FEBS Letters 397 (1996) 210–214 FEBS 17802

Coexpression of a human P450 (CYP3A4) and P450 reductase generates a highly functional monooxygenase system in *Escherichia coli*

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Received 27 September 1996; revised version received 10 October 1996

Abstract The catalytic activities of recombinant cytochrome P450s expressed in *E. coli* have been impeded by the absence of endogenous P450 reductase. To solve this problem, we coexpressed P450 reductase with CYP3A4. Membranes from this strain contained 215 pmol P450/mg protein and a reductase activity of 1315 nmol cytochrome *c* reduced/min per mg. We detected 6β-hydroxylation of testosterone and oxidation of nifedipine in vivo with turnover numbers of 15.2 and 17.3 min^{-1}, respectively. These values compare favourably with those obtained using an optimally reconstituted system. Our data demonstrate that a catalytically efficient human P450 system can be generated in *E. coli*.

Key words: Recombinant cytochrome P450, human; Drug metabolism; Bioreactors; Heterologous expression; *E. coli*

1. Introduction

Mammalian cytochrome P450 monooxygenases comprise a superfamily of membrane-bound hemoproteins that catalyse the metabolism of a wide variety of endogenous and exogenous compounds, including steroids, therapeutic drugs and carcinogens [1–3]. To be catalytically active, microsomal P450s require a supply of electrons, which are provided by NADPH-cytochrome P450 oxidoreductase (P450 reductase; EC 1.6.2.4) [4].

The study of human cytochrome P450s has been greatly facilitated by their expression in heterologous systems, including mammalian cells [5–7], insect cells [8], yeast [9,10] and *E. coli* [11–16]. While *E. coli* expression systems have the advantages of being easier and less expensive to handle, and generally yield higher quantities of recombinant proteins, one limitation is the absence of an endogenous P450 reductase, resulting in very low P450 enzyme activities in membranes derived from these expression strains [16]. For example, while the steroidogenic P450c17 has been demonstrated to couple, albeit inefficiently, with an endogenous NADPH-flavodoxin reductase in *E. coli* [17] the testosterone 6β-hydroxylase activity of recombinant cytochrome P450 3A4 (CYP3A4) was found to be barely detectable in *E. coli* (the turnover number was 1.6×10^{-5} min^{-1}, approximately a million-fold lower than the activity obtained in a reconstituted system [18]). Consequently, the catalytic parameters of recombinant mammalian P450s have mainly been determined in reconstituted systems in which exogenous P450 reductase is supplied. Such reconstituted systems can be rather complex, as some P450s are also reported to require other components such as cytochrome b_5, glutathione, detergent and a particular phospholipid composition for maximal activity. This is exemplified by studies with the abundant isoform CYP3A4, which catalyses the metabolism of a large number of drugs and steroids, and the activity of which has been reported to be stimulated by cytochrome b_5 for metabolism of certain substrates [11,18,19].

Recently, the ethanol inducible cytochrome P450 2E1 (CYP2E1) has been coexpressed with rat P450 reductase in *E. coli* [20]. The resulting bacterial membranes contained P450 and P450 reductase, and solubilised membranes were shown to catalyse oxidation of *p*-nitrophenol and *N*-nitrosodimethylamine. However, no substrate oxidation could be observed in intact *E. coli* and P450 yield was low (0.8 nmol/l culture). In the present study, we have established a highly active P450 monooxygenase system in *E. coli* cells by coexpression of CYP3A4 and P450 reductase.

