Biochemical Characterization of Rat P450 2C11 Fused to Rat or Bacterial NADPH-P450 Reductase Domains†

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ABSTRACT: cDNAs coding for rat P450 2C11 fused to either a bacterial (the NADPH-cytochrome P450 BM3 reductase domain of P450 BM3) or a truncated form of rat NADPH-P450 reductases were expressed in *Escherichia coli* and characterized enzymatically. Measurements of NADPH cytochrome *c* reductase activity showed fusion-dependent increases in the rates of cytochrome *c* reduction by the bacterial or the mammalian flavoprotein (21 and 48%, respectively, of the rates observed with nonfused enzymes). Neither the bacterial flavoprotein nor the truncated rat reductase supported arachidonic acid metabolism by P450 2C11. In contrast, fusion of P450 2C11 to either reductase yielded proteins that metabolized arachidonic acid to products similar to those obtained with reconstituted systems containing P450 2C11 and native rat P450 reductase. Addition of a 10-fold molar excess of rat P450 reductase markedly increased the rates of metabolism by both fused and nonfused P450s 2C11. These increases occurred with preservation of the regioselectivity of arachidonic acid metabolism. The fusion-independent reduction of P450 2C11 by bacterial P450 BM3 reductase was shown by measurements of NADPH-dependent H_2O_2 formation [73 ± 10 and 10 ± 1 nmol of H_2O_2 formed min^{-1} (nmol of P450)$^{-1}$ for the reconstituted and fused protein systems, respectively]. These studies demonstrate that (a) a self-sufficient, catalytically active arachidonate epoxygenase can be constructed by fusing P450 2C11 to mammalian or bacterial P450 reductases and (b) the P450 BM3 reductase interacts efficiently with mammalian P450 2C11 and catalyzes the reduction of the heme iron. However, fusion is required for metabolism and product formation.

Cytochrome P450 enzymes (P450s)[1] are involved in the metabolism of a variety of exogenous and endogenous (steroids, vitamins, fatty acids, plant hormones, etc.) compounds (*1*−*4*). Molecular biology has contributed extensively to our understanding of the genetics, molecular, and enzymatic properties of these enzymes. Among these advances, the design and the expression of recombinant proteins containing fused P450 heme and NADPH-P450 reductase domains (*5*−*7*) have provided useful tools for enzymatic and mechanistic studies of P450 heme/flavin electron transport, catalytic turnover, and product formation (*8*, *9*). Fused P450/reductase constructs have been modeled after P450 BM-3 (Cyp102), a soluble enzyme isolated from *Bacillus megaterium* (*10*) that contains, in a single 119 kDa polypeptide, a P450 heme domain with its carboxy terminus fused to the amino terminus of a P450 reductase domain (*10*, *11*). P450 BM3 catalyzes the epoxidation and ω-3 hydroxylation of arachidonic acid (AA) (*12*) and the ω-1, ω-2, and ω-3 hydroxylation of medium and long chain saturated fatty acids (*13*−*15*). During catalytic turnover by P450 BM3, substrate, oxygen, and NADPH are utilized in an approximately 1:1:1 molar ratio, demonstrating an efficient coupling of electron transport to oxygen reduction and fatty acid oxidation by this self-contained, high-turnover, catalytic unit. More recent examples of eukaryotic fused P450 monooxygenases are the nitric oxide synthases (*16*, *17*) and a fungal fatty acid ω-1/ω-3 hydroxylase (*18*).

Microsomal P450 catalyzes the in vivo metabolism of AA to a series of bioactive eicosanoids, including EETs, DHETs, and 19- and 20-(OH)-AA (*1*, *19*−*22*). These metabolites have been shown to influence vascular physiology (*1*, *19*−*22*) and to be implicated in the pathophysiology of experimental spontaneous and salt-sensitive hypertension (*19*−*23*). However, a lack of suitable experimental models, i.e., cultured cells expressing significant levels of relevant P450 isoforms, has limited the application of mechanistic approaches to the study of the functional significance of this branch of the AA cascade. To overcome some of these limitations, cell

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[1] Abbreviations: P450, cytochrome P450; OR, rat liver NADPH-P450 reductase; ORtr, a truncated form of OR that lacks its membrane insertion peptide; BM3OR, bacterial NADPH-P450 reductase; [2C11−OR], a protein containing fused P450 2C11 and OR domains; [2C11−ORtr], a protein containing fused P450 2C11 and ORtr domains; [2C11−BM3OR], a protein containing fused P450 2C11 and BM3OR domains; [2C11−FMN], a protein containing a P450 2C11 fused to the FMN domain of the OR; AA, arachidonic acid; EET, *cis*-epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; 20-OH-AA, 20-hydroxyeicosatetraenoic acid; DLPC, dilauroylphosphatidylcholine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography; RP, reverse phase; PVDF, poly(vinylidene difluoride); SOD, superoxide dismutase; aa, amino acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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transfection techniques are being extensively applied to the characterization of cDNA-dependent cellular phenotypes (*24*, *25*). As part of our studies of the cellular roles of the P450-derived eicosanoids, our goal is to develop self-sufficient catalytic units consisting of eukaryotic P450 epoxygenases fused to P450 reductase, to utilize these constructs for cell and/or organ transfection, and to characterize cDNA-dependent, AA monooxygenase-associated phenotypes. We report here the design, construction, and biochemical characterization of self-contained catalytic units consisting of P450 2C11, the predominant 2C isoform in the rat liver (*26*), fused to either mammalian or bacterial P450 reductases and capable of AA metabolism in the presence of only NADPH and oxygen.

MATERIALS AND METHODS

Plasmid and Enzymes Used. pCWori$^+$ containing the P450 3A4 ORF fused to a truncated form of P450 reductase (ORtr) by means of a Ser−Thr linker (3A4/ORtr) was a gift of Dr. F. P. Guengerich (Department of Biochemistry, Vanderbilt University Medical School, Nashville, TN) (*27*, *28*). The pBluescript and pCR2.1 cloning vectors were from Stratagene (LaJolla, CA) and InVitrogene (Carlsbad, CA), respectively. The rat liver NADPH-P450 reductase (OR) cDNA was a gift of Dr. C. Kasper (McArdle Laboratory, University of Wisconsin). The P450 BM3 (BM3) cDNA was a gift of Dr. Julian Peterson (Department of Biochemistry, Southwestern Medical Center, Dallas, TX). The P450 BM3 reductase domain (BM3OR) was obtained by *Rsa*I/*Xho*I digestion of the BM3 cDNA (in pIBI) (*27*), cloned into the *Eco*RV and *Xho*I sites of the pET-30b vector (Novagen, WI), and expressed in BL 21 *E. coli*. Recombinant BM3OR and OR were affinity-purified using a His-Tag system (Novagen, WI). The FAD domain of the BM3OR was a gift of Dr. Julian Peterson.

Construction of Plasmids Coding for P450 2C11 Fused to either Rat or Bacterial P450 Reductase. P450 3A4 was removed from a fused P450 3A4−P450 reductase construct in pCWori$^+$ by *Nde*I and *Sal*I digestion. After gel purification, the linearized plasmid was ligated to a *Nde*I−*Sal*I 2C11 fragment obtained by PCR amplification of the rat P450 2C11 cDNA (in pBluescript) using the following primers: (a) a 30-mer sense oligonucleotide containing the italicized *Nde*I site as part of the P450 2C11 ATG initiation codon (5′-GGAATTC *CATATG*GATCCAGTCCTAGTCCT-3′) and (b) a 31-mer antisense primer containing the italicized *Sal*I site upstream of a TAA stop codon (5′-ACGC*GTCGAC*G-GCAGATGAGAGCT TAGAGAG-3′). Prior to ligation, the PCR product was sequenced to document the fidelity of PCR amplification. The resulting PCWori$^+$, in which the 3′-end of P450 2C11 was fused to the 5′-end of the ORtr by means of a Ser−Thr linker [2C11−ORtr], was sequenced and expressed. A modified version of this plasmid, in which the FMN domain of the ORtr was removed, was prepared by *Sal*I−*Bgl*I digestion of [2C11−ORtr] in PCWori$^+$, followed by purification, filling with Kleenow polymerase, and circularization. The FMN domain of ORtr was defined as the polypeptide positioned between residues 57 and 245 of the published protein sequence (*30*).

