High-level expression in *Escherichia coli* of enzymatically active fusion proteins containing the domains of mammalian cytochromes P450 and NADPH–P450 reductase flavoprotein

(hybrid protein/steroid 17 α -hydroxylation/ ω -oxidation)

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Contributed by Ronald W. Estabrook, August 13, 1992

ABSTRACT This report describes the properties of two mammalian cytochromes P450 that have been expressed at high levels in *Escherichia coli* as enzymatically active fusion proteins containing the flavoprotein domain of rat NADPH–cytochrome P450 reductase (EC 1.6.2.4). Fusion proteins were prepared by engineering the cDNAs for the steroid-metabolizing bovine adrenal P450 17A with the cDNA for rat liver NADPH–P450 reductase with the introduction of a Ser-Thr linker to give a protein we have named rF450[mBov17A/mRatOR]L1. Similarly, the cDNA for the ω -hydroxylase of rat liver (P450 4A1) was linked with the cDNA for rat liver NADPH–P450 reductase to give rF450[mRat4A1/mRatOR]L1. A procedure involving disruption of transformed *E. coli* by sonication, isolation of membranes by differential centrifugation, solubilization with detergent, and affinity chromatography provided significant amounts of purified fusion proteins of $\approx$ 118 kDa. The purified fusion proteins had turnover numbers for the metabolism of steroids (rF450[mBov17A/mRatOR]L1) or fatty acids (rF450[mRat4A1/mRatOR]L1) ranging from 10/min to 30/min in the absence of added phospholipid. Addition of purified rat liver cytochrome b_5 stimulated the 17,20-lyase reaction for the conversion of 17-hydroxypregnenolone to dehydroepiandrosterone, and addition of purified rat NADPH–cytochrome P450 reductase enhanced the formation of ω – 1 metabolites from lauric and arachidonic acids. NADPH oxidation was tightly coupled to substrate hydroxylation with the purified fusion proteins.

Cytochromes P450 (P450s) of mammalian tissues can be categorized into two groups (1) depending on their intracellular compartmentation: P450s associated with the endoplasmic reticulum are one type, and these microsomal P450s require only an NADPH-reactive, FAD- and FMN-containing flavoprotein for the transfer of electrons from NADPH to a P450; P450s associated with mitochondria are a second type, and these mitochondrial P450s function with a mini electron-transfer chain composed of an NADPH-reactive FAD-containing flavoprotein and a P450-reactive iron-sulfur protein. Many different P450s have been used to reconstitute enzymatic activities by incubating a purified P450 with its designated purified electron-transfer partner(s) in the presence of a suitable phospholipid (2).

Miura and Fulco (3) were the first to describe the presence of a P450 as a fusion protein—i.e., a single protein containing both the heme domain of a P450 and the flavoprotein domain equivalent to the microsomal FAD- and FMN-containing NADPH–P450 reductase. They isolated and purified this soluble P450_{BM-3}, named P450 102, from *Bacillus megaterium* and characterized it as an ω -hydroxylase of fatty acids. P450

102 is the most enzymatically active form of any known P450 (turnover number > 1500/min).

Murakami *et al.* (4) genetically engineered the cDNA of rat liver P450c (P450 1A1) with the cDNA of rat NADPH–P450 reductase to construct a P450 fusion protein which they expressed in yeast. Subsequent studies by this group (5) extended their technique to the expression in yeast of fusion proteins of P450s active in steroid metabolism by using the cDNAs of bovine P450 17A or bovine P450 21 and the cDNA of the yeast flavoprotein NADPH–P450 reductase. An enhanced enzymatic activity for the fusion protein containing bovine P450 17A in the hydroxylation of progesterone was reported (6).

Studies of P450s have been greatly facilitated by the successful application of heterologous expression techniques (7). This approach has been particularly useful when applied to P450s engineered for site-specific mutations (8) or for the generation of large quantities of difficult-to-obtain biologically active proteins, such as human P450s (9).

