Cytochrome P450 4A4: Expression in *Escherichia coli*, Purification, and Characterization of Catalytic Properties†

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ABSTRACT: Rabbit lung prostaglandin ω-hydroxylase (P450 4A4) was expressed in *Escherichia coli* using the isopropyl β-D-thiogalactopyranoside (IPTG) inducible expression vector pCWori+, containing the full-length cDNA encoding the P450 4A4. The first seven codons were changed to reflect *E. coli* codon bias [a modification of the method of Barnes et al. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 5597–5601]; only the second residue of P450 4A4 was altered (Ser to Ala), while the remaining mutations were silent. This strategy was adopted in order to minimize changes in the structure of the expressed enzyme. Induction by IPTG of the apoprotein peaked after 6 h, and by including the heme precursor δ-aminolevulinic acid, enzymatic activity peaked 12 h after addition of IPTG. The isolated membrane fraction, free of cell debris, contained 12–15 nmol of P450/L of media. The expressed enzyme was purified to electrophoretic homogeneity, and kinetic and spectrophotometric data indicate that this expressed, purified enzyme is equivalent to the enzyme purified from rabbit lung. The K_m for PGE_1 was determined to be 3.0 μM, which is the same as that obtained for the enzyme purified from lung [Williams et al. (1984) *J. Biol. Chem. 259*, 14600–14608]. The CO-reduced difference spectrum of purified P450 4A4 exhibited a λ_{max} at 450 nm, and the absolute absorbance spectrum of the pyridine hemochromogen revealed a typical *b* type heme. To characterize P450 4A4 further, the catalytic activities with prostaglandin E_1 (PGE_1), arachidonate, 15-hydroxyeicosatetraenoic acid (15-HETE), and palmitate were investigated. PGE_1 and arachidonate ω-hydroxylation activities were highly dependent on the concentration of NADPH–cytochrome P450 oxidoreductase, with maximal activities being achieved at a 10–20-fold excess of reductase. Interestingly, activities with arachidonate, palmitate, and 15-HETE, but not with PGE_1, were found to be highly dependent on the amount of L-α-dilauroylphosphatidylcholine (DLPC) in the reaction mixtures. Using optimal amounts of DLPC, initial velocity kinetic experiments were performed. Surprisingly, in spite of structural dissimilarity among these substrates, the K_m values did not differ significantly. Cytochrome b_5 had basically no effect on the K_m values but doubled V_{max} values for PGE_1, palmitate, and 15-HETE and tripled the V_{max} for arachidonate. The V_{max} for arachidonate was found to be the highest, 37 pmol min^{-1} $pmol^{-1}$, and for PGE_1 the lowest, 8.4 pmol min^{-1} $pmol^{-1}$, determined in the presence of cytochrome b_5. The V_{max}/K_m values were determined to be 22, 10, 6.1, and 2.8 for arachidonate, palmitate, 15-HETE, and PGE_1, respectively, in the presence of cytochrome b_5. These results demonstrate that P450 4A4 utilizes arachidonate efficiently and suggest that this enzyme is contributing to the physiological ω-hydroxylation of arachidonate.

Cytochromes P450 of the 4A family catalyze the ω-hydroxylation of a variety of fatty acids, prostaglandins (PGs),[1] and eicosanoids. The catalysis of prostaglandin ω-hydroxylation was first demonstrated in lung microsomes of pregnant or progesterone-treated rabbits and was shown to be CO-inhibitable (Powell & Solomon, 1978; Powell, 1978). Williams et al. (1984) purified cytochrome P450 4A4 from lungs of pregnant rabbits, and Yamamoto et al. (1984) simultaneously purified this enzyme from lungs of progesterone-treated rabbits. A cDNA clone encoding the prostaglandin ω-hydroxylase was isolated from a rabbit lung cDNA library, classified as a member of the P450 4A gene subfamily (Matsubara et al., 1987), and later designated as P450 4A4 by the Nebert et al. (1991) nomenclature system. Interestingly, rabbit kidney microsomes catalyze the ω- and (ω–1)-hydroxylation of various fatty acids but have only negligible activity with PGE_1. This suggested that rabbit kidney expresses forms of the P450 4A family that are distinct from P450 4A4. From the kidney cortex of a bis(2-ethylhexyl) phthalate (DEHP) treated rabbit, two P450s (now known to be P450 4A6 and 4A7) were purified, and it was suggested from the N-terminal amino acid sequence (Kusunose et al., 1989) that these two enzymes were new forms of the P450 4A family. Within the year, three distinct cDNA clones were isolated from a rabbit kidney cDNA library by Johnson's and Masters' laboratories (Johnson et al., 1990) and were confirmed to be members of the P450 4A gene subfamily but distinct from P450 4A4. These clones were designated by Nebert et al. (1991) as P450 4A5, 4A6, and 4A7 (Yokotani et al., 1989, 1991; Johnson et al., 1990).

Although these enzymes exhibit 85% sequence identity according to the amino acid sequences deduced from these cDNA clones, it was shown that each enzyme revealed a unique, but overlapping, substrate specificity by means of the expression of the cDNA clones encoding the P450 4A4, 4A5, 4A6, and 4A7 in COS-1 cells (Johnson et al., 1990; Roman et al., 1990, 1991, 1992, 1993). In this expression system P450 4A5, 4A6, and 4A7 catalyze preferentially the ω-hy-

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[1] Abbreviations: PGs, prostaglandins; PGE_1, prostaglandin E_1; 15-HETE, 15-hydroxyeicosatetraenoic acid; 20-HETE, 20-hydroxyeicosatetraenoic acid; DLPC, L-α-dilauroylphosphatidylcholine or dilauroyl-L-α-lecithin; IPTG, isopropyl β-D-thiogalactopyranoside; δ-ALA, δ-aminolevulinic acid; PMSF, phenylmethanesulfonyl fluoride.