To generate a cDNA coding for a P450 2C11 fused to the full-length P450 reductase [2C11−OR], the 5′-end of the OR cDNA was modified by PCR to introduce an oligonucleotide coding for a Pro−Ser−Thr linker and to replace the cDNA's ATG initiation codon with a *Sal*I site. A *Sal*I−*Avr*II fragment, containing an ATG-minus OR reading frame, was generated by PCR amplification of the OR clone using the following primers: (a) a 26-mer sense oligonucleotide with the italicized *Sal*I site (5′-CC*GTCGAC*TGGGGACTCT-CACGAAGA-3′) and (b) a 22-mer antisense primer with the italicized *Avr*II site and the TAA stop codon (bold) (5′-*CCTAGG*TGA**TTA**CAGGGAG CGA-3′). The purified PCR product was sequenced and ligated to a *Sal*I−*Avr*ll digest of the [2C11−ORtr] in PCWori$^+$. After confirming ligation and cloning integrity, the resulting [2C11−OR] plasmid was used for expression.

Similar approaches were utilized for the construction of PCWori$^+$ expression plasmids containing P450 2C11 fused to the N-amino terminal of the P450 BM3 reductase [2C11−BM3OR] (*31*). The 5′-end of the BM3OR cDNA was modified by PCR to incorporate an oligonucleotide coding for a Pro−Ser−Arg linker and an *Ava*I site and using the following primers: (a) a 23-mer sense oligonucleotide containing the italicized *Ava*I site (5′-CC*CTCGAG*CAAA-AAGGCAGAAAA-3′) and (b) a 20-mer antisense primer containing the italicized *Xba*I site (5′-*TCTAGA*GGATCT-GCTGTCAC-3′). After purification and sequence analysis, the fragment was ligated into a *Sal*I−*Xba*I digest of the [2C11-OR] construct in PCWori$^+$.

To improve bacterial translation and protein expression, we modified the 5′-end of the P450 2C11 cDNA by replacing the cDNA's ATG initiation codon with the first 41 nucleotides of the published sequence for P450 BM3 (*32*). The added sequence contained the Shine−Dalgarno sequence, the cDNA initiation codon, and the codons corresponding to the first eight amino acid residues in P450 BM3 (*32*). For these purposes, we synthesized complementary 47-mer oligonucleotides containing the italicized start codon and Shine−Dalgarno sequences: (A) (5′-**GATCC**AAGTGA*AAGAGG*-GATAAC*ATG* ACAATTAAAGAAATGCCTCA**G**-3′) (B) (5′-**G**TTCACT*TTCTCC*CTATTG*TAC*TGTTAAT TTCTT-TACGGAGT**CCTAG**-3′). After phosphorylation, the oligonucleotides were annealed by heating the mixture at 90 °C and slowly cooling to room temperature in the presence of 20 mM Tris-HCl pH 7.5, 2 mM $MgCl_2$, and 50 mM NaCl. Annealing generated a *Bam*HI cohesive end (bold). After gel purification, the double-stranded DNA fragment was ligated into pCW:2C11/RatORtr previously digested with *Bam*HI and dephosphorylated. In view of low ligation yields, after 12 h at 16 °C the ligation mixture was PCR-amplified using two primers: (a) a 22-mer sense oligonucleotide (5′-*GATATC*AAGTGAAAGAGGGATA-3′) containing an *Eco*RV site (italicized) replacing the *Bam*HI site of primer (A) and (b) a 20-mer antisense oligonucleotide reverse complement to P450 2C11 sequence (5′-AAATT*CTTAAG*TACTTGGTT-3′) containing an *Afl*II site (italicized). Gel-purified PCR products were ligated into the pCR2.1 vector and sequenced. The *EcoR*V−*Afl*II fragment was released from pCR2.1 plasmid and ligated into either [2C11−ORtr], [2C11−OR], or [2C11−BM3OR] constructs opened by *Bam*HI (blunted) and *Afl*II digestion.

Expression and Purification of Fusion Proteins. DH5α *E. coli* were transformed with the pCWori$^+$ vector containing the [2C11−ORtr], [2C11−OR], or [2C11−BM3OR] inserts

in the presence of ampicillin. Cells were grown in modified TB containing thiamin (1 mM), potassium phosphate buffer, pH 7.7 (100 mM), IPTG (1 mM), δ-aminolevulinic acid (1 mM), and a rare salt solution (*33*). After 48 h at 28 °C, cells were harvested by centrifugation (2800*g*, 12 min, 4 °C); lysed in 0.1 M Tris-HCl, pH 7.4, containing 10 mM CHAPS (Sigma Chemical Co., MO), 20% glycerol, and 1 mM EDTA; and centrifuged for 10 min at 2800*g*. The resulting pellets were resuspended in the lysis buffer and utilized to determine expression levels by CO-difference spectroscopy (*34*).

For the isolation of the membrane fraction, cells expressing the different P450 2C11 constructs were collected by centrifugation, and the cell pellets were suspended in TSE buffer (50 mM Tris-acetate, pH 7.6, containing 250 mM sucrose and 0.25 mM EDTA). A solution of freshly prepared lysozyme was added (0.25 mg/mL, final concentration), and the mixture was then gently shaked at 4 °C for 30−45 min. After centrifugation (2800*g* for 12 min at 4 °C), the resulting spheroplasts were suspended in 0.1 M NaPi buffer, pH 7.4, containing 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT, 1 mM PMSF, 0.1 μg/mL leupeptin, and 0.04 U/mL aprotinin, and disrupted by sonication at 4 °C. After a 10-min centrifugation at 4000*g*, the supernatants were centrifuged at 100000*g* for 1 h. The resulting membrane pellets were suspended in 10 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose and used for either protein purification or enzymatic characterization.

For protein purification, the membrane fractions were suspended in 10 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose and solubilized by the dropwise addition of 10% sodium cholate (w/v) (Sigma Chemical Co., MO) to obtain a final concentration of 1%. After a 12−16 h incubation at 4 °C, insoluble proteins were removed by centrifugation (1 h, 140000*g*), the P450 and sodium cholate concentrations of the high-speed supernatant were adjusted to 1 μM and 0.4%, respectively, and the resulting suspension was immediately loaded onto an octyl-sepharose 4B (Pharmacia Biotech AB, Uppsala, Sweden) column (5 × 10 cm). Extensive column washing was followed by elution of bound recombinant P450s in the presence of 0.4% (v/v) Emulgen 911 (Kao Chemical Co., Tokyo, Japan). After dialysis versus 10 mM sodium phosphate buffer (pH 7.5) containing 20% (v/v) glycerol, 0.2% (w/v) sodium cholate, 0.1 mM DTT, and EDTA (buffer A) (16 h, 100 vol), recombinant fusion proteins were loaded onto a hydroxylapatite (Bio-Rad Laboratories, Richmond, CA) column (2 × 12 cm) equilibrated with buffer A. The column was washed with 10 vol of the equilibrating buffer, and the bound P450s were then eluted in the presence of 0.3 M sodium phosphate (pH 7.4) in buffer A and dialyzed [vs 100 vol of 10 mM sodium phosphate buffer (pH 7.4) containing 20% glycerol and 0.2% (v/v) sodium cholate]. The specific contents of the purified proteins were 10, 4, 6, and 5 nmol of P450s 2C11 per mg of protein, respectively, for P450s 2C11, [2C11−ORtr], [2C11−BM3OR], and [2C11−FMN].

Enzymatic Activities, Product Characterization. Incubations were performed exactly as previously described (*35*). Briefly, purified recombinant P450 2C11, [2C11−ORtr], [2C11−OR], and [2C11−BM3OR] (10−40 pmol) were mixed with a solution of dilauroylphosphatidylcholine (2 mg/mL; 5−25 μL) and, when needed, 0.5−10 μL of a 20 μM solution of NADPH-P450 reductase (to obtain P450/NADPH-P450 reductase molar ratios between 1:1 and 1:20). After 10 min at room temperature, the mixtures were diluted 10-fold by the addition of 50 mM Tris-HCl buffer (pH 7.5) containing 0.15 M KCl, 10 mM $MgCl_2$, 8 mM sodium isocitrate, and isocitrate dehydrogenase (0.5 IU/mL) and preincubated 1 min at 35 °C prior to the addition of a 20 mM solution of [1-^{14}C]sodium arachidonate in 0.1 M Tris-HCl buffer (pH 8.0) (20−50 μCi/μmol; 50−100 μM, final concentration) (*35*). Reactions were initiated with NADPH (1 mM, final concentration).

E. coli membrane fractions, containing expressed recombinant proteins, were suspended in 50 mM Tris-HCl buffer (pH 7.5) containing 0.15 M KCl, 10 mM $MgCl_2$, 8 mM sodium isocitrate, and isocitrate dehydrogenase (0.5 IU/mL) to a final concentration of 1 mg of protein/mL. After 1 min at 35 °C, [1-^{14}C]arachidonic acid (20−50 μCi/μmol; 50−100 μM final concentration) was added, and the reactions were initiated by the addition of NADPH (1 mM, final concentration) and continued at 35 °C under constant mixing. Aliquots of the reaction mixture were withdrawn at different time points, and the organic soluble products were extracted into acidified ethyl ether and resolved and quantified by RP-HPLC with on-line liquid scintillation counting (Flo-one β Counter; Radiomatic Instruments, Tampa, FL) (*35*).