Recently, the high-level expression in *Escherichia coli* of bovine adrenal P450 17A (10) and human liver P450 1A2 (9) has been described. These studies are extended here to the expression, purification, and enzymatic characterization of two fusion proteins containing the functional domains of different microsomal P450s linked to the microsomal flavoprotein domain of NADPH–P450 reductase. We introduce a nomenclature for these fusion proteins, rF450[mBov17A/mRatOR]L1 and rF450[mRat4A1/mRatOR]L1, where r = recombinant; F = fused; 450 = P450; m = modified cDNA; Bov or Rat = species; 17A or 4A1 = family name of P450; OR = NADPH–P450 reductase; and L1 = linker type.

MATERIALS AND METHODS

Plasmids and *E. coli* Strains Used. A construct expressing bovine P450 17A in vector pCWori⁺ (pCWmod17) was obtained from the laboratory of Michael Waterman (10); a 2.1-kilobase (kb) insert in pUC19 containing the cDNA for the open reading frame for rat liver P450 4A1 was obtained from G. Gordon Gibson (11), University of Surrey, U.K.; the cDNA for rat liver NADPH–P450 reductase (pOR263) was obtained from Charles Kasper (12), University of Wisconsin.

Recombinant DNA Manipulations. Initial experiments were directed to the construction by PCR mutagenesis of a plasmid encoding bovine P450 17A fused to rat liver NADPH–P450 reductase. The coding sequence of the amino terminus of bovine P450 17A had been modified (10). Mutagenesis was performed to modify the coding sequence for the carboxyl terminus of bovine P450 17A and the coding sequence for the amino terminus of rat liver NADPH–P450 reductase in a manner similar to that described by Murakami *et al.* (4) in

order to allow fusion of these sequences with one encoding a dipeptide linker, Ser-Thr (Fig. 1). To digest the plasmid (pCWmod17) at the methylated *Stu* I site the plasmid was transformed into the *dcm*⁻ *E. coli* strain GM48 and plasmid DNA was prepared. A 40-base-pair (bp) fragment was deleted by digesting the plasmid with *Stu* I and *Hind*III. Rat liver NADPH-P450 reductase cDNA (pOR263) was amplified by PCR using primers deleting the amino-terminal membrane-anchoring region and incorporating a *Sal* I site encoding Ser-Thr as a linker. The 3' reductase primer incorporated a *Hind*III site after the TAG stop codon. The PCR fragment was subcloned into the *Sma* I site of pTZ19R. The 5' and 3' ends of the amplified sequence were sequenced to the two internal *Nco* I sites. Bovine P450 17A was amplified by PCR with a 3' primer deleting the TGA stop codon and incorporating the same *Sal* I site encoding Ser-Thr as the reductase 5' primer. The PCR product was digested with *Bam*HI and *Sal* I. The *Sal* I-*Hind*III reductase domain was ligated to the *Bam*HI-*Sal* I P450 domain fragment and pTZ19R digested with *Bam*HI and *Hind*III. A clone containing the *Bam*HI-*Hind*III fragment was sequenced from the *Stu* I site to the *Sal* I site. The positive clone was digested with *Nco* I to remove the 1.4-kb internal *Nco* I-*Nco* I fragment from the PCR-generated reductase domain. The 1.4-kb *Nco* I fragment was isolated from the plasmid preparation of the original rat liver reductase cDNA. The *Nco* I fragment was ligated with the *Nco* I-deleted vector construct. A construct with a replaced *Nco* I fragment in the correct orientation was identified and transformed into *E. coli* GM48, and the resulting plasmid preparation was digested with *Stu* I and *Hind*III. The 1.9-kb *Stu* I-*Hind*III fragment was ligated with the original pCWmod17 digested with *Stu* I and *Hind*III. Positive colonies were identified by restriction digestion. All regions constructed by PCR amplification thus were sequenced to ensure the integrity of the amplified sequence. To the degree possible, unmodified regions amplified by PCR were replaced with original plasmid-derived DNA.

The rat P450 4A1-reductase fusion product was prepared by PCR mutagenesis (Fig. 2). A portion of the coding sequence of the amino terminus of rat P450 4A1 was deleted and replaced with a 27-bp fragment from the modified bovine P450 17A (Fig. 2B). The carboxyl terminus was modified (Fig. 2A) by incorporating an *Xho* I site encoding Ser-Thr as a linker replacing the TAA stop codon to allow fusion with the modified DNA sequence of NADPH-P450 reductase as described above. This PCR product was digested with *Nde* I and *Xho* I. The plasmid encoding rF450[mBov17A/mRatOR]L1 described above was digested with *Nde* I and *Sal* I to remove the P450 domain. The digested plasmid containing only the reductase domain was ligated with the *Nde* I-*Xho* I PCR-amplified rat P450 4A1 domain. A construct with the replaced *Nde* I-*Xho* I fragment in the correct orientation was identified by restriction digestion and transformed into *E. coli*. Positive colonies were identified as described above.

