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Self-Sufficient Catalytic System of Human Cytochrome P450 4A11 and NADPH-P450 Reductase

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Abstract − The human cytochrome P450 4A11 is the major monooxygenase to oxidize the fatty acids and arachidonic acid. The production of 20-hydroxyeicosatetraenoic acid by P450 4A11 has been implicated in the regulation of vascular tone and blood pressure. Oxidation reaction by P450 4A11 requires its reduction partners, NADPH-P450 reductase (NPR). We report the functional expression in *Escherichia coli* of bicistronic constructs consisting of P450 4A11 encoded by the first cistron and the electron donor protein, NPR by the second. Typical P450 expression levels of wild type and several N-terminal modified mutants was observed in culture media and prepared membrane fractions. The expression of functional NPR in the constructed P450 4A11: NPR bicistronic system was clearly verified by reduction of nitroblue tetrazolium. Membrane preparation containing P450 4A11 and NPR efficiently oxidized lauric acid mainly to ω-hydroxylauric acid. Bicistronic coexpression of P450 4A11 and NPR in *E. coli* cells can be extended toward identification of novel drug metabolites or therapeutic agents involved in P450 4A11 dependent signal pathways.

Keywords: P450 4A11, Bicistronic expression, Lauric acid, Arachidonic acid

INTRODUCTION

The cytochrome P450 (CYP or P450) are the superfamily of heme-containing monooxygenase enzymes, found throughout all nature including mammals, plants, and microorganisms (Guengerich, 2002). Mammalian P450 enzymes are involved in oxidative metabolism of a wide range of endo- and exogenous chemicals (Ortiz de Montellano, 2005). The broad substrate specificity of these enzymes includes steroids, eicosanoids, pharmaceuticals, carcinogens, pesticides, and pollutants (Guengerich, 2008).

The cytochrome P450 4 enzymes catalyze the hydroxylation of saturated and unsaturated fatty acids of various chain lengths (Ortiz de Montellano, 2008). These enzymes possess a high preference for oxidation of the terminal carbon of fatty acid chain (Hoch *et al*., 2000; Baer and Rettie, 2006). In mammals, the terminal hydroxylation provides a means to avoid potentially toxic level of excess free fatty acids and is the first step in the formation of dicarboxylic acids for peroximal β-oxidation (Hsu *et al*., 2007). Importantly, the cytochrome P450 4 family enzymes also provide pathways for the degradation of signaling molecules such as prostanoids and leukotriens, as well as for the production of bioactive metabolites from arachidonic acid such as 20-hydroyeicosatetraenoic acid (20-HETE) (Capdevila *et al*., 2000).

The prosthetic heme group in the cytochrome P450 4 family enzymes is covalently attached to the glutamic acid residue of I-helix (LeBrun *et al*., 2002). The role of this covalent heme bond in 4 family enzymes was proposed to be a strategy to accommodate a constricted access channel in the enzymes to the ferryl species that disfavors presentation of any other than the terminal methyl oxidation (CaJacob *et al*., 1988). However, the physiological relevance of the heme-covalent bond in 4 family enzymes is still unclear (Ortiz de Montellano, 2008). In addition, it should be noted that covalent heme binding is not a prerequisite for high terminal hydroxylation regiospecificity (Ortiz de Montellano, 2008). *Candida albicans* CYP52A21, which does not bind its prosthetic heme covalently, cata-

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lyzes the terminal hydroxylation of lauric acid predominantly (Kim *et al*., 2007).