Other Methods. Polyclonal antibodies against purified recombinant P450 2C11 were raised in female White New Zealand rabbits (*36*) and purified by affinity chromatography on a Protein G Superose HR 10/2 column (Pharmacia Biotech AB, Uppsala, Sweden). After $(NH_4)_2SO_4$ precipitation and dialysis against 20 vol of 20 mM sodium phosphate buffer (pH 7.0) (*36*), samples of anti-2C11 rabbit IgG were loaded (at 1 mL/min) onto the affinity column, and the column then was washed for 10 min with 20 mM sodium phosphate buffer (pH 7.0). The anti-2C11 antibodies were eluted with a linear buffer gradient from initially 100% 20 mM sodium phosphate buffer (pH 7.0) to 100% 100 mM glycine (pH 2.7) over 20 min at 1 mL/min. Antigen−antibody reactions were detected using a SuperSignal Substrate Western Blotting kit (Pierce, Rockford, IL) and the manufacturer's instructions. Densitometric analyses were done by using a Raiser camera and Alpha Imager 950 software package. Protein concentrations were determined using the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories). Cytochrome P450 concentrations were determined according to Omura and Sato (*34*). NADPH-cytochrome *c* reductase activities were measured at 25 °C in 50 mM potassium phosphate buffer (pH 7.7) containing 40 μM bovine heart cytochrome *c* and 0.1 mM EDTA. Reactions were initiated by the addition of NADPH (50 mM, final concentration), and the reduction of cytochrome *c* was monitored at 550 nm. Hydrogen peroxide was determined as described (*37*). Briefly, recombinant enzymes (0.1 μM), suspended in 50 mM Tris-HCl buffer (pH 7.5) containing 0.15 M KCl, 10 mM $MgCl_2$, 8 mM sodium isocitrate, and isocitrate dehydrogenase (0.5 IU/mL) were incubated at 35 °C with constant mixing and in the absence or the presence of AA (100 μM, final concentration) and NADPH. Aliquots of the incubation mixtures were withdrawn at different points in time and, after the addition of TCA, $Fe(NH_4)_2(SO_4)_2$, and KSCN, the H_2O_2-dependent formation of $Fe(SCN)_3$ was measured at 480 nm (*37*).

Table 1: Expression Levels of Recombinant P450 2C11 Constructs in *E. coli*

recombinant enzymes	cell suspensions (nmol/L)[a]	membrane fraction (nmol/mg)
2C11	206 ± 52	6.9
[2C11−ORtr]	182 ± 8	2.1
[2C11−OR]	23 ± 11	undetectable
[2C11−BM3OR]	163 ± 27	2.8
[2C11−FMN]	211 ± 25	1.8

[a] Values are averages calculated from at least three different experiments ± SE.

RESULTS AND DISCUSSION

Expression of 2C11 Fusion Proteins in E. coli. The characterization of wild type and modified P450 isoforms requires access to quantities of recombinant protein that are sufficient for purification and enzymatic studies. Because of their inherent simplicity, low cost, and short time required from cloning to expression, bacterial expression systems are now widely utilized for the expression of mammalian P450 (*38*−*40*). However, as recognized early on, the expression of most mammalian P450 requires extensive modifications to the protein N-terminal amino acid sequence (*38*−*40*). In most cases, these modifications are designed to replace the protein's first 8−25 amino acids and to add a bacterial ribosome recognition site to the cDNA (*40*). Nevertheless, the nature and the extent of these modifications remain mostly empirical and nearly P450 isoform-specific. For the *E. coli* expression of P450 2C11 cDNA constructs, we employed a strategy similar to that described in ref *40*. Briefly, the cDNA ATG initiation codon was removed and replaced by a sequence coding for the P450 BM3 translation initiator consensus (Shine−Dalgarno), its ATG initiation codon, and the sequence coding for the protein's first eight amino acids. The thus modified P450 2C11 cDNA was then cloned into the pCWori$^+$ vector and expressed in DH5α *E. coli*. As shown in Table 1, and with the exception of the [2C11−OR] construct, all cDNAs were expressed at a high level (between 163 and 211 nmol of P450 protein/L of culture). For the [2C11−OR] protein, the presence of the OR membrane insertion peptide markedly reduced the capacity of the *E. coli* to biosynthesize the corresponding fused protein (Table 1). Thus, for example, as compared to the construct containing a truncated OR sequence ([2C11−ORtr]), this hydrophobic peptide, positioned between the hemoprotein and the flavoprotein domains, reduced overall expression levels by ∼8-fold (Table 1).

Lysozyme digestion of cDNA expressing *E. coli*, followed by sonication and high-speed centrifugation (105000*g*; 60 min), showed that, with the exception of the [2C11−OR] where low expression levels precluded accurate spectrophotometric detection, all recombinant proteins were recovered associated with the membrane fractions isolated by high-speed centrifugation (Table 1). These results differ from those obtained with P450s 2E1 and 2B4, in which approximately 73 and 80% of the corresponding recombinant proteins were recovered in the cell cytosol (*40*). The reported higher solubility of the recombinant P450s 2E1 and 2B4 is likely due to differences in the extent of modifications introduced into their N-amino terminals, i.e., the replacement of the proteins first 20 N-terminal amino acids with the P450 BM3 first 16 amino acids (*40*), instead of the 8 residues utilized in this work.

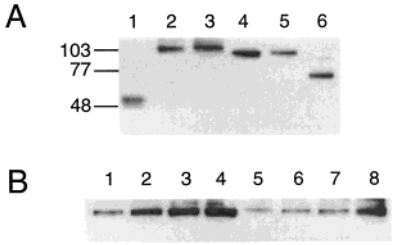

FIGURE 1: Immunoelectrophoresis characterization of the purified recombinant forms of P450 2C11. Purified P450s 2C11, [2C11−ORtr], [2C11−OR], [2C11−BM3OR], a modified [2C11−BM3OR] protein containing six additional aa residues in the P450−BM3OR linker (*29*), and [2C11−FMN] (lanes 1−6, respectively; 1 pmol of P450 each) (A) or samples of purified [2C11−OR] (lanes 1−4, respectively; 5, 10, 20, and 40 μg of protein each) and of [2C11−ORtr] (lanes 5−8, respectively; 5, 10, 20, and 40 μg of protein containing 1.2, 2.4, 4.8, and 9.6 pmol of P450 2C11 each) (B) were submitted to SDS−PAGE (100 × 60 × 1 mm, 10% acrylamide gel slabs). After transfer to PVDF membranes, the blots were incubated, first, with a polyclonal antibody raised against purified recombinant P450 2C11, and second, with a peroxidase-coupled anti-rabbit IgG. The sample's chemioluminescense was detected using a Super Signal kit and Kodak X-ray film. P450 2C11 contents were estimated by densitometric analysis. See Materials and Methods for further details. Shown are the relative electrophoretic mobilities of protein molecular weight standards.

To obviate alternate, non-P450 OR-catalyzed pathways of P450 2C11 reduction (*41*), the expressed proteins were solubilized and purified as described in Materials and Methods. SDS−PAGE analysis of the purified enzymes indicated that they were obtained with overall purities of 80, 60, 80, and 40% for P450s 2C11, [2C11−ORtr], [2C11−BM3OR], and [2C11−FMN], respectively (not shown). Immunoelectrophoresis using polyclonal anti-2C11 antibodies (*36*) showed that all purified enzymes contained an anti-2C11 inmunoreactive protein band with average MWs corresponding to those predicted from the individual cDNA-translated protein sequences (58, 128, 135, 122, and 80 kDa for P450s 2C11, [2C11−ORtr], [2C11−OR], [2C11−BM3OR], and [2C11−FMN], respectively) (Figure 1A). Included in Figure 1A (lane 5) are the inmunoreactive properties of a modified [2C11−BM3OR] protein with a P450−BM3OR linker containing six additional residues (Ile−Pro−Leu−Gly−Gly−Ile) (*29*). Low expression levels and the unstable nature of the [2C11−OR] protein made its further purification and spectral quantification impractical. As an alternative, we compared by Western blot analysis the anti 2C11-immunoreactivity of this protein with that of the [2C11−ORtr] enzyme and estimated its P450 2C11 specific content by densitometric analysis (Figure 1B). It was calculated that the P450 2C11 content in the [2C11−OR] membrane fractions isolated after lysozyme digestion, sonication, and high-speed centrifugation corresponded to approximately one-fifth of that obtained with the [2C11−ORtr] cDNA expression (Figure 1B).