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droxylation and medium-chain fatty acids (laurate), and P450 4A6 and 4A7 also ω-hydroxylate arachidonate. P450 4A4, expressed in COS-1 cells, catalyzes preferentially the ω-hydroxylation of 20-carbon fatty acids (arachidonate) and prostanoids (PGs) but not laurate. In particular, the capacity for arachidonate ω-hydroxylation is intriguing from the physiological point of view since it has been reported recently that ω-hydroxyarachidonate [20-hydroxyeicosatetraenoic acid (20-HETE)] is involved in hemodynamic functions (Escalante et al., 1989; Ma et al., 1993). On the other hand, there is also uncertainty as to which of the kidney cytochrome P450 ω-hydroxylases catalyzes the formation of 20-HETE physiologically and, indeed, in which segment(s) of nephron and in which cell type(s) these reactions occur. Omata et al. (1992) have microdissected the rat nephron and identified the proximal convoluted tubular segments as having arachidonate ω-hydroxylase activity, but the localization of the individual cytochromes P450 has not been performed.

Expression in COS-1 cell is a powerful technique; however, due to the transient and low level of expression of the enzymes, the results obtained by this technique are qualitative and do not yield reliable kinetic results. In order to characterize P450 4A4 further and, specifically, to determine which substrate is more efficiently hydroxylated by P450 4A4, it is essential to obtain sufficient quantities of purified enzyme for kinetic experiments. *Escherichia coli* is attractive as an alternative expression system owing to its ability to express high levels of foreign proteins.

Herein we report the modification of the cDNA encoding P450 4A4, expression in *E. coli*, and purification of the expressed enzyme to homogeneity. Using purified P450 4A4, the optimal reconstitution conditions were determined, and initial velocity kinetic studies were performed with PGE_1, arachidonate, 15-HETE, and palmitate as substrates. These kinetic experiments demonstrate that P450 4A4 is catalytically efficient in arachidonate ω-hydroxylation. The physiological significance of this enzyme and the nature and mechanism of its hormonal regulation by steroids remain to be determined.

MATERIALS AND METHODS

Expression Plasmid Construction. The plasmid pCWori+ (Genger & Dahlquist, 1991) was used to express a modified prostaglandin ω-hydroxylase. A cDNA encoding a full-length P450 4A4, previously cloned in Johnson's and Masters' laboratories (Roman et al., 1990, 1991, 1992, 1993), was mutated by means of polymerase chain reaction mutagenesis (Higuchi et al., 1988). The first seven codons were modified from 5′-ATG AGC GTG TCT GCG CTG AGC-3′ to 5′-ATG GCT GTT TCT GCT TTA AGT-3′; only the second amino acid residue of P450 4A4 is altered (Ser to Ala). Following subcloning and DNA sequencing of the amplification product, the expression plasmid was constructed by ligation of *Nde*I/*Hin*dIII-cleaved vector DNA with the subcloned fragment.

Bacterial Expression and Cellular Fractionation. JM109 cells, transformed with the P450 4A4 expression plasmid, were grown to an OD_{550} of 0.8–1.0 in terrific broth (TB) containing 50 μg/mL ampicillin at 37 °C. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5–1.0 mM. If δ-aminolevulinic acid (δ-ALA) was added, this was done 30 min before addition of IPTG. At various times after addition of IPTG, the cells were harvested. Following lysozyme treatment and sonication, the cell debris and unbroken cells were removed by low-speed centrifugation after which the supernatant was centrifuged at 100000*g*, leaving a pellet designated as the membrane fraction. With preparations used for the purification of the P450 4A4, cells were incubated first with 75 mg/L δ-ALA for 30 min and then with IPTG for 12 h before harvesting.

Solubilization and Purification of P450 4A4. The membrane fraction was suspended in 20 mM potassium phosphate buffer (pH 7.4), 20% glycerol, 1.0 mM DTT, 1.0 mM EDTA, and 0.1 mM phenylmethanesulfonyl fluoride (PMSF) with a protein concentration of 8.0–10 mg/mL. Solubilization was effected with 1.5% Triton X-100 and 0.1% cholate at 4 °C for 1 h. After centrifugation at 100000*g*, the supernatant was applied to a DE-52 column (1.7 × 8.0 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.4), 20% glycerol, 1.0% Triton X-100, 1.0 mM DTT, 1.0 mM EDTA, and 0.1 mM PMSF. The enzyme did not bind to the column and was eluted with the equilibration buffer. Active fractions were pooled, and the pH was adjusted to 6.7. Following dialysis against 3.0 mM potassium phosphate buffer (pH 6.7), 20% glycerol, 0.1 mM DTT, and 0.1 mM EDTA, this sample was applied to a CM-Sepharose column (1.0 × 2.5 cm). The column was washed with 3.0 mM potassium phosphate buffer (pH 6.7), 20% glycerol, 0.05% cholate, 0.1 mM DTT, and 0.1 mM EDTA, and the P450 4A4 was eluted subsequently with 50 mM potassium phosphate buffer (pH 7.4), 1.0 M NaCl, 20% glycerol, 0.05% cholate, 0.1 mM DTT, and 0.1 mM EDTA. The purified P450 4A4 was stored at −80 °C.