Many metabolism studies of mammalian P450 enzymes employ the recombinant P450 expression in bacterial system due to the low costs, facility of handling, and high enzyme yields (Guengerich *et al*., 1996). However, mammalian P450 enzymes are integral membrane proteins and require an NADPH-P450 reductase (NPR), a 78 kD flavoprotein, to deliver NADPH for reduction of the heme iron during the enzymatic cycle (Guengerich, 1993). Therefore, a bicistonic system to co-express P450 and NPR independently in the inner membrane of *Escherichia coli* has been popular for the analysis of P450 dependent drug metabolism and carcinogenesis (Parikh *et al*., 1997). In this study, we constructed a bicistronic expression system including human P450 4A11 and NADPH-P450 reductase in *E. coli* and demonstrated its specific catalytic activity. This study can provide the system close to the physiological condition to elucidate the enzymatic role of human P450 4A11.

MATERIALS AND METHODS

Chemicals

Lauric acid, sodium dithionite, nitroblue tetrazolium, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and $NADP^+$ were purchased from Sigma (St. Louis, MO) or Aldrich Chemical Co. (Milwaukee, WI). N,O-bis (trimethylsilyl) trifluoroacetamide was purchased from Pierce (Rockford, IL).

Construction of P450 4A11 bicistronic expression plasmids

The pBL plasmid including the human cytochrome P450 4A11 gene was kindly provided by Prof. Jorge H. Capdevila at Vanderbilt University Medical School. The open reading frame for P450 4A11, and an added 6× His-C-terminal tag, were amplified using PCR (forward-primer: agtccagtccagcatatgagtgtctctgtgctgagccccagcagactcctggg, reverse-primer: agtccagtccagtctagattagtgatggtgatggtgatgaagctggtccttgtcttcac). The 1.5 kb amplified PCR fragment was purified by agarose gel electrophoresis (Fig. 1) and cloned into the pCW (Ori^+) bicistronic vector (containing NADPH-P450 reductase gene) using the *NdeI* and *XbaI* restriction sites as previously described (Kim and Guengerich, 2004). The ligation mixture was transformed into *Escherichia coli* DH10b ultra competent cells (Invitrogen, Carlsbad, CA) and then the cloned pCW bicistronic plamid DNA was purified using a Promega Wizard miniprep kit (Promega, Madison, WI).

Construction of human P450 4A11 N-terminal modified mutants

N-terminus of P450 4A11 was modified to increase the expression level of P450 4A11 holoenzyme as described previously (Dong *et al*., 1996). Second codon (Ser) of a native P450 4A11 (4A11-N) gene was mutated to Ala

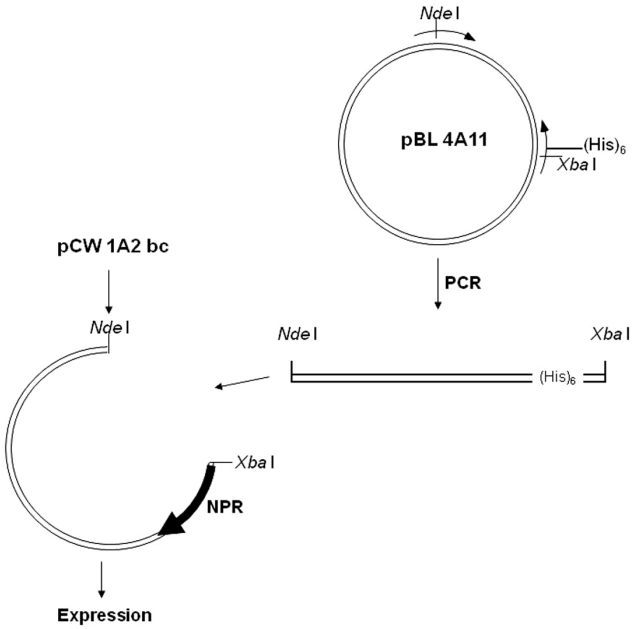

Fig. 1. Generalized construction scheme for P450 4A11:NPR bicistronic expression plamid. The open reading frame for P450 4A11, and an added 6×His-C-terminal tag, were amplified from pBL4A11 and cloned into pCW1A2bc plasmid truncated with P450 1A2 region.

