BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **230,** 211–214 (1997)
ARTICLE NO. RC965918

Characterization of a Fusion Protein between Human Cytochrome P450 1A1 and Rat NADPH-P450 Oxidoreductase in *Escherichia coli*

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Received November 18, 1996

A cDNA of fusion protein between human cytochrome P450 1A1 and rat NADPH-P450 reductase was genetically engineered and expressed in *Escherichia coli* DH5α cells under the control of an inducible *tac* promoter (Y. J. Chun, T. Shimada, and F. P. Guengerich, (1996) *Arch. Biochem. Biophys.* 330, 48-58). ***E. coli* membranes of transformed cells showed much higher P450 1A1-dependent monooxygenase and NADPH-P450 reductase activities than pCW control vector or P450 1A1 expression vector-transformed cells. Ethoxyresorufin *O*-deethylase and methoxyresorufin *O*-demethylase were 22-fold and 11-fold higher than the control activity, respectively. α-Naphthoflavone and β-naphthoflavone strongly inhibited P450 1A1 activity of the fusion protein, with α-naphthoflavone being more potent than β-naphthoflavone. Divalent cations (e.g. Ca^{2+} and Mg^{2+}) increased P450 1A1 activity as well as NADPH-P450 reductase activity. These results demonstrate that this fusion protein in *E. coli* membrane may be a useful model for elucidating details of protein-protein interactions between P450 and NADPH-P450 reductase in the endoplasmic reticulum of mammalian cells.** © 1997 Academic Press

Cytochrome P450 enzymes are integral membrane proteins responsible for the metabolism of numerous endogenous and exogenous chemicals (1-3). Among various P450s, P450 1A1 has been of considerable interest because of its inducibility by several environmental toxicants and a possible relationship to human lung cancer (4,5). Human P450 1A1 is primarily expressed in extrahepatic tissues such as placenta, lung, and peripheral blood cells (6). Many polycyclic aromatic hydrocarbons seem to be substrates or inducers of human P450 1A1.

In order to study human drug metabolism, it is necessary to develop high-level P450 expression systems to obtain sufficient amounts of P450s. Heterologous P450 expression systems have been proved to be very important in P450 research (7). Among several other expression systems, bacterial expression systems using *Escherichia coli* cells provide a number of advantages in terms of high yield, low cost, and ease of handling (8-10). However, although the activity of some P450s can be supported by flavodoxin and NADPH-flavodoxin reductase in *E. coli,* electron transfer from reductase to P450 is much less efficient than with mammalian NADPH-P450 reductase (11,12). To solve this problem, fusion proteins between P450 and its reductase have been genetically engineered (10,13,14). Recently, an enzymatically active fusion protein between the cDNA for human P450 1A1 and rat NADPH-P450 reductase was expressed in *E. coli* (15). Some enzymatic properties of this fusion protein are characterized here using membranes of transformed cells.

MATERIALS AND METHODS

Materials. Restriction endonucleases were purchased from New England Biolab. Co. (Beverly, MA). Bacterial media for *E. coli* were from Difco (Detroit, MI). Resorufin and alkoxyresorufins, $NADP^+$, NADPH, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, δ-aminolevulinic acid, and isopropyl-1-thio-β-D-galactopyranoside (IPTG) were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of reagent grade and commercially available.

Construction and expression of fusion protein in E. coli. The procedure for construction and expression of fusion protein is described elsewhere (15). Briefly, the coding region of human cytochrome P450 1A1 (16) was fused with rat NADPH-P450 reductase coding region using a Ser-Thr dipeptide linker by PCR. The fusion plasmid was transformed into *E. coli* DH5α cells and ampicillin-resistant colonies were selected. The fusion plasmid was purified from these colonies,

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TABLE 1

P450-Dependent Monooxygenase and NADPH-Cytochrome *c* Reductase Activities of *E. coli* Membrane-Transformed Expression Vectors

