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Temperature Effect on the Functional Expression of Human Cytochromes P450 2A6 and 2E1 in *Escherichia coli*

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Human cytochromes P450 (CYP) 2A6 and 2E1 are of great interest because of their important roles in the oxidation of numerous drugs and carcinogens. Bacterial expression systems, especially *Escherichia coli* cells, have been widely used for the production of various CYP enzymes in order to obtain high yield of proteins. The expression methods usually employ longer culture time (30-72 h) at lower temperature (usually under 30°C). Expression levels of CYPs 2A6 and 2E1 at 37°C were compared to those at 28°C, which is a usual temperature used in most bacterial expression systems for human CYP expression. Within 18 h the expression levels of CYPs 2A6 and 2E1 reached up to 360 and 560 nmol per liter culture at 37°C, respectively, which are compatible with those of 36 h culture at 28°C. The activities of CYPs expressed at 37°C were also comparable to those expressed at 28°C. The present overexpression system can be useful for rapid production of large amounts of active human CYPs 2A6 and 2E1 in *E. coli*.

Key words: Human CYP2A6, Human CYP2E1, CYP Expression, Temperature effect

INTRODUCTION

Cytochromes P450 (CYP or P450) are the major enzymes involved in the oxidation of xenobiotic chemicals and endogenous substrates (Wrighton and Stevens, 1992), and multiple forms of CYPs are present in mammals (Guengerich, 1995). Human CYPs 2A6 (Yamazaki *et al.*, 1992; Yun *et al.*, 1991) and 2E1 (Yamazaki *et al.*, 1992; Guengerich *et al.*, 1991) participate in the metabolism of a variety of compounds including the activation of potentially carcinogenic aryl and heterocyclic amines. These CYPs are also of considerable interest due to their relevance in progression of cancer (Fujieda *et al.*, 2004; Tsukino *et al.*, 2002; Oyama *et al.*, 1997).

Human CYP2A6 is a major member of the hepatic family of enzymes, that metabolizes pharmaceutical agents such as coumarin, methoxyflurane, halothane, losigamone, letrozole, valproic acid, disulfiram, and fadrozole, and activates some procarcinogens such as 4-methylnitrosoamino-1-(3-pyridyl)-1-butanone and *N*-nitrosodiethylamine (Oscarson, 2001). Especially, CYP2A6 is a major metabolic enzyme of nicotine and is suggested to be related to smoking behavior (Tricker, 2003).

CYP2E1 is a toxicologically relevant enzyme because of its unique ability to convert numerous substrates in the environment to cytotoxins (Lieber, 1997). CYP2E1 is involved in the activation of a wide variety of xenobiotics, including a number of protoxins and procarcinogens. More than 70 organic compounds such as alcohols, ketones, dialkyl-nitrosamines, ethylcarbamate, halogenated solvents or anesthetics (enflurane, halothane) are known to be metabolized by CYP2E1 to toxic materials (Guengerich, 1995; Lieber, 1997). CYP2E1 is mainly a hepatic enzyme, but it is also present in other tissues including lung, kidney, nasal mucosa, lymphocytes, and bone marrow (Raucy *et al.*, 1997; Roberts *et al.*, 1994).

Recombinant human CYP enzymes have been proven to be useful for drug metabolism research and thereby many heterologous expression systems have been devel-

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oped for several human CYPs (Guengerich and Parikh, 1997).

Expression of mammalian CYPs in *Escherichia coli* has proven to be a valuable means to characterize these enzymes and has also been widely used for the production of various CYP enzymes in order to obtain high yield of proteins. Moreover, to achieve maximal catalytic activity as well as protein expression, various strategies are employed such as co-expression of CYP with NADPH-P450 reductase (Iwata *et al.*, 1998), *N*-terminal modification (Pritchard *et al.*, 1997), and use of fusion proteins (Parikh and Guengerich, 1997). It has also been reported that the expression of CYP3A7 was elevated by the presence of molecular chaperone GroEL, known to assist the correct folding of proteins in *E. coli* (Inoue *et al.*, 2000). Recently, we have shown that human molecular chaperones, Hsp40 and Hsp70 can be used for rapid production of large amounts of active human CYPs 1A2 and 3A4 in *E. coli* (Ahn *et al.*, 2004a, 2004b; Ahn and Yun, 2004).

