

The Flavin-Containing Reductase Domain of Cytochrome P450 BM3 Acts as a Surrogate for Mammalian NADPH-P450 Reductase

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Abstract

Cytochrome P450 BM3 (CYP102A1) from *Bacillus megaterium* is a self-sufficient monooxygenase that consists of a heme domain and FAD/FMN-containing reductase domain (BMR). In this report, the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) by BMR was evaluated as a method for monitoring BMR activity. The electron transfer proceeds from NADPH to BMR and then to BMR substrates, MTT and CTC. MTT and CTC are monotetrazolium salts that form formazans upon reduction. The reduction of MTT and CTC followed classical Michaelis-Menten kinetics ($k_{cat}=4120 \text{ min}^{-1}$, $K_m=77 \text{ }\mu\text{M}$ for MTT and $k_{cat}=6580 \text{ min}^{-1}$, $K_m=51 \text{ }\mu\text{M}$ for CTC). Our continuous assay using MTT and CTC allows the simple, rapid measurement of BMR activity. The BMR was able to metabolize mitomycin C and doxorubicin, which are anticancer drug substrates for CPR, producing the same metabolites as those produced by CPR. Moreover, the BMR was able to interact with CYP1A2 and transfer electrons to promote the oxidation reactions of substrates by CYP1A2 and CYP2E1 in humans. The results of this study suggest the possibility of the utilization of BMR as a surrogate for mammalian CPR.

Key Words: CYP102A1, NADPH-cytochrome P450 reductase, Reduction, Reductase domain, Tetrazolium salts

INTRODUCTION

Microsomal NADPH-cytochrome P450 reductase (CPR) enzymes are FMN/FAD-containing flavin redox proteins that play important roles in mediating electron transfer from NADPH to other microsomal proteins, such as cytochromes P450 (CYP or P450) and heme oxygenase (Sevrioukova and Peterson, 1995). CPR also transfers electrons to nonphysiological chemical substrates, such as potassium ferricyanide and 2,6-dichlorophenolindophenol (Jamakhandi *et al.*, 2005).

Flavocytochrome P450 BM3 (CYP102A1), a fatty acid monooxygenase from *Bacillus megaterium*, is the first known bacterial P450 enzyme that belongs to the microsomal P450 class, resembling the eukaryotic P450 systems in both structure and function (Munro *et al.*, 2002). CYP102A1 is a self-sufficient enzyme that contains an equimolar ratio of heme, FAD, and FMN in a single polypeptide. That is, the reductase domain of CYP102A1 (BMR) is fused to the heme domain, which is a characteristic that is different from mammalian P450 enzymes and allows a more efficient electron transfer. However, under limited trypsinolysis, the holoenzyme can be

prepared as two separate proteins, a hemoprotein that contains the heme and a flavoprotein that contains both FMN and FAD (Narhi and Fulco, 1987; Miles *et al.*, 1992). Due to the several advantages of using bacterial P450 as a model system to understand the biochemical characteristics of microsomal CPR, the reductase domain of CYP102A1 has been intensively studied by many researchers (Sevrioukova *et al.*, 1996a; Hazzard *et al.*, 1997; Davydov *et al.*, 2010).

The amino acid sequence alignment of CPR and BMR shows approximately 31% identity and 42% similarity (Fig. 1). The identical sequences that are highly conserved between these two enzymes from different species are proven to be involved in the binding of flavins and pyridine nucleotides (Ruettinger *et al.*, 1989). The structures and the interactions of these proteins with P450 enzymes appear to be conserved throughout evolution (Sevrioukova and Peterson, 1995).

Recombinant BMR, which was heterologously expressed in *Escherichia coli* and purified, has been shown to be capable of supporting the activity of the heme domain of CYP102A1 (BMP) (Boddupalli *et al.*, 1992) and of the efficient reduction of CYP2B4 (Davydov *et al.*, 2000) and CYP3A4 (Fernando *et al.*,

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Open Access <http://dx.doi.org/10.4062/biomolther.2012.20.6.562>

pISSN: 1976-9148 eISSN: 2005-4483

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Received Sep 7, 2012 Revised Sep 27, 2012 Accepted Oct 4, 2012

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2008) through mechanisms that are similar to those of CPR. It was also reported that BMR has been used to construct functionally active chimeric proteins with several human P450s, including CYP3A4 (Dodhia *et al.*, 2006). With regard to the catalytic activity of the BMR, it is known that BMR can catalyze the reduction of cytochrome c, ferricyanide, and 2,6-dichlorophenolindophenol (DCIP) (Sevrioukova *et al.*, 1996b).

