Molecular Characterization of Specifically Active Recombinant Fused Enzymes Consisting of CYP3A4, NADPH-Cytochrome P450 Oxidoreductase, and Cytochrome b_5[†]

Hideyuki Inui,[‡,||] Atsuyo Maeda,[§,||] and Hideo Ohkawa*[,‡,§,⊥]

Research Center for Environmental Genomics, Kobe University, Nada-ku, Kobe 657-8501, Japan, and Graduate School of Science and Technology, Kobe University, Nada-ku, Kobe 657-8501, Japan

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ABSTRACT: Microsomal cytochrome P450 3A4 (CYP3A4) catalyzes monooxygenase reactions toward a diverse group of exogenous and endogenous substrates and requires cytochrome b_5 (b_5) in the oxidation of the typical substrate testosterone. To analyze the molecular interaction among CYP3A4, NADPH-cytochrome P450 oxidoreductase (P450 reductase), and b_5, we constructed several fused enzyme genes and expressed them in *Saccharomyces cerevisiae*. The recombinant fused enzymes CYP3A4−truncated (t)-P450 reductase−t-b_5 (3RB) and CYP3A4−t-b_5−t-P450 reductase (3BR) in yeast microsomes showed a higher specific activity in 6β-hydroxylation of testosterone than did the reconstitution premixes of CYP3A4, P450 reductase, and b_5. The purified fused enzymes exhibited lower K_m values and substantially increased V_{max} values in 6β-hydroxylation of testosterone and oxidation of nifedipine. Moreover, the fused enzymes showed significantly higher activities in cytochrome *c* reduction than the reconstitution premixes. Although the affinity of 3RB toward cytochrome *c* was twice as high as that of 3BR, 3BR and 3RB showed nearly the same affinity toward NADPH/NADH. In addition, the heme of the CYP3A4 moiety of 3RB was reduced preferentially and more rapidly than that of 3BR, whereas the heme of the b_5 moiety of 3BR was selectively reduced compared with that of 3RB. These results suggest that the conformation of the 3RB molecule was the most suitable for high activity because of appropriate ordering of the CYP3A4, P450 reductase, and b_5 moieties for efficient electron flow. Thus, we believe that the b_5 moiety plays an important role in the efficient transfer of the second electron in the vicinity of the CYP3A4 moiety.

Cytochrome P450s (P450s or CYPs)[1] occur widely in bacteria, plants, and animals, and in eukaryotes, P450s are membrane-bound on microsomes and in mitochondria. The microsomal P450s form electron transport chains together with NADPH-cytochrome P450 oxidoreductase (P450 reductase), cytochrome b_5 (b_5), or both. P450 reductase transfers two electrons from NADPH/NADH to the heme of P450 for reduction of the ferrous−dioxygen complex after the binding of a substrate to P450. Both b_5 and NADH-cytochrome b_5 oxidoreductase (b_5 reductase) also contribute to the electron flow toward P450s (*1*).

Microsomal b_5 is a membrane-bound protein consisting of a hydrophilic N-terminal heme-containing segment and a hydrophobic C-terminal membrane-anchoring segment. b_5 accepts an electron from either P450 reductase or b_5 reductase and then transfers electrons to several enzymes involved in the biosynthesis of fatty acids (*2*) and cholesterol (*3*). Also, b_5 participates in the monooxygenase reactions catalyzed by certain microsomal P450s. In the P450-dependent monooxygenase reactions, b_5 has been proposed to be involved in (a) electron donation through reduced b_5 to P450s, (b) an "effector" function that alters the substrate binding site of P450 (*4*, *5*), (c) promotion of the efficiency of electron flow for substrate monooxygenation by decreasing the loss of reducing equivalents resulting in the formation of hydrogen peroxide (*6*), or (d) the molecular interaction of P450 with other proteins such as P450 reductase. These effects of b_5 are highly dependent on the P450 species (e.g., CYP2B4, CYP2E1, or CYP3A4) as well as the nature of the substrate (*7*−*10*).

In humans, CYP3A4 is the most abundant P450 species in the liver, constituting approximately 30% of the total P450 species, although there is considerable interindividual variability in the level of hepatic CYP3A4 (*11*). In addition, CYP3A4 metabolizes a wide range of substrates with various

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* Corresponding author. Tel: +81-84-936-2111, ext. 4675. Fax: +81-84-936-2459. E-mail: hohkawa@fubac.fukuyama-u.ac.jp.

[‡] Research Center for Environmental Genomics.

[§] Graduate School of Science and Technology.

[||] These authors contributed equally to this work.

[⊥] Present address: Hideo Ohkawa, Research Center for Green Science, Fukuyama University, Gakuen-cho 1, Hiroshima 729-0292, Japan.

[1] Abbreviations: b_5, cytochrome b_5; b_5 reductase, NADH-cytochrome b_5 oxidoreductase; t-b_5, truncated form of cytochrome b_5 (Met_1 to Asp_{104}); DLPC, L-1,2-dilauroyl-*sn*-glycerol-3-phosphocholine; DLPS, L-1,2-dilauroyl-*sn*-glycerol-3-phosphoserine; DOPC, L-1,2-dioleoyl-*sn*-glycerol-3-phosphocholine; Fd, ferredoxin; FNR, ferredoxin $NADP^+$ reductase; P450s or CYPs, cytochrome P450s; P450 reductase, NADPH-cytochrome P450 oxidoreductase; t-P450 reductase, truncated form of P450 reductase (Ala_{42} to Trp_{691}); Pd, putidaredoxin; PdR, putidaredoxin reductase; plasmids (see Figure 1): control, pAAH5N; 3A4, pAAH3A4; 3, pAAH3A4; b_5, pAAHb5; R, pARR3N; 3CR, pARR3A4; 3R, pAAHF3R; 3BCR, pAAHF3BCR; 3RCB, pAAHF3RCB; 3BR, pAAHF3BR; 3RB, pAAHF3RB.

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molecular structures and sizes. However, reconstituted systems containing purified CYP3A4 and P450 reductase show little catalytic activity toward a variety of substrates, although adding components such as b_5 (*12*), lipids (*13*, *14*), divalent cations (*15*), mixed salts (*16*, *17*), glutathione (*18*, *19*), or CYP1A2 (*20*) to these system stimulates the catalytic activity of CYP3A4.

Murakami et al. constructed a cDNA that contained the fused sequences of rat CYP1A1 and rat P450 reductase and expressed it in *Saccharomyces cerevisiae* (*21*). The fused enzyme transferred electrons intramolecularly from the reductase moiety to the P450 moiety and exhibited high monooxygenase activity. Since Murakami's report, a number of fused enzymes between various P450 species and P450 reductases have been constructed and their activities evaluated. A b_5 and P450 reductase fused enzyme with an intermolecular membrane-anchoring domain has also been constructed (*22*, *23*). The CYP17A and CYP3A4 showed enhanced monooxygenase activities. A b_5-like domain has been found in a number of enzymes, including nitrate reductase (*24*), sulfite oxidase (*25*), and fatty acid desaturase (*26*), but the position of the b_5-like domain in these enzymes was not conserved.