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4A11-N:  MSVSVLSPSRLLGDVSGILQAASLLILLLLLIKAVQLYLHRQWL
4A11-A:  MAVSVLSPSRLLGDVSGILQAASLLILLLLLIKAVQLYLHRQWL
4A11-L:                  MALLLAVFKAVQLYLHRQWL
4A11-S:                      MAKAVQLYLHRQWL
4A11-M:                MALLLAVFLLLLLIKAVQLYLHRQWL
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Fig. 2. N-terminal amino acid sequences of constructed P450 4A11 bicistronic clones. 4A11-N, native P450 4A11 gene; 4A11-A, Second codon (Ser) of a native P450 4A11 gene was mutated to Ala; 4A11-S; the putative membrane anchoring Leu-rich region was truncated, 4A11-L, and 4A11-M; bovine P450 N-terminal sequence (MALLLAVF) was incorporated .

(4A11-A), or the putative membrane anchoring Leu-rich region was truncated (4A11-S) and bovine P450 N-terminal sequence (MALLLAVF) was incorporated (4A11-L, 4A11-M) (Fig. 2) (Clark and Waterman, 1992).

Expression and membrane preparation

Expression and purification of human P450 4A11 protein were carried out as previously described with some modifications (Kim and Guengerich, 2004; Chae *et al.,* 2008). The *E. coli* strains DH5α transformed with pCW 4A11:NPR bicistronic vectors were inoculated into TB medium containing 100 μg/ ml ampicillin and 1.0 mM IPTG. The expression cultures were grown at 37°C for 3 h and then at 28°C with shaking at 200 rpm in 1 liter Fernbach flasks. Bacterial inner membranes containing P450 4A11 and NADPH-P450 reductase were isolated from 1 L TB/ampicillin (100 g ml^{-1}) expression cultures of *E. coli* DH5α (Gillam *et al*., 1995). The 105 g pellet was resuspended in a final volume of 10 ml of 200 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v) and homogenized with a dounce homogenizer. The homogenized membrane fraction was stored on ice until analysis.

Spectroscopic characterization

Spectroscopic characterization has been previously described (Guengerich, 2001). Briefly, sodium dithionite was added to reduce the ferric P450 membrane fraction after dilution in 100 mM potassium phosphate buffer (pH 7.4). The CO-ferrous P450 complexes were generated by passing CO gas through solutions of the ferrous P450. UV-visible spectra from 400 to 500 nm were collected on a CARY 100 spectrophotometer at room temperature.

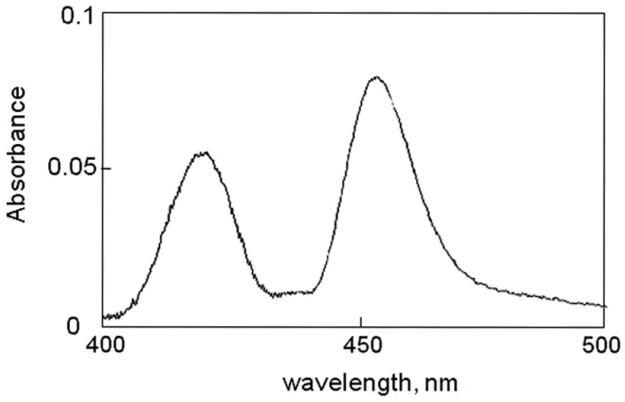

Fig. 3. $Fe2^{+}$ vs Fe^{2+}•CO difference spectrum of a P450 4A11:NPR bicistronic membrane preparation. Spectrum was recorded following 1:20 dilution of prepared P450 4A11:NPR bicistronic membrane fraction.

Nitroblue tetrazolium (NBT) assays

The reductase activity of NPR in *E. coli* whole cells was analyzed using NBT assays with some modification. Medium in 1 ml culture of whole cells was removed with centrifugation and 1 mM NADPH was added to initiate NBT assay. The increase in absorbance at 535 nm was monitored on a CARY 100 spectrophotometer at room temperature. Calculation of the specific activity for NPR assumed an absorption coefficient of 12.8 mM^{-1} cm^{-1} (Murphy *et al*., 1998).