The first purpose of this work was to provide a method of monitoring the reduction activity of BMR. Microsomal CPR has been previously examined to assess its ability to reduce several artificial substrates, such as ferricyanide, cytochrome c, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Yim *et al.*, 2004), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Yim *et al.*, 2005), and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Kim *et al.*, 2009). In the present study, we performed a continuous spectrophotometric assay with two tetrazolium salts, MTT and CTC, to evaluate the reductase activity of BMR. Tetrazolium salts are used extensively to measure cell viability, metabolic activity, and oxidative reactions. The reduction of tetrazolium salts produces colored products called formazans that are easily observed with the naked eye (Altman, 1976; Bernas and Dobrucki, 1999), and the reduction of MTT and CTC can be assessed spectrophotometrically by measuring the change in their absorbance, as described previously (Yim *et al.*, 2005; Kim *et al.*, 2009). This method offers advantages of a rapid, simple measurement of BMR reductase activity with the use of commercially available substrates.

The second aim of this research was to study the biochemical characteristics of BMR. In efforts to elucidate the biochemical characteristics of BMR, we tested the ability of BMR to metabolize specific anticancer drugs, such as mitomycin C (Pan *et al.*, 1984; Siegel *et al.*, 1992) and doxorubicin, which are reported to be the substrates of CPR (Mizutani *et al.*, 2003). Furthermore, oxidation reactions of 7-ethoxycoumarin (Kim *et al.*, 2006) and phenacetin (Yun *et al.*, 2000) were performed with human CYP1A2, described as the major P450 enzyme involved in the oxidation of 7-ethoxycoumarin and phenacetin, together with BMR to determine whether BMR can interact with human P450 enzymes and transfer electrons to these enzymes.

MATERIALS AND METHODS

Materials

MTT, CTC, mitomycin C, doxorubicin and NADPH were obtained from the Sigma Chemical Co (St. Louis, MO). All other chemicals were of analytical grade.

Spectrophotometric analysis

The absorption spectra were recorded using a Shimadzu UV-1601 spectrophotometer (Tokyo, Japan).

Expression and purification of BMR

The pET-21a(+)-BMR plasmid was transformed into *E. coli* DH5 α F'-IQ cells. Overnight cultures (20 ml) grown in Luria-Bertani broth with ampicillin (100 μ g/ml) selection at 37°C were used to inoculate a 200 ml culture of Terrific broth containing 100 μ g/ml ampicillin, 1.0 mM thiamine, trace elements, 50 μ M FeCl₃, 1 mM MgCl₂, and 2.5 mM (NH₄)₂SO₄. The cells were grown at 37°C and 210 rpm to an OD₆₀₀ between 0.6-0.8. Protein expression was induced by adding 1.0 mM isopropyl- β -D-

thiogalactopyranoside (IPTG), and the cultures were grown at 28°C and 200 rpm for 23 h. The cells were harvested by centrifugation (15 min, 5,000 x g, 4°C). The cell pellet was resuspended in TES buffer {100 mM Tris-HCl (pH 7.6), 500 mM sucrose, and 0.5 mM EDTA} and lysed by sonication (Sonicator, Heat Systems - Ultrasonic, Inc., Newport, OR, USA). After the lysate was centrifuged at 100,000 x g, the soluble cytosolic fraction was collected and purified using 2',5'-ADP-agarose as described previously (Jamakhandi *et al.*, 2005). BMR was quantitated by flavin absorbance ($\epsilon=21\text{mM}^{-1}\text{cm}^{-1}$) (French and Coon, 1979).

Enzymatic activities of the reductase domain of BMR (BMR)

All of the continuous spectrophotometric assays for BMR activity were performed using standard 1 cm cuvettes in a total reaction volume of 1 ml. The ability of BMR to reduce MTT and CTC was determined using 200 μ M of each substrate and 20 pmol BMR in 100 mM potassium phosphate (pH 7.6 and pH 7.4, respectively). The change in absorbance was measured following the addition of 100 μ M NADPH (at 610 nm for MTT and 450 nm for CTC). The MTT and CTC reduction rates were calculated using extinction coefficients of 11.3 $\text{mM}^{-1}\text{cm}^{-1}$ and 0.016 $\text{mM}^{-1}\text{cm}^{-1}$ for reduced MTT and CTC, respectively.