In this study, we constructed a number of fused enzyme genes consisting of human CYP3A4, yeast P450 reductase, and human b_5 cDNAs and expressed them in *S. cerevisiae*. We prepared the purified enzymes from microsomes of recombinant yeast cells and subjected them to enzymatic assays for testosterone hydroxylation and nifedipine oxidation activities. On the basis of the assay results, we discuss the roles of the combination of CYP3A4, P450 reductase, and b_5, particularly the roles of b_5, in P450-dependent monooxygenase activity and in the interaction among P450, P450 reductase, and b_5 moieties of the fused enzymes.

EXPERIMENTAL PROCEDURES

Chemicals and Biochemicals. Testosterone and nifedipine were purchased from Nacalai Tesque Co. (Kyoto, Japan). 6β-Hydroxy testosterone was provided by Sumitomo Chemical Co., Ltd. (Osaka, Japan). Oxidized nifedipine was purchased from ULTRAFINE Chemicals (Manchester, UK). Sucrose monolaurate was purchased from Dojindo Laboratories (Kumamoto, Japan). Diethylaminoethyl-cellulose DE52 and Toyopearl HW-55F were obtained from Whatman International Ltd. (Maidstone, UK) and Tosoh Co. (Tokyo, Japan), respectively. L-1,2-Dilauroyl-*sn*-glycerol-3-phosphocholine (DLPC), L-1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and L-1,2-dilauroyl-*sn*-glycerol-3-phosphoserine (DLPS) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL).

Construction of Yeast Expression Plasmids for Fused Enzyme Genes Consisting of Human CYP3A4, Yeast NADPH-Cytochrome P450 Oxidoreductase, and Human Cytochrome b_5 cDNAs. The cDNAs of human CYP3A4, yeast P450 reductase, and b_5 were provided by Sumitomo Chemical Co., Ltd. PCR fragments and the constructed fused enzyme genes were sequenced with a 5500-L Hitachi DNA sequencer (Tokyo, Japan). Each of the expression plasmids (Figure 1) was introduced into *S. cerevisiae* AH22 cells [*MATa*, *leu2*−*3*, *leu2*−112, his4−519, can1, (*cir*$^+$)] by the lithium chloride method (*27*) to obtain the corresponding recombinant yeast strains.

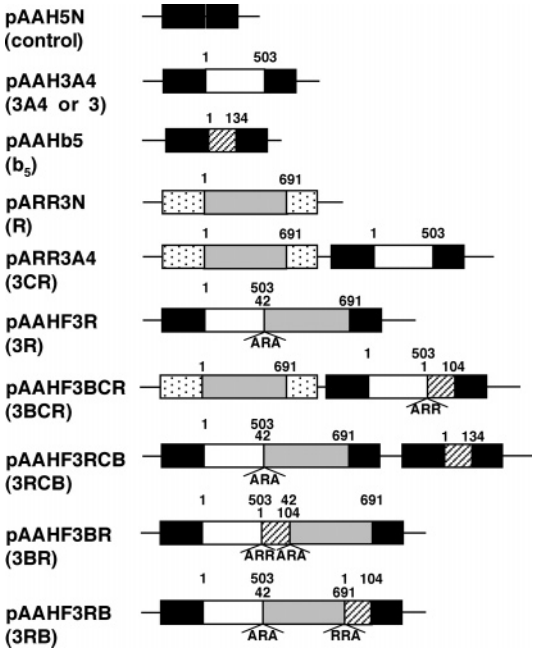

FIGURE 1: Structure of recombinant plasmids for the expression of CYP3A4, P450 reductase, and b_5 genes. The open, gray, and shaded boxes indicate the genes corresponding to the protein-coding regions for CYP3A4, P450 reductase, and b_5, respectively. The solid and dotted boxes indicate alcohol dehydrogenase gene promoter and terminator regions, and yeast P450 reductase gene promoter and terminator regions, respectively. The numbers above the coding regions indicate those of amino acid residues from the amino-terminus of CYP3A4, P450 reductase, and b_5. The letters under the coding regions indicate additional amino acids derived from the linker region. The abbreviations used in this study are indicated below the name of the plasmid.

Microsomal Preparation. Each of the recombinant yeast strains was cultivated in SD medium (5.4% yeast nitrogen base without amino acids, 8% glucose, and 160 μg/mL histidine) (*28*) at 30 °C for 38−40 h. Recombinant yeast microsomal fractions were prepared as described previously (*29*). Microsomal fractions were homogenized in a small volume of the suspension buffer (0.1 M potassium phosphate buffer [pH 7.4], 20% [v/v] glycerol, 1 mM phenylmethylsulfonyl fluoride, and 4 mM 2-mercaptoethanol). Protein concentrations were measured by the Lowry method, using bovine serum globulin as a standard (*30*). P450 content was determined using an extinction coefficient of 91 mM^{-1} cm^{-1} ($\Delta_{\epsilon 450}-_{\epsilon 490}$ for $Fe^{2+}CO$ vs Fe^{2+}) (*31*, *32*), and b_5 content was determined using an extinction coefficient of 185 mM^{-1} cm^{-1} ($\Delta_{\epsilon 426}-_{\epsilon 409}$ for Fe^{2+} vs Fe^{3+}) (*33*).

SDS-PAGE and Western Blot Analysis. Microsomes prepared from the recombinant yeast cells were subjected to electrophoresis in a 10% polyacrylamide gel. The separated proteins were stained with Coomassie Brilliant Blue. In western blot analyses, proteins were transferred electrophoretically from the gel to a PVDF-Plus membrane (Micron Separations, Westborough, MA), and the protein bands were detected using primary rabbit polyclonal antibodies against rat CYP3A2 IgG (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) and secondary rabbit antibody conjugated with alkaline phosphatase (Sigma-Aldrich Co., St. Louis, MO).

Purification of Fused Enzymes. Each of the microsomal fractions prepared from the recombinant yeast strains was solubilized by stirring at 4 °C for 30 min in buffer A (20

mM potassium phosphate buffer [pH 7.4], 20% [v/v] glycerol, and 2 mM EDTA) containing 0.5% (w/v) sodium cholate and 0.1% (w/v) sucrose monolaurate. The solubilized microsomes were each centrifuged for 1 h at 115,000*g*. The supernatant obtained was applied to a DEAE-cellulose column (26 × 250 mm) and washed with buffer B (buffer A containing 0.1% [w/v] sodium cholate and 0.02% [w/v] sucrose monolaurate). Fractions containing the fused enzymes were eluted with 400 mL of a 0−0.8 M gradient of potassium chloride in buffer B. Afterward, fractions of the fused enzymes were applied to a Toyopearl HW-55F gel filtration column (26 × 600 mm) and then eluted with buffer B. The fractions containing the fused enzymes were collected and used for enzyme assays.