Measurement of P450 4A11 oxidation activity by GC-MS

The reaction mixtures consisted of 0.10 M P450 in bicistronic membranes (including NPR), 100 mM potassium phosphate buffer (pH 7.4), and an NADPH-generating system [0.5 mM $NADP^{+}$, 10 mM glucose 6-phosphate, and 1.0 IU glucose 6-phosphate dehydrogenase ml^{-1}], and varying concentrations of lauric acid in a total volume of 0.50 ml. Incubations were done for 10 min at 37°C and terminated with addition of 0.5 ml of CH_2Cl_2. The CH_2Cl_2 extract was dried under N_2 and then converted to trimethylsilyl derivatives by incubation with 50 μl of *N,O-bis* (trimethylsilyl)trifluoroacetamide at 70°C for 10-15 min. The derivatized samples were allowed to cool, vortexed, and transferred to sealed Teflon-capped glass vials for either manual or autoinjection into the GC-MS. Analyses were performed on an Agilent 6890 gas chromatograph coupled to an Agilent 5973 Network Mass Selective Detector in the flame ionization mode as previously described (Kim *et al*., 2007)

RESULTS

Expression of P450 4A11 wild type and modified mutants

P450 expression levels of wild type and modified mutants were spectrally determined in the whole cell culture (Fig. 3). The maximum level of P450 holoenzyme was observed 48-60 hr. Recombinant protein of two truncated mutants (4A11-L, 4A11-S) were not expressed while

Table I. Time-dependent expression of P450 4A11 holoenzyme in bicistronic system

Time (h)	24	36	48	60	72
4A11-N	59	79	101	91	57
4A11-A	13	21	64	67	52
4A11-L	ND	ND	ND	ND	ND
4A11-S	ND	ND	ND	ND	ND
4A11-M	76	134	150	94	ND

nmol P450 holoenzyme per liter culture.

4A11-M clone (including Leu-rich region following the bovine P450 sequence) showed the maximal expression level of 150 nmol P450 holoenzyme per liter culture medium (Table I). The typical expression of native form of P450 4A11 was observed with 100 nmol P450 holoenzyme per liter culture medium (Table I). The reductase activity of NPR in the bicistronic system was verified by the reduction of nitroblue tetrazolium. A clear reduction activity in *E. coli* whole cells containing bicistronic operons was observed but no color change in the control cells including pCW' (Ori^+) vector (Fig. 4). The calculated reductase activity of NPR was about 7.6 nmol reduced NBT/min/ml whole cell culture. These results confirm the clear expression of functional NPR in the constructed system.

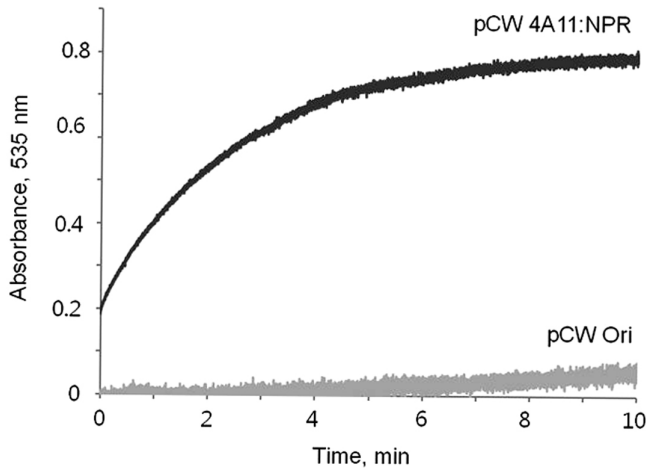

Fig. 4. Activity measurement of NPR in P450 4A11:NPR bicistronic expression system. After 1 mM NADPH was added to the sample cuvette, the absorbance at 533 nm was monitored until there was no further increase.

