, except for elaidic acid which shows a metabolite profile more similar to those of saturated fatty acids.

Fatty acid oxidation by genetically engineered human P450 2E1 and 4A11

Saturated (from C12 to C18), and unsaturated (C18:1 and C18:2) fatty acids were incubated in the presence of genetically engineered human P450 2E1 and 4A11 at 37°C for 60 min.

Figure 2A shows that the CYP2E1 enzyme was able of hydroxylating saturated and unsaturated fatty acids at the subterminal (ω–1) position, while CYP4A11 (Fig. 2B) shows mainly hydroxylated fatty acids at the terminal ω position, and to a lesser extent at the (ω–1) position. The turnover numbers of heterologously expressed CYP2E1 for (ω–1)-hydroxylation decreased from 3.6 to 0.13 min^{-1} when the alkyl chain length increased from lauric to stearic acids. The turnover of oleic and linoleic acids was 0.5 min^{-1}. These values increased when cytochrome b5 was added in the incubation medium.

Concerning the ω-hydroxylation of fatty acids, the turnover numbers of heterologously expressed CYP4A11 decreased from 7.2 to 0.85 min^{-1} when the alkyl chain length increased from lauric to palmitic acids. Although stearic acid was not a substrate of CYP4A11, the turover values for unsaturated C18 fatty acids (1.6 and 1.2 min^{-1} for oleic and linoleic acids, respectively) were higher than for palmitic acid. These values increased by 1.65 ± 0.17-fold when cytochrome b5 was added in the incubation medium. The CYP4A11 enzyme slightly metabolized lauric and myristic acids at the (ω–1) positions, with turnover values of 0.56 and 0.6 min^{-1}, respectively.

Metabolism of methylester palmitate

Methylated palmitic acid was incubated with human liver microsomes (FH3) or genetically engineered human P450s (2E1 and 4A11) in order to study the role of the free carboxylic group in the substrate access channel of the cytochrome P450 active site. As shown in **Fig. 3B**, no hydroxylated metabolite was detected when incubating methylated palmitic acid with human liver microsomes. Figure 3B shows only two peaks, corresponding to palmitic acid (peak 3) resulting from hydrolysis of ester group by esterases largely present in microsomal preparations, and the residual methylated palmitic acid (peak 4).

In the same way, the incubation of methylester palmitate with genetically engineered cells P450 2E1 and 4A11 did not allow the production of hydroxylated metabolites (data not shown).

DISCUSSION

The interplay of multiple enzymes involved in the oxidative metabolism of fatty acids makes difficult the understanding of the physiological role and effects of individual