In the present study, we have examined the effect of temperature on the expression of human CYPs 2A6 and 2E1. It was observed that the culture at 37°C with shorter culture time (18 h) can achieve a satisfactory production level of the CYPs 2A6 and 2E1 with compatible catalytic activities, when compared to the culture at the lower temperature (28°C) with long culture time (36-48 h).

MATERIALS AND METHODS

Materials

Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, coumarin, chlorzoxazone, and $NADP^+$ were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant rat NADPH-P450 reductase was expressed in *E. coli* and was purified as described (Hanna *et al.*, 1998).

Expression of recombinant CYPs 2A6 and 2E1 and preparation of membranes

The cDNA for human CYPs 2A6 and 2E1 with *N*-terminal modification (Soucek, 1999; Gillam *et al.*, 1994), were kindly provided by Professor F. Peter Guengerich (Vanderbilt University, Nashville, TN, USA).

E. coli DH5aF'IQ cells were transformed with the expression plasmid. Transformed cells were grown in Terrific Broth (TB) containing 0.2% bactopeptone (w/v). TB was supplemented with 100 μg ampicillin/mL, 1.0 mM thiamine, trace elements (Sandhu *et al.*, 1993), 50 μM $FeCl_3$, 1.0 mM $MgCl_2$, and 2.5 mM $(NH_4)_2SO_4$. When the optical density of the culture at 600 nm was approximately 0.4~0.5, protein expression was induced by adding 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 1.5 mM δ-aminolevulinic acid (δ-ALA). At every 3 or 6 h, a portion of culture was sampled and expression level of CYP was quantitated by Fe^{2+}-CO *versus* Fe^{2+} difference spectra (Omura and Sato, 1964). Expression was allowed to proceed for 24 h at 37°C and for 54 h at 28°C, respectively. Bacterial membrane fractions were prepared as described previously (Dong *et al.*, 1996).

Enzymatic assay

Whole cells and membrane fractions were analyzed for expressed CYP levels by Fe^{2+}-CO *versus* Fe^{2+} difference spectroscopy in a Shimadzu UV-1650 PC spectrophotometer as per the reported procedure (Omura and Sato, 1964). Briefly, the reaction mixture comprised of 100 mM Tris-Cl, pH 7.4, containing 20% glycerol (v/v), 10 mM CHAPS (3-[(3-chloamidopropyl)dimethyl-ammonio]-1-propanesulfonate), and 1.0 mM Na-EDTA. The catalytic activities of CYPs 2A6 and 2E1 were measured in membrane fractions. Activities of coumarin 7-hydroxylation and chlorzoxazone 6-hydroxylation were determined for CYP2A6 (Yun *et al.*, 1991) and CYP2E1 (Peter *et al.*, 1990), respectively, as described. The activity assay was performed in 100 mM potassium phosphate buffer (pH 7.4). The reaction volume was 500 μL. Membrane fraction (0.40 μM CYP1A2) and NADPH-cytochrome P450 reductase (2.0 μM) were mixed in the presence of coumarin (20 μM) or chlorzoxazone (200 μM) as substrate. The reaction was started by the addition of NADPH-generating system. In the case of coumarin 7-hydroxylation assay, after incubating the sample at 37°C for 10 min, the reaction was terminated by the addition of 60 ml of 15% (w/v) aqueous CCl_3COOH and 1.0 mL of CH_2Cl_2. Aliquots (0.5 mL) of the organic extracts were then added to 3 mL of 30 mM sodium borate buffer (pH 9.0). After vortex mixing and centrifugation, the supernatants were analyzed fluorometrically (λ_{ex} of 358 nm and λ_{em} of 458 nm) and the readings were compared with those of 7-hydroxycoumarin standards (Yun *et al.*, 1991). For the chlorzoxazone 6-hydroxylation assay, after incubating the sample at 37°C for 10 min, the reaction was terminated by adding 50 μL of 43% (w/v) H_3PO_4 and 1.5 mL of CH_2Cl_2. After vortex mixing and centrifugation, the organic extracts were collected and desiccated under N_2 stream. The residues were analyzed by HPLC, as described (Peter *et al.*, 1990).