Vector/activity[a]	Methoxyresorufin	Ethoxyresorufin	Pentoxyresorufin	Benzyloxyresorufin	NADPH cyt. *c* reductase
pCW	14 ± 1[b]	24 ± 2	16 ± 1	9 ± 1	4 ± 1
	(100)[c]	(100)	(100)	(100)	(100)
1A1	25 ± 9	40 ± 8	24 ± 3	22 ± 5	3 ± 1
	(180)	(170)	(150)	(240)	(75)
1A1OR	158 ± 2	515 ± 16	35 ± 6	42 ± 2	830 ± 86
	(1100)	(2200)	(220)	(470)	(21000)

[a] Enzyme activities were measured as described in Materials and Methods.
[b] Results represent means ± SD of triplicate determinations.
[c] The values in parentheses represent % compared with pCW-transformed cells.

and the size of plamid was confirmed by restriction analysis. Positive colonies were grown for 48 h at 28 °C at 125 rpm in Terrific Broth containing 0.2% bactopeptone (w/v), ampicillin (100 μg/ml), 1.0 mM thiamine, 0.5 mM δ-aminolevulinic acid, and trace elements (17). Induction of *tac* promoter was done with 1.0 mM IPTG. Subsequent membrane preparation was performed as described previously (18).

Enzyme assays. Methoxy-, ethoxy-, pentoxy-, and benzyloxyresorufins were used as substrates to determine P450-dependent monooxygenase activities (19). Briefly, the reaction mixture (2 ml) consisted of 0.1 M potassium phosphate buffer, pH 7.4, containing 2 mg of bovine serum albumin/ml, 10 μM dicumarol, 5 mM glucose 6-phosphate, 1 U of glucose 6-phosphate dehydrogenase, 5 μM NADPH, and 2 μM substrate (20). Microsomal P450 content was measured by the method of Omura and Sato (21). NADPH-cytochrome *c* reductase activity was measured as a surrogate for NADPH-P450 reductase activity, as described previously (22). Protein concentrations were determined according to the method of Lowry using bovine serum albumin as a standard (23).

Kinetic analyses. Estimates of apparent *K*m and *V*max were obtained by graphic analysis of Lineweaver-Burk plots or by fitting the data to the Michaelis-Menten equation.

RESULTS AND DISCUSSION

We had successfully expressed human P450 1A1:rat NADPH-P450 reductase fusion protein in *E. coli* previously (15). Membranes containing expressed fusion protein showed much higher P450 activities as well as reductase activity than those of pCW control vector or pCW/1A1 P450 1A1 expression vector-transformed cells, including 7-ethoxyresorufin *O*-deethylase (EROD) and methoxyresorufin *O*-demethylase (MROD) (Table 1). EROD was 22-fold higher than control and MROD was 11-fold higher. However, the activities for P450 2B-specific monooxygenases such as pentoxyresorufin *O*-dealkylase or benzyloxyresorufin *O*-debenzylase were not much higher than control. Interestingly, NADPH-cytochrome *c* reductase activity was 210-fold greater than control. These results suggest that the P450 1A1 and reductase domains in the fusion protein exist in fully active forms in the membrane. Nerurkar *et al.* (24) have suggested that MROD may be selective for P450 1A2. However, human P450 1A1 also showed strong MROD activity (although the activity was 2-fold less than EROD activity). Thus, our present results indicated that MROD may not be selective for human P450 1A2. *K*m and *V*max value for EROD activity were 0.52 μM and 380 pmol/min/mg microsomal protein, respectively. For MROD activity, the apparent *K*m was 0.08 μM and the *V*max was 210 pmol/min/mg microsomal protein (Figure 1 and Table 2).