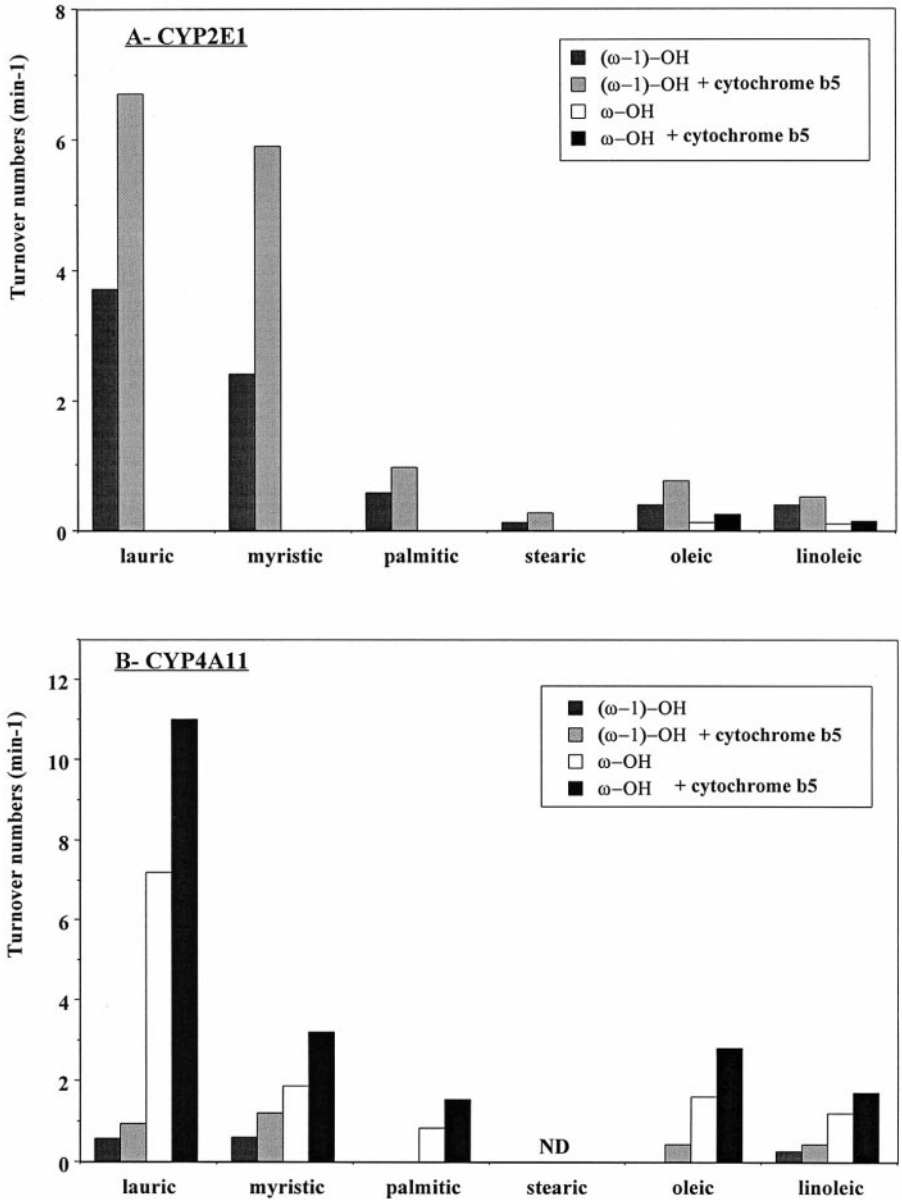

Fig. 2. Incubation of various fatty acids (0.1 mm; specific activity 2.5 mCi/mmol) with human recombinant cytochromes P450 2E1 (A) and 4A11 (B) at 37°C for 60 min, in presence or absence of cytochrome b5 (molar ratio of 1:2 for P450:b5); ND, not detectable.

metabolites generated during the oxidative cascade of saturated and unsaturated fatty acids. The liver is mainly involved in detoxification processes because it contains several inductible P450 isoforms which are able to oxidize a large number of very lipophilic endogenous and exogenous compounds. The catalytic capability and the substrate specificity of individual P450 capable of oxidizing fatty acids might provide information as to their role in the catabolism of fatty acids, and in the synthesis of bioactive oxidized molecules. In this study, two P450 isoforms have been investigated in the hydroxylations of fatty acids, namely CYP2E1 and CYP4A that are shown to be highly regioselective for catalyzing (ω−1) and ω-hydroxylations, respectively.

Two different criteria determine the preferred site of hydroxylation of fatty acids. One is thermodynamic, i.e., the ease of hydrogen atom abstraction by the ferryl oxygen complex of P450 that reflects the strength of the C–H bond. The other criterion is steric, i.e., the proximity of the H atom to the ferryl oxygen. The thermodynamic considerations favor oxidation of in-chain carbons over oxidation at the terminam methyl. Both experimental (this study) and computational studies (27) have clearly demonstrated the regioselectivity of lauric acid hydroxylation by CY4A11. The terminal methyl is strictly positioned close to the active ferryl-oxo species by steric hindrance of the active site of CY4A11, especially three amino-acid residues (27). In opposite, the regioselectivity of hydroxylation by CYP2E1 is governed by the thermodynamic criterion, i.e., the easiest abstraction of H atom from CH_2 group of (ω−1) position than from terminal methyl. Accord-