Determination of kinetic parameters

The reaction mixtures included 20 pmol BMR in 100 mM potassium phosphate and varying concentrations of the substrate in a total volume of 1 ml. The reactions were initiated by adding 100 μ M NADPH. The kinetic parameters (K_m and k_{cat}) were determined by nonlinear regression using Graph-Pad Prism software (San Diego, CA).

HPLC analysis of mitomycin C and doxorubicin metabolites

The reactions were performed using 50 pmol CPR or BMR with two different substrates, mitomycin C (Pan *et al.*, 1984) and doxorubicin (Mizutani *et al.*, 2003), in 100 mM potassium phosphate buffer, as described previously. We used substrate concentrations of 200 μ M for mitomycin C and doxorubicin.

For the assay of mitomycin C reduction, the reaction mixture contained 50 pmol CPR, 100 mM potassium phosphate buffer (pH 5.8), and 200 μ M substrate in a total volume of 0.25 ml. The stock of each substrate was prepared by dissolving in dimethyl sulfoxide at a concentration of 10 mM and was then added to the reaction mixture at the specified concentration. The sample was preincubated for 10 min. The reaction was initiated by the addition of an NADPH-generating system (final concentrations per ml: 10 mM glucose 6-phosphate, 0.5 mM NADP, and 1 IU yeast glucose 6-phosphate dehydrogenase), and the incubation was continued in a water bath at 37°C. After a 30 min incubation, the reaction was terminated by adding 125 μ l methanol. Aliquots of the terminated reaction solution were injected directly onto the HPLC column for the analysis of metabolites using a Gemini C18 column (4.6 \times 150 mm, 5 μ m; Phenomenex, Torrance, CA). The mobile phase consisted of 10 mM potassium phosphate buffer pH 7.2 (buffer A) and methanol plus 10 mM potassium phosphate buffer pH 7.2 (1:1 v/v) (buffer B). The column was eluted at a flow rate of 1 ml/min using a linear gradient from 5% buffer B to 90% buffer B for 25 min. The absorbance was monitored at 313 nm.

For the assay of doxorubicin reduction, the reaction mixture

contained 50 pmol CPR, 100 mM potassium phosphate buffer (pH 7.4), and 200 μM of the substrate in a total volume of 0.25 ml. The stock of substrate was prepared by dissolving in water at a concentration of 5 mM and was then added to the reaction mixture at the specified concentration. The sample was preincubated for 10 min. The reaction was initiated by the addition of the NADPH-generating system (final concentrations per ml: 10 mM glucose 6-phosphate, 0.5 mM NADP, and 1 IU yeast glucose 6-phosphate dehydrogenase), and the incubation was continued in a water bath at 37°C. After a 30 min incubation, the reaction was terminated and extracted by adding 500 μl cold dichloromethane and centrifuged at 1,000 x g for 10 min. The organic layers were combined, and the solvent was removed under N₂ gas. The products were analyzed by HPLC using a Gemini C18 column (4.6×150 mm, 5 μm; Phenomenex, Torrance, CA, USA) with a mobile phase of CH₃CN: 0.05 mM sodium phosphate plus 0.1% SDS pH 3.7 (40:60). The column was eluted at a flow rate of 1 ml/min, and the products were detected at 252 nm.

P450 catalytic activity assays

7-Ethoxycoumarin (Kim *et al.*, 2006) and phenacetin (Yun *et al.*, 2000) were used to examine whether the separately prepared flavoprotein moiety of CYP102A1 can interact with human P450 enzymes and mediate electron transfer.

The 7-ethoxycoumarin oxidation reaction included 50 pmol CYP1A2, 100 pmol CPR or BMR in 0.25 ml 100 mM potassium phosphate buffer (pH 7.4) and 0.5 mM of the substrate. The sample was preincubated for 10 min. The reaction was initiated by the addition of the NADPH-generating system (final concentrations per ml: 10 mM glucose 6-phosphate, 0.5 mM NADP, and 1 IU yeast glucose 6-phosphate dehydrogenase), and the incubation was continued in a water bath at 37°C. After a 30 min incubation, the reaction was terminated and extracted by adding 500 μl cold dichloromethane and centrifuged at 10³ x g for 10 min. The organic layers were combined, and the solvent was removed under N₂ gas. The metabolites of 7-ethoxycoumarin were analyzed by HPLC using a Gemini C18 column (4.6×150 mm, 5 μm; Phenomenex, Torrance, CA) with a mobile phase of H₂O:CH₃CN (55:45, v/v) containing 10 mM HClO₄. The column was eluted at a flow rate of 1 ml/min, and the products were detected at 330 nm.