Fusion proteins for other P450s, including the human P450s 1A2 and 3A4, were constructed by using a strategy similar to that described for the preparation of the fusion protein of rat

509 57
Bovine P450 17A | | Rat NADPH-P450 OR
GlySerThrProSerThrIleGlnThr
GGTAGCACCCCTCGACTATCCAAACA
Sal I

FIG. 1. DNA sequence and deduced amino acid sequence of the region of fusion between the bovine P450 17A domain and the rat NADPH-cytochrome P450 reductase domain. Numbering refers to the last amino acid of bovine P450 17A (509) and the beginning of rat NADPH-cytochrome P450 reductase (oxidoreductase, OR) (57). The *Sal* I site is underlined.

509 57
A Rat P450 4A1 | | Rat NADPH-P450 OR
LysLysLeuHisSerThrIleGlnThr
GGTAGCACCCCTCGACTATCCAAACA
Sal I *Xho I*
B Bovine P450 17A Rat P450 4A1
Met Ala Leu Leu Leu Ala Val Phe Leu 23 24 25 26 27
ATG GCT CTG TTA TTA GCA GTT TTT CTG **GTT** **CTG** **CTG** **CTG** **GTC**

FIG. 2. Modifications of the amino and carboxyl termini of rat P450 4A1. (A) Region of fusion between the rat P450 4A1 domain and the rat NADPH-cytochrome P450 domain (cf. Fig. 1). The hybrid *Sal* I/*Xho* I site is underlined. (B) Modification of the amino terminus of P450 4A1 and fusion to the 27-bp fragment encoding 9 amino acids of modified bovine P450 17A. Numbers in bold are those of rat P450 4A1. Alignment of the amino-terminal regions of the modified bovine 17A (10) and rat P450 4A1 was based upon the data of Nelson and Strobel (13).

P450 4A1. The properties of these fusion proteins will be described in subsequent publications.

Cell Growth and Harvesting. Growth conditions for the expression of P450 fusion proteins were similar to those described (9, 10). The cells were harvested, washed with 10 mM potassium phosphate buffer, pH 7.5/0.15 M NaCl, suspended in a Dounce homogenizer with 2 volumes (vol/wt) of TSE buffer (75 mM Tris-HCl, pH 7.5/250 mM sucrose/0.25 mM EDTA), and divided into 100-ml aliquots (33 g wet weight) and frozen at -80°C.

Cell Disruption and Preparation of Membranes. Membranes were prepared from 100 ml of thawed cells to which 0.5 ml of 200 mM phenylmethanesulfonyl fluoride was added. Cells were disrupted at 0°C by sonic treatment with a Fisher Scientific 550 Sonic Dismembrator fitted with a 1/4-inch 20-stud (1/2 inch in diameter) tip for a total of 2 min, using a cycle of 20 sec on and 35 sec off at a setting of 6.5. The disrupted cells were diluted with 3 volumes of TGE buffer [75 mM Tris-HCl, pH 7.5/10% (vol/vol) glycerol/0.25 mM EDTA] and centrifuged at 4000 × *g* for 10 min, and the supernatant was centrifuged at 100,000 × *g* for 60 min. The pellet was suspended in 10–15 ml of TGE buffer, divided into aliquots, and stored frozen at -80°C.