Testosterone 6β-Hydroxylation and Nifedipine Oxidation Activities. For purified fused enzyme assays, a standard reaction mixture (200 μL) contained 0.1 M potassium phosphate buffer (pH7.4), 3 mM magnesium chloride, 3 mM reduced glutathione, 5 mM glucose-6-phosphate, 1 U of glucose-6-phosphate dehydrogenase, 0.5 mM NADPH, 0.5 mM NADH, 1 mg/mL liposomes (DLPC, DOPC, and DLPS [1:1:1, w/w/w]), 50 μM testosterone or 100 μM nifedipine, and a portion of the purified fused enzyme. Equimolar amounts of exogenous P450 reductase and b_5 with respect to CYP3A4 and its fused enzymes were added to reaction mixtures (e.g., CYP3A4 (*3*) + P450 reductase + b_5 [B]). The purified fused enzyme in buffer B was mixed with liposomes, reduced glutathione, and substrate, and then preincubated at 37 °C. The reaction mixture was diluted by the addition of the remaining components and incubated at 37 °C for 10 min. Residual detergents in the final assay mixtures were less than 0.015% (w/v) for sodium cholate and 0.003% (w/v) for sucrose monolaurate. Liposomes were not included in reaction mixtures containing unpurified fused enzymes. The enzymatic reaction was stopped by vigorous mixing with 2 volumes of dichloromethane for testosterone or with 2 volumes of dichloromethane, 0.2 M sodium chloride, and 0.1 M sodium carbonate for nifedipine. The organic phases were evaporated, and the resulting residues were solubilized with 50% (v/v) methanol/water for HPLC analysis on a COSMOSIL 5C18-ARII column (4.6 × 150 mm; Nacalai Tesque Co.). The column was eluted with 55% (v/v) methanol/water for testosterone or 51% (v/v) for nifedipine at a flow rate of 1.0 mL/min at 40 °C. Testosterone and its metabolites were detected by the absorbance at 240 nm (*20*), whereas nifedipine and its metabolite were detected by the absorbances at 238 and 204 nm, respectively.

Substrate Binding Spectra. Substrate perturbation difference spectroscopy was carried out on samples (1 mL) containing CYP3A4 or the fused enzyme, 1 mg/mL liposomes, 3 mM magnesium chloride, and 0.1 M potassium phosphate buffer (pH7.4). The substrate was titrated into the sample to a final concentration of 0.2 mM. The spectra were recorded at 15 °C on a U-3000 spectrophotometer.

Cytochrome c Reduction Activity. Cytochrome *c* reduction was followed by measuring the absorbance change at 550 nm on a Hitachi U-3000 spectrophotometer, using an extinction coefficient of 19.1 mM^{-1} cm^{-1} (*34*). The reaction mixture (1 mL) contained CYP3A4 or the fused enzyme, 1 mg/mL liposomes, 0−0.2 mM cytochrome *c*, 0−0.1 mM NADPH, 0−0.1 mM NADH, 0.1 M potassium phosphate buffer (pH7.4), and 0.06 mM EDTA. The reaction was carried out at 25 °C.

Heme Reduction Activity. The reduction of ferric P450 to the ferrous−carbon monoxide form was carried out at 28 °C under an anaerobic carbon monoxide environment, whereas the reduction of b_5 was carried out under aerobic conditions. Both reactions were monitored with a U-3000 spectrophotometer. Reduction activities were measured for mixtures (1 mL) containing 0.6 μM CYP3A4 or the fused enzymes, 1 mg/mL liposomes, 30 mM magnesium chloride, 100 μM testosterone, and 0.1 M potassium phosphate buffer (pH 7.4). The reactions were initiated by adding 0.5 mM NADPH and 0.5 mM NADH. The P450 and b_5 reductions were monitored at 448 and 426 nm, respectively (*35*).

RESULTS

Construction of Yeast Expression Plasmids for CYP3A4, P450 Reductase, b_5, and Their Fused Enzyme cDNAs. We constructed yeast expression plasmids for human CYP3A4 (pAAH3A4), yeast P450 reductase (pARR3N), human b_5 (pAAHb5), and their fused enzyme cDNAs (Figure 1). pARR3A4 was constructed for the coexpression of CYP3A4 and P450 reductase cDNAs. pAAHF3R contains the fused enzyme gene encoding CYP3A4 (Met_1 to Ala_{503}) and the truncated form (t) of P450 reductase (Ala_{42} to Trp_{691}; t-P450 reductase), which included three additional amino acids (Ala-Arg-Ala) derived from the linker DNA at the junction between CYP3A4 and P450 reductase cDNA. The t-P450 reductase contained FAD-, FMN-, and NADPH-binding regions but not the amino-terminal hydrophobic-membrane anchor (41 amino acid residues), which is not necessary for electron transfer from NADPH to P450 (*36*). We constructed plasmid pAAHF3BCR to coexpress P450 reductase and the CYP3A4−t-b_5 (Met_1 to Asp_{104}; t-b_5) fused enzyme, which included three additional amino acids (Ala-Arg-Arg) at the CYP3A4−t-b_5 fusion junction. t-b_5, constructed analogous to the construction of t-P450 reductase, contained a heme domain but not the carboxyl-terminal hydrophobic membrane anchor. On the basis of the similarity of amino acid sequences between rat and human b_5, 30 amino acids from the C-terminus of human b_5 were predicted to be the membrane-anchoring domain (*37*, *38*), although Gilep et al. used 40 amino acids of human b_5 for the fusion with P450 reductase (*22*, *23*). The plasmid pAAHF3RCB was used for the coexpression of the CYP3A4−t-P450 reductase fused enzyme and b_5 (Met_1 to Asp_{134}). The plasmid pAAHF3BR contains the gene encoding the CYP3A4−t-b_5−t-P450 reductase fused enzyme, which included three additional amino acids at the CYP3A4−t-b_5 junction and the t-b_5−t-P450 reductase junction (Ala-Arg-Arg and Ala-Arg-Ala, respectively). The plasmid pAAHF3RB contains the cDNA for the fused enzyme consisting of CYP3A4, t-P450 reductase, and t-b_5, which included three additional amino acids (Ala-Arg-Ala and Arg-Arg-Ala) at the CYP3A4−t-P450 reductase junction and t-P450 reductase−t-b_5 junction, respectively.