The reaction mixture for the O-deethylation assay consisted of 50 pmol CYP1A2, 100 pmol CPR or BMR in 100 mM potassium phosphate buffer (pH 7.4), the NADPH-generating system, and 2 mM phenacetin in a total volume of 0.25 ml. The reaction mixture was incubated for 30 min at 37°C, terminated with 50 μl HClO₄ (17%), and centrifuged (1,000 x g for 10 min). Then, 0.5 ml of a mixture of CHCl₃ and 2-propanol (6:4, v/v) was added to the supernatant to extract the products, followed by centrifugation (twice at 1,000 x g). The organic layers were combined, and the solvent was removed under N₂ gas. The metabolites of phenacetin were analyzed by HPLC using a Gemini C18 column (4.6×150 mm, 5 μm; Phenomenex, Torrance, CA) with a mobile phase of H₂O:CH₃OH:CH₃CO₂H (65:35:0.1, v/v/v). The column was eluted at a flow rate of 1 ml/min, and the products were detected at 254 nm.

The catalytic activity of human CYP2E1 using chlorzoxazone was measured as described previously (Guengerich *et al.*, 1991).

RESULTS AND DISCUSSION

Amino acid sequence alignment and purification of CPR and BMR

The amino acid sequence alignment of CPR and BMR shows approximately 31% identity and 42% similarity (Fig. 1), and the regions of the three cofactor-binding domains (FMN-, FAD-, and NADPH-) are highly conserved between these two enzymes. The purified BMR protein yielded the expected single band on SDS-PAGE of 65 kDa. The absorption spectra of purified BMR show that the absorption maximum for the purified BMR enzyme is located at 452 nm (results not shown).

BMR reduction activity of tetrazolium salts

Two tetrazolium salts, MTT and CTC, were used as the substrates in an assay to provide a monitoring system that measures the reduction activity of BMR. The MTT and CTC reduction rates were measured spectrophotometrically with 20 pmol BMR in potassium phosphate buffer.

The chemical structures of MTT and its reduced product, MTT formazan, are shown in Fig. 2A. The MTT reduction catalyzed by BMR was determined at an absorbance of 610 nm with a substrate concentration that ranged from 1 to 500 μM (Fig. 3). Following the addition of NADPH, the increase in absorbance at 610 nm for the first 30 sec was used to calculate the reduction rate at the corresponding substrate concentration.

The chemical structures of CTC and its reduced product are shown in Fig. 2B. The increase in absorbance at 450 nm

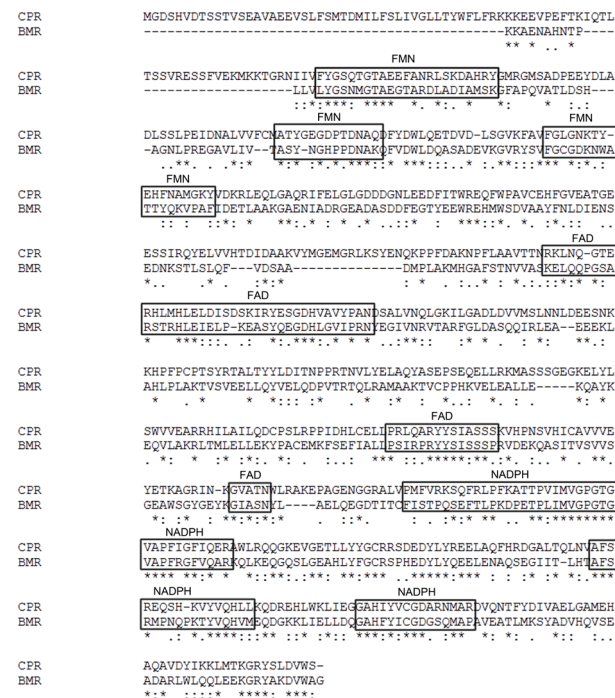


Fig. 1. Amino acid sequence alignment of CPR and BMR. The reductase domain of CYP102A1 (lower line) was aligned with CPR (upper line). The amino acids that match between two species are indicated with the asterisks. The residues corresponding to the putative FMN-, FAD-, and NADPH-binding domains are shown in bold with boxes.

was measured, to calculate the BMR-catalyzed reduction of CTC at substrate concentrations between 1 and 500 μM (Fig. 3). Following the initiation of the reaction by adding 100 μM NADPH, the increase in absorbance at 450 nm for the first 5 to 20 sec was used to calculate the reduction rate at the respective substrate concentration.