The absolute spectrum of recombinant [2C11−ORtr] and of [2C11−BM3OR] were similar with Soret, α-, and β-bands at 414, 570, and 537 nm, respectively. A broad absorption band centered at around 480 nm was indicative of the spectral contributions of the fused OR FMN and FAD prosthetic groups. Reduction with sodium dithionite resulted in the attenuation and displacement of the Soret band to 416 nm

Table 2: Rates of Cytochrome *c* Reduction by Recombinant Enzymes

enzyme system	cytochrome *c* reduced[a] − SOD	+ SOD
OR	2925 ± 120	2878 ± 64
2C11 + OR	2270 ± 100	2147 ± 94
[2C11−ORtr]	4316 ± 102	4091 ± 143
BM3OR	3140 ± 102	3354 ± 185
2C11 + BM3OR	1879 ± 49	1623 ± 98
[2C11−BM3OR]	3808 ± 120	2979 ± 162
[2C11−FMN] + OR	2027 ± 149	1925 ± 132

[a] In nmol of cytochrome *c* reduced min^{-1} (nmol of flavoprotein)$^{-1}$ at 25 °C. Values are the averages calculated from at least three different experiments ± SE.

and the replacement of the α- and β-bands for a broad absorption band centered at around 542 nm. The addition of CO to a solution of reduced [2C11−ORtr] or [2C11−BM3OR] leads to the rapid development of a strong absorption band at 450 nm, with shoulders at 422 and 563 nm, indicative of the formation of the CO-bound complex of reduced [2C11−ORtr] or [2C11−BM3OR]. Experiments in which CO was added to the cuvette prior to reduction showed that, for both proteins, heme reduction was rapid and completed within the first few seconds of dithionite addition (*34*).

Cytochrome c Reduction. To characterize the integrity of NADPH/FAD/FMN electron transfer and to investigate whether protein steric factors affected cytochrome *c*/reductase interactions, we determined the cytochrome *c* reductase activity of the fused proteins for comparison to that of OR and BM3OR. As shown in Table 2, the addition of purified P450 2C11 had only a minor effect on the rates of cytochrome *c* reduction catalyzed by the mammalian and bacterial oxido reductases. These results show that the presence of purified P450 2C11 does not alter the rates of FAD/FMN/cytochrome *c* electron transfer. On the other hand, and as compared with the corresponding purified enzymes, fusion of the flavoproteins' amino-end to P450 2C11 carboxyl-end increased the cytochrome *c* reductase activity of both the mammalian and the bacterial oxido reductases (40 and 21% increase in activity for the [2C11−ORtr] and [2C11−BM3OR] fusion proteins, respectively) (Table 2). With the exception of the [2C11−BM3OR] protein, the P450 2C11 rate effects were, for the most part, SOD-insensitive (Table 2) suggesting direct donor−acceptor, protein/protein, electron transfer and not a bulk solvent $O_2^{\bullet-}$ mediated process. While the mechanisms and/or the paths of electron transfer from the OR prosthetic groups to the heme of cytochrome *c* are not understood, the observed P450 2C11 fusion-dependent increases in flavoprotein turnover suggest that fused P450 heme/cytochrome *c* heme interactions may be responsible for these augmented rates of NADPH/cytochrome electron transport. However, clotrimazole (between 1 and 5 μM), a strong cytochrome P450 heme ligand and potent inhibitor, had no effect on the rates of cytochrome *c* reduction by the fused proteins (not shown). As with P450 2C11, the presence of the bacterial FMN domain fused to the carboxyl-end of P450 2C11 had no effects on the rate of cytochrome *c* reduction by the OR (Table 2). Finally, highly variable, preparation-dependent rates of cytochrome *c* reduction were observed with *E. coli* membranes containing recombinant [2C11−OR] proteins.

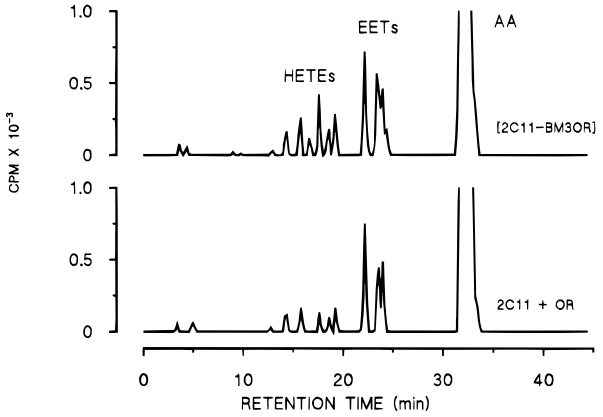

FIGURE 2: Chromatographic resolution of the AA metabolites generated by P450 2C11 reconstituted with rat P450 reductase or fused to a bacterial P450 reductase. Reaction mixtures containing either a combination of P450 2C11 and OR (0.02 μM each, final concentration) (bottom frame) or, alternatively, the [2C11−BM3OR] fusion protein (0.1 μM, final concentration) (top frame) were incubated at 35 °C for 15 min and in the presence of DLPC (50 μg/mL) and a buffer system containing NADPH, an NADPH-regenerating system, and [1-^{14}C]AA (20 μCi/μmol; 100 μM). Reactions were terminated by the addition of acidified ethyl ether, and the organic soluble products were extracted and resolved by RP-HPLC as described in Materials and Methods. Shown are the radiochromatograms derived from incubation mixtures containing 4 and 20 nmol of P450 2C11 and [2C11−BM3OR], respectively.

Metabolism of Arachidonic Acid. To evaluate and compare the enzymatic activities of the fused proteins to that of nonfused P450 2C11, we first reconstituted the AA monooxygenase activity of purified P450 2C11 in the presence of DLPC, NADPH, and variable P450/reductase molar ratios (*42*). Incubation of recombinant P450 2C11 with an equimolar concentration of OR and AA (70−100 μM, final concentration) resulted in the NADPH- and time-dependent formation of metabolites with the chromatographic properties of mixtures of authentic 14,15-, 11,12-, 8,9-, and 5,6-EETs (*35*, *36*) (Figure 2). Approximately 27% of the total P450 2C11 AA oxidation products eluted with HPLC retention times similar to those of mixtures of HETEs, including 12- and 15-HETE, and are yet to be fully characterized (Figure 2). Catalytic activity was P450- and OR-dependent and required the presence of 150 mM KCl for optimal turnover. As with other P450 AA monooxygenases (*8*, *43*), turnover rates increased almost linearly with increasing OR and reached a maximum at a molar ratio of OR to P450 2C11 of approximately 10 (Table 3). Those OR-dependent rate effects indicate that during the catalysis of AA oxidation, electron transfer and heme reduction are rate limiting. While the truncated form of the OR, the ORtr, was unable to support metabolism by purified recombinant P450 2C11 (*44*, *45*), its fusion to the carboxyl-terminal of P450 2C11 yielded a self-sufficient catalyst that metabolized AA at approximately one-fourth the rate of the reconstituted system but requiring only air and NADPH for full activity (Table 3). As with purified P450 2C11, metabolism by the [2C11−ORtr] protein was also stimulated by the addition of a 10-fold molar excess of purified P450 OR (22-fold over the rates in the absence of exogenously added OR; Table 3). These results are similar to data obtained with several nonfused P450 isoforms (*42*) and indicate that (a) the presence of an ORtr molecule connected to the P450 2C11 carboxy-terminal does not preclude catalytically productive interactions with additional

Table 3: Rates of Arachidonic Acid Metabolism by the 2C11 Recombinant Enzymes[a]

recombinant protein	type of system	added reductase	added reductase:P450	reaction rates[c]
2C11	reconst[b]	OR	1:1	3.7 ± 0.7
2C11	reconst	OR	10:1	34.5 ± 1.2
2C11	reconst	BM3OR	1:1	ND[d]
2C11	reconst	BM3OR	10:1	ND
[2C11−ORtr]	fused	none	1:1	1.0 ± 0.2
[2C11−ORtr]	fused	OR	10:1	22.2 ± 2.2
[2C11−BM3OR]	fused	none	1:1	0.20 ± 0.02
[2C11−BM3OR]	fused	BM3OR	10:1	6.8 ± 1.0

[a] Enzymes (0.05−0.2 μM P450) were incubated (at 35 °C) with [1-^{14}C]AA (70−100 μM) and in the presence of NADPH (1 mM) and DLPC (50−100 μg/mL) as indicated in Materials and Methods and in ref *35*. Initial reaction rates were calculated from the liner portion of plots of product formation versus incubation time. Values are the averages calculated from at least three different experiments ± SE. [b] Reconstituted. [c] In nmol min^{-1} (nmol of P450)$^{-1}$. [d] ND, not detectable.