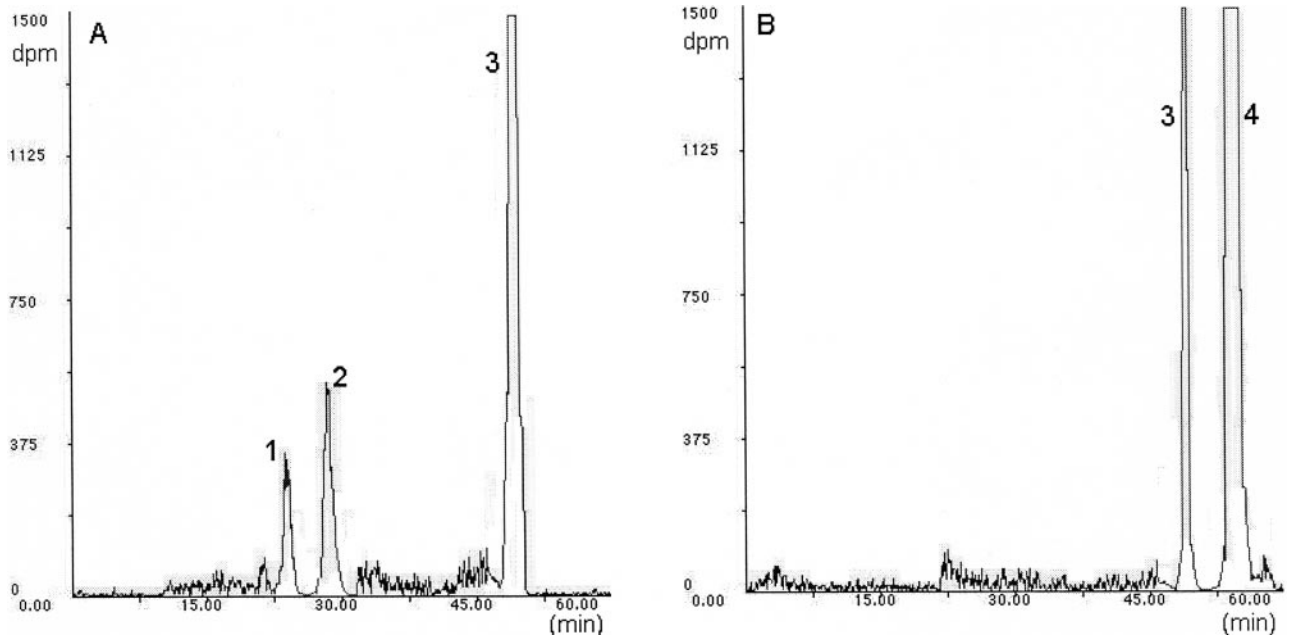

Fig. 3. HPLC analysis of metabolites formed from [^{14}C]palmitic acid (A) or methylated palmitic acid (B), both (0.1 mm; 2.5 mCi/mmol) incubated with human liver microsomes (0.3 mg; FH3 sample). Peaks 1 and 2 were identified as (ω–1) and ω-hydroxylated palmitic acid; peaks 3 and 4 corresponded to palmitic acid and methylated palmitic acid, respectively.

ingly, the regioselectivity of hydroxylation depends upon the length of the alkyl chain and the position of the double bond in-chain.

CYP2E1 enzyme hydroxylated saturated fatty acids specifically at the (ω–1) position. Among the saturated fatty acids studied, high turnover numbers up to 6 min^{-1} were measured with lauric and myristic acids, and much lower turnover numbers with the other fatty acids. This result suggests that lauric acid was the most appropriate fatty acid as substrate for CYP2E1. This result was in agreement with previous studies performed using rat and human liver (28). Interestingly, lauric acid is also the best substrate for plant fatty acid ω-hydroxylase (CYP94A1) heterologously expressed in yeast (29). As it was previously reported (30–32), the addition of cytochrome b5 increased the catalytic efficiency of the CYP2E1 enzyme by 1.8-fold, as cytochrome b5 had a strong stimulatory effect on CYP2E1-mediated two-electron oxidations. CYP2E1 binds and oxidizes a wide variety of small molecules such as ethanol, alcohols, halogenated alcanes and alkenes, *N*-nitrosodimethylamine, acetaminophen, *p*-nitrophenol, and chlorzoxazone. Lauric acid, by its alkyl tail, allows an optimal binding of substrate in the active site of CYP2E1. Indeed, lauric acid is metabolized with a high turnover number of 6 min^{-1}, i.e., in the same order of magnitude as those of two selective substrates, chlorzoxazone and *p*-nitrophenol (turnover of 20 min^{-1}) (33, 34).