The kinetic analyses were performed using MTT and CTC as the electron acceptors, and the results are summarized in Table 1. The k_{cat} values for BMR were 4,120 min^{-1} for MTT and 6,580 min^{-1} for CTC. The K_{m} values for BMR were 77 μM for MTT and 51 μM for CTC.

Metabolism of anticancer drugs by BMR

To examine whether BMR can metabolize known substrates of CPR, HPLC analyses were performed following reactions with two anticancer drugs. The reaction mixtures included 50 pmol BMR with the two different substrates in 100 mM potassium phosphate buffer. Mitomycin C (Pan *et al.*, 1984; Siegel *et al.*, 1992) and doxorubicin (Mizutani *et al.*, 2003) were selected as the substrates, as they are reported to be metabolized by CPR.

The results of the HPLC analyses of mitomycin C metabolism (Fig. 4A and B) show that BMR is able to produce the major metabolite that is also produced by CPR, though at a lower rate (15 pmol product/min/nmol BMR) in comparison to purified CPR (22 pmol product/min/nmol CPR). The retention times for mitomycin C and the major metabolite were 24.5 min and 26.3 min, respectively. Furthermore, the LC-MS comparison (results not shown) with the authentic standard compound confirmed that the metabolite was 2,7-diamino 1-hydroxymitosene, a known CPR-catalyzed reduction product of mitomy-

cin C (Fernando *et al.*, 2008). The results of the HPLC analyses following doxorubicin metabolism (Fig. 4C and D) show that BMR produces the major metabolite at a rate of 0.026 min^{-1} , which is 82% of the turnover catalyzed by CPR. The major metabolite produced by both CPR and BMR has a retention time of 16.8 min.

Use of BMR as electron donor in human P450-catalyzed reactions

To examine whether the separately prepared flavoprotein moiety (BMR) of CYP102A1 can interact with human P450 enzymes and mediate electron transfer, a 7-ethoxycoumarin oxidation assay was performed, followed by HPLC analysis (Fig. 5A and B). 7-ethoxycoumarin is metabolized by several types of human P450s, typically CYP1A2, and, in the presence of CYP1A2 and CPR, 7-ethoxycoumarin is metabolized to two different hydroxyl products, 7-OH coumarin and 3-OH 7-ethoxycoumarin (Kim *et al.*, 2006). The retention times for 7-ethoxycoumarin, 3-OH 7-ethoxycoumarin, and 7-OH coumarin are 7 min, 5.2 min, and 2.7 min, respectively (Fig. 5A).

The ratio of CYP1A2 and BMR used was 1:2, which is the optimal ratio for the catalytic assay (results not shown). The HPLC chromatogram shows that two major metabolites, 3-OH 7-ethoxycoumarin and 7-OH coumarin, are produced upon reaction with CYP1A2 and BMR. The formation rates for 3-OH 7-ethoxycoumarin and 7-OH coumarin were 0.018 (± 0.002) and 0.013 (± 0.002) nmol product per min per nmol P450, respectively. However, the amount of these products was much lower than those of the reaction with CPR: in the case of the CPR-supported reaction, the formation rates for 3-OH 7-ethoxycoumarin and 7-OH coumarin were 2.0 (± 0.1) and 12 (± 2)

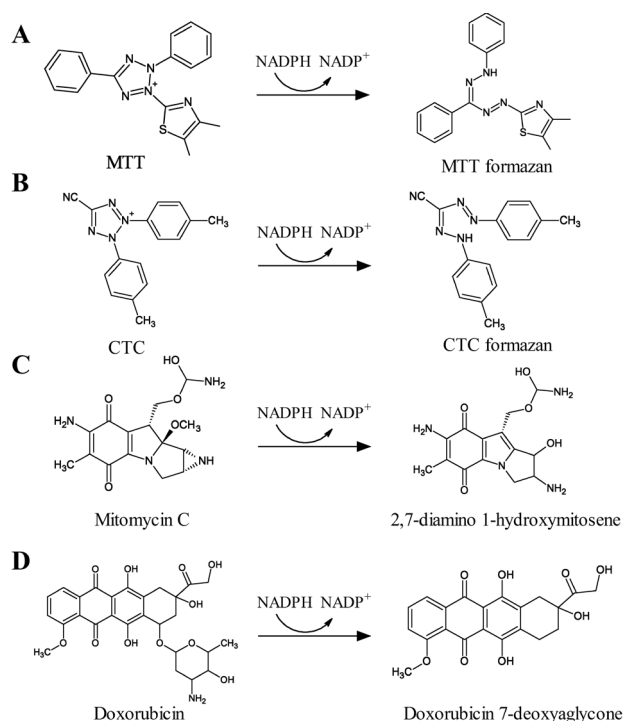


Fig. 2. Chemical structures of MTT (A), CTC (B), mitomycin C (C), and doxorubicin (D). Each corresponding reduction product is also shown.