The effect of α-naphthoflavone (α-NF) has been demonstrated with both microsomes and purified P450 1A family enzymes (25,26). α-NF (5 μM) inhibited 70% of EROD activity of benzo[*a*]pyrene-induced mouse liver microsomes, and 53% of that of cDNA-expressed mouse P450 1A1 (27). The effects of α-NF and β-naphthoflavone (β-NF) were also investigated with the fusion protein in the present study (Table 3). Both chemicals decreased the EROD activity of fusion protein in a dose dependent manner, with α-NF a stronger inhibitor of P450 1A1; 74% of activity was inhibited by 50 nM α-NF, whereas 65% was inhibited by same concentration of β-NF. These results indicate that human P450 1A1 is about 100-fold more sensitive to benzoflavone inhibition than the mouse enzyme. Benzoflavones are quite interesting because they usually act as strong inhibitors of P450 1A1 *in vitro,* but can induce P450 1A1 gene expression when administered to experimental animals. How do benzoflavones inhibit P450 1A1 activity *in vitro?* Siess *et al.* (28) suggested that benzoflavones are competitive inhibitors of EROD activity. Bauer *et al.* (29) found that α-NF was rapidly oxidized to α-NF-5,6-oxide and α-NF-5,6-dihydrodiol by recombinant human P450 1A1. Although we do not have enough data for α-NF oxidation by P450 1A1 fusion protein, presumably the same metabolites are formed. Recently, we have reported that treatment with β-NF increases NADPH-P450 reductase activity of the fusion protein. Other P450 1A1 substrates, such as benzo[*a*]pyrene and 7-ethoxyresorufin, also increased reductase activity (30). Interestingly, β-NF enhances reductase activity of the NADPH-P450 reductase domain while

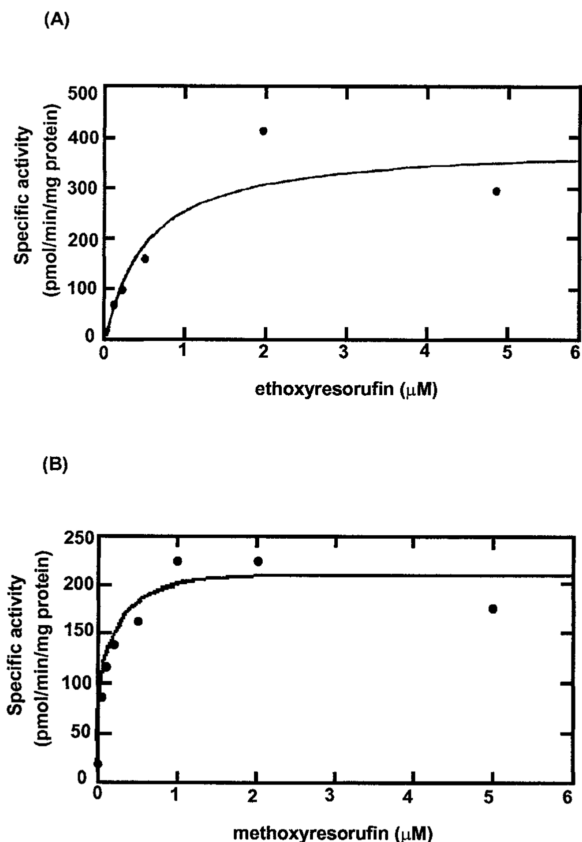

FIG. 1. Typical Michaelis-Menten plots for the metabolism of alkoxyresorufins in the presence of fusion protein. The reaction mixture (2 ml) consisted of 0.1 M potassium phosphate buffer, pH 7.4, containing 2 mg of bovine serum albumin/ml, 10 μM dicumarol, 5 mM glucose 6-phosphate, 1 U of glucose 6-phosphate dehydrogenase, 5 μM NADPH, and *E. coli* membranes (20 μg) with varying substrate concentrations (0-5 μM). (A) Ethoxyresorufin, (B) methoxyresorufin.

inhibiting P450 1A1 activity. Thus, it seems that benzoflavones and other substrates interact with fusion protein and change the structure of protein to increase electron transfer from NADPH-P450 reductase domain for enhancement of metabolism.