Purification of NADPH–Cytochrome P450 Oxidoreductase. Pig liver NADPH-cytochrome P450 oxidoreductase was purified as described by Yasukochi and Masters (1976) using 2′,5′-ADP–Sepharose affinity chromatography. A modified procedure was used to purify the reductase expressed in *E. coli* containing the cDNA for rat reductase (Shen et al., 1989) in the expression plasmid vector pIN-III-ompA3 (Ghrayeb et al., 1984). According to the original procedure (Yasukochi & Masters, 1976), the reductase is eluted with 2′-AMP. For the reductase expressed in *E. coli*, however, we used $NADP^+$ for the elution. The reductase eluted with $NADP^+$ was applied to a DE-52 column which was then wahsed with buffer containing 0.1% cholate until the absorbances at 260 and 280 nm of the eluate were negligibly low. Reductase was eluted with buffer containing 0.1% cholate and 0.5 M NaCl, and this active fraction was dialyzed against 50 mM potassium phosphate buffer (pH 7.4), 0.1 mM EDTA, and 0.1 mM DTT. The flavin content was determined using the extinction coefficient $\epsilon_{454nm} = 10.8\ mM^{-1}\ cm^{-1}$, subtracting absorbance at 700 nm.

Purification of Other Enzymes. Rabbit lung P450 4A4 was purified as described by Williams et al. (1984) as modified by Muerhoff et al. (1990). Pig liver cytochrome b_5 was purified as described by Strittmatter et al. (1978).

Enzyme Assays. The ω-hydroxylations of various substrates were assayed by procedures described by Williams et al. (1984), Muerhoff et al. (1990), and Okita et al. (1991) with some modifications. In brief, the reaction mixture contained 50 mM potassium phosphate (pH 7.4), 0.5 mM EDTA, 2.5 mM $MgCl_2$, 20 mM isocitrate, 48–60 milliunits/mL isocitrate dehydrogenase, and 1.0 mM NADPH in 0.2 or 1.0 mL. The amounts of P450 4A4, pig liver NADPH–cytochrome P450 oxidoreductase or *E. coli* expressed, purified NADPH–cytochrome P450 reductase, pig liver cytochrome b_5, and L-α-dilauroylphosphatidylcholine (DLPC) are indicated in the figure legends. Variations in the amounts of DLPC, reductase, cytochrome b_5, and P450 were determined empirically as described in Results and in the figure legends. The reactions

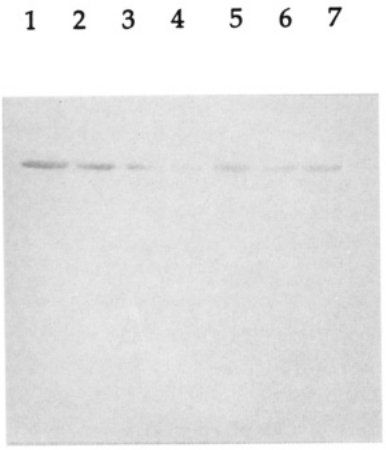

FIGURE 1: Induction of P450 4A4 by IPTG: Immunoblot analysis of membrane fractions. Lanes 1–4 contained 3.0, 1.4, 0.7, and 0.4 pmol of purified P450 4A4, respectively, from rabbit lung; lanes 5–7 contained 10 μg of protein of *E. coli* membrane fractions that were prepared after 30, 18, and 6 h of culture with 1.0 mM IPTG, respectively.

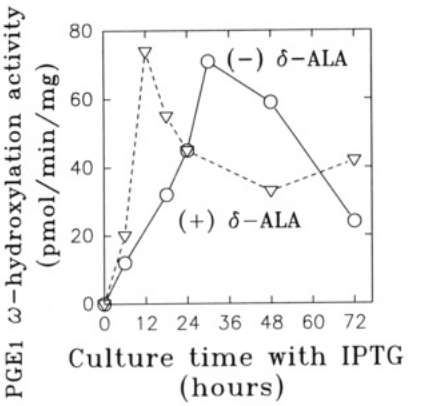

FIGURE 2: PGE_1 ω-hydroxylation activities of membrane fractions. Membrane fractions were prepared at various times after addition of 1.0 mM IPTG without δ-ALA (open circles) and with 75 mg of δ-ALA/L of culture media (open triangles). PGE_1 ω-hydroxylation activities were assayed in a 1.0-mL reaction mixture containing 1.0 mg of protein of the membrane fraction, 26.5 pmol of NADPH–cytochrome P450 reductase, 30 pmol of cytochrome b_5, and 50 nmol of PGE_1 and the same buffer and NADPH-regenerating system as described in Materials and Methods.

were initiated by the addition of NADPH, when reductase eluted with 2′-AMP was used, or by the addition of a mixture of the isocitrate, isocitrate dehydrogenase, and NADPH, when reductase eluted with $NADP^+$ was used. The conversion of substrate to the ω-hydroxylated product was analyzed as described by Okita et al. (1991) with a Beckman System Gold HPLC equipped with an on-line radioisotope flow detector.

Other Methods. Protein concentration was determined by the method of Lowry et al. (1951) or by the bicinchroninic acid method (Smith et al., 1985) with human serum albumin as the standard. SDS–PAGE was performed as described by Laemmli (1970). Immunoblot analysis was carried out essentially as described by Burnette (1981), sequentially using goat polyclonal antibody to P450 4A4 and a rabbit anti-goat IgG antibody conjugated to alkaline phosphatase. Immunoreactive protein was visualized using 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP) and *p*-nitro blue tetrazolium chloride (NBT) as substrates for alkaline phosphatase. The concentration of cytochrome P450 was determined by the CO-reduced difference spectral method of Omura and Sato (1964) using an extinction coefficient $\epsilon_{450-490nm}$ = 91 mM^{-1} cm^{-1}. The total heme content was determined by the pyridine hemochromogen method reported by Paul et al. (1953), using an extinction coefficient of 34.7 mM^{-1} cm^{-1} at 557 nm (data not shown).