Expression of CYP3A4, P450 Reductase, b_5, and Their Fused Enzyme cDNAs in Yeast Microsomes. S. cerevisiae AH22 cells were transformed with each of the expression plasmids. CYP3A4 and b_5 produced in each of the recombinant yeast strains exhibited characteristic absorption spectra: CYP3A4 showed a typical P450 spectrum with a peak

Table 1: Contents of P450 and b_5 and Activities of Cytochrome *c* Reduction in the Recombinant Yeast Microsomes

	content		cytochrome *c* reduction activity
enzyme	P450 pmol (mg protein)$^{-1}$	b_5 pmol (mg protein)$^{-1}$	nmol min^{-1} (mg protein)$^{-1}$
control	4	6	25
3A4	130	8	35
b_5	5	120	40
R	5	5	150
3CR	57	7	150
3R	71	9	220
3BCR	55	55	190
3RCB	57	62	260
3BR	49	59	520
3RB	44	50	380

at 448 nm in the reduced carbon monoxide difference spectrum, and b_5 showed a typical b_5 absorption spectrum with a peak at 426 nm and a valley at 409 nm in the NADH-dependent difference spectrum (data not shown). Table 1 summarizes the P450 and b_5 contents as well as the cytochrome *c* reduction activities in the microsomes of the recombinant yeast strains. AH22/pAAH3A4 microsomes (3A4 or 3) and AH22/pAAHb5 microsomes (b_5) produced the highest levels of P450 and b_5, respectively. However, the yeast strains coexpressing 3A4 and P450 reductase or expressing the fusion genes with the other protein genes produced lower levels of P450 and b_5. The P450 reductase moiety of each of the fused enzymes reduced horse heart cytochrome *c* in the presence of NADPH. AH22/pAAHF3R microsomes (3R) exhibited a higher cytochrome *c* reduction activity than did AH22/pARR3N (R). These results are consistent with those of Hayashi et al. (*39*).

The fused proteins in the microsomes were analyzed by western blot analysis with an anti-CYP3A2 IgG, which recognized human CYP3A4 (Figure 2) (*40*). The protein bands derived from CYP3A4 (3A4, 51 kDa), the CYP3A4−t-b_5 fused enzyme (3B, 61 kDa), the CYP3A4−t-P450 reductase fused enzyme (3R, 132 kDa), the CYP3A4−t-b_5−t-P450 reductase fused enzyme (3BR, 142 kDa), and the CYP3A4−t-P450 reductase−t-b_5 fused enzyme (3RB, 142 kDa) were detected in each of the recombinant yeast microsomes. However, degraded fused proteins were also detected in each of the recombinant yeast microsomes. A band derived from each of the fused proteins containing P450 reductase and degraded fused proteins was also detected in each of the recombinant yeast microsomes with an anti-P450 reductase IgG (data not shown).

6β-Hydroxylation of Testosterone in the Yeast Microsomes. We next measured 6β-hydroxylation of testosterone in microsomes prepared from each of the recombinant yeast strains (Figure 3). The CYP3A4-dependent metabolic activity of testosterone was enhanced by the addition of P450 reductase and b_5. In addition, the microsomes of 3CR fortified with exogenously added purified b_5 (3CR+B) showed an increased metabolic activity. The fused enzyme 3R exhibited a higher activity than did 3 + R or 3CR. Moreover, 3R fortified with b_5 (3R + B), 3BCR, and 3RCB in the microsomes were more active in the metabolism of testosterone than 3 + R + B. These results clearly indicate that the fusion of CYP3A4 with P450 reductase or b_5 increased the P450-dependent enzyme activity and that the CYP3A4 and P450 reductase fused enzyme was more active in the hydroxylation than the CYP3A4 and b_5 fused enzyme, that is, 3RCB exhibited a higher activity than did 3BCR.

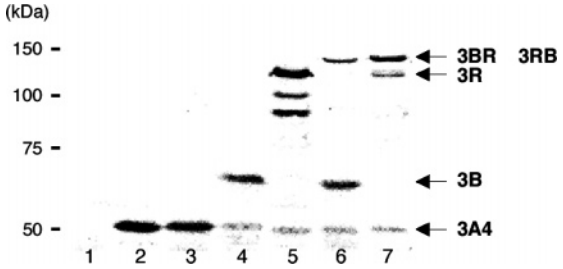

FIGURE 2: Western blot analysis of the recombinant yeast microsomes. Microsomal proteins (10 μg) were analyzed in 10% SDS−PAGE. CYP3A4 fused proteins were detected with the anti-CYP3A2 antibody. Lanes 1−7 show the control, 3A4, 3CR, 3BCR, 3RCB, 3BR, and 3RB, respectively.

However, 3RB in the microsomes showed the highest metabolic activity among the recombinant enzymes produced in the yeast microsomes. The activity of 3RB was 2 times higher than that of 3R + B and 3RCB in the microsomes. 3RB was also 2-fold more active than 3BR, although these fused enzymes were slightly degraded in the microsome preparations (Figure 2). Therefore, enzyme assays with purified fused enzymes were used for further characterization.

Purification of the Fused Enzymes. The microsomes prepared from the recombinant yeast strains were solubilized in the presence of sodium cholate and sucrose monolaurate, after which the fused enzymes in the microsomes were purified by a combination of anion exchange column chromatography and gel filtration chromatography. SDS−PAGE of the purified fused enzymes 3BR and 3RB gave a single band with an apparent molecular mass of 140 kDa (Figure 4). The oxidized spectra of the purified fused enzymes 3BR and 3RB showed a large absorption peak at 420 nm and a small absorption peak at 550 nm, similar to the oxidized spectrum of 3A4 (Figure 5). The reduced carbon monoxide difference spectra of 3A4, 3BR, and 3RB showed a characteristic absorption peak at 448 nm (Figure 5).

Catalysis of 6β-Hydroxylation of Testosterone and Oxidation of Nifedipine by the Purified Enzymes. The fused enzymes purified from the yeast microsomes catalyzed 6β-hydroxylation of testosterone and oxidation of nifedipine. Both enzymatic reactions showed saturation kinetics of the Michaelis−Menten type, as indicated by the straight line in the Lineweaver−Burk plot (Figure 6). Compared with 3 + R + B, 3BR and 3RB showed a substantial decrease in K_m value and an increase in V_{max} value in both metabolic activities, indicating that 3BR and 3RB were suitable combinations of CYP3A4, P450 reductase, and b_5 for metabolizing testosterone and nifedipine.

Substrate Binding. Substrate binding constants (K_s) for each of the fused enzymes were determined spectroscopically (Figure 7). Each of the fused enzymes exhibited typical type I spectra, and K_s values were estimated from double-reciprocal plots of $\Delta A_{390-420}$ versus testosterone concentration or nifedipine concentration. Similar K_s values were obtained for the substrates (Figure 7). Moreover, the substrate binding profiles of the fused enzymes were quite similar to the binding profile of CYP3A4, in spite of the fusion with P450 reductase and b_5.