Purification of Fusion Proteins. Membranes were thawed and diluted to a protein concentration of about 2.5 mg/ml with 10 mM potassium phosphate buffer, pH 7.5/20% glycerol/0.1 mM dithiothreitol. The first step in the solubilization of the membranes was achieved by the dropwise addition of a 10% (vol/vol) solution of Emulgen 911 (Kao, Chemical Division, Tokyo) to give a final concentration of 1%. The suspension was stirred for 1 hr at 4°C and then centrifuged for 1 hr at 100,000 × *g*. The supernatant, containing the solubilized membrane proteins, was decanted and applied to a Whatman DE-52 column equilibrated with buffer A (50 mM potassium phosphate, pH 7.8/20% glycerol/0.5 mM EDTA/0.1 mM dithiothreitol/0.001 mM FMN/0.2% Emulgen 911). The fusion protein bound to the resin and was eluted with a linear gradient of KCl in buffer A at about 0.2 M KCl. Fractions containing the eluted fusion protein were pooled and applied to an affinity column of adenosine 2',5'-bisphosphate (2',5'-ADP)-Sepharose 4B (14) that had been equilibrated with buffer B [10 mM potassium phosphate, pH 7.5/20% glycerol/0.5 mM EDTA/0.1 mM dithiothreitol/0.001 mM FMN/0.05% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)]. The fusion protein bound tightly to the affinity column and was washed with >5 column volumes of buffer B to remove as much Emulgen 911 as possible. Then the fusion protein was eluted with buffer B supplemented with 2 mM 2'-AMP and 0.25 M KCl. The pooled fractions were concentrated to 5–10 ml in an Amicon concentrator with a PM-10 ultrafiltration membrane and then

dialyzed overnight in the cold against buffer B. The dialyzed sample was applied to an LKB Ultragel AcA 34 column equilibrated with buffer B. The P450 fusion protein was eluted as a single fraction resolved from proteins of lower molecular weight. The fraction was dialyzed overnight against buffer B minus CHAPS, frozen, and stored at -80°C .

Spectrophotometric and HPLC Assay Techniques. The concentration of P450 was determined spectrophotometrically with an Aminco DW2 double-beam spectrophotometer (9). Rates of NADPH-cytochrome *c* reduction were measured at 550 nm (14), and rates of NADPH oxidation were measured at 340 nm. The flavin content of the purified enzyme fractions was determined fluorometrically by using purified FAD and FMN (15). The conditions for measuring metabolism of various substrates are described in the figure legends. Product formation was measured by HPLC analysis with a Waters 380 reversed-phase apparatus coupled to a Radiometer Flow-1 radioactive detector as described (9). Protein concentration was estimated by the Bradford method (16) with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Characterization of the Expressed Protein. Growth of transformed *E. coli* for 72 hr at 27°C in Fernbach flasks results in a level of expression of rF450[mBov17A/mRatOR]L1 of about 700 nmol/liter of growth medium (18 g wet weight of cells). This yield is similar to that obtained with *E. coli* expressing only the bovine 17 α -hydroxylase (rP450[mBov17A]) (10).

Membranes containing the fusion protein were prepared by high-speed centrifugation and found to contain about 0.4 nmol of P450 per mg of protein. NADPH-cytochrome *c* reductase activity was about 0.3–0.6 μmol of cytochrome *c* reduced per min per mg of protein. Approximately 60–70% of the spectrophotometrically detectable P450 was destroyed during the breakage of the cells by sonication, even though protease inhibitors were included in the reaction mixture.

The membrane fraction possessed an NADPH-dependent activity for the 17 α -hydroxylation of progesterone and pregnenolone in the absence of any added purified NADPH-P450 reductase flavoprotein. The 17 α -hydroxylase activity was similar to that seen when the membrane-bound recombinant bovine P450 17 α -hydroxylase (rP450[mBov17A]) was assayed in the presence of a 2- or 3-fold excess of added purified recombinant NADPH-P450 reductase (rmRatOR) (10). When the membrane-bound form of rF450[mBov17A/mRatOR]L1 was used, the 17,20-lyase reaction for the conversion of 17 α -hydroxypregnenolone to dehydroepiandrosterone was very slow relative to the rate of 17-hydroxylation of progesterone. Further, addition of a four-fold excess of purified rmRatOR had little or no influence on the rate of 17 α -hydroxylation of either progesterone or pregnenolone.

SDS/PAGE (Fig. 3) revealed an intensely stained band at about 118 kDa in the membranes containing rF450[mBov17A/mRatOR]L1 (lane 3) compared with membranes prepared from *E. coli* transformed with the same vector without an insert (lane 2). In addition this membrane fraction showed two intensely stained bands at 38 and 35.5 kDa (lane 3). These two proteins (labeled A and B in Fig. 3) that were coexpressed with rF450[mBov17A/mRatOR]L1 remain unidentified. Western blot analysis using an antibody against rat liver NADPH-P450 reductase revealed an immunoreactive protein at 118 kDa in the membranes containing rF450[mBov17A/mRatOR]L1. Of interest is the absence of immunoreactive protein at about 70 kDa, indicating the absence of proteolytically released NADPH-P450 reductase protein that remained associated with these membranes. A comparable pattern of antibody reactivity was noted when Western blots were probed with an antibody against P450 17A.