2. Materials and methods

2.1. Construction of coexpression plasmid

The *E. coli* K12 strain used throughout was JM109. The vector pCW has previously been employed for the successful expression of several mammalian P450s including CYP3A4 [11] and was used here for the expression of P450 reductase and CYP3A4. The plasmid pJR4 comprises the pCW vector containing the human P450 reductase cDNA translationally fused to the bacterial *pelB* signal sequence. The sequence of the 5′ end of the cDNA is: [5′ATGAAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCCAGCCGGCGATGGCCATGGATATCGGATCCGAATTCCGCAACATG-human P450 reductase cDNA (~2 kb)3′], where the *pelB* leader sequence is shown underlined and the native P450 reductase ATG start codon is shown in bold. NF14 (pCW containing a CYP3A4 sequence which had been optimized for expression in *E. coli*) was constructed in such a way as to be identical to that made by Gillam et al. [11]. Plasmid pB215 was constructed by replacing the *SalI-BglII* fragment of NF14 containing a transcription terminator with a *SalI-BglII* double-stranded oligonucleotide containing a *trpA* transcription terminator of the following sequence (only top strand shown): 5′TCGACAGCCCGCCTAATGAGCGGGCTTTTTTTTA3′, thereby removing one of the two vector-derived *BglII* sites. A *BclI-BglII* fragment from pJR4 containing the *pelB*-reductase cDNA with its expression signals was subcloned into pB215 at the unique *BglII* site to create pB216.

2.2. Coexpression of CYP3A4 and P450 reductase in E. coli

Expression conditions were a modification of those described elsewhere [11]. JM109 cells were transformed with pB216 and transformants isolated on LB agar plates containing 50 μg/ml ampicillin. Transformants were streaked to single colonies which were used to

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PII S0014-5793(96)01196-9

inoculate 5 ml starter cultures in LB broth containing ampicillin, and were grown with shaking at 37°C overnight. Induction of expression was carried out in Terrific Broth modified and supplemented as described [11], but also containing 0.5 mM δ-aminolevulinic acid and inoculated with 1/100 volume overnight culture. Cells were grown shaking at 200 rpm, 30°C until A_{600nm} 0.5–0.7 prior to induction with 1 mM IPTG. Growth and expression of the recombinant proteins was then continued for 20–24 h at 30°C, with shaking at 200 rpm.

2.3. Harvesting cultures, membrane fractionation and determination of expression levels

Cells were harvested and resuspended in 2.5 ml 100 mM Tris-acetate (pH 7.6), 0.5 M sucrose, 0.5 mM EDTA (2×TSE) as described [10]. An equal volume of ice-cold water was then added. Membrane fractions were isolated from the cells as described [10]. The P450 and P450 reductase content of the membranes was determined. The CYP3A4 content was determined using Fe^{2+}-CO vs. Fe^{2+} difference spectra by mixing 100 μl cell or membrane suspension in 1×TSE with 1900 μl 100 mM Tris-Cl (pH 7.4), 10 mM CHAPS, 20% v/v glycerol, 1 mM EDTA, and a few crystals of sodium dithionite added. The spectrum was recorded as described [21]. The yield of active P450 reductase was estimated in membranes using a spectrophotometric assay to measure cytochrome *c* reductase [22].

2.4. Immunodetection of recombinant CYP3A4 and P450 reductase

Immunoblotting was performed as described [23]. Primary antibodies were a mixture of rabbit anti-rat CYP3A and anti-rat P450 reductase immunoglobulins and the secondary antiserum was HRP-linked donkey anti-rabbit immunoglobulin. P450 reductase and CYP3A4 proteins were detected using enhanced chemiluminescence (ECL; Amersham).

2.5. Metabolism of CYP3A4 substrates

2.5.1. Testosterone 6β-hydroxylase assays. Assays were carried out with cells and membrane fractions. In both cases, approx. 100 pmol P450 was incubated with shaking in TSE containing 30 mM $MgCl_2$. The final testosterone concentration was 0.2 mM. Where membranes were used, an NADPH generating system was added (final concentration 1 mM NADP, 5 mM glucose 6-phosphate, 1 unit glucose-6-phosphate dehydrogenase). Reactions were carried out at 37°C for 5 min, then stopped by addition of 1 ml ice-cold methanol and placed on ice for 10 min. Following centrifugation, supernatants were diluted with an equal volume of ice-cold water, and the testosterone metabolites extracted using Isolute C18 columns (IST Ltd), and eluted in 1 ml methanol. The methanol was evaporated in a SpeedVac and the metabolites were separated by HPLC on a Spherisorb ODS-2 (5 μm) 250×4.6 mm column using a gradient based on water, methanol and acetonitrile, at a flow rate of 1 ml/min, and detected at 240 nm. The yield of the 6β-hydroxytestosterone was calculated by reference to a known concentration of this metabolite. The HPLC method was supplied by Glaxo-Wellcome, and testosterone metabolites by Steraloids Inc. (a gift from Sterling Winthrop).