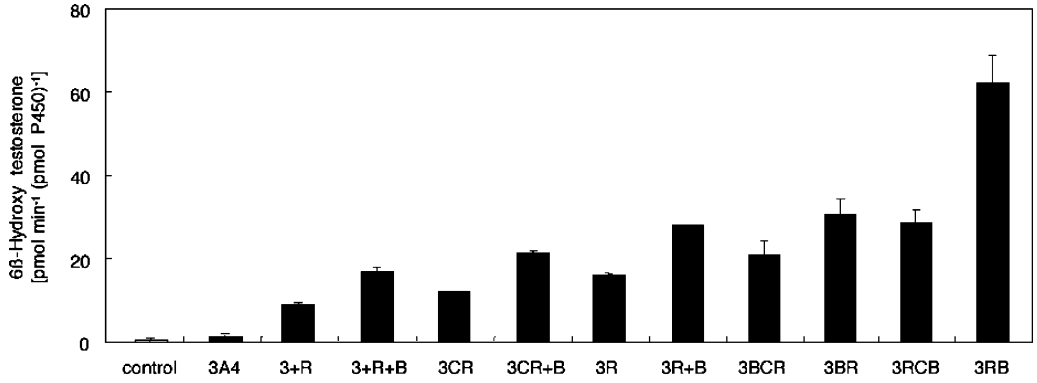

FIGURE 3: Effects of b_5 and the expression form on the catalytic activity of CYP3A4 toward testosterone. All values were the means of triplicate experiments ± SD.

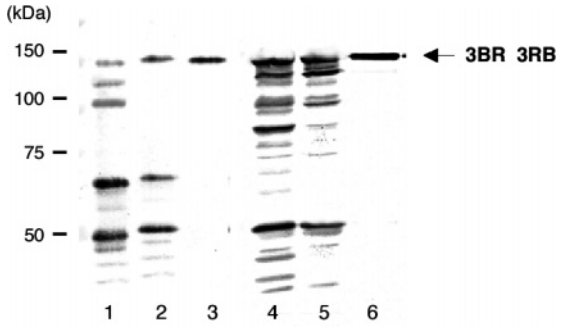

FIGURE 4: SDS−PAGE of the purified fused enzymes. Each protein (50 μg) was separated by 10% SDS−PAGE. Lanes 1−3 and 4−6 show each purification step for 3BR and 3RB, respectively. Lanes 1 and 4 are the solubilized supernatant. Lanes 2 and 5 are the DEAE cellulose fraction. Lanes 3 and 6 are the gelumination fraction. An arrow shows the fused proteins consisting of CYP3A4, b_5, and P450 reductase.

Cytochrome c Reduction Activity. To examine the electron transfer activity of P450 reductase, we measured the cytochrome *c* reduction activity in the fused enzymes. Each of the fused enzymes showed Michaelis−Menten type kinetics for NADPH/NADH and cytochrome *c*, as indicated by the straight lines in the Lineweaver−Burk plots (Figure 8). Although 3BR and 3RB showed slightly different cytochrome *c* reduction activities, their activities were different from the activity of 3 + R + B. 3RB had a smaller K_m value for cytochrome *c* than did 3BR, although the K_m value of 3RB for NADPH/NADH was nearly the same as that of 3BR. Thus, we deemed 3RB to have the most suitable combination of CYP3A4, P450 reductase, and b_5 for the electron acceptor, even though there were no significant differences in affinity toward the electron donors.

NADPH/NADH-Dependent Heme Reduction Activity. The hemes of CYP3A4 and b_5 in the fused enzymes 3BR and 3RB were reduced more quickly than those in 3 + R + B (Figure 9), and 3RB reduced the heme in CYP3A4 much faster than did 3BR. In contrast, 3BR reduced the heme in b_5 much faster than did 3RB. Thus, 3RB was the most efficient for electron transfer to the heme in CYP3A4, and 3BR was the most efficient for b_5.

These results confirm that the conformation of CYP3A4, P450 reductase, and b_5 moieties in the 3RB molecule produces the most efficient electron flow because of the appropriate ordering of three components, and therefore, 3RB has the highest activity.

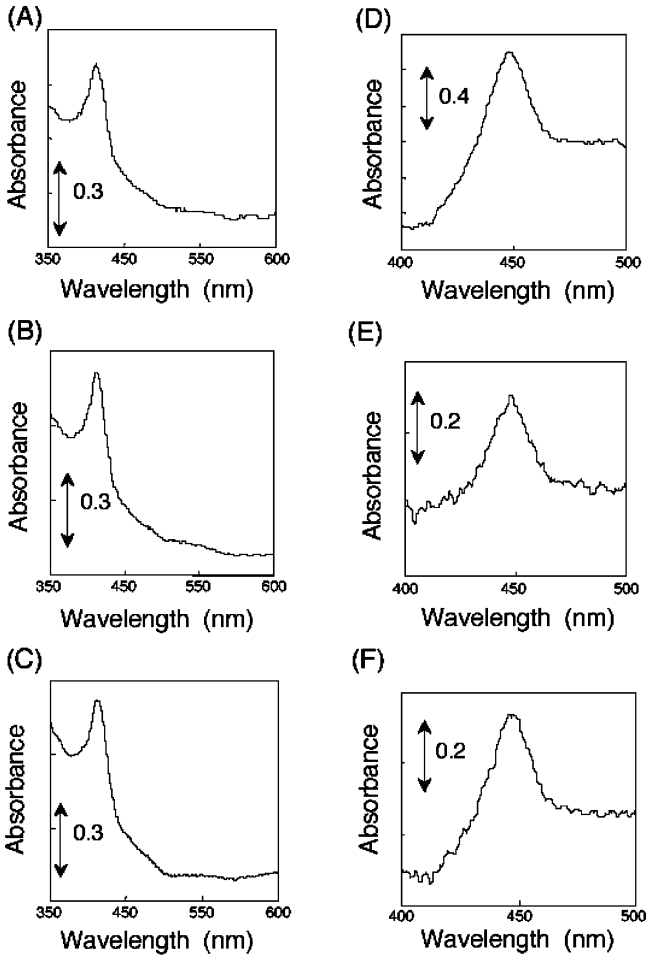

FIGURE 5: Oxidized spectra (A, B, and C) and the reduced carbon monoxide difference spectra (D, E, and F) of the purified fused enzymes. (A) and (D), (B) and (E), and (C) and (F) depend on 3A4, 3BR, and 3RB, respectively.

DISCUSSION

To characterize the function of the combination of CYP3A4, P450 reductase, and b_5, particularly the role of b_5, in the electron-transfer system, we constructed fused enzymes containing these three components. Gilep et al. reported that the fused enzyme between the rat full-length form of b_5 and P450 reductase with an internal membrane anchor showed higher activities in the CYP3A4-dependent hydroxylation of testosterone and the CYP17A-dependent hydroxylation of progesterone than the separate proteins (*22*).