Other methods

Protein concentrations were estimated by following bicinchoninic acid procedure according to the manufacturer's directions (Pierce, Rockford, IL). Rabbit anti-human CYP2A6 or CYP2E1 was used as primary antibody in the immunoblotting experiments. The specificity and properties of similar preparations have been reported elsewhere (Yun *et al.*, 1991; Yamazaki *et al.*, 1996). The antiserum was adsorbed with *E. coli* proteins before use to remove any background staining.

RESULTS AND DISCUSSION

Several strategies for the expression of recombinant human CYPs in bacteria have been developed and are widely used (Guengerich and Parikh, 1997; Iwata *et al.*, 1998). Since human recombinant CYP17 was expressed in *E. coli* at 28°C (Barnes *et al.*, 1991), expression of recombinant CYPs have been performed at lower temperature, usually lower than 30°C. However, we found that expression of human recombinant CYPs 1A2 (Ahn *et al.*, 2004a, 2004b) and 3A4 (Ahn and Yun, 2004) could produce active CYPs within shorter culture time. We could observe the CO-spectrum within 3 h culture time at 37°C for the both enzymes. In the present study, we have examined the effect of temperature on the expression of human CYPs 2A6 and 2E1.

The induction time-dependent expression levels of recombinant CYPs 2A6 and 2E1 in *E. coli* cells were measured at 37°C and compared to those cultured at 28°C (Fig. 1 and 2). The production of CYPs 2A6 and 2E1 was elevated with increasing induction time and maximal yield was obtained at around 18 h, as indicated by Fe^{2+}-CO *versus* Fe^{2+} difference spectra of whole *E. coli* cells. Expression levels of CYPs 2A6 and 2E1 at 37°C were compared at those at 28°C, which was the usual temperature

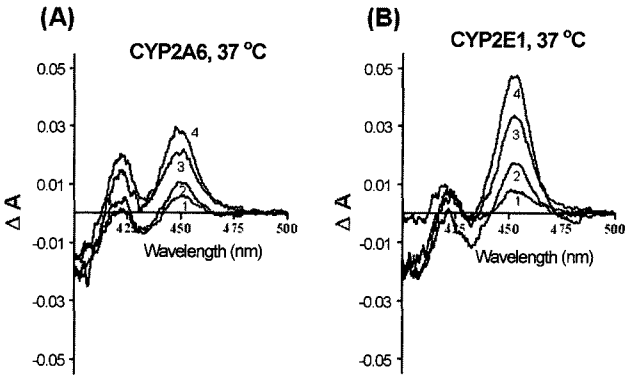

Fig. 1. Induction time-dependent expression level of recombinant human CYPs 2A6 and 2E1. The Fe^{2+}-CO *versus* Fe^{2+} difference spectra with increasing induction time show increased expression of CYPs 2A6 (A) and 2E1 (B) at 37°C in whole *E. coli* cells. Each spectrum was obtained after incubation of 3 (1), 6 (2), 12 (3), and 18 (4) h. Error bars represent ± S.D with three independent determinations.

used in the present system. The expression levels of CYPs 2A6 and 2E1 at 37°C reached up to 360 and 560 nmol (liter culture)$^{-1}$, respectively, within 18 h, which are compatible with those of 36 h culture at 28°C. (Fig. 2 and Table I).