Lauric acid hydroxylation activities of membrane preparation containing P450 4A11 and NADPH-P450 reductase

Characterization of the constructed P450 4A11 and NPR bicistronic system was done with membrane preparations. The bicistronic membrane preparation catalyzed the oxidation reaction of the lauric acid (Fig. 5). The major product in the reaction is ω-hydroxylated lauric acid (11.78 min) and the minor product, (ω-1)-hydroxylated lauric acid (10.85 min) is negligible, which is similar to the reaction production pattern by purified P450 4A11 reconstitution system previously reported (Hoch *et al*., 2000).

Steady-state kinetics of P450 4A11

The substrate concentration-dependent oxidation of lauric acid is shown Fig. 6. Michaelis-Menten equation was used to obtain the data fitting. The k_{cat} value was approximately 34 min^{-1} with a calculated K_m value of 12.5 μM (Fig. 6).

DISCUSSION

In this study, we used a bicistronic system that produces human P450 4A11 and an auxiliary protein, NPR, from a single mRNA. The coding region for the two proteins was connected by the T7 gene leader including a Shine-Delgarno ribosomal binding sequence. Expression levels of various N-terminal modified P450 4A11 enzymes ranges from 50-150 nmol P450 holoenzyme per liter culture. Expression of any P450 holoenzyme was not observed in two clones with truncation of poly-Leu region at

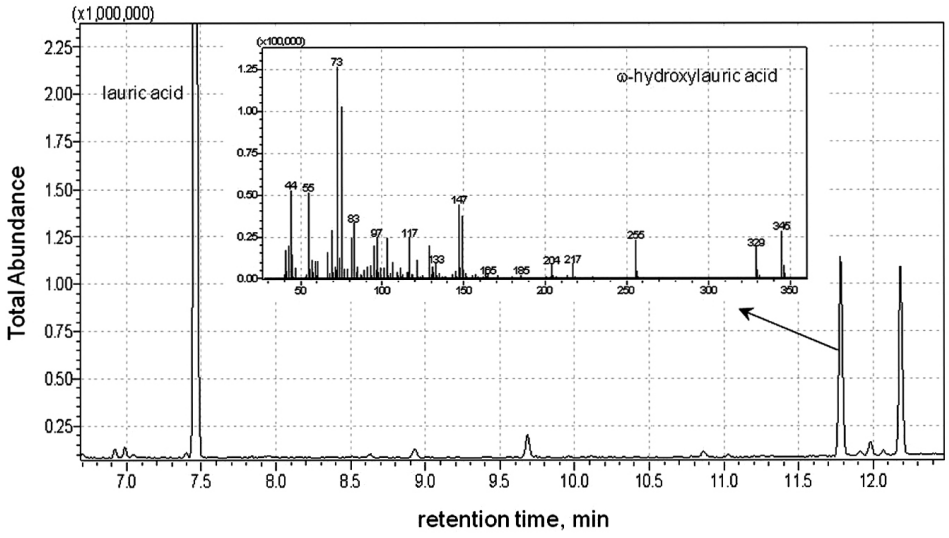

Fig. 5. GC-MS analysis of lauric acid hydroxylation by P450 4A11:NPR bicistronic system.