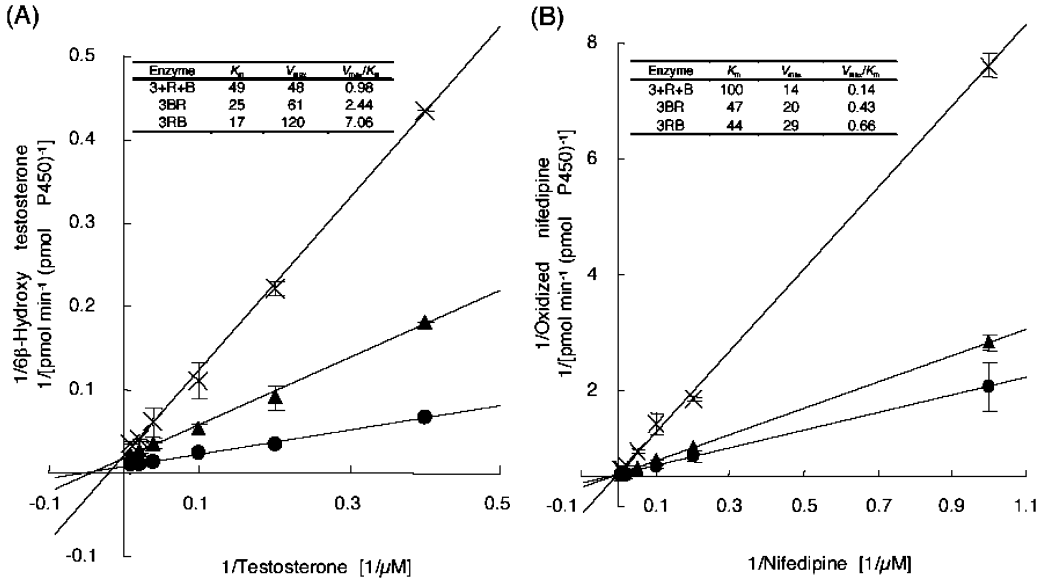

FIGURE 6: Testosterone hydroxylation (A) and nifedipine oxidation (B) of the purified fused enzymes. The concentration of testosterone or nifedipine was changed from 1 to 0.1 mM (A) or from 1 to 0.2 mM (B). All values were the means of triplicate experiments ± SD. (×), 3 + R + B; (▲), 3BR; (●), 3RB. K_m (μM) and V_{max} (pmol product/min/pmol P450) values are described in the figure.

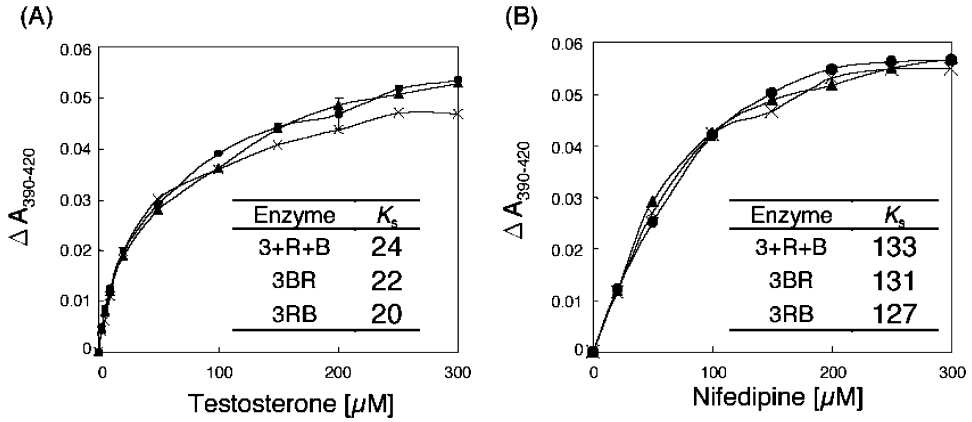

FIGURE 7: Substrate binding to the fused enzymes. Plots of $\Delta_{390-420}$ vs testosterone concentration (A) or nifedipine concentration (B). All values were the means of triplicate experiments ± SD. (×), 3 + R + B; (▲), 3BR; (●), 3RB. The *K*s (μM) value is described in the figure.

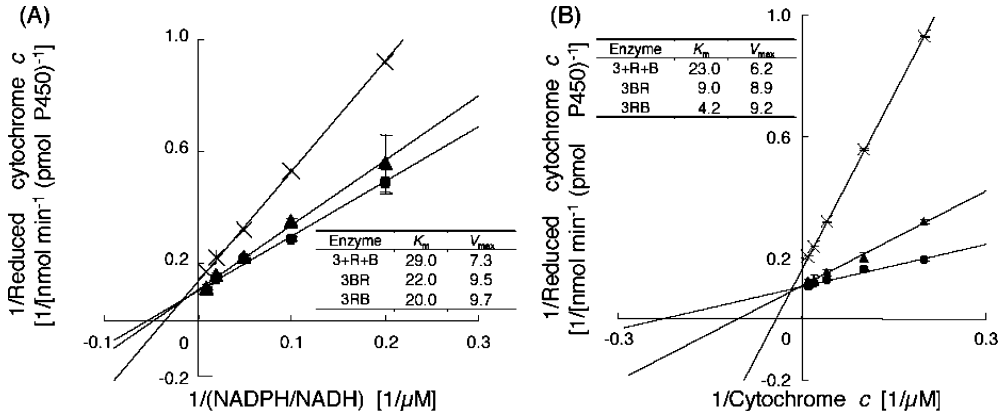

FIGURE 8: Cytochrome *c* reduction activity in the purified fused enzymes. The concentration of NADPH and NADH (A) or cytochrome *c* (B) was changed from 1 to 0.1 mM or from 1 to 0.2 mM, respectively. All values were the means of triplicate experiments ± SD. (×), 3 + R + B; (▲), 3BR; (●), 3RB. K_m (μM) and V_{max} (nmol product/min/pmol P450) values are described in the figure.

The fused enzymes of the present study contain a membrane anchor in the N-terminal region but not in the junction of the fused enzymes. These fused enzymes showed high metabolic activities toward testosterone and nifedipine, as compared with the CYP3A4 reconstitution systems, in which CYP3A4 showed slight activity in the 6β-hydroxylation of testosterone. However, adding P450 reductase or b_5 to the system increased the metabolic activity (Figure 3). Thus, it appears that CYP3A4 was not always efficiently accepting electrons from endogenous P450 reductase or b_5, consistent with the findings of an earlier report (*18*). For the fused enzymes of the present study, 3R metabolized testosterone