2.5.2. Erythromycin N-demethylase assays. Bacterial membrane fractions were incubated with 0.5 mM erythromycin in 50 mM HEPES buffer (pH 7.5) containing 150 mM KCl and 10 mM $MgCl_2$ in the presence of NADPH generating system (as above), for 20 min at 37°C. The formation of formaldehyde was detected using the Nash reagent [24,25].

2.5.3. Nifedipine oxidase assays. Cells or membrane fractions were incubated with 0.2 mM nifedipine in TSE containing 30 mM $MgCl_2$ at 37°C for 10 min with shaking. Where membrane fractions were used, NADPH generating system was included (as above). The reactions were stopped by adding ice-cold methanol (30% v/v final concentration) and perchloric acid (1.5% v/v final concentration) and the precipitated protein collected by centrifugation. The supernatants were transferred to HPLC vials and nifedipine and its oxidised metabolite were separated isocratically on a Spherisorb ODS-2 (5 μm) 250×4.6 mm column using a mobile phase of methanol, acetonitrile and water (25:30:45 v/v), and detected at 254 nm by HPLC. The amount of product formed was calculated by reference to a standard containing a known concentration of oxidised nifedipine.

Table 1
Cytochrome P450 content and cytochrome *c* reductase activities of JM109 NF14 and JM109 pB216 cells and/or membranes

	P450 content		Reductase activity
	nmol/l culture[a]	pmol/mg[b]	(nmol cyt. *c* reduced/min per mg)
pCW	n.d.	n.d.	38 ± 16
NF14	222 ± 35	350 ± 50	30 ± 3
pJR4	n.d.	n.d.	1355 ± 380
pB216	200 ± 45	215 ± 35	1315 ± 321

P450 contents were measured by Fe^{2+}-CO vs. Fe^{2+} difference spectra. Contents are expressed as means of 4 experiments ± S.D. Reductase activities were calculated by measuring the rate of reduction of cytochrome *c* per mg protein in membrane fractions, and values are given as means of four experiments ± S.D. n.d., no detectable activity.
[a]Content was measured in 50 μl cells in 1×TSE (~0.5 ml culture).
[b]Content was assessed per mg protein in membrane fractions derived from recombinant bacteria.

3. Results

3.1. Construction of a plasmid for coexpression of CYP3A4 and P450 reductase

Preliminary experiments to optimise expression of P450 reductase in *E. coli* indicated that high levels of IPTG-inducible expression were achieved from pJR4. In this construct the human reductase cDNA was translationally fused at its N-terminus to the bacterial *pelB* leader sequence and expressed from the $P_{tac}P_{tac}$ promoter of pCW; these expression levels were found to be approx. 40-fold higher than those obtained from the comparable construct lacking the *pelB* leader (data not shown). These results are consistent with the previously described high-level expression P450 reductases with *ompA* and *pelB* leader sequences [26,27]. A coexpression plasmid was constructed by subcloning the *pelB*-reductase cDNA into the optimised CYP3A4 expression plasmid NF14 [11] as follows. pB215 was constructed by modification of NF14 (see Section 2 and Fig. 1 for details). A fragment from pJR4 containing the *pelB*-reductase cDNA and its $P_{tac}P_{tac}$ promoter was then subcloned into pB215, creating a plasmid, pB216, in which the two cDNAs, each bearing the P_{tac} P_{tac} promoter, were arranged head-to-tail (see Fig. 1).

3.2. Coexpression of P450 reductase and CYP3A4 from pB216

The culture conditions used were similar to those established to be optimal for expression of CYP3A4 from NF14 [11], except that cultures were routinely supplemented with δ-aminolevulinic acid, as this was found to increase expression of CYP3A4. Similar observations have also been made by other workers for several P450 isoforms [19].