Compared to many other P450 enzymes, those of the 4A family catalyzed the hydroxylation of a relatively homogeneous group of substrates, i.e., fatty acids, with a regioselectivity for the ω-hydroxylation. Enzyme activity decreases when fatty acid chain length increases with a maximum of activity observed with a dodecyl chain. The ratio of ω-hydroxylase to (ω–1)-hydroxylase activity was shown to be dependent on the alkyl chain length and the configuration of double bond. These results are in agreement with those reported by Hardwick et al. (35, 36) concerning rat CYP4A1. In the same way, Aoyama et al. (16) compared the catalytic activities of vaccinia virus-expressed CYP4A1 and CYP4A3 towards lauric and palmitic acids. In the present study, the use of human recombinant P450 4A11 catalyzed the formation of ω and (ω–1)-hydroxylated metabolites in the ratio ω/ω–1 of approximately 12 and 3.8 for lauric and myristic acids, respectively, suggesting a high selectivity of CYP4A11 with lauric acid as substrate. Such a selectivity has been reported previously (16, 35–37). Most studies of fatty acid oxidation were performed using *cis* isomers, because they are the major natural products. Recently, the metabolism of an unsaturated fatty acid with *cis*/*trans* (Z/E) configuration has been investigated (12) in order to characterize the geometry of the substrate access channel of the active site of P450 2E1. The microsomal hydroxylation of elaidic acid (E C18:1 Δ9) was compared to that of oleic acid (Z C18:1 Δ9) in human liver microsomes. The relative ratios of the two hydroxylated metabolites ω/ω–1 were different (1.32 ± 1 and 5.2 ± 2.6 for elaidic and oleic acids, respectively). On the other hand, they were quite similar in liver microsomes of control rats (1.14 ± 0.1 and 1.2 ± 0.01 for elaidic and oleic acids, respectively). It appears that the geometry of the double bond (*cis*/*trans*) did not modify the ability of the fatty acid to bind to the substrate access channel of cytochrome P450 active site. If the *trans* isomer was (ω–1)-hydroxylated at the same rate as the *cis* substrate, the V_m/K_m ratio reflecting the intrinsic clearance, was 5-fold higher for elaidic than for oleic acid. This result has been also reported in plants (29, 38). The double bond configu-

ration did not modify the regiospecificity of oxidation of unsaturated fatty acids by plant and human cytochrome P450s. Fatty acids of variable chain length are hydroxylated with remarkable selectivity by human P450 2E1 and 4A11, as these substrates presented significant conformational flexibility and a single polar functional group at the opposite end of the molecule from the hydroxylation site. Furthermore, the regioselectivity of the CYP4A11 enzyme for ω-hydroxylation of lauric acid is particularly remarkable as the C–H bonds of the terminal methyl group are much less reactive than those of the adjacent ω-1 methylene group as previously reported for CYP4A1 (39). These two chemical characteristics of fatty acids, namely an optimal alkyl chain length and a terminal carboxylic group, are required for their regiospecific hydroxylations by P450 2E1 and 4A11.