OR molecules and (b) the existence of multiple electron pathways and/or P450 [2C11−ORtr]−OR molecular interactions leading to productive electron flow from the flavoprotein to the P450 heme iron.

As shown in Table 3, the P450 BM3 flavoprotein domain (BM3OR) (*29*), even when added in a 10-fold molar excess, was unable to support AA oxidation by purified P450 2C11 (Table 3). On the other hand, fusion of the BM3OR N-amino terminal to the carboxyl-end of P450 2C11 yielded a protein that metabolized AA at a rate approximately one-fifth of that of the [2C11−ORtr] enzyme (Figure 2, Table 3). As with 2C11 and [2C11−ORtr], the presence of a 10-fold molar excess of purified OR, but not BM3OR, markedly increased the catalytic turnover of the [2C11−BM3OR] protein (Table 3). To the best of our knowledge, this is the first demonstration of catalytically productive electron transfer between the heme iron of a mammalian P450 and a soluble flavoprotein of bacterial origin such as the BM3OR. Furthermore, the demonstrated inability of the purified BM3OR to support catalytic turnover by P450 2C11 suggests that this lack of activity is either caused by limited productive interactions between these proteins or electronic and/or mechanistic reasons. In this regard, P450 2C11 fused to the FMN domain of the BM3OR was also unable to support AA metabolism in the presence of OR or BM3OR (not shown). Importantly, regardless of the nature and/or concentration of the proximal electron donor to the heme iron, for example, the fused ORtr or BM3OR flavoproteins or, alternatively, the purified OR, the regiochemistry of AA oxidation by these enzymes was similar and under the control of P450 2C11 (*36*) (Table 4). Published results have also demonstrated that the fusion of P450 and OR does not result in significant changes in catalytic efficiency or product chemistry (*46, 47*).

To characterize the contribution of intramolecular and intermolecular electron transfer processes to catalytic activity, we studied the kinetics of AA oxidation by the fused hemoprotein systems, incubated in the presence of excess NADPH and AA (*40*). The rationale for these studies was that, as previously shown with a bovine P450 17A fused protein (*6*), an intramolecular electron transfer process should result in turnover numbers that are enzyme concentration-independent while, on the other hand, during intermolecular transfers, enzyme turnover is protein concentration-dependent

Table 4: Regioselectivity of AA Epoxidation by the Recombinant 2C11 Enzymes[a]

		(% distribution)		
enzyme system	epoxygenase activity[b]	14,15-EET	11,12-EET	8,9- and 5,6-EET
2C11 + OR	73	40	38	22
[2C11−ORtr]	70	44	35	21
[2C11−OR]	73	44	41	15
[2C11−BM3OR]	64	38	31	31
[2C11−FMN] + OR	67	46	33	21

[a] Values calculated from a representative experiment. Approximately 30% of the reaction products generated from AA by the different enzyme systems eluted with RP-HPLC retention times similar to those of HETE mixtures and remain uncharacterized. [b] Epoxygenase is the activity of P450 2C11 that leads to formation of EETs. Epoxygenase activity is expressed as percent of the total organic soluble products.

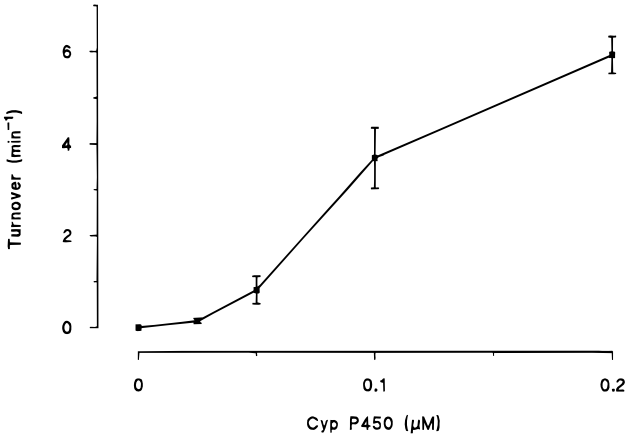

FIGURE 3: P450 concentration-dependent changes in the rates of AA metabolism by a reconstituted system containing P450 2C11 and OR. Equimolar mixtures of P450 2C11 and OR were incubated at the indicated P450 2C11 concentrations in the presence of DLPC (50 μg/mL), NADPH (1 mM), an NADPH-regenerating system, and AA (100 μM; 10−20 μCi/μmol) for 5 min at 35 °C. Products were extracted, resolved, and quantified as described in Materials and Methods.

and should increase exponentially with increasing enzyme concentrations. As a control, we first reconstituted the AA monooxygenase in the presence of increasing equimolar concentrations of purified P450 2C11 and OR. At enzyme concentrations below 100 nM, increasing P450 and OR concentrations resulted in exponential increases in catalytic activity (Figure 3). On the other hand, at P450 and OR concentrations above 100 nM, second-order kinetics was rapidly lost (Figure 3), indicating that at high reaction rates turnover becomes increasingly P450−OR collision-independent and limited by (a) redox transfer rates, (b) AA availability, or (c) rates of product formation and release. In the presence of NADPH, the specific rates of AA metabolism by either the [2C11−ORtr] or the [2C11−BM3OR] proteins were protein concentration-dependent (Figures 4 and 5), indicating that electron transfer between the fused flavoproteins and P450 2C11 occurred by combinations of intra- and intermolecular pathways. Thus, for example, over a 8- and 3-fold range of enzyme concentrations, AA oxidation (expressed in nmol of product formed/nmol of P450) by the [2C11−ORtr] and [2C11−BM3OR] proteins increased by nearly 9- and 5-fold, respectively (Figures 4 and 5). On the basis of the above, we concluded that, at difference with bovine P450 17A fused to ORtr (*6*), the [2C11−ORtr] and

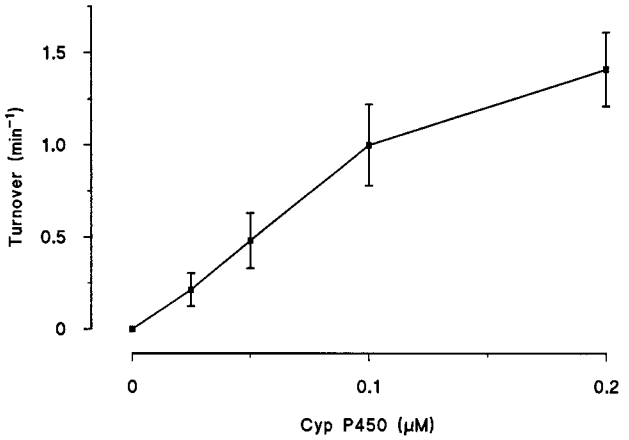

FIGURE 4: P450 concentration-dependent changes in the rates of AA metabolism by the purified P450 [2C11−ORtr] protein. P450 [2C11−ORtr] was incubated at the indicated P450 2C11 concentrations with [1-^{14}C]AA (100 μM; 20−50 μCi/μmol) in the presence of NADPH (1 mM) and an NADPH-regenerating system for 10 min at 35 °C. Products were extracted, resolved, and quantified as described in Materials and Methods.

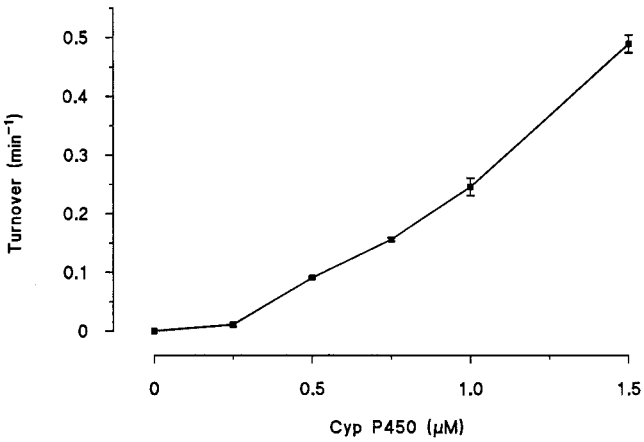

FIGURE 5: P450 concentration-dependent changes in the rates of AA metabolism by the purified P450 [2C11−BM3OR] protein. P450 [2C11−BM3OR] was incubated at the indicated P450 2C11 concentrations with [1-^{14}C]AA (100 μM; 50 μCi/μmol) in the presence of NADPH (1 mM) and an NADPH-regenerating system for 10 min at 35 °C. Products were extracted, resolved, and quantified as described in Materials and Methods.