CYP3A4 content was determined in bacterial cells using Fe^{2+}-CO vs. Fe^{2+} difference spectra. Typical spectra for whole cells containing pB216 and the control plasmids pCW, pJR4 and NF14 are shown in Fig. 2A. For the assessment of cytochrome *c* reductase activities, bacterial membrane fractions were prepared since a high background cytochrome *c* reductase activity was detected in whole cell or spheroplast preparations (data not shown). The expression levels of P450 reductase and CYP3A4 obtained are shown in Table 1. Table 1 shows that the CYP3A4 yield measured in JM109 pB216 cells was typically 200 nmol/l culture and membranes contained 215 pmol P450/mg protein. The P450 reductase activity was 1315 nmol cytochrome *c* reduced/min per mg membrane protein.

A typical Western blot showing the recombinant proteins is shown in Fig. 2B. P450 reductase and CYP3A4 were detected

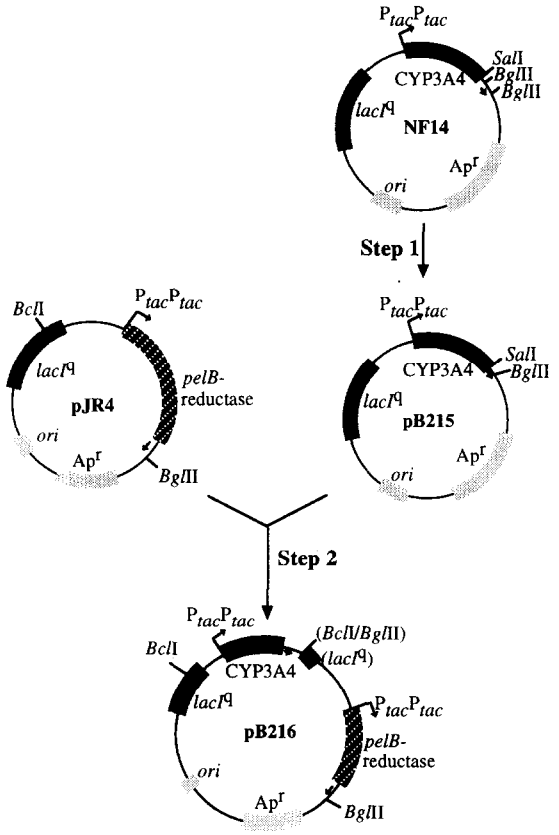

Fig. 1. Construction of pB216. pB216 was constructed by insertion of a *pelB*-reductase-containing fragment from pJR4 into the modified NF14 construct, pB215. pB215 was constructed by replacement of the *SalI-BglII* fragment of NF14 with an oligonucleotide containing a transcription terminator (shown by a small arrow), thereby removing one of the two *BglII* restriction sites downstream of the CYP3A4 cDNA (step 1). The remaining *BglII* site could then be used for subsequent cloning of the *pelB*-reductase cDNA from pJR4 as a *BclI-BglII* fragment without abolishing the transcription terminator of CYP3A4 expression (step 2).

in the membrane fraction derived from pB216 (track 8), while P450 reductase alone and CYP3A4 alone were detected in those samples derived from pJR4 (track 6) and NF14 (track 4), respectively. Some basal expression was observed in the absence of IPTG (tracks 5,7). However, the amounts of active P450 reductase and spectrally active CYP3A4 derived from these fractions were very low compared to the induced cultures (data not shown). The specific contents of recombinant CYP3A4 and P450 reductase in JM109 pB216 membrane fractions appeared similar to those detected in a sample of human liver microsomes (tracks 8,9).

3.3. Assays of CYP3A4 activity in whole cells and membranes

JM109 pB216 cells were found to metabolise testosterone and nifedipine (Table 2). No 6β-hydroxytestosterone formation was detected after incubation of testosterone with JM109 pCW (vector only) or JM109 pJR4 (P450 reductase only) or JM109 NF14 (CYP3A4 only). Testosterone 6β-hydroxylase activity was still not detectable in these strains after increasing the incubation time of the assay from 5 min to 60 min. These data demonstrate that *E. coli* is unable to catalyse 6β-hydroxylation of testosterone in the presence of either CYP3A4 or P450 reductase alone. On coexpression of CYP3A4 with P450 reductase, cells catalysed testosterone 6β-hydroxylation with a turnover rate of 17.3 min^{-1}. Similarly, membranes derived from JM109 pB216 cells catalysed the formation of 6β-hydroxytestosterone with a turnover number of 25.5 min^{-1}. In the absence of Mg^{2+}, the activity was found to be approx. 10-fold lower (data not shown). Other reports have described turnover rates for this enzyme activity of ~10–12 min^{-1} using reconstituted systems containing bacterially expressed CYP3A4, P450 reductase and cytochrome b_5 [11,18]. The turnover rate in human liver microsomes has been estimated to be 6 min^{-1} [28].