RESULTS

Induction of P450 4A4 in E. coli by IPTG and Effect of δ-ALA. The plasmid pCWori+ (Genger & Dahlquist, 1991; Barnes et al., 1991) was used to express a modified P450 4A4 cDNA (Nishimoto et al., 1992, 1993). The first seven codons were modified (see Materials and Methods) using polymerase chain reaction mutagenesis (Higuchi et al., 1988) to reflect *E. coli* codon bias. Only the second amino acid residue of the cDNA was altered (Ser to Ala) by this process.

Immunoblot analysis of the P450 4A4 expression in *E. coli* demonstrated that the enzyme was localized mainly in the membrane fraction with only a trace amount of protein in the cytosolic fraction (data not shown). The immunoreactive protein comprised approximately 0.6–0.8% of the total protein in the membrane fraction, 12–15 nmol of P450 4A4/L of culture. Expression of the immunoreactive P450 4A4 apoprotein, induced by IPTG, reached a nearly maximal level 6 h after the addition of IPTG, with almost no increase at later times as shown in the Western blot of Figure 1, lanes 5–7. Interestingly, the maximum level of PGE_1 ω-hydroxylase activity lagged behind the peak apoprotein synthesis by about 24 h as shown in Figure 2 (open circles), indicating an insufficient rate of incorporation of heme. In order to increase *de novo* heme synthesis, δ-aminolevulinic acid (δ-ALA) was added, and as shown in Figure 2 (open triangles), it accelerated the maximum synthesis of the active enzyme to 12 h after addition of IPTG. After 12 h, the specific activity of this expressed enzyme decreased.

Table I: Purification of Expressed Cytochrome P450 4A4 from 4 L of *E. coli*[a]

fraction	cytochrome P450 (nmol)	yield (%)
membrane fraction	50–55	100
solubilized membrane	12–15	20–25
DE-52 cellulose	7–10	13–18
CM-Sepharose	5–8	8–13

[a] The P450 content was determined by the CO-reduced difference spectra.

Purification of the E. coli Expressed P450 4A4. A summary showing a range of results obtained with three purifications is given in Table I. Because of the high, inhibitory detergent concentration required for solubilization of the membrane fraction and elution from DE-52, values obtained for enzymatic activity and protein concentration were considered to be inaccurate and are not shown. Several different detergents and combinations of detergents at various concentrations were tested for solubilizing the expressed P450 4A4, and the best result, yielding the highest recovery of active enzyme, was obtained with 1.5% Triton X-100 plus 0.1% cholate. The enzyme was about 50% pure after the DE-52 step. CM-Sepharose chromatography effected final purification as well as removal of Triton X-100. The purified P450 4A4 was electrophoretically homogeneous (data not shown) but had a low specific content of 6.0 nmol of P450/mg of protein (relative to the theoretical content of ~18 nmol/mg).

Spectral Properties of E. coli Expressed, Purified P450 4A4. The absolute absorbance spectrum of the expressed, purified P450 4A4, as well as the dithionite-reduced spectrum, typically showed a low-spin heme iron form (data not shown) which is the same as has been found for the enzyme purified from rabbit lung. The CO-reduced difference spectrum of

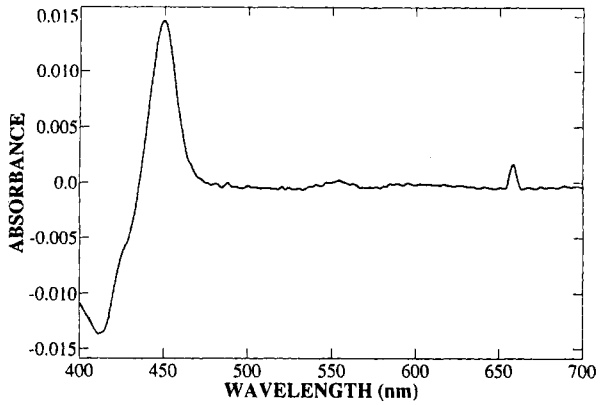

FIGURE 3: CO-reduced difference spectrum of expressed, purified P450 4A4 from *E. coli*. After a few grains of dithionite were added and the sample was divided into reference and sample curvettes and balanced, the CO-reduced difference spectrum was measured 10 min after CO bubbling of the enzyme in the sample curvette. The calculated P450 content was 6.0 nmol/mg.

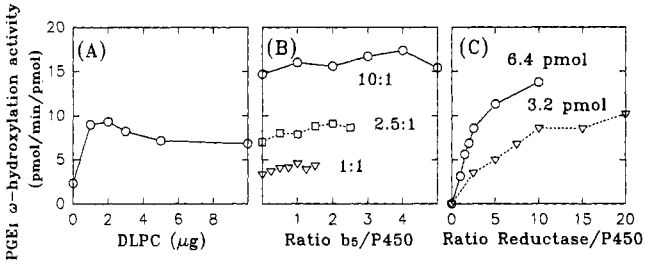

FIGURE 4: Effects of DLPC, cytochrome b_5, and pig liver NADPH–cytochrome P450 oxidoreductase on PGE_1 ω-hydroxylation catalyzed by *E. coli* expressed, purified P450 4A4. Assay conditions: (A) 3.0 pmol of purified P450 4A4, 30 pmol of P450 reductase, 9.0 pmol of cytochrome b_5, 4.0 nmol of PGE_1, and the indicated amounts of DLPC in 200-μL reaction mixtures; (B) 6.4 pmol of P450 4A4 and 64 pmol of reductase (10:1), 16 pmol of reductase (2.5:1), or 6.4 pmol of reductase (1:1), each with 2.0 μg of DLPC, 4.0 nmol of PGE_1, and the indicated amounts of cytochrome b_5 in 200-μL reaction mixtures; (C) 3.2 pmol (- - -) or 6.4 pmol (—) of purified P450 4A4, 2.0 μg of DLPC, 4.0 nmol of PGE_1, and various amounts of reductase in 200-μL reaction mixtures. The data points are averages of two different analyses.