The negatively charged carboxylic group allows the fatty acid to be anchored in the substrate access channel of both P450s, with this polar group recognition site at the outside border of the access channel, exposing it to aqueous environment (39). By methylation of the ester group, this apolar group is exposed to an environment that is energetically unfavorable, as previously confirmed with CYP4A1/NADPH P450 reductase fusion protein (39). Data reported in this study are in full agreement with the conceptual model of P450 consisting of a substrate access channel and a substrate binding pocket (28). The marked difference in regiospecificity of catalytic hydroxylations of fatty acids by CYP2E1 and CYP4A11 should probably be ascribed to the property of the substrate binding pocket. It can be speculated that the positioning of the substrate in the active site near the ferrylaxo complex should proceed in at least two ways, either by the methyl terminal for CYP4A11 or by the methylene group for CYP2E1. Both CYP2E1 and CYP4A11 catalyze ω- and (ω–1)-hydroxylations, but with a very contrasted selectivity of carbon mainly oxidized. Our results suggest that these two possibilities of positioning the substrate exist for all the fatty acids tested with CYP4A11. In contrast, the alkyl chain length seems to have an effect on the regioselectivity in CYP2E1 reaction. Indeed, lauric acid is only (ω–1)-hydroxylated, although both ω- and (ω–1)-hydroxylated metabolites are generated from oleic and linoleic acids by CYP2E1.