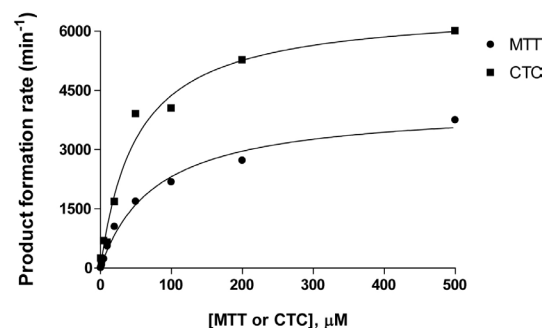


Fig. 3. Concentration-dependent reduction of MTT and CTC by BMR. The reduction of MTT was measured spectrophotometrically at 610 nm using varying concentrations of MTT (1–500 μM) in the presence of NADPH for 20 sec. The reduction of CTC was measured spectrophotometrically at 450 nm with varying concentrations of MTT (1–500 μM) in the presence of NADPH for 5 sec. The kinetic parameters of MTT reduction and CTC reduction were determined by nonlinear regression using Graph-Pad Prism software (San Diego, USA) and are shown in Table 1.

Table 1. Kinetic analysis of MTT and CTC reduction catalyzed by BMR

| | k_{cat} (min^{-1}) | K_{m} (μM) | $k_{\text{cat}}/K_{\text{m}}$ ($\text{min}^{-1} \cdot \mu\text{M}^{-1}$) |
|-----|--|----------------------------------|--|
| MTT | 4,120 \pm 210 | 77 \pm 11 | 53 \pm 8 |
| CTC | 6,580 \pm 670 | 51 \pm 14 | 130 \pm 40 |

nmol product per min per nmol P450, respectively.

The catalytic activity of CYP1A2 with CPR or BMR was also examined using phenacetin, a typical CYP1A2 substrate. Phenacetin is oxidized by CYP1A2 with CPR (Fig. 5C) or BMR (Fig. 5D) to produce two major metabolites, acetaminophen and acetol. The identities of the major metabolites and the substrate were verified by comparing the results by HPLC (Fig. 5) and LC-MS (results not shown). The retention times for phenacetin, acetol, and acetaminophen are 11.6, 8.5, and 2.7 min, respectively. These results imply the interaction of BMR with CYP1A2 and electron transfer between them. In the case of chlorzoxazone, which is a typical substrate for CYP2E1, the BMR was able to transfer electrons to CYP2E1 to produce the 6 β -OH metabolite (Fig. 5F).

Assays of 7-ethoxycoumarin oxidation, phenacetin oxidation, and chlorzoxazone hydroxylation were performed in presence and absence of DLPC, which is typically used to facilitate the proper interaction between human P450 enzymes and CPR. The results showed that the production of the metabolites was slightly higher (approximately 10-20%) in the presence of DLPC (results not shown). Because human CYP1A2 is a membrane-anchored protein, this result may be due to the conformational stabilization of human CYP1A2 in the presence of the lipid component of the reaction mixture, thus promoting a better interaction with BMR.

CYP102A1 consists of a heme domain (BMP), a reductase domain (BMR), and a linker region between the domains. When the domains were expressed and purified separately,

the affinity for one another and the catalytic activity of the domains decreased dramatically (Boddupalli *et al.*, 1992). This observation revealed the importance of the region linking the two domains and suggested that the intrachain electron transfer is much more efficient than that of the interchain electron transfer. It is also known that isolated BMR was less stable (Munro *et al.*, 1996) and susceptible to temperature-induced activity loss when compared to isolated BMP (Jamakhandi *et al.*, 2005).

In the present study, a continuous spectrophotometric method was used to measure the reduction activity of the reductase domain of CYP102A1 using MTT and CTC as substrates. MTT and CTC have several advantages as the substrates for BMR. First, the solutions of both of these tetrazolium salts are stable for several days at room temperature. Second, MTT and CTC are freely soluble in water, up to 5 mM and 50 mM, respectively. Third, MTT and CTC are suitable substrates for a colorimetric high-throughput assay, as both MTT and CTC form highly colored reduced products (formazans) and show obvious color changes (from yellow to blue for MTT and colorless to red for CTC), which can be easily observed with the naked eye. In addition, the CTC formazan is fluorescent, thereby allowing more-sensitive spectrofluorometric assays.