2C22/BM3OR proteins catalyze flavin−heme iron electron transfer via a combination of inter- and intramolecular pathways (*6*) and that the relative contribution of these pathways to overall catalytic turnover is enzyme concentration-dependent.

The capacity of the [2C11−BM3OR] enzyme to increase its catalytic activity in response to increasing concentrations of OR (Table 3), but not BM3OR (not shown), supports the idea that while there may be multiple P450−OR pathways for intermolecular electron transfer, intramolecular transfer in fused P450 2C11/reductase enzymes occurs throughout unique, fusion-dependent, P450-reductase interactions. Recent studies have indicated that the size of the linker connecting the heme and the flavoprotein domains of P450 BM3 is an important determinant of overall catalytic turnover (*31*, *48*); however, varying the length of this linker from 4 to 6 residues (*31*, *48*) had no effect on the AA monooxygenase activity of the [2C11−BM3OR] enzyme (not shown).

H_2O_2 Formation. To investigate the degree of P450 2C11 monooxygenase coupling and the characteristics of the

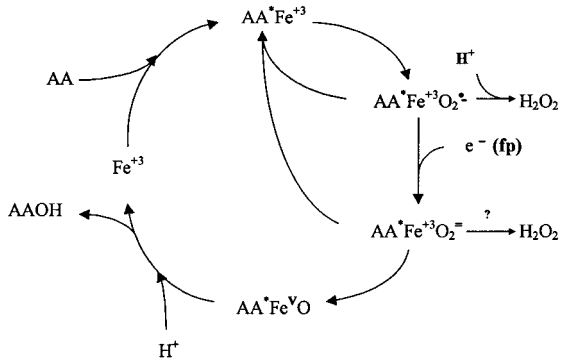

FIGURE 6: Schematic representation of the catalytic cycle followed by P450 2C11 fusion proteins during the metabolism of AA. For simplicity, a single resonance form is shown for the heme iron−oxygen complexes described. *Fe, the heme iron of fusion proteins; AA*Fe, the AA-bound form of fused P450 2C11; *$Fe^{3+}O_2^-$, the oxy complex of fused P450 2C11; *$Fe^{3+}O_2^{2-}$, the peroxy complex of fused P450 2C11; *$Fe^{V}O$, the oxo complex of fused P450 2C11; fp, nonfused flavoprotein.

NADPH/O_2 electron flow catalyzed by reconstituted and fused protein systems, we measured their rates of NADPH-dependent H_2O_2 formation. The capacity of several P450 isoforms to catalyze the NADPH-dependent formation of H_2O_2 has been demonstrated (*8*). As indicated in Figure 6, H_2O_2 can be generated as the result of either the direct enzyme-catalyzed two-electron reduction of O_2 or from the dismutation of P450-generated superoxide anion ($O_2^{\bullet-}$). Regardless of the mechanism, the P450-catalyzed, NADPH-dependent reduction of O_2 to H_2O_2 (NADPH oxidase reaction) diverts electrons from substrate oxidation (monooxygenase reaction) and changes the stoichiometry of NADPH oxidation, O_2 utilization, and substrate oxidation.

During earlier measurements, we observed the presence in the AA substrate of $(NH_4)_2Fe(SO_4)_2$/KSCN reactive substances. The concentration of these reactive substances was variable, NADPH-independent, and associated with the AA substrate batch. To control for substrate-associated hydroperoxides, all measurements were carried out in the presence and the absence of catalase. Only catalase-sensitive H_2O_2 levels are reported here. Initial rates of H_2O_2 formation were obtained within the first 2 min of incubation since, for all the recombinant proteins, the linearity between incubation time and product formation was lost rapidly thereafter. As it has been shown for several reconstituted systems (*8*), purified recombinant P450 2C11 reconstituted in the presence of an equimolar concentration of OR catalyzed the rapid, NADPH-dependent reduction of molecular oxygen to H_2O_2 (Table 5). Furthermore, H_2O_2 formation by the reconstituted P450 2C11 system was increased by the addition of AA (100 μM, final concentration) and more so by the presence of a 10-fold molar excess of OR (Table 5). We believe that these rate increases are due to, on one hand, substrate-binding induced changes in the heme iron redox potential (*49*, *50*) and, on the other hand, to OR-dependent mass action-induced changes in P450−OR interactions. Comparison of the data in Tables 3 and 5 shows that in the presence of AA, OR-dependent increases in H_2O_2 formation are accompanied by significant increases in rates of AA metabolism. In the presence of AA and a 10-fold molar excess of OR, P450 2C11 doubles its AA monooxygenase catalytic efficiency, as indicated by a significant decrease in the H_2O_2/AA metabolite ratio (Table 5). We propose that this results from

Table 5: Rates of OR-Dependent H_2O_2 Formation by Recombinant P450 Proteins[a]

enzyme system	2C11 + OR		[2C11−ORtr]	
OR/P450 molar ratio	1:1	10:1	1:1	10:1
minus AA	40 ± 9	142 ± 6	46 ± 2	64 ± 4
plus AA	55 ± 9	197 ± 8	45 ± 6	77 ± 2
H_2O_2/AA metabolites	15	6	45	3

[a] Enzymes were incubated with NADPH in the presence or in the absence of AA exactly as described in Table 2 and Materials and Methods. Catalase-sensitive H_2O_2 concentrations, determined from at least three different experiments, are given as nmol of H_2O_2 formed (nmol of P450)$^{-1}$ min^{-1} at 35 °C and are the averages ± SE.

Table 6: Rates of BM3OR-Dependent H_2O_2 Formation by Recombinant P450 Proteins[a]

enzyme system	2C11 + BM3OR		[2C11−BM3OR]	
BM3OR/P450 molar ratio:	1:1	10:1	1:1	10:1
minus AA	73 ± 10	131 ± 2	10 ± 1	100 ± 5
plus AA	142 ± 13	147 ± 6	13 ± 1	183 ± 9
H_2O_2/AA metabolites	NA[b]	NA	65	915

[a] Enzymes were incubated with NADPH in the presence or in the absence of AA exactly as described in Tables 2 and 5 and in Materials and Methods. Catalase-sensitive H_2O_2 concentrations, determined from at least three different experiments, are given as nmol of H_2O_2 formed (nmol of P450)$^{-1}$ min^{-1} at 35 °C and are the averages ± SE. [b] NA, not applicable.

an OR concentration-dependent increase in electron transfer to the one-electron reduced form of substrate and oxygen-bound P450 2C11 (the AA*$Fe^{3+}O_2^{\bullet-}$complex; Figure 6).

As compared with the reconstituted system, fusion to the ORtr did not significantly change the rate of H_2O_2 generation by P450 2C11 (Table 5). Furthermore, NADPH-dependent peroxide generation by the [2C11−ORtr] enzyme was substrate-independent and substantially less affected by the presence of exogenously added OR (Table 5). Again, comparison of the data in Tables 3 and 5 indicated that (a) fusion does not improve the catalytic efficiency of P450 2C11 for AA metabolism, that is, the [2C11−ORtr] protein diverts an important part of the NADPH-supplied electron to H_2O_2, as revealed by high H_2O_2 molar ratios (Table 5); (b) the lack of a substrate effect on the rates of H_2O_2 formation suggests that most of the peroxide formed is derived from the one-electron reduction of P450 and the dismutation of the $O_2^{\bullet-}$ released from the P450 2C11 oxy complex (*51*) ($Fe^{3+}O_2^{\bullet-}$, Figure 6); and (c) the marked increase in the efficiency of the AA monooxygenase observed after the addition of a 10-fold molar excess of OR to the [2C11−ORtr] protein (Table 5) suggests that the exogenously added OR facilitates the donation of the second electron to the substrate-bound oxy-P450 complex, (AA*$Fe^{3+}O_2^{\bullet-}$; Figure 6), the required step for the subsequent generation of the iron-bound oxidant species (AA*$Fe^{V}O$, Figure 6).