the purified P450 4A4 exhibited a maximum absorbance at 450 nm as shown in Figure 3. The spectrum of the pyridine hemochromogen of this purified enzyme showed a typical peak of *b*-type heme (data not shown). The total heme content of this sample was determined to be 12.5 nmol/mg by means of the pyridine hemochromogen spectrum, but 6.0 nmol/mg by the CO-reduced difference spectrum. The higher heme content as determined by the pyridine hemochromogen method has been found with two different preparations of enzyme and suggests the presence of improperly or adventitiously bound heme. Therefore, the P450 content determined by the CO-reduced difference spectrum was used for all calculations of enzyme activity.

Dependence of PGE_1 ω-Hydroxylation Activity on Phospholipid and NADPH–Cytochrome P450 Reductase and Effect of Cytochrome b_5. To characterize further the expressed, purified P450 4A4, the effects of varying amounts of DLPC, cytochrome b_5, and P450 reductase on the ω-hydroxylation of PGE_1 were studied. As shown in Figure 4A, 1–2 μg of DLPC in a 200-μL reaction mixture gave the highest activity, and 2 μg was used for all other experiments.

The PGE_1 ω-hydroxylation activity catalyzed by P450 4A4 was not stimulated significantly by cytochrome b_5 regardless of the amount of reductase in the reaction mixture (Figure

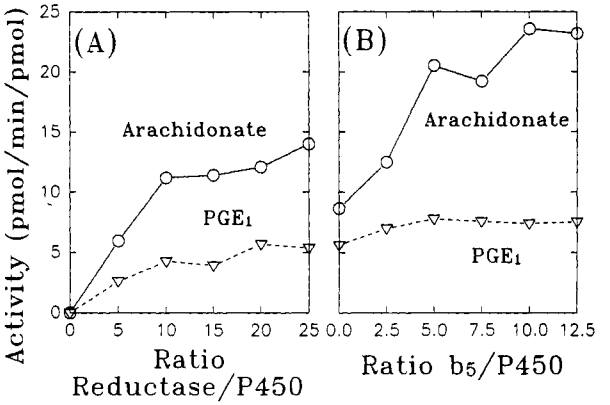

FIGURE 5: Effect of (A) *E. coli* expressed, purified NADPH–cytochrome P450 reductase and (B) cytochrome b_5 on PGE_1 (- - -) and arachidonate hydroxylation activities (—) catalyzed by P450 4A4. Assay conditions: (A) 1.0 pmol of P450 4A4, 2.0 nmol of substrate, 2.0 μg of DLPC for PGE_1 or 1.0 μg of DLPC for arachidonate, and the indicated amounts of reductase in 200-μL reaction mixtures; (B) 1.0 pmol of P450 4A4, 20 pmol of reductase, 4.0 nmol of substrate, 2.0 μg of DLPC, and the indicated amounts of cytochrome b_5 in 200-μL reaction mixtures. The data points are averages of two different analyses.

4B). Shown in Figure 4B is the titration with cytochrome b_5 at three different ratios of reductase to P450 4A4: 1:1, 2.5:1, and 10:1.

The molar ratio of cytochrome P450 reductase to the P450 4A4 was varied using two different amounts of purified P450 4A4, 16 nM (3.2 pmol) and 32 nM (6.4 pmol). As shown in Figure 4C, PGE_1 ω-hydroxylase activity increased with increasing reductase. Furthermore, the turnover number, expressed as picomoles per minute per picomole of P450, increased with the higher amount of P450 4A4 (see Discussion).

Dependence of Arachidonate ω-Hydroxylation Activity on NADPH–Cytochrome P450 Reductase and Cytochrome b_5. As shown in Figure 5A, arachidonate ω-hydroxylation activity catalyzed by P450 4A4 was also highly dependent on the amount of reductase in this reconstituted system as found with PGE_1 ω-hydroxylation activity. Nearly maximal activities were obtained at a 10–20-fold excess of reductase over P450 4A4 in 200-μL reaction mixtures containing 1.0 pmol of P450 4A4, 2.0 nmol of substrate, and 2.0 μg of DLPC for PGE_1 or 1.0 μg of DLPC for arachidonate. For the remaining experiments reported herein, a 20-fold excess of reductase was utilized.

In contrast to the slight stimulatory effect of cytochrome b_5 on PGE_1 ω-hydroxylation activity (Figures 4B and 5B, open triangles), the arachidonate ω-hydroxylation activity was stimulated nearly 3-fold by cytochrome b_5 as shown in Figure 5B (open circles) under conditions similar to those used for PGE_1, namely, a 20-fold excess of reductase to P450 4A4, 2.0 μg of DLPC, and 4.0 nmol of substrate. This stimulation of arachidonate ω-hydroxylation does not depend upon the molar ratio of reductase to P450 4A4; in the presence of 2.5-fold excess of reductase to P450 4A4, cytochrome b_5 gave the same effect (data not shown).