Although rCPR has a membrane anchor domain at its N-terminus, BMR is a soluble protein without a membrane anchor domain. Regardless, the BMR can be developed as a surrogate for the mammalian CPR.

In conclusion, the ability of BMR to metabolize the sub-

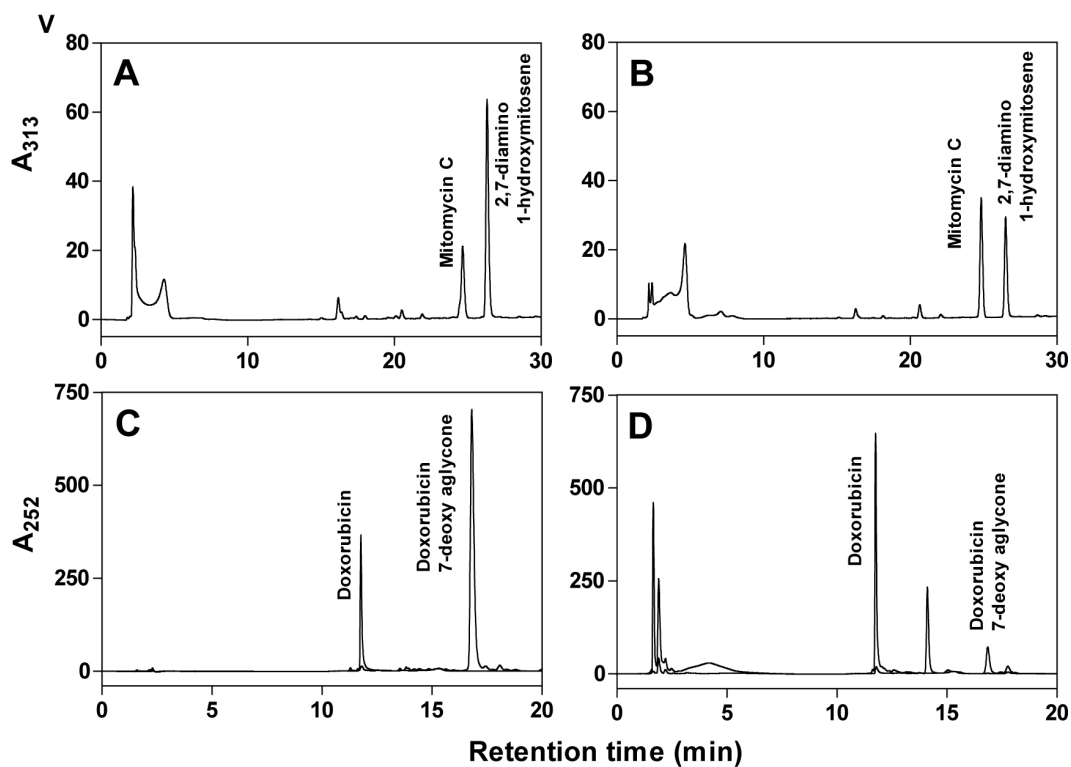


Fig. 4. HPLC analysis of mitomycin C and doxorubicin metabolism by purified rat CPR (A, C) and BMR (B, D) respectively. Mitomycin C (200 μ M) was incubated with CPR or BMR in 100 mM potassium phosphate buffer (pH 5.8) for 30 min at 37°C. The HPLC eluates were detected at 313 nm. The retention times for mitomycin C and the major metabolite are 24.5 min and 26.3 min, respectively (A, B). Doxorubicin (200 μ M) was incubated with CPR or BMR in 100 mM potassium phosphate buffer (pH 7.4) for 30 min at 37°C. The HPLC eluates were detected at 252 nm. The retention times for doxorubicin and the major metabolite are 11.6 min and 16.8 min, respectively.

strates of CPR was examined, and the results showed that BMR is able to metabolize mitomycin C and doxorubicin, the anticancer drug substrates for CPR, producing the same major metabolite. Moreover, BMR is able to interact with human CYP1A2 and CYP2E1 and mediate electron transfer. The results of this study suggest that BMR has biochemical properties that are similar to those of mammalian CPR and that BMR can be used as a surrogate for CPR. Several substrates of CPR, including CTC, MTT, mitomycin C, and doxorubicin, can be used for the BMR.

ACKNOWLEDGMENTS

This work was supported by a grant from the Next-Gener-

ation BioGreen 21 Program (SSAC, grant# PJ008058), Rural Development Administration, Republic of Korea.