However, further incubation decreased the level of CYP production. The expressions of CYPs 2A6 and 2E1 were also confirmed by western blot analysis (Fig. 3). These

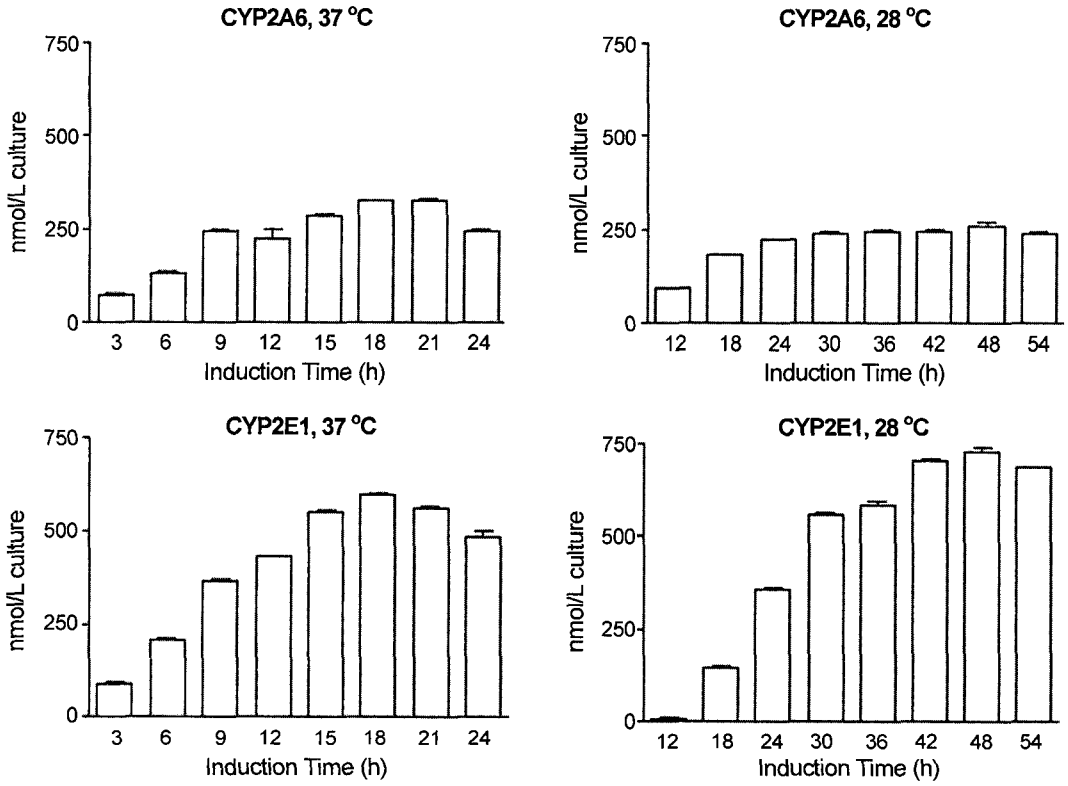

Fig. 2. Effect of temperature on the expression levels of recombinant human CYPs 2A6 and 2E1. Induction time-dependent expression levels of CYPs 2A6 (A and B) and 2E1 (C and D) were measured at 37 (A and C) and 28°C (B and D), respectively, at the indicated culture time. Error bars represent ± S.D with three independent determinations.