Purification of the Fusion Protein. The ability to grow large amounts of *E. coli* provides the advantage of producing large

FIG. 3. SDS/PAGE characterization of the proteins expressed in *E. coli*. Lanes 1 and 7, molecular size markers; lane 2, membranes prepared from *E. coli* transformed with pCWori⁺ without an insert (20 μg of protein); lane 3, membranes prepared from *E. coli* transformed with pCWori⁺ containing the cDNA for rF450[mBov17A/mRatOR]L1 (25 μg of protein); lane 4, purified rF450[mBov17A/mRatOR]L1 (4 μg of protein); lane 5, purified rmRatOR (6 μg of protein); lane 6, purified rP450[mBov17A] (5 μg of protein). Proteins were stained with Coomassie brilliant blue. Arrows indicate positions of the fusion protein, the purified recombinant rat and bovine proteins, and two unidentified proteins (A and B) that are coexpressed with the fusion protein.

quantities of membranes that can be used for purification. Purification of P450 fusion proteins from *E. coli* membranes proved to be remarkably simple. As described in *Materials and Methods* a four-step procedure was all that was required. The first step involved the solubilization of the membranes with detergent. Second was the resolution of proteins by a DE-52 column chromatography. The fusion proteins bound to the column, permitting separation from constitutive bacterial cytochromes and other pigments of *E. coli* membranes. Third was the use of an affinity column made of 2',5'-ADP-Sepharose 4B, which bound the flavoprotein domain in a manner similar to that seen in the purification of liver NADPH-P450 reductase flavoprotein (14). In this way the fusion proteins could be rapidly purified to near homogeneity. Further, this affinity chromatography step allowed the displacement of Emulgen 911 by CHAPS. Although Emulgen 911 is an effective agent for solubilizing fusion proteins from *E. coli* membranes, it was recognized to be a rather potent inhibitor of enzymatic activities for steroid 17 α -hydroxylation. To ensure removal of Emulgen 911, in the fourth step the eluate was concentrated in an Amicon concentrator and resolved by gel filtration on Ultragel AcA 34. With this procedure 50–60 mg of pure fusion protein can be obtained from cells harvested from 10 liters of growth medium. An SDS/PAGE comparison of pure rF450[mBov17A/mRatOR]L1 with purified rP450[mBov17A] and purified rmRatOR is shown in Fig. 3.

Spectrophotometric studies of the pure enzyme showed that rF450[mBov17A/mRatOR]L1 was isolated in the low-spin oxidized form with an absorbance maximum at about 420 nm. In addition, there was a weak absorbance at about 480 nm contributed by the flavin domain. As expected, addition of progesterone to the enzyme resulted in a spectral shift associated with substrate binding. Of interest was the nearly stoichiometric binding of the enzyme by progesterone. The subsequent addition of NADPH resulted in a loss of absorbance at about 480 nm, associated with the bleaching of the flavin component, and in the appearance of an absorbance band at about 415 nm, attributable to reduced P450. Gassing of the sample with carbon monoxide resulted in the rapid formation of the absorbance band at 450 nm characteristic of the carbon monoxide complex of reduced cytochrome P450. The subsequent addition of a few crystals of sodium dithionite resulted in no further increase in absorbance at 450 nm,

FIG. 4. Comparison of the rate of progesterone (P4) and pregnenolone (P5) metabolism by pure rF450[mBov17A/mRatOR]L1. Enzyme (0.2 nmol/ml) was incubated at 37°C with 10 μ M steroid in 50 mM Tris-HCl, pH 7.5/10 mM MgCl₂. The reaction was initiated by the addition of NADPH (1 mM). Samples were removed at the times indicated, rapidly mixed with dichloromethane, extracted, and analyzed by the HPLC method. TN, turnover number based on P450 content.

showing that the heme domain of the fused enzyme was fully reduced enzymatically. These results are equivalent to those reported by Peterson and Boddupalli (17), who studied the spectrophotometric properties of the bacterial fusion protein P450_{BM-3}.