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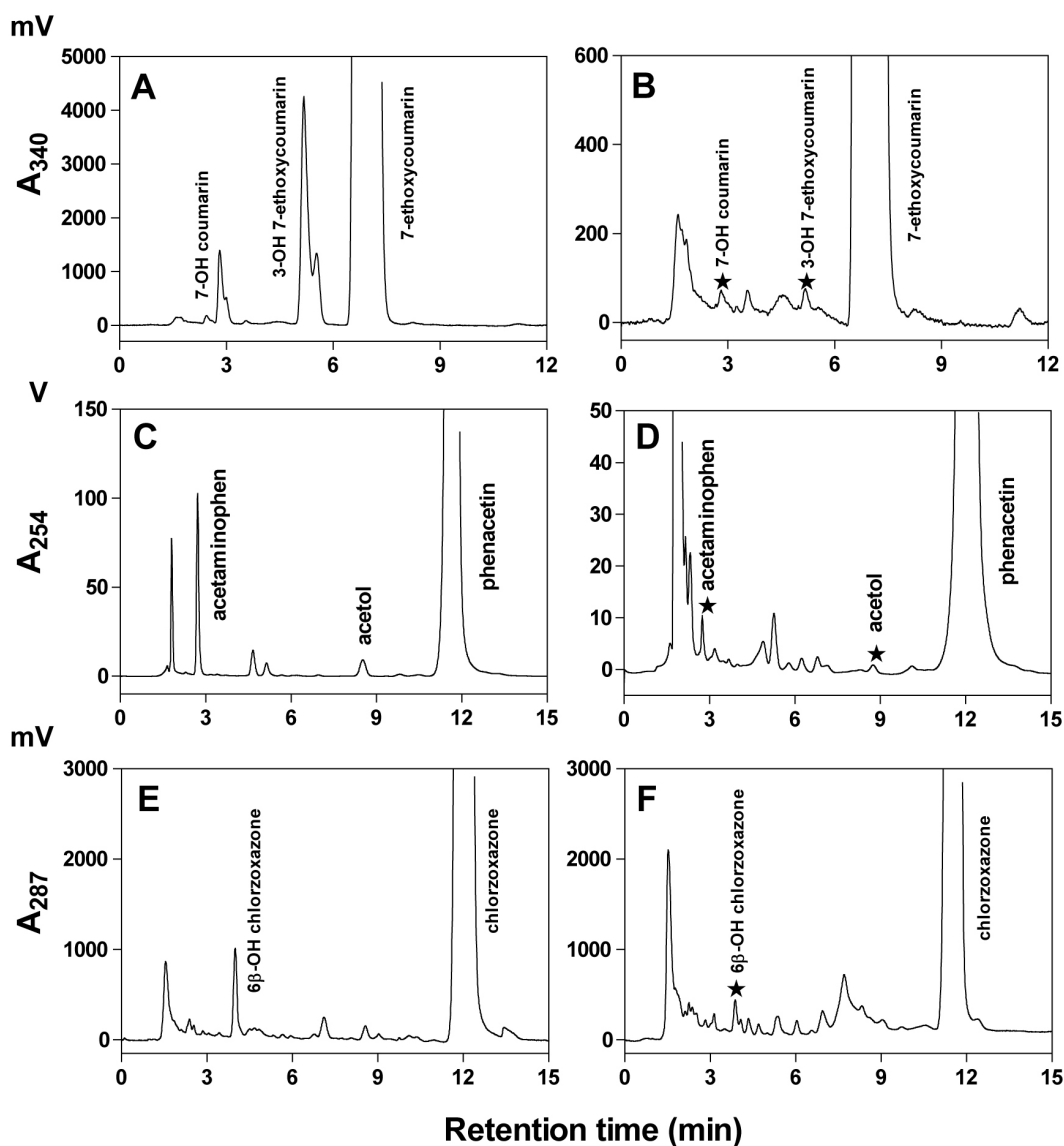


Fig. 5. HPLC analysis of the metabolites of 7-ethoxycoumarin, phenacetin, and chlorzoxazone produced by human CYP1A2 with CPR (A, C, E) and BMR (B, D, F). The reactions were performed with substrate (A, B - 500 μ M 7-ethoxycoumarin; C, D - 2 mM phenacetin; E, F - 500 μ M chlorzoxazone), 50 pmol human CYP1A2, and 100 pmol CPR or BMR in 100 mM potassium phosphate buffer (pH 7.4) for 30 min at 37°C. The HPLC eluates were detected at each of the indicated wavelengths on the Y axis.

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