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REFERENCES

1. Nelson, D. R., L. Koymans, T. Kamataki, J. J. Stegeman, R. Feyereisen D. J. Waxman, M. R. Watesman, O. Gotoh, D. J. Coon, R. W. Estabrook, I.C. Gunsalus, and D. W. Nebert. 1996. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics.* **6:** 1–42.
2. Guengerich, F. P. 1989. Characterization of human cytochrome P-450 enzymes. *Annu. Rev. Pharmacol. Toxicol.* **29:** 241–264.
3. Kroetz, D. L., P. Yook, P. Costet, P. Bianchi, and T. Pineau. 1998. Peroxisome proliferator-activated receptor alpha controls the hepatic CYP4A induction adaptive response to starvation and diabetes. *J. Biol. Chem.* **273:** 31581–31589.
4. Guengerich, F. P., D. H. Kim, and M. Iwasaki. 1991. Role of human cytochrome P450 IIE1 in the oxidation of many low molecular weight cancer suspects. *Chem. Res. Toxicol.* **4:** 168–179.
5. Koop, D. P., and D. J. Tierney. 1990. Multiple mechanisms in the regulation of ethanol inductible cytochrome P450IIE1. *Bioessays.* **12:** 429–435.
6. Raucy, J. L., J. C. Kramer, and J. M. Lasker. 1993. Bioactivation of halogenated hydrocarbons by cytochrome P450 2E1. *Crit. Res. Toxicol.* **23:** 1–20.
7. Amet, Y., F. Berthou, T Goasduff, J. P. Salaün, L. le Breton, and J. F. Ménez. 1994. Evidence that cytochrome P450 2E1 is involved in the (ω–1)-hydroxylation of lauric acid in rat liver microsomes. *Biochem. Biophys. Res. Commun.* **203:** 1168–1174.
8. Clarke, S. E., S. J. Baldwin, J. C. Bloomer, A. D. Ayrton, R. S. Sozio, and R. J. Chenery. 1994. Lauric acid as a model substrate for the simultaneous determination of cytochrome P450 2E1 and 4A in hepatic microsomes. *Chem. Res. Toxicol.* **7:** 836–842.
9. Fukuda, T., Y. Imai, M. Komori, M. Nakamura, E. Kusunose, K. Satouchi, and M. Kusunose. 1994. Different mechanisms of regioselection of fatty acid hydroxylation by laurate (omega–1)-hydroxylating P450s, P450 2C2 and P450 2E1. *J. Biochem.* **115:** 338–344.
10. Amet, Y., F. Berthou, S. Baird, Y. Dréano, J. P. Bail, and J. F. Ménez. 1995. Validation of the (ω–1)-hydroxylation of lauric acid as an *in vitro* substrate probe for human liver CYP2E1. *Biochem. Pharmacol.* **50:** 1775–1782.
11. Adas, F., F. Berthou, D. Picart, P. Lozac'h, F. Beaugé, and Y. Amet. 1998. Involvement of cytochrome P450 2E1 in the (ω–1)-hydroxylation of oleic acid in human and rat liver microsomes. *J. Lipid Res.* **39:** 1210–1219.
12. Adas, F., D. Picart, F. Berthou, B. Simon, and Y. Amet. 1998. Liquid chromatography-mass spectrometry and gas chromatography-mass spectrometry of ω and (ω–1)-hydroxylated metabolites of elaidic and oleic acids in human and rat liver microsomes. *J. Chromatogr.* **714:** 133–144.
13. Simpson, A. E. C. M. 1997. The cytochrome 4 (CYPA) family. *Gen. Pharmacol.* **28:** 351–359.
14. Bains, S. K., S. M. Gardiner, K. Mannweiler, D. Gillett, and G. G. Gibson. 1985. Immunochemical study on the contribution of hypolipidaemic-induced cytochrome P-452 to the metabolism of lauric acid and arachidonic acid. *Biochem. Pharmacol.* **34:** 3221–3229.
15. Sharma, D. K., M. V. Doig, D. F. W. Lewis, and G. Gibson. 1989. Role of hepatic and renal cytochrome P450 IVA1 in the metabolism of lipid substrats. *Biochem. Pharmacol.* **38:** 3621–3629.
16. Aoyama, T., J. P. Hardwick, S. Imaoka, Y. Funae, H. V. Gelboin, and F. J. Gonzalez. 1990. Clofibrate-inductible rat hepatic P450s IVA1 and IVA3 catalyze the ω- and (ω–1)-hydroxylation of fatty acids and the ω-hydroxylation of prostaglandins E1 and $F_{2\alpha}$ *J. Lipid Res.* **31:** 1477–1482.
17. Sharma, R., B. G. Lake, J. Foster, and G. G. Gibson. 1988. Microsomal cytochrome P-452 and peroxisome proliferation by lipidaemic agents in rat liver. A mechanistic interrelationship. *Biochem. Pharmacol.* **37:** 1193–1201.
18. Powell, K. C., I. Wolf, and J. M. Lasker. 1996. Identification of CYP4A11 as the major lauric acid ω-hydroxylase in human liver microsomes. *Arch. Biochem. Biophys.* **335:** 219–226.
19. Dreyer, C., H. Keller, A. Mahfoudi, V. Laudet, G. Krey, and W. Wahli. 1993. Positive regulation of the peroxisomal beta-oxidation pathway acids through activation of peroxisome proliferator-activated receptors (PPAR). *Biol. Cell.* **77:** 67–76.
20. Keller, H., C. Dreyer, J. Medin, A. Mahfoudi, K. Ozato, and W. Wahli. 1993. Fatty acids and retinoids control lipids metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. *Proc. Natl. Acad. Sci. USA.* **90:** 2160–2164.
21. Falck, J. R., S. Lumin, I. Blair, E. Dishman, M. V. Martin, D. J. Waxman, F. P. Guengerich, and J. H. Capdevila. 1990. Cytochrome P-450-dependent oxidation of arachidonic acid to 16-, 17-, and 18-hydroxyeicosatetraenoic acids. *J. Biol. Chem.* **265:** 10244–10249.
22. Capdevila, J. H., J. R. Falck, and R. W. Estabrook. 1992. Cytochrome P450 and the arachidonate cascade. *FASEB J.* **6:** 731–736.
23. Zeldin, D. C., R. N. Dubois, J. R. Falck, and J. H. Capdevila. 1995. Molecular cloning, expression and characterization of an endogenous human cytochromes P450 arachidonic acid epoxygenase isoform. *Arch. Biochem. Biophys.* **322:** 76–86.
24. Berthou, F., D. Ratanasavanh, C. Riché, D. Picart T. Voisin, and A. Guillouzo. 1989. Comparison of caffeine metabolism by slices, microsomes, and hepatocyte cultures from adult human liver. *Xenobiotica.* **19:** 401–417.
25. Bradford, M. M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of proteins-dye binding. *Anal. Biochem.* **72:** 248–254.