Effect of DLPC on Initial Velocity Kinetics with Arachidonate. The P450 4A4 activity with PGE_1 does not vary significantly with concentrations of DLPC ranging from 1 to 10 μg as described above (Figure 4A). In contrast, we found that arachidonate ω-hydroxylation activity is highly dependent on the DLPC amount. As shown in Figure 6, using three different amounts of DLPC, 2, 5, and 10 μg, the results of initial velocity kinetics of arachidonate ω-hydroxylation were

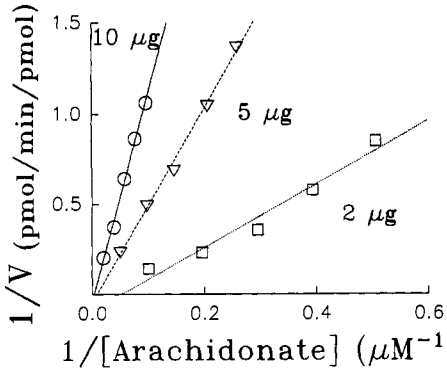

FIGURE 6: Effect of the amount of DLPC on initial velocity kinetic studies of P450 4A4 mediated arachidonate hydroxylation activity. The data are presented as double-reciprocal plots and the lines determined by linear regression. Assay conditions: 3.0 pmol of P450 4A4, 60 pmol of pig liver reductase, various amounts of arachidonate, and 2, 5, or 10 μg of DLPC in 200-μL reaction mixtures.

Table II: Amounts of DLPC in the Reaction Mixture for Initial Velocity Kinetic Experiments[a]

arachidonate							
(−) b_5		(+) b_5		palmitate, (−/+) b_5		15-HETE, (−/+) b_5	
substrate (nmol)	DLPC (μg)	substrate (nmol)	DLPC (μg)	substrate (nmol)	DLPC (μg)	substrate (nmol)	DLPC (μg)
1.1	0.85	1.5	1.0	1.9	1.2	1.0	0.80
0.81	0.70	1.0	0.80	1.3	1.0	0.66	0.65
0.59	0.60	0.60	0.60	0.96	0.85	0.52	0.55
0.40	0.50	0.50	0.50	0.73	0.70	0.42	0.50
0.30	0.45	0.45	0.45	0.61	0.60	0.35	0.45

[a] Substrate amount (nmol) and DLPC amount (μg) in 200-μL reaction mixtures are indicated in left and right columns, respectively. The concentrations of other components are described in Materials and Methods and the legend of Figure 7. These amounts of DLPC were used for the kinetic data shown in Figure 7 and Table III.

drastically affected by DLPC. Furthermore, the results of the linear regression of the data are not interpretable as the lines do not intersect the appropriate axes. Eadie–Hofstee and Hanes plots of these data were likewise uninterpretable. In a series of studies with arachidonate, palmitate, and 15-HETE, we discovered that the DLPC amount must be varied as the substrate amount is varied in order to obtain optimal activities. To obtain an estimate of the appropriate amount of DLPC to use in initial velocity kinetic studies, experiments were performed with two different concentrations of substrate, a high and low concentration, and the amount of DLPC was varied for each concentration of substrate. With 1.5 and 0.3 nmol of arachidonate, the optimal amounts of DLPC were 1.0 and 0.45 μg, respectively, in a 200-μL reaction mixture. These values were plotted as nanomoles of arachidonate versus optimal micrograms of DLPC, and a line was drawn between these two points. For each of the other substrate concentrations used in initial velocity kinetic studies, the "optimal" amount of DLPC was extrapolated from the above described line. While the assumption of linearity may not be accurate, reasonable initial velocity kinetic data were obtained as shown below. The actual amounts of DLPC in the reaction mixtures used for initial velocity experiments are shown in Table II.

Initial Velocity Kinetic Studies. Using appropriate amounts of DLPC (see Table II), initial velocity kinetic experiments were performed with arachidonate, PGE_1, 15-HETE, and palmitate in the absence and presence of cytochrome b_5, and the results are shown for arachidonate in the Hanes plot of Figure 7. The K_m and V_{max} values for arachidonate, as well as for each of the other substrates, are in Table III.

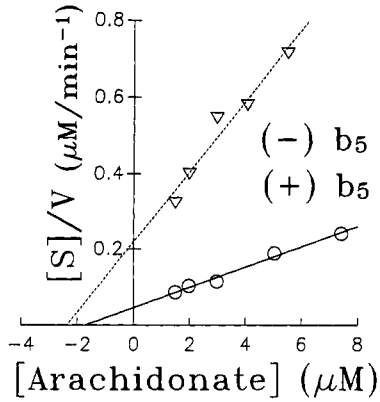

FIGURE 7: Initial velocity kinetics of P450 4A4 with arachidonate. The data are presented as Hanes plots and the lines determined by linear regression. Assay conditions: 1.0 pmol of P450 4A4, 20 pmol of *E. coli* expressed, purified reductase, 10 pmol of cytochrome b_5 (when added), and various amounts of DLPC as given in Table II in 200-μL reaction mixtures.

Table III: Initial Velocity Kinetic Constants with Various Substrates[a]

substrate	b_5	V_{max} (pmol min⁻¹ pmol⁻¹)	K_m (μM)	V_{max}/K_m
PGE_1	(−)	4.0	2.7	1.5
	(+)	8.4	3.0	2.8
15-HETE	(−)	8.6	2.2	3.9
	(+)	15	2.5	6.1
palmitate	(−)	18	3.6	4.9
	(+)	36	3.6	10
arachidonate	(−)	11	2.4	4.6
	(+)	37	1.7	22

[a] The V_{max} and K_m values were determined by linear regression of Hanes plots.