JM109 pB216 cells and membranes also catalysed oxidation of nifedipine (Table 2). The turnover number was typically 15.2 min^{-1} for cells and 12.7 min^{-1} for membranes. No activity was detected in cells expressing either P450 reductase or CYP3A4 alone. Membranes were also found to mediate metabolism of erythromycin in the presence of NADPH. The turnover number was typically 2.3 min^{-1} (Table 2). We were unable to measure this activity with intact cells, as the background release of formaldehyde from control cells in the absence in erythomycin was found to be too high (data not shown).

4. Discussion

We describe the generation of a functional P450 monooxygenase system in *E. coli*, by coexpression of human CYP3A4 and P450 reductase. To our knowledge this is the first instance

Table 2
CYP3A4-dependent metabolism of testosterone, nifedipine and erythromycin by JM109 pB216 cells and membranes

	Turnover (min^{-1})				
	Testosterone		Nifedipine		Erythromycin[a]
	Cells	Membranes	Cells	Membranes	
pCW	<0.5	<0.5	<2	<2	<0.3
NF14	<0.5	<0.5	<2	<2	<0.3
pJR4	<0.5	<0.5	<2	<2	<0.3
pB216	17.3 ± 3.3	25.5 ± 4.3	15.2 ± 1.3	12.7 ± 0.9	2.3 ± 0.7

Metabolism of three known CYP3A4 substrates by cells or membranes containing recombinant CYP3A4 and P450 reductase was assessed. Turnover numbers are recorded as nmol product formed/min per nmol P450, and are shown ± S.D. The products which were detected were 6β-hydroxytestosterone, oxidised nifedipine and formaldehyde. Where no activities were detected, detection levels are shown. For testosterone metabolism, no 6β-hydroxytestosterone was formed even after 60 min incubation with cells or membranes lacking either CYP3A4 or P450 reductase (data not shown).

[a]For erythromycin metabolism, activities could only be recorded with membranes, as the background level of formaldehyde formation by whole cells was very high.