Measurement of the FMN and FAD content of a sample of the purified fusion protein indicated a stoichiometry of 1:1 for FMN/FAD and a content of 5.5 nmol of FAD per mg of protein. However, the NADPH-cytochrome *c* reductase activity of the pure fusion protein showed a rate of only 5–6 μ mol of cytochrome *c* reduced per min per mg of protein. This is equivalent to that obtained with 1.3 nmol/mg of NADPH-P450 reductase protein. Difference spectrophotometry indicated a P450 content of about 7.5 nmol/mg of the purified enzyme protein. The estimated molecular mass of 118 kDa predicts a P450 content of about 8.4 nmol/mg. Comparison of the FAD and FMN content of the enzyme (5.5 nmol/mg) with the P450 content (7.5 nmol/mg) suggests a near equal stoichiometry for the two domains and the presence of a minimal amount of apoenzyme deficient in bound heme, as well as bound FMN and FAD.

Enzymatic Characteristics of the Purified P450 Fusion Enzyme rF450[mBov17A/mRatOR]L1. Incubation of the pure

rF450[mBov17A/mRatOR]L1 with progesterone or pregnenolone in the presence of NADPH and a regenerating system resulted in the rapid 17 α -hydroxylation of steroids (Fig. 4). Turnover numbers as high as 30/min have been obtained for the 17 α -hydroxylation of progesterone. The turnover number of the fusion enzyme increased significantly during purification; e.g., a turnover number of about 3/min was routinely obtained for the membrane-bound form of the fusion protein catalyzing the 17 α -hydroxylation of progesterone. Addition of phospholipid (dilauroyl phosphatidylcholine, 100 μ g/ml) significantly inhibited the rate of progesterone metabolism by the purified rF450[mBov17A/mRatOR]L1.

The bovine P450 17A is characterized by a 17,20-lyase activity for the conversion of 17-hydroxypregnenolone to dehydroepiandrosterone but not for the conversion of 17-hydroxyprogesterone to androstenedione (18, 19). Purified rF450[mBov17A/mRatOR]L1 showed only very low activities for the metabolism of 17-hydroxypregnenolone or 17-hydroxyprogesterone (turnover numbers of about 0.5/min). As expected, 17-hydroxypregnenolone was metabolized to dehydroepiandrosterone. Unexpected was the conversion of 17-hydroxyprogesterone, at a comparable rate, to three polar metabolites which remain to be characterized. The addition of purified NADPH-P450 reductase had no measurable effect on the rates of 17 α -hydroxylation of progesterone or pregnenolone or the 17,20-lyase reactions. However, the addition of pure rat liver cytochrome *b*₅ did stimulate significantly the conversion of 17-hydroxypregnenolone to dehydroepiandrosterone. The basis for this stimulation will be the subject of a subsequent manuscript.

Substrate Stimulation of NADPH Oxidation. The purified bacterial P450 fusion protein P450_{BM3} has been shown to be "tightly coupled" (20)—i.e., the oxidation of NADPH and the uptake of oxygen are dependent upon the presence of substrate. When the rate of NADPH oxidation by pure rF450[mBov17A/mRatOR]L1 was measured, a similar tight coupling was observed (Fig. 5). The addition of progesterone stimulated the rate of NADPH oxidation 3- to 4-fold. That this stimulation of the rate of NADPH oxidation was associated with an increased turnover of the P450 was confirmed by the inhibition observed following the addition of ketoconazole (24). Similar results have been obtained using pregnenolone as substrate. Control experiments using NADH rather than NADPH showed no measureable rate of NADH

FIG. 5. The coupling of NADPH oxidation with addition of steroid substrate and the effect of the inhibitor ketoconazole. A sample of purified rF450[mBov17A/mRatOR]L1 (0.28 nmol of P450) was added to a cuvette containing 3 ml of 50 mM Tris-HCl, pH 7.5/10 mM MgCl₂/35 μ M NADPH and the rate of decrease in absorbance at 340 nm recorded. At the times indicated 10 μ M progesterone or 13 μ M ketoconazole was added.

oxidation and no effect of added steroid. The slow rate of NADPH oxidation observed in the absence of added steroid varied for different preparations and may have been due in part to residual detergent that remained associated with the purified protein. Preliminary experiments have shown the formation of hydrogen peroxide concomitant with the oxidation of NADPH. The source of this hydrogen peroxide remains to be identified.