Surprisingly, in spite of the structural dissimilarity among these four types of fatty acids, the K_m values for these substrates are very similar and are essentially the same in the presence or absence of cytochrome b_5. However, the V_{max} values were markedly increased upon the inclusion of cytochrome b_5, 3-fold with arachidonate and 2-fold with the other substrates. The catalytic efficiency, V_{max}/K_m, values shown in Table III indicate that arachidonate is the most efficient substrate for P450 4A4.

There was no activity with laurate as a substrate, with a detection limit of 0.05 pmol min^{-1} $pmol^{-1}$, 2 orders of magnitude less than the activities obtained with the other substrates. Studies varying the type of detergent and concentration were performed, and no condition employed resulted in the hydroxylation of laurate.

DISCUSSION

Although the capability of liver and kidney cortex to ω-oxidize medium-chain-length fatty acids has been known for over 30 years (Robbins, 1961), the characterization of the enzyme systems involved has been performed only recently. This fact is in spite of the successful reconstitution of a cytochrome P450 mediated ω-hydroxylation system by Lu and Coon in 1968. Studies by Orrenius and co-workers (Jakobsson et al., 1970; Ellin et al., 1972, 1973) presented no compelling evidence for the involvement of distinct cytochromes P450 in the ω- vs (ω − 1)-hydroxylation of lauric acid. However, later experiments in our laboratory by Okita et al. (1981) not only suggested that distinct cytochromes P450 were involved in these regioselective activities but also showed that the prostanoids, PGE_1 and PGA_1, could be

substrates for these enzymes in porcine kidney microsomal fractions. Following the report of Powell and Solomon (1978) and Powell (1978) that a gestational age dependent ω-hydroxylation, inhibitable by carbon monoxide, of prostaglandins and thromboxane B_2 occurs in the lungs of pregnant rabbits, our laboratory (Williams et al., 1984) and that of Kusunose (Yamamoto et al., 1984) reported the isolation and purification of a cytochrome P450 from lung microsomes capable of catalyzing the ω-hydroxylation of prostaglandins E_1 and A_1. Subsequent studies from both laboratories have led to the cloning and sequencing of this cytochrome P450 classified as a member of the cytochrome P450 4A gene subfamily and designated as *CYP4A4* (Matsubara et al., 1987; Roman et al., 1990, 1993) and of three distinct cytochromes P450 in kidney designated as *CYP4A5*, *CYP4A6*, and *CYP4A7* (Yokotani et al., 1989, 1991; Johnson et al., 1990). Members of the *CYP4A* gene subfamily have been expressed in African green monkey kidney cells (COS-1) through the collaborative efforts of the Johnson's and Masters' laboratories. These experiments have permitted the determination of substrate specificities among these three expressed kidney P450 clones, 4A5, 4A6, and 4A7, and of the expressed prostaglandin ω-hydroxylase, P450 4A4 (Johnson et al., 1990; Roman et al., 1990, 1991, 1992, 1993; Masters et al., 1991, 1993), since COS-1 cells do not catalyze these activities in the absence of the transfected cDNAs.

Only in the last two years have mammalian cytochromes P450 been expressed in *E. coli*. P450 2E1 (Larson et al., 1991), P450 17 (Barnes et al., 1991), and P450 11A1 (Wada et al., 1991) were expressed in *E. coli* as membrane-bound forms using the expression plasmid vectors pKKHC, pCWori+, and pTrc99A, respectively. Especially in the case of the P450 17, the first seven codons were changed to optimize the bacterial translation efficiency. Although these changes resulted in a modification of only the second amino acid residue, with the remaining mutations being silent, the level of expressed enzyme dramatically increased. P450 1A2 (Fisher et al., 1992) and P450 2C3 (Richardson et al., 1993) also have been expressed in *E. coli* using the pCWori+ vector. In both cases, the cDNA that encodes the N-terminal eight amino acid residues (P450 1A2) or nine amino acid residues (P450 2C3) was replaced with a modified sequence derived from P450 17 (Barnes et al., 1991), and the enzymes were expressed as chimeric proteins.

In designing the construct utilized for expression of P450 4A4 in *E. coli* in the current studies, the assumption was made, based on the comparison of limited kinetic studies, that N-terminal modification (Wada et al., 1991; Barnes et al., 1991; Fisher et al., 1992; Richardson et al., 1993) or even the absence of portions of the N-terminus (Sakaki et al., 1985; Larson et al., 1991) would have no effect on enzymatic properties of these cytochromes P450. In fact, these modifications did not cause significant changes in K_m values (Li & Chiang, 1991; Richardson et al., 1993). However, in the absence of data directly comparing recombinant, expressed forms containing full-length and truncated N-termini, the potential effects of mutations of the N-terminus on enzymatic properties need to be considered.