26. Berthou, F., Y. Dréano, C. Belloc, L. Kangas, J. C. Gautier, and P. Beaune. 1994. Involvement of cytochrome P450 3A enzyme family in the major metabolic pathways of toremifene in human liver microsomes. *Biochem. Pharmacol.* **47:** 1883–1895.
27. Chang, Y-T., and G. H. Loew. 1999. Homology modeling and substrate binding study of human CYP4A11 enzyme. *Proteins.* **34:** 403–415.
28. Wang, M. H., D. Wade, L. Chen, S. White, and C. S. Yang. 1995. Probing the active sites of rat and human cytochrome P450 2E1 with alcohols and carboxylic acids. *Arch. Biochem. Biophys.* **317:** 299–304.
29. Tijet, N., C. Helvig, F. Pinot, R. Le Bouquin, A. Lesot, F. Durst, J. P. Salaün, and I. Beneviste. 1998. Functionnal expression in yeast and characterization of a clofibrate-inductible plant cytochrome P-450 (CYP 94A1) involved in cutin monomers synthesis. *Biochem. J.* **332:** 583–589.
30. Zerilli, A., D. Ratanasavanh, D. Lucas, T. Goasduff, Y. Dréano, C. Ménard, D. Picart, and F. Berthou. 1997. Both cytochromes P450 2E1 and 3A are involved in the ω-hydroxylation of *p*-nitrophenol, a catalytic activity known to be specific for P450 2E1. *Chem. Res. Toxicol.* **10:** 1205–1212.
31. Chen, W., R. M. Peter, S. McArdle, K. E. Thummel, R. O. Sigle, and S. D. Nelson. 1996. Baculovirus expression and purification of human and rat cytochrome P450 2E1. *Arch. Biochem. Biophys.* **335:** 123–130.
32. Patten, C. J., and Koch P. 1995. Baculovirus expression of human P450 2E1 and cytochrome b5: spectral and catalytic properties and effect of b5 on the stoechiometry of P450 2E1 catalyzed reactions. *Arch. Biochem. Biophys.* **317:** 504–513.
33. Zerilli, A., D. Lucas, Y. Dréano, D. Picart, and F. Berthou. 1998. Effect of pyrazole and dexamethasone administration on cytochrome P450 2E1 and 3A isoforms in rat liver and kidney: lack of specificity of p-nitrophenol as a substrate of P450 2E1. *Alcohol. Clin. Exp Res.* **22:** 652–657.
34. Lucas, D., R. Ferrara, P. Bodénez, E. Gonzalez, A. Albores, M. Manno, and F. Berthou. 1999. Chlorzoxazone, a specific probe for phenotyping cytochrome P450 2E1 (CYP2E1) in humans. *Pharmacogenetics.* **9:** 377–388.
35. Hardwick, J. P., B. J. Song, E. Hubertman, and F. J. Gonzalez. 1987. Isolation, complementary DNA sequence, and regulation of rat hepatic lauric acid omega-hydroxylase (cytochrome P-450 LA omega). Identification of a new cytochrome P-450 gene family. *J. Biol. Chem.* **262:** 801–810.
36. Hardwick, J. P. 1991. CYP4A subfamily: Functional analysis by immunohistochemistry and *in situ* hybridization. *Methods Enzymol.* **26:** 273–283.
37. Palmer, C. N. A., T. H. Richardson, K. J. Griffin, M. H. Hsu, A. S. Muerhoff, J. E.. Clark, and E. F. Johnson. 1993. Characterization of a cDNA encoding a human kidney cytochrome P-450 4A fatty acid ω-hydroxylation and the cognate enzyme expressed in *Escherichia coli*. *Biochim. Biophys. Acta.* **1172:** 161–166.
38. Weissbart, D., J. P. Salaün, F. Durst, P. Pflieger, and C. Miokowski. 1992. Regioselectivity of a plant lauric acid omega hydroxylase. Omega hydroxylation of cis and trans unsaturated lauric acid analogs and epoxygeneration of the terminal olefin by plant cytochrome P-450. *Biochim. Biophys. Acta.* **1124:** 135–142.
39. Alterman, M. A., C. S. Chaurasia, P. Lu, J. P. Hardwick, and R. P. Hanzlik. 1995. Fatty acid discrimination and omega-hydroxylation by cytochrome P450 4A1 and a cytochrome P450 4A1/NADPH-P450 reductase fusion protein. *Arch. Biochem. Biophys.* **320:** 289–296.