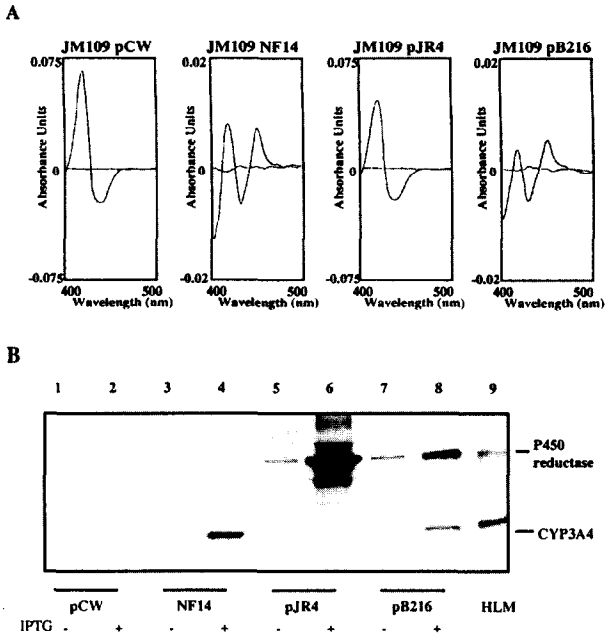

Fig. 2. Expression of P450 reductase and CYP3A4 in *E. coli*. (A) Cytochrome P450 content was measured in whole bacterial cells (equivalent to 0.5 ml bacterial culture) by Fe^{2+}-CO vs Fe^{2+} difference spectra. (B) Levels of recombinant CYP3A4 and P450 reductase in *E. coli* membranes determined by Western blotting. JM109 cells were transformed with pCW (control plasmid), NF14 (CYP3A4 only), pJR4 (P450 reductase only) or pB216 (CYP3A4/P450 reductase coexpression). Tracks were loaded alternately with membrane samples derived from cultures which had been uninduced (lanes 1,3,5,7) or induced (lanes 2,4,6,8) with IPTG. Lanes 1,2 contained membranes from JM109 pCW cells, lanes 3,4 from JM109 NF14 cells, lanes 5,6 from JM109 pJR4 cells, and lanes 7,8 from JM109 pB216 cells. Track 9 contains human liver microsomal protein. All lanes were loaded with 10 μg membrane protein. Recombinant CYP3A4 and P450 reductase were detected using a combination of anti-CYP3A and anti-P450 reductase antibodies.

in which a mammalian xenobiotic-metabolising P450 has been shown to be significantly catalytically active in intact *E. coli*. The yields of recombinant proteins were high: 215 pmol P450/mg protein, and a reductase activity of 1315 nmol cytochrome *c* reduced/min per mg were obtained. These levels are significantly higher than those of CYP3A4 and P450 reductase measured in human liver microsomes (90 pmol/mg protein and 230 nmol cytochrome *c* reduced/min per mg protein respectively [29,30]). The yield of P450 achieved in this strain is approx. 200-fold higher than that achieved in a strain coexpressing CYP2E1 and P450 reductase [20].

JM109 pB216 cells catalysed CYP3A4-mediated reactions, indicating that electron transfer occurred efficiently in vivo. This was exemplified by turnover numbers which were in excess of those estimated in human liver. Activities measured towards the substrates we tested were found to be comparable to those obtained by other workers using purified recombinant CYP3A4 in reconstituted systems [11,19]. The reconstituted systems described contained components other than those included here, such as detergent, an optimised phospholipid mixture, glutathione, and cytochrome b_5, in order to obtain maximal CYP3A4 activity towards testosterone and nifedipine [11,18,28]. It was therefore extremely interesting that, in our studies, high levels of CYP3A4 activity towards these substrates were observed in the absence of exogenously added cytochrome b_5. It has been postulated that cytochrome b_5 binds to CYP3A4 in the presence of P450 reductase, substrate and divalent cations such as Mg^{2+} to facilitate reduction by P450 reductase thereby increasing the rate of P450-mediated metabolism of certain substrates [18,28]. We found that while the testosterone 6β-hydroxylase activity of membranes containing CYP3A4 and P450 reductase was increased 10-fold by Mg^{2+}, the nifedipine oxidase activity was already high and was not significantly affected by the addition of Mg^{2+}. In addition, the enzyme activities towards both of these substrates were not stimulated by Mg^{2+} when assayed in intact cells, perhaps due to high intracellular pools of divalent cations. Taken together, these results suggest that there is an endogenous bacterial protein which can substitute for cytochrome b_5 to stimulate CYP3A4 activity, but that, if this is the case, the complex formed between this protein, P450 reductase and CYP3A4 has different substrate-specific requirements for Mg^{2+} ions than the complex involving cytochrome b_5. In future experiments we will address this issue by coexpression of cytochrome b_5 together with CYP3A4 and P450 reductase in *E. coli*.

In summary, we have successfully achieved high level coexpression of CYP3A4 and P450 reductase in *E. coli*. The resulting cells and membranes derived from them are proficient for metabolism of several known CYP3A4 substrates. The specific activities achieved from our coexpression strain compare favourably to results previously obtained with complex reconstituted systems. This approach will be used as a model system for future coexpression of further P450 isoforms with P450 reductase in *E. coli*, and we expect that it will significantly aid the use of cytochrome P450s in biocatalysis and in drug development.

Acknowledgements: This work was sponsored within a LINK programme by: the BBSRC, the DTI, Zeneca, Astra Hässle, Astra Charnwood, Servier, Wyeth, SmithKline Beecham, Glaxo Wellcome, Novo Nordisk, Pfizer, Janssen, Roche, Parke-Davis, Sanofi and Lilly. Their generous support and their helpful discussions are gratefully acknowledged.

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