The strategy utilized in the present study involved biasing the nucleotides in the initial seven codons for expression of rabbit microsomal P450 4A4, resulting in only a single amino acid change from Ser to Ala in the second position (see Materials and Methods) which increases translation efficiency in *E. coli*. This approach was taken in order to preserve the original structure of the P450 so as not to perturb its structure–function characteristics. In making these modifications, a number of silent mutations were introduced, and it was necessary to construct a *Hin*dIII restriction endonuclease site at the 3′ end for ligation into the vector. This strategy resulted in the expression of P450 4A4 in *E. coli*, thus permitting its purification to electrophoretic homogeneity and its kinetic characterization. Immunoblot analysis of the whole cells and membrane fractions revealed that the majority of P450 4A4 expressed, subsequent to IPTG induction, was associated with the membrane fraction (data not shown) and represented 0.6–0.8% of the total protein in the fraction (Figure 1). The determination of PGE_1 ω-hydroxylation activity revealed that maximal activity was achieved after 30–36 h of induction with IPTG (Figure 2), although the immunodetectable P450 4A4 had reached a maximal level within 6 h post-IPTG (Figure 1), suggesting limiting quantities of heme in the cells. This was verified by the addition of δ-ALA, which accelerated the appearance of maximal enzyme activity to 12 h. The lower amount of CO-binding heme (~6 nmol/mg) compared to the amount of heme measured by the pyridine hemochromogen technique (~12 nmol/mg) suggested that up to 50% of the heme was incorporated into apoprotein in a nonproductive manner. All estimates of enzyme activity were made on the basis of CO-binding heme content. The absolute absorbance spectrum (data not shown) was typical of a low-spin heme, as was the case for the P450 4A4 purified from lungs of pregnant rabbits (Williams et al., 1984), and there was no spectral shift upon reduction by dithionite, thereby precluding contamination by any of the major *E. coli* cytochromes, e.g., cytochrome b_{556} (Kita et al., 1978), cytochrome b_{562-o} (Kita et al., 1984a), and cytochrome b_{558-d} (Kita et al., 1984b), which exhibit a red shift upon reduction. The lack of a Soret absorbance maximum at 401 nm (Murphy et al., 1973) in the pyridine hemochromogen specturm (not shown) precludes the presence of siroheme.

Due to the difference in electronic and steric features of the various substrates of cytochrome P450 4A4 tested, namely, PGE_1, 15-HETE, palmitate, and arachidonate, it was important to establish the conditions for reconstitution, especially regarding the requirements for phospholipid and cytochrome b_5. In these experiments, the phospholipid, DLPC, was utilized in the reaction medium as the lipid component. As shown in Figure 4A, the optimal concentration of DLPC for reconstitution of PGE_1 ω-hydroxylation was 1–2 μg in the 200-μL reaction mixture. In all subsequent studies, 2 μg of DLPC was utilized for reconstitution of PGE_1 ω-hydroxylation activity. When the cytochrome b_5 concentration (Figure 4B) was varied with PGE_1 as substrate, no dramatic increase in turnover number was obtained when reductase and cytochrome P450 4A4 were maintained at constant levels, in contrast to previous studies (Williams et al., 1984) in which a 4-fold stimulation upon the addition of cytochrome b_5 was observed. It can only be assumed that differences in assay conditions are responsible for the disparate results. In the same set of experiments, however, there was a dramatic increase in turnover number as the reductase:P450 4A4 ratio was increased (Figure 4B,C). The data are tantalizing in that they indicate that reductase is rate-limiting for formation of a catalytically competent reductase–P450 complex under the conditions of these measurements and that the addition of cytochrome b_5 does not alleviate the dependence upon reductase. In addition, at any ratio of reductase to cytochrome P450, the addition of more P450 resulted in an increase in activity per picomole of cytochrome P450 (Figure 4C). Miwa et al. (1979) have proposed that the equilibrium between

microsomal cytochrome P450 and NADPH–cytochrome P450 reductase determines the rate of a monooxygenase reaction. Our current findings are also of interest in explaining the apparent discrepancies between the data of Williams et al. (1984) and Yamamoto et al. (1984) in the rates of PGE_1 and PGA_1 ω-hydroxylation catalyzed by equivalent forms of P450 (P450 4A4) in purified, reconstituted systems, i.e., that optimal conditions were not realized in all cases with each of the substrates tested. With P450 4A4 purified from rabbit lung, we obtain results identical to those reported herein for recombinant, expressed P450 4A4 using optimized assay conditions (data not shown). The optimal levels of DLPC vary somewhat with each substrate, suggesting that their relative hydrophobicities and structural characteristics influence the catalytic efficiency. The catalytic efficiencies (V_{max}/K_m), derived from these data and shown in Table III, are PGE_1 = 2.8, 15-HETE = 6.1, palmitate = 11, and arachidonate = 22, determined in the presence of cytochrome b_5 under the same reconstitution conditions. These data are surprising in view of the fact that this enzyme was originally isolated as a prostaglandin ω-hydroxylase from lung microsomes and serve to emphasize the importance of determining the kinetic parameters under optimal conditions and expressing the results as catalytic efficiencies, i.e., V_{max}/K_m. Such detailed kinetic results have been pursued to a limited degree with other cytochromes P450, expressed and purified to homogeneity, with the result that the most catalytically efficient substrate is determined among a group of candidates. The candidate substrates, in this case, have been chosen on the basis of physiological stimulation of eicosanoid ω-hydroxylation, particularly by steroids and glucocorticoids, and/or by their efficacy as a substrate for the purified P450 4A4. The search for the "physiological substrate" is at best a difficult one, but clues can be followed as to regulatory stimuli and effects.

In the current studies, it is of interest for physiological reasons that arachidonic acid is an excellent substrate for P450 4A4. Pursuant to this observation, the hydroxylation of arachidonate was measured with varying concentrations of cytochrome b_5, which was found to stimulate this hydroxylation activity by over 3-fold. Analysis of the kinetics reveals a K_m of 1.7 μM and a V_{max} of 37 min^{-1} for arachidonate, values which indicate that the P450 4A4 contributes to the physiological metabolism of arachidonate. Also, the formation of 15-HETE in mucus-secreting cells of the lung, increased in the bronchial tree in asthmatics, suggests that significant levels could be reached to provide substrate for P450-mediated ω-hydroxylation catalyzed by P450 4A4 (Okita et al., 1987).

It is apparent that careful examination of assay conditions has been important in determining the "natural" substrate for P450 4A4, but further experimentation is necessary to define the complex interactions among the proteins involved in these monooxygenation reactions in vivo. The availability of cytochromes P450, in the amounts expressed in *E. coli* with appropriate vectors, will aid in the performances of studies leading to the mapping of the topology of the active site.

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