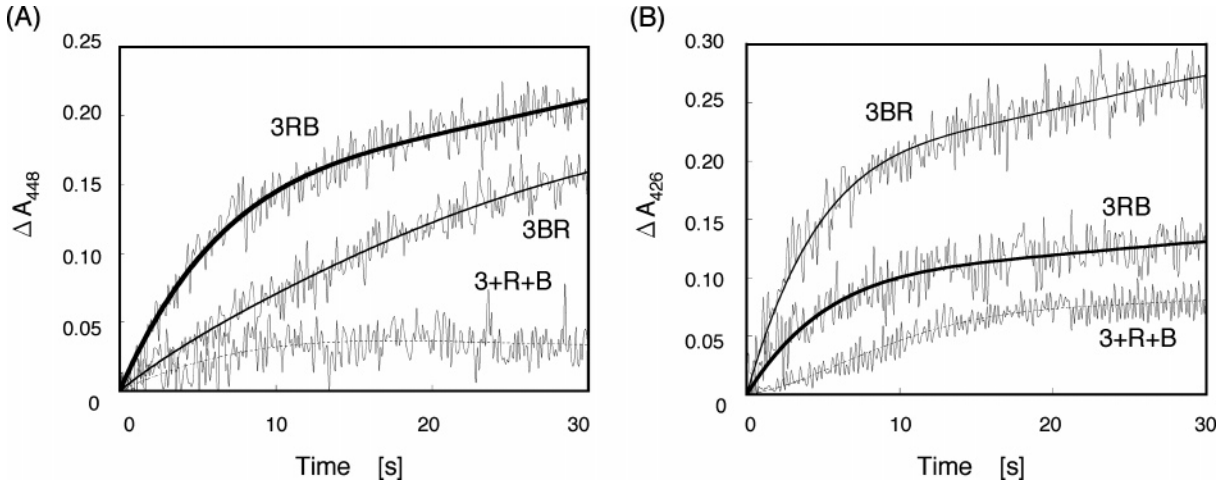

FIGURE 9: Kinetics of reduction of CYP3A4 (A) and b_5 (B). Traces (Δ_{448} for P450 or Δ_{426} for b_5) are shown for a system containing 0.6 μM CYP3A4 or the fused enzymes and other reaction components cited in Experimental Procedures. The 1.2 μM P450 reductase and 0.6 μM b_5 were contained in the reaction mixture for the measurement with CYP3A4. The bold, thin, and dotted lines indicate 3RB, 3BR, and 3 + R + B, respectively.

more actively than did 3 + R or 3CR (Figure 3), indicating that 3R was the competent fused enzyme, as reported previously (*41*). The activity of the fused enzyme was also enhanced by adding b_5, as with 3R + B (Figure 3). Therefore, the fused enzymes consisting of CYP3A4, P450 reductase, and b_5 would have higher activity because the fusion appears to effect electron transfer via b_5 as well as P450 reductase. It is probable that P450 reductase and b_5 in the fused enzyme could transfer more electrons to CYP3A4 than exogenous addition of b_5 because of their constitutive spatial proximity to CYP3A4.

The importance of the positions of P450 reductase and b_5 in the primary structure of the recombinant CYP3A4 fused enzymes was evaluated by comparing the enzyme activities of 3BR and 3RB. In all conformations, both 3BR and 3RB were able to catalyze testosterone hydroxylation and nifedipine oxidation, and neither 3BR nor 3RB induced a change in the tertiary structure that was unfavorable for catalytic reaction. However, 3RB showed higher catalytic activity than did 3BR, indicating that the order of the three proteins in 3RB was more favorable for catalytic activity than the order in 3BR. Thus, we used purified fused enzymes to analyze why 3RB showed higher catalytic activity than that of 3BR.

Spectral characterization revealed that all combinations of proteins resulted in similar values of K_s, suggesting that the fused enzymes did not affect the binding affinity of CYP3A4 for the substrates (Figure 7). This is consistent with the results reported for the fusion of CYP3A4 and yeast or rat P450 reductase toward the binding affinity of testosterone (*39*, *41*).

The cytochrome *c* reduction activities of 3BR and 3RB were higher than that of 3 + R + B, suggesting more rapid electron transfer in the fused enzyme toward the electron acceptor cytochrome *c* but lesser effect for the electron donors NADPH/NADH (Figure 8). Thus, efficient electron transfer from P450 reductase to the substrate rather than efficient interaction between NADPH/NADH and P450 reductase was responsible for the increase in metabolic activities. In contrast, the cytochrome *c* reduction activity of 3RB was higher than that of the 3BR, indicating that the CYP3A4 moiety in 3RB accepts electrons from P450 reductase more readily than does the CYP3A4 moiety in 3BR. We speculated that the higher metabolic activity of 3RB was due to efficient electron transport to CYP3A4 caused by the conformational changes. The kinetics results shown in Figure 9 confirmed that electron transfer from NADPH/NADH to CYP3A4 was more efficient in 3RB than in 3BR. Interestingly, 3BR transferred more electrons to b_5 than did 3RB, suggesting that greater electron leakage to b_5 in 3BR than 3RB was the reason 3BR showed a lower activity than 3RB because the first electron flow from NADPH via P450 reductase to P450 is one of the rate-limiting factors in the hydroxylation of testosterone and oxidation of nifedipine by CYP3A4 (*17*). It is likely that 3RB showed the highest metabolic activity because the first electron was transferred more rapidly to CYP3A4 than subsequent electrons. This result is consistent with a high potential for electron transfer from P450 reductase to cytochrome *c* in 3RB. In contrast, 3BR may intramolecularlly transfer a second electron much more rapidly than 3RB because b_5 in 3BR was more actively reduced than b_5 in 3RB (Figure 9). Sibbesen et al. and Lacour et al. reported that the order of electron flow from electron donor to P450 in the fused enzymes was importantly related to activity (*42*, *43*). Sibbesen et al. constructed triple fusion enzymes consisting of P450cam, putidaredoxin (Pd), and putidaredoxin reductase (PdR) (*42*). The fusion protein PdR-Pd-P450cam showed a higher activity than that of Pd-PdR-P450cam. The order of the components in PdR-Pd-P450cam corresponded to the electron flow: NADH ($E_{m,7} \sim -310$ mV) to PdR ($E_{m,7} \sim -250$ mV) (*44*) to Pd ($E_{m,7} \sim -230$ mV) (*45*) to P450cam ($E_{m,7} \sim -173$ mV). In contrast, Lacour et al. reported a fused enzyme consisting of rat CYP1A1, ferredoxin (Fd), and ferredoxin $NADP^+$ reductase (FNR) (*43*) in which the redox potentials were not optimal [NADPH ($E_{m,7} \sim -320$ mV) to FNR ($E_{m,7}$, ~ -360 mV) (*46*) to Fd ($E_{m,7}$, ~ -400 mV) (*47*)]. Although CYP1A1-Fd-FNR showed a higher metabolic activity than the other constructs (e.g., CYP1A1-FNR-Fd), its activity was much lower when compared with the activity of a CYP1A1−P450 reductase fused enzyme. In the present study, we believe the order of electron flow in 3RB is suitable for high activity: NADPH ($E_{m,7} \sim -320$ mV) to P450 reductase (FAD: $E_{m,7} \sim -290$ mV; FMN: $E_{m,7} \sim$

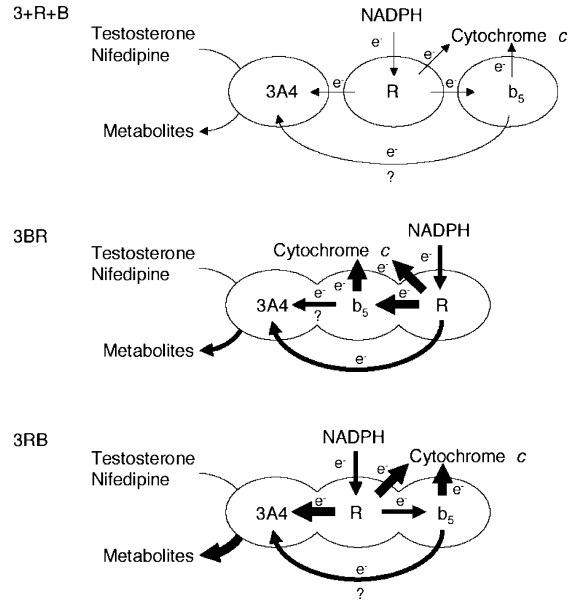

FIGURE 10: Proposed electron flow from NADPH/NADH to CYP3A4 in the reconstitution system and the recombinat fused enzymes 3BR and 3RB.