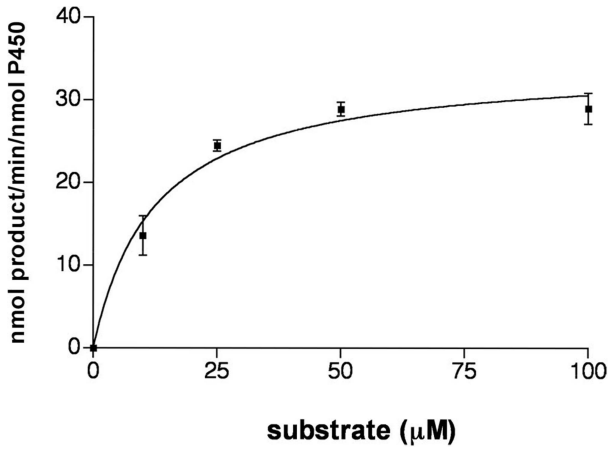

Fig. 6. Steady-state kinetics of lauric acid hydroxylation by P450 4A11:NPR bicistronic membrane. Each point presented is a mean ± SD of triplicate assays. The estimated parameters were k_{cat} 34 ± 2 nmol of total hydroxylated lauric acid formed/min/nmol of P450, Km 13 ± 3 μM, and k_{cat}/K_m 2.7 ± 0.6.

N-terminus. These results suggested that poly-Leu region in P450 4A11 may be necessary for the right conformational folding or location in membrane. Previously, Gillam *et al.* reported the similar expression patterns in various modified P450 2D6 clones (Gillam *et al*., 1995). P450 peaks of DB3 and DB7 (P450 2D6 enzymes shortened in the poly-Leu region) were too small to be quantified. Dong *et al.* reported the high level of P450 1A2 expressions in *E. coli* were obtained with addition of the specific N-terminal sequence (MALLLAF) (Dong *et al*., 1996). The same sequence modification applied to P450 4A11 substantially increased the expression yield of holoenzyme (Table I). The reduction assay of nitroblue tetrazolium verified the reductase activity in the bicistronic system (Fig. 4). A clear reduction activity in the bicistronic system containing two cistronic operons was observed but no color change in the control. Enzymatic activity of lauric acid hydroxylation (k_{cat} =34 min^{-1}) by the bicistronic membrane preparation was consistent with value from the reconstitution system previously reported (Hoch and Ortiz De Montellano, 2001). Theoretical ratio of P450 4A11 to NPR may be around 1:1 but this ratio is substantially higher with respect to NPR than 20:1 ratio measured in human liver microsomes, implying that electron transport is not rate-limiting (Parikh *et al*., 1997).

Metabolic products of arachidonic acid by human P450 4A11 have important physiological roles. The ω-hydroxylated product, 20-hydroxyeicosatetraenoic acid (20-HETE), is a vasoconstrictor (Ma *et al*., 1993; Imig and Navar, 1996), whereas the (ω-1) hydroxylated product, 19-HETE and some of the arachidonic acid epoxides are vasodilators (Zeldin, 2001). Therefore, the ω-hydroxylation regiospecificity of P450 4A11 is of critical physiological importance. In this study, P450 4A11 and NPR bicistronic system also predominantly hydroxylated the ω-position of the fatty acids (Fig. 5). Glu residue, which is involved in the heme-covalent binding in P450 4A11 enzymes, is responsible for the narrow architecture of active site in the protein to maintain the structure features required for ω-hydroxylation.

The bicistronic expression approach has advantages in the field of drug metabolism study (Parikh *et al*., 1997); the natural environment for the reconstitution of two proteins is accommodated and there is no need to purify the proteins for enzyme reactions. Pharmaceutical companies as well as academic laboratories have become interested in using P450-NPR membranes to probe drugs for potential routes of metabolism. Mutagenesis studies can provide information on the structure-function relationships of P450 proteins. However, it requires a high-throughput assay system including the complete reconstitution of P450, and NPR. Therefore, the development of this system presents the prospect of its use in the mutagenesis study for structure-function relationships. Human has 57 human P450s and many of them still remain “orphans” in the sense that their function, expression sites, and regulation are still largely not elucidated (Stark and Guengerich, 2007). Characterization of these orphan P450s can provide the unraveled roles of human P450 enzyme so far. However, it has been a challenge to establish a strategy for finding the substrates and metabolites of orphan P450s. NPR are known to be able to transfer electrons to most of orphan P450 enzymes. Therefore, the application of bicistronic systems in orphan human P450s could provide an alternative strategy to make the initial substrate screening possible.

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