Fatty Acid Metabolism by rF450[mRat4A1/mRatOR]L1. The expression construct for rF450[mBov17A/mRatOR]L1 described above contains unique restriction sites which allow the facile interchange of other P450 domains for the synthesis of other fusion proteins. The domain for rat P450 4A1 was interchanged with the bovine P450 17A1 domain to generate a mammalian P450 fusion protein (rF450[mRat4A1/mRatOR]L1) capable of catalyzing the ω -hydroxylation of fatty acids. This fusion protein was expressed at high levels in *E. coli* and purified in a manner similar to that described above. Rat liver P450 4A1 can catalyze the ω -oxidation of a number of fatty acids—principally lauric and myristic acids, as well as arachidonic acid (11). Studies of lauric acid metabolism by the purified rat liver P450 4A1 prepared from clofibrate-treated animals showed (11) the formation of not only ω -hydroxylauric acid but also the $\omega - 1$ metabolite, although it remains controversial whether the same P450 catalyzes both the ω and the $\omega - 1$ reactions (21). When rF450[mRat4A1/mRatOR]L1 was incubated with lauric acid in the presence of an excess of NADPH, the ω -hydroxy lauric acid metabolite was rapidly formed (Fig. 6). Prolonged incubation did show the formation of a small amount of a second metabolite whose retention time on reversed-phase HPLC chromatography was similar to that of ($\omega - 1$)-hydroxylauric acid. However, when purified NADPH-P450 reductase was added to the reaction mixture, the rate of laurate metabolism was increased nearly 3-fold and there was a marked increase in the rate of formation of the metabolite identified as ($\omega - 1$)-hydroxylauric acid (Fig. 6). Of interest is the precursor-product relationship of ω -hydroxylaurate to the ($\omega - 1$)-hydroxy lauric acid metabolite. Similar results have been obtained during measurements of arachidonic acid metabolism by rF450[Rat4A1/mRatOR]L1. Most surprising was the failure of added purified rat cytochrome *b*₅

FIG. 6. Stimulation of lauric acid ω -hydroxylation by addition of purified NADPH-P450 reductase (mRatOR) to rF450[mRat4A1/mRatOR]L1. —, Purified fusion protein rF450[mRat4A1/mRatOR]L1 (0.4 nmol/ml) was incubated at 37°C with 20 μ M [¹⁴C]lauric acid in 50 mM Tris-HCl, pH 7.5/10 mM MgCl₂. The reaction was started by addition of NADPH (1 mM), and 0.5-ml aliquots were removed for HPLC analysis at the times indicated. - - -, The experiment was repeated with the addition of purified rat NADPH-P450 reductase [flavoprotein (FP) 0.8 nmol/ml]. Concentrations of laurate and its ω - and ($\omega - 1$)-hydroxy metabolites were measured. In each experiment, the concentration of ω -hydroxylaurate peaks and then declines while the concentration of ($\omega - 1$)-hydroxylaurate continues to increase, indicating a precursor-product relationship.

to enhance the rate of lauric acid or arachidonic acid metabolism (22, 23). Indeed, an inhibition of the ω -hydroxylation reaction was observed.

The ability to prepare large amounts of enzymatically active fusion proteins containing different P450s combined with the required flavoprotein reductase provides a self-contained biocatalytic unit which can be applied to a number of commercial processes. In addition, these fusion proteins will serve as models for assessing how domains interact in multidomain proteins. Fusion proteins of the type described here possess unique enzymatic properties that may provide further insight into the function of P450s.

We thank Dr. Andrew Parkinson of the University of Kansas Medical Center for a generous gift of purified rat liver cytochrome *b*₅ and Ms. Margarita Guijarro for excellent technical assistance. This work was supported in part by grants from the National Institutes of Health (GM16488) and the Robert A. Welch Foundation (I-0959) and by a Sponsored Research Agreement between Dallas Biomedical Inc. and the University of Texas Southwestern Medical Center at Dallas.

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