As discussed, the P450 2C11 AA monooxygenase could not be reconstituted in the presence of a 1:1 or a 10:1 molar excess of the bacterial flavoprotein reductase, the BM3OR. However, under similar conditions, the BM3OR was an effective P450 2C11 reductase and actively supported H_2O_2 formation by this enzyme in an AA and BM3OR concentration-dependent fashion (Table 6). When reconstituted with the BM3OR in a 1:1 molar ratio, P450 2C11 catalyzed H_2O_2 generation at nearly double the rate of the mammalian flavoprotein (∼1.8-fold faster) (Tables 5 and 6). As with the OR, the rates of H_2O_2 formation were increased by the addition of AA and a 10-molar excess of BM3OR (Table 6), reflecting AA-dependent changes in P450 redox potential and BM3OR mass action kinetic effects. The observation that a reconstituted system composed of P450 2C11 and P450 BM3OR actively catalyzes the NADPH-dependent reduction of O_2 to H_2O_2, but not AA oxidation, indicates that the bacterial flavoprotein supports the one-electron reduction of substrate-free or bound P450 2C11 but not that of the oxy complex of ferrous P450 2C11 (Figure 6) (*51*). The above interpretation implies that the BM3OR-supported P450 2C11-dependent formation of H_2O_2 results from the one-electron reduction of O_2 and $O_2^{\bullet-}$ dismutation (*51*).

Fusion of the BM3OR to P450 2C11 markedly reduced the rate of P450 2C11-dependent H_2O_2 formation by the [2C11−BM3OR] protein incubated in either the presence or the absence of AA (Table 6). As illustrated in Table 3, only after fusion was the BM3OR capable of supporting AA metabolism, albeit at rates lower that those of the [2C11−ORtr] enzyme. A comparison of the molar ratio between the H_2O_2 formed and the extent of AA metabolism (the H_2O_2/AA ratio) shows that the [2C11−ORtr] and [2C11−BM3OR] proteins metabolize AA with similar catalytic efficiencies (Tables 5 and 6). The addition of a 10-fold molar excess of BM3OR to the [2C11−BM3OR] protein resulted in a greatly stimulated rate of H_2O_2 formation (10-fold increase; Table 6), an effect that was further augmented by the inclusion of AA (100 μM, final concentration) (Table 6). Finally, and at difference with the OR (Table 3), the addition of exogenous BM3OR to incubates containing [2C11−BM3OR] and AA did not alter the rates of AA metabolism by the [2C11−BM3OR] protein but led to high rates of H_2O_2 formation and a low catalytic efficiency (Table 6). Taken together, these experiments showed that (a) fusion reduces the rate at which the BM3OR transfers the first electron to the heme iron of substrate-bound or substrate-free P450 2C11 (Figure 6). However, fusion is required for the BM3OR-catalyzed transfer of the second electron to the AA-bound oxy-P450 complex, oxygen activation, and metabolism; (b) as with the reconstituted system, exogenously added (nonfused) BM3OR actively catalyzes the one-electron reduction of the [2C11−BM3OR] heme iron and H_2O_2 formation via superoxide dismutation; and (c) through unique conformational and/or changes in redox properties, fusion of the N-terminal of BM3OR to the carboxyl-end of P450 2C11 opens a catalytically productive pathway of electron flow to the P450. This presumably intramolecular pathway of electron transfer is not functional when the purified BM3OR is incubated with either the P450 2C11 or the [2C11−BM3OR] proteins.

The mechanisms of H_2O_2 formation by these proteins, that is, P450 2C11-dependent one- or two-electron reduction of O_2 (to either $O_2^{\bullet-}$ or O_2^{2-} **)** remain to be established; however, a one-electron reduction, $O_2^{\bullet-}$-mediated pathway for the P450-catalyzed formation of H_2O_2 has been proposed (*51*). Nevertheless, regardless of the nature of the immediate H_2O_2 precursor, the observed high levels of NADPH oxidase activity support the idea that, for all the three enzyme systems studied, reduction of the oxy-P450 complex (*51*) and/or oxygen insertion into the substrate carbon template are the rate-limiting steps during AA monooxygenation. Additional

Table 7: Effects of Catalase and SOD in Arachidonic Acid Epoxidation by Recombinant [2C11−ORtr][a]

conditions	epoxygenase activity[b]	(% distribution) 14,15-EET	11,12-EET	8,9- and 5,6-EET
control	70	44	35	21
+ SOD	68	43	37	20
+ CAT	70	47	34	19
SOD + CAT	65	45	37	18

[a] SOD and catalase (5 U/mL, final concentrations) were added to the incubates prior to the addition of AA and NADPH. Values shown are from one representative experiment. Approximately 30% of the total reaction products eluted with RP-HPLC retention times similar to those of HETE mixtures and remain uncharacterized. [b] Epoxygenase is the activity of P450 2C11 that leads to formation of EETs. The epoxygenase activity is expressed as percent of the total organic soluble products.

supporting evidence for the above was provided by the fact that for all the enzymes studied, the addition of a 10-molar excess of purified OR led to an increased catalytic efficiency, as determined by decreased H_2O_2/AA metabolite molar ratios.

Figure 6 integrates the above ideas into our current understanding of the P450 catalytic cycle. AA binding to fused or nonfused P450 2C11 is followed by rapid heme iron reduction and oxygen binding to generate the oxy-P450 complex ($AA^*Fe^{3+}O_2^{\bullet-}$) (*51*). At this point, the oxy-P450 complex partitions between release of $O_2^{\bullet-}$ and AA bound enzyme or its enzymatic reduction to the peroxy-P450 complex ($AA^*Fe^{3+}O_2^{2-}$) (Figure 6). As indicated above, the high rates of H_2O_2 formation by these P450 2C11 proteins indicate that reduction of the $AA^*Fe^{3+}O_2^{\bullet-}$ complex is the rate limiting step for AA oxygenation. We believe that the principal effect of an excess OR is to increase catalytic turnover by electron transfer to the oxy-complex. This reductive step is likely to follow a molecular path distinct from that utilized by the fused flavoproteins during the donation of the first electron. Finally, and as with most P450s, protonation and oxygen−oxygen bond excision leads to the release of H_2O, product and the resting enzyme (Figure 6).

High rates of H_2O_2 generation catalyzed by the fused and nonfused recombinant proteins raised the possibility of a P450-active site independent oxidation of AA by reactive oxygen species. To investigate the roles that high levels of H_2O_2 could play in the oxygenation of AA and the chemistry of its products, we incubated the [2C11−ORtr] protein with AA and NADPH in the presence or absence of SOD, catalase, or a mixture of SOD and catalase. Product extraction and analysis demonstrated that these enzymes had no significant effects on either the turnover rate or the chemistry of the reaction products (Table 7), confirming that AA metabolism requires a P450-active site controlled delivery of the reactive oxygen species.

Our results show that the overall AA monooxygenase activities of the fused proteins more or less mimic those of the reconstituted system composed of P450 2C11, OR, and DLPC. A unique exception is that of the BM3OR, in which the bacterial reductase actively catalyzes the one-electron reduction of the substrate-free or bound heme iron but is unable to support AA metabolism by P450 2C11. Crystallographic and NMR studies have suggested that substrate binding and one-electron reduction of the heme iron of P450 BM3 result in significant changes in protein conformation (*15*, *52*−*54*). These changes included a partial closing of the substrate access channel (*54*) and a movement of the substrate oxygen acceptor carbon atom toward the enzyme's heme iron center (*52*). These studies indicate the possibility that electron transfer within the BM3OR−P450 BM3 complex may be controlled by a combination of redox and conformational factors and that the role of these factors may differ during the first and second electron donation steps. It is of interest that fusion is required for electron transfer to P450 2C11 by the BM3OR only after the one-electron reduction of the iron and that it is not required to facilitate productive redox interactions between otherwise incompatible membrane-bound and soluble proteins.

In summary, these studies utilized P450 2C11 fusion constructs to characterize the enzymology of electron transfer and AA catalytic turnover between the major 2C isoform in rat liver and the P450 oxido reductases of mammalian and bacterial origin. They indicate the potential for multiple and distinct interactions between P450 and its flavoprotein oxidoreductase that result in productive catalytic turnover. We have also documented, for the first time, the capacity of the bacterial BM3OR to interact with a mammalian P450 isoform in a redox productive fashion to (a) actively support the NADPH-dependent reduction of the heme iron of P450 2C11 and of molecular oxygen and the generation of H_2O_2 and (b) reconstitute, in a fusion-dependent fashion, the AA monooxygenase activity of P450 2C11 to products that are structurally identical to those generated by its mammalian counterpart. These results raise the interesting possibility that the pathways of electron flow from NADPH to P450 2C11 are multiple and determined by unique P450−OR molecular interactions. These interactions appear to be highly dependent on the redox status of the two P450/substrate/oxygen electron acceptor complexes that are generated during the enzyme's catalytic cycle.

Finally, the data also demonstrate the feasibility of designing and constructing 2C AA epoxygenases in which catalytic turnover is only NADPH- and lipid-dependent. Published data have demonstrated that this type of an approach can also be successfully applied to the rat P450 4A isoforms for the generation of self-sufficient AA ω/ω-1 hydroxylases (*9*). We have recently engineered P450 BM3 to function as a regio- and enantioselective 14(*S*),15(*R*)-epoxygenase and utilized it for studies of the role of this epoxygenase in cell physiology (*55*). However, the soluble nature and the narrow catalytic versatility of this bacterial enzyme limit its usefulness as a general tool for cell transfection studies. We expect that the approach documented here will permit the extension of these studies to include additional self-contained epoxygenases of mammalian origin.