The effects of divalent cations, e.g. Ca^{2+} and Mg^{2+}, on reductase activity and P450 1A1 activity were determined (Table 4). Voznesensky and Schenkman (31) reported that the reduction of P450 1A2 in reconstitution system or β-NF-induced rabbit liver microsomes was facilitated when ionic strength was increased. Moreover, the reduction of several other purified P450s was also stimulated by increasing ionic strength. These results suggest that electrostatic forces may inhibit P450s in their interaction with the reductase. Because NADPH-P450 reductase and P450s contain many charged residues (32,33), electrostatic force may be important in formation of a stable protein structure. Divalent metal ions (Ca^{2+} and Mg^{2+}) stimulated reductase activity as well as EROD activity in a dose dependent manner. Ca^{2+} (20 mM) enhanced EROD activity 2.2-

TABLE 2

Apparent Kinetic Parameters for P450-Dependent Monooxygenases of Fusion Protein Expressed in *E. coli*

	Kinetic parameter[a]	
Substrate	K_m (μM)	V_{max} (pmol/min/mg protein)
Ethoxyresorufin	0.52[b]	380
Methoxyresorufin	0.08	210

[a] Apparent K_m and V_{max} were determined as described in Materials and Methods.
[b] Data represent means of triplicate determinations.

TABLE 3

Inhibition of EROD Activity of Fusion Protein Expressed in *E. coli* by Benzoflavones

Inhibitor (nM)	EROD activity (pmol/min/mg protein)	% Uninhibited activity
No additionl max (pmol/min/mg protein)	% Uninhibited activity	
No addition	448 ± 3[a]	100
α-Naphthoflavone		
5	351 ± 4	78
25	189 ± 25	42
50	116 ± 18	26
100	100 ± 13	22
β-Naphthoflavone		
5	415 ± 45	93
25	230 ± 24	51
50	157 ± 4	35
100	144 ± 16	32

[a] Results represent means ± SD for triplicate determinations.

TABLE 4

Effect of Divalent Cations on EROD and NADPH-Cytochrome *c* Reductase Activity of Fusion Protein in *E. coli* Membrane

Divalent cation	EROD activity (% of control)	NADPH-cyt *c* reductase (% of control)
Untreated control	100[a]	100
[Ca^{2+}]		
5 mM	120	120
10 mM	200	140
20 mM	220	210
[Mg^{2+}]		
5 mM	260	110
10 mM	320	200
20 mM	340	220

[a] Results represent means of triplicate determinations.

fold and reductase activity 2.1-fold. Mg^{2+} seems to have stronger effect than Ca^{2+}. The same concentration of Mg^{2+} activated EROD 3.4-fold and reductase activity 2.2-fold. We assumed that the effect of divalent cations on P450 1A1 activity is, at least partially, caused by increasing electron transfer from NADPH-P450 reductase by decreasing electrostatic forces. However, direct effect of divalent cations on P450 1A1 domain could not be ruled out so far. Yamazaki *et al.* (34) found that divalent cations increase P450 3A4 activity in a reconstituted system and demonstrated that high concentration of divalent cations may stimulate the transfer of electrons to or from cytochrome b_5 and that divalent cations stimulate electron transfer from NADPH-P450 reductase to several acceptors including P450 3A4. Charge repulsion may influence the interaction between cytochrome b_5, and NADPH-P450 reductase. In addition, electron transfer from the microsomal reductase to P450, cytochrome b_5 and cytochrome *c* was stimulated by divalent cations (35,36). However, in the case of P450 1A1, the role of cytochrome b_5 is negligible because electron transfer from reductase to acceptor was increased by divalent cations without cytochrome b_5. Cytochrome b_5 is not required for reconstitution of P450 1A1 activity with purified fusion protein (15), whereas many activities of the P450 3A4 fusion protein require the presence of cytochrome b_5 (13). Therefore, the differences in mechanisms between P450s should be considered.

In summary, we report some enzymatic properties of expressed fusion protein between human P450 1A1 and rat NADPH-P450 reductase in *E. coli.* This fusion protein system may be useful in elucidating protein-protein interactions in complex P450 systems.

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