Table I. Expression yield of recombinant human CYPs 2A6 and 2E1 expressed in *E. coli*[a]

CYP	Culture temperature	Expression level		
		Whole cells (nmol/liter culture)	Membranes (nmol/liter culture)	Membrane CYP content (nmol/mg protein)
CYP2A6	37°C	327 ± 7[b]	155 ± 14	0.61 ± 0.05
CYP2A6	28°C	260 ± 10	87 ± 5	0.37 ± 0.02
CYP2E1	37°C	560 ± 5	223 ± 43	0.84 ± 0.17
CYP2E1	28°C	731 ± 12	332 ± 54	1.45 ± 0.24

[a]Expression was carried out at 37°C for 18 h or 28°C for 36 h in *E. coli* DH5aF'IQ in the presence of 1.0 mM IPTG and 1.5 mM δ-ALA.
[b]Results are expressed as means ± SD, with three independent experiments.

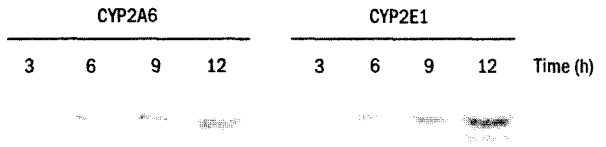

Fig. 3. Immunoblot analysis of expression of CYPs 2A6 and 2E1 in whole *E. coli* cells as induction over time. The blot was probed with rabbit anti-human CYP2A6 or CYP2E1 primary antibody and HRP-linked rat anti-rabbit IgG secondary antibody.

Table II. Activities of recombinant human CYPs 2A6 and 2E1 expressed at 37°C or 28°C in *E. coli*[a]

CYP	Culture temperature	Activity (nmol product formed/min/mg protein)	
		Coumarin 7-hydroxylation	Chlorozoxazone 6-hydroxylation
CYP2A6	37°C	2.2 ± 0.3[b]	not determined
CYP2A6	28°C	2.0 ± 0.2	not determined
CYP2E1	37°C	not determined	2.3 ± 0.3
CYP2E1	28°C	not determined	2.8 ± 0.2

[a]Activity was determined with bacterial membranes and represented in nmol product formed/min/mg protein.
[b]Results are expressed as means ± SD, with three independent experiments.

results indicate that active holoenzyme of recombinant human CYPs 2A6 and 2E1 can be expressed in *E. coli*.

We assayed the catalytic activities of CYPs 2A6 and 2E1 in membrane fractions and the results are summarized in Table II. The activities of CYPs expressed at 37°C were comparable to those of CYPs expressed at 28°C. This result indicates that expression of CYPs at 37°C can be applicable to obtain functional recombinant human CYPs 2A6 and 2E1 in *E. coli*.

CONCLUSION

We report here the successful expression system for the production of recombinant human CYPs 2A6 and 2E1 in *E. coli* at 37°C. Production levels of active CYPs 2A6 and 2E1 at 37°C were comparable to those expressed at 28°C. The present system is very rapid when compared with other reported bacterial expression methods, which usually employ long culture time (48~72 h) and lower temperature (usually under 30°C) for the expression of proteins. The catalytic activities of CYPs 2A6 and 2E1 produced at 37°C were also compatible to those expressed at 28°C.

Taken together, it can be suggested that effective over-expression system for each recombinant human CYP should be investigated for rapid and large production by studying several parameters, such as culture temperature (as in this work), molecular chaperones (Inoue *et al.*, 2000; Ahn *et al.*, 2004b; Ahn and Yun, 2004), modification of *N*-terminal sequences (Barnes *et al.*, 1991), use of signal sequences (Pritchard *et al.*, 1997), and possibly proper host cells for expression. In conclusion, several recombinant human CYPs including CYP1A2 (Ahn *et al.*, 2004a, 2004b), CYP3A4 (Ahn and Yun, 2004), CYP2A6 (this work), and CYP2E1 (this work) can be expressed in *E. coli* cells at 37°C, rather than lower temperature (<30 °C), for rapid production of functional CYP enzymes.

ABBREVIATIONS

CYP or P450, cytochrome P450; *E. coli, Escherichia coli*; IPTG, isopropyl-β-D-thiogalactopyranoside; δ-ALA, δ-aminolevulinic acid

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