REFERENCES

1. Makita, K., Falck, J. R., and Capdevila, J. H. (1996) *FASEB J. 10*, 1456−1463.
2. Kagawa, N., and Waterman, M. R. (1995) in *Cytochrome P450: Structure and Biochemistry* (de Montellano, P. O., Ed.) pp 419−442, Plenum Press, New York.
3. Fu, O. K., Portale, A. A., and Miller, W. L. (1997) *DNA Cell Biol. 16*, 1499−1507.
4. Li, J., Nagpal, P., Vitart, V., McMorris, T. C., and Chory, J. (1996) *Science 272*, 398−401.

5. Murakami, H., Yabusaki, Y., Sakaki, M., and Ohkawa, H. (1987) *DNA 6*, 189−197.
6. Shet, M. S., Fisher, C. W., Arlotto, M. P., Shackleton, C. H., Holmans, P. L., Martin-Wixtrom, C. A., Saeki, Y., and Estabrook R. W. (1994) *Arch. Biochem. Biophys. 311*, 402−417.
7. Fisher, C. W., Shet, M. S., and Estabrook, R. W. (1996) *Methods Enzymol*. *272*, 15−25.
8. Shet, M. S., Faulkner, K. M., Holmans, P. L., Fisher, C. W., and Estabrook R. W. (1995) *Arch. Biochem. Biophys*. *318*, 314−321.
9. Fisher, C. W., Shet, M. S., Claudle, D. L., Martin-Wixtrom, C. A., and Estabrook (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10817−10821.
10. Narhi, L. O., and Fulco, A. J. (1986) *J. Biol. Chem*. *261*, 7160−7169.
11. Wen, L. P., and Fulco A. J. (1987) *J. Biol. Chem*. *262*, 6676−6682.
12. Graham-Lorence, S., Truan, G., Peterson, J. A., Falck, J. R., Wei, S., Helvig, C., and Capdevila, J. H. (1997) *J. Biol. Chem*. *272*, 1127−1135.
13. Miura, Y., and Fulco, A. J. (1975) *Biochim. Biophys. Acta 388*, 305−317.
14. Ho, P. P., and Fulco A. J. (1976) *Biochim. Biophys. Acta 431*, 249−256.
15. Ravichandran, K. G., Boddupalli, S. S., Hasermann, C. A., Peterson, J. A., and Deisenhofer, J. (1993) *Science 261*, 731−736.
16. McMillan, K., Bredt, D. S., Hirsch, D. J., Snyder, S. H., Clarck, J. E., and Master, B. S. I. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11141−11145.
17. Geller, D. A., Lowenstein, C. J., Shapiro, R. A., Nussler, A. K., DiSilvio, M., Wang S. C., Nakayama, O. K., Simmons, R. L, Snyder, S. H., and Billiar, T. R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 3491−3495.
18. Nakayama, N., Takemae, A., and Shoun H. (1996) *J. Biochem. 119*, 435−440.
19. Oliw, E. H., Bylund, J., and Herman, C. (1996) *Lipids 31*, 1003−1021.
20. MacGiff, J. C., Steinberg, M., and Quilley, J. (1996) *Trends Cardiovasc. Med*. *6*, 4-10.
21. Harder, D. R., Lange, A. R., Gebremedhin, D., Birks, E. K., and Roman, R. J. (1997) *J. Vasc. Res*. *32*, 79−92.
22. McGiff, J. C., and Quilley, J. (1999) *Am. J. Physiol. 277*, R607-R623.
23. Stec, D. E., Deng, A. Y., Rapp, J. P., and Roman, R. J. (1996) *Hypertension 27*, 564−568.
24. Karara, A., Makita, K., Jacobson, H. R., Falck, J. R., Guengerich, F. P., DuBois, R. N., and Capdevila, J. H. (1993) *J. Biol. Chem. 268*, 13565−13570.
25. Cederbaum, A. I. (1998) *Biofactors 8*, 93−96.
26. Guengerich, F. P., Dannan, G. A., Wright, S. T., Martin, M. V., and Kaminsky, L. S. (1982) *Biochemistry 21*, 6019−6030.
27. Gillam, E. M., Baba, T., Kim, B. R., Ohmori, S., and Guengerich, F. P. (1993) *Arch. Biochem. Biophys. 305*, 123−131.
28. Gillam, E. M., Guo, Z., Ueng, Y. F., Yamazaki, H., Cock, I., Reilly, P. E., Hooper, W. D., and Guengerich, F. P. (1995) *Arch. Biochem. Biophys. 317*, 374−384.
29. Boddupali, S. S., Oster, T., Estabroock, R. W., and Peterson, J. A. (1992) *J. Biol.* Chem. *267*, 10375−10380.
30. Porter, T. D., and Kasper, C. B. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 973−977.
31. Govindaraj, S., and Poulos, T. L. (1995) *Biochemistry 34*, 11221−11226.
32. Ruettinger, R. T., Wen, L. P., and Fulco, A. J. (1989) *J. Biol. Chem*. *264*, 10987−10995.
33. Bauer, S., and Shiloach, J. (1974) *Biotechnol. Bioeng*. *16*, 933−941.
34. Omura, T., and Sato, R. (1964) *J. Biol. Chem*. *239*, 2370−2378.
35. Capdevila, J. H., Falck, J. R., Dishman, E., and Karara, A. (1990) *Methods Enzymol*. *187*, 385−394.
36. Capdevila, J. H., Wei, S., Yan, Y., Karara, A., Jacobson, H. R., Falk, J. R., Guengerich, F. P., and Dubois, R. N. (1992) *J. Biol. Chem. 267*, 21720−21726.
37. Hildebrandt, A. G., and Roots, I. (1975) *Arch. Biochem. Biophys. 171***,** 385−397.
38. Barnes, H. J., Arlotto, M. P., and Waterman, M. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 5597−5601.
39. Barnes, H. (1996) *Methods Enzymol*. *272*, 3−14.
40. Pernecky, S. J., and Coon M. J. (1996) *Methods Enzymol*. *272*, 25−34.
41. Jenkins, C. M., and Waterman, M. R. (1998) *Biochemistry 37*, 6106−6113.
42. Helvig, C., Dishman, E., and Capdevila, J. H. (1998) *Biochemistry 37*, 12546−12558.
43. Shet, M. S., Fisher, C. W., Holmans, P. L, and Estabrook R. W. (1996) *Arch. Biochem. Biophys. 330*, 199−208.
44. Lu, A. Y. H., Junk, K. W., and Coon, M. J. (1969) *J. Biol. Chem. 244*, 3714−3721.
45. Black, S. D., and Coon, M. J. (1982) *J. Biol. Chem*. *257*, 5929−5938.
46. Parikh, A., and Guengerich, F. P. (1997) *Protein Expression Purif*. *3*, 346−354.
47. Chaurasia, C. S., Alterman, M. A., Lu, P., and Hanziik, P. P. (1995) *Arch. Biochem. Biophys. 317*, 161−169.
48. Govindaraj, S., and Poulos, T. L. (1996) *Protein Sci. 5*, 1389−1393.
49. Gunsalus, I. C., Meeks, J. R., Lipscomb, J. D., Debrunner, P., and Munck, E. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., Ed.) Chapter 14, pp 559−613, Academic Press, New York.
50. Makino, R., Iizuka, T., Sakaguchi, K., and Ishimura, Y. (1982) in *Oxygenase and Oxygen Metabolism* (Nozaki, M., Yamamoto, S., Ishimura, Y., Coon, M. J., Ernster, L., and Estabrook, R. W., Eds.) pp 467−477, Academic Press, New York.
51. Loida, P. J., and Sligar, S. G. (1993) *Biochemistry 32*, 11530−11538.
52. Li, H., and Poulos, T. L. (1997) *Nat. Struct. Biol*. *4*, 140−146.
53. Li, H. Y., and Poulos, T. L. (1995) *Acta Crystallogr. D51*, 21−32.
54. Modi, S., Primrose, W. U., Boyle, J. M. B., Gibson, C. F., Lian, L.-Y., and Roberts, G. C. K. (1995) *Biochemistry 4*, 8982−8988.
55. Chen, J. K., Wang, D. W., Falck, J. R., Capdevila, J., and Harris, R. C. (1999) *J. Biol. Chem*. *274*, 4764−4769.

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