−270 mV) to ferric P450 ($E_{m,7} \sim -200$ mV) for the first electron and ferrous P450 ($E_{m,7} \sim +50$ mV) for the second electron from P450 reductase or b_5 ($E_{m,7} \sim +20$ mV) (*48*, *49*). However, electrons may in part flow from P450 reductase to b_5 in 3BR but flow less to CYP3A4. In addition, the distance between P450 reductase and CYP3A4 may affect the enzyme activities of 3BR. The previous results together with results from this work suggest that the protein order of 3RB leading to the change of protein conformation for an efficient electron transfer is the most favorable for high activity because of an appropriate conformation for efficient electron flow.

We propose that the enzyme activity of 3RB is higher than that of 3BR because of a stronger and more rapid transfer of electrons from NADPH via P450 reductase (Figure 10). The recombinant fusion technology described in this article should be useful for creating recombinant enzymes with a high metabolic activity in electron transport systems.

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REFERENCES

1. Hildebrandt, A., and Estabrook, R. W. (1971) Evidence for the participation of cytochrome b_5 in hepatic microsomal mixed-function oxidation reactions, *Arch. Biochem. Biophys. 143*, 66−79.
2. Oshino, N., Imai, Y., and Sato, R. (1971) A function of cytochrome b_5 in fatty acid desaturation by rat liver microsomes, *J. Biochem. (Tokyo) 69*, 155−167.
3. Fukushima, H., Grinstead, G. F., and Gaylor, J. L. (1981) Total enzymic synthesis of cholesterol from lanosterol. Cytochrome b_5-dependence of 4-methyl sterol oxidase, *J. Biol. Chem. 256*, 4822−4826.
4. Tamburini, P. P., MacFarquhar, S., and Schenkman, J. B. (1986) Evidence of binary complex formations between cytochrome P-450, cytochrome b_5, and NADPH-cytochrome P-450 reductase of hepatic microsomes, *Biochem. Biophys. Res. Commun. 134*, 519−526.
5. Imaoka, S., Inoue, K., and Funae, Y. (1988) Aminopyrine metabolism by multiple forms of cytochrome P-450 from rat liver microsomes: simultaneous quantitation of four aminopyrine metabolites by high-performance liquid chromatography, *Arch. Biochem. Biophys. 265*, 159−170.
6. Pompon, D. (1987) Rabbit liver cytochrome P-450 LM2: roles of substrates, inhibitors, and cytochrome b_5 in modulating the partition between productive and abortive mechanisms, *Biochemistry 26*, 6429−6435.
7. Vatsis, K. P., Theoharides, A. D., Kupfer, D., and Coon, M. J. (1982) Hydroxylation of prostaglandins by inducible isozymes of rabbit liver microsomal cytochrome P-450. Participation of cytochrome b_5, *J. Biol. Chem. 257*, 11221−11229.
8. Canova-Davis, E., and Waskell, L. (1984) The identification of the heat-stable microsomal protein required for methoxyflurane metabolism as cytochrome b_5, *J. Biol. Chem. 259*, 2541−2546.
9. Renaud, J. P., Cullin, C., Pompon, D., Beaune, P., and Mansuy, D. (1990) Expression of human liver cytochrome P450 IIIA4 in yeast. A functional model for the hepatic enzyme, *Eur. J. Biochem. 194*, 889−896.
10. Patten, C. J., Ishizaki, H., Aoyama, T., Lee, M., Ning, S. M., Huang, W., Gonzalez, F. J., and Yang, C. S. (1992) Catalytic properties of the human cytochrome P450 2E1 produced by cDNA expression in mammalian cells, *Arch. Biochem. Biophys. 299*, 163−171.
11. Shimada, T., Yamazaki, H., Mimura, M., Inui, Y., and Guengerich, F. P. (1994) Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians, *J. Pharmacol. Exp. Ther. 270*, 414−423.
12. Guengerich, F. P., Martin, M. V., Beaune, P. H., Kremers, P., Wolff, T., and Waxman, D. J. (1986) Characterization of rat and human liver microsomal cytochrome P-450 forms involved in nifedipine oxidation, a prototype for genetic polymorphism in oxidative drug metabolism, *J. Biol. Chem. 261*, 5051−5060.
13. Imaoka, S., Imai, Y., Shimada, T., and Funae, Y. (1992) Role of phospholipids in reconstituted cytochrome P450 3A form and mechanism of their activation of catalytic activity, *Biochemistry 31*, 6063−6069.
14. Yun, C. H., Song, M., and Kim, H. (1997) Conformational change of cytochrome P450 1A2 induced by phospholipids and detergents, *J. Biol. Chem. 272*, 19725−19730.
15. Yamazaki, H., Ueng, Y. F., Shimada, T., and Guengerich, F. P. (1995) Roles of divalent metal ions in oxidations catalyzed by recombinant cytochrome P450 3A4 and replacement of NADPH−cytochrome P450 reductase with other flavoproteins, ferredoxin, and oxygen surrogates, *Biochemistry 34*, 8380−8389.
16. Yun, C. H., Song, M., Ahn, T., and Kim, H. (1996) Conformational change of cytochrome P450 1A2 induced by sodium chloride, *J. Biol. Chem. 271*, 31312−31316.
17. Yamazaki, H., Nakano, M., Imai, Y., Ueng, Y. F., Guengerich, F. P., and Shimada, T. (1996) Roles of cytochrome b_5 in the oxidation of testosterone and nifedipine by recombinant cytochrome P450 3A4 and by human liver microsomes, *Arch. Biochem. Biophys. 325*, 174−182.
18. Gillam, E. M., Baba, T., Kim, B. R., Ohmori, S., and Guengerich, F. P. (1993) Expression of modified human cytochrome P450 3A4 in *Escherichia coli* and purification and reconstitution of the enzyme, *Arch. Biochem. Biophys. 305*, 123−131.
19. Kim, B. R., and Kim, D. H. (1998) Influence of glutathione on the catalytic activity of reconstituted cytochrome P450 3A4, *Biochem. Biophys. Res. Commun. 242*, 209−212.
20. Yamazaki, H., Nakajima, M., Nakamura, M., Asahi, S., Shimada, N., Gillam, E. M. J., Guengerich, F. P., Shimada, T., and Yokoi, T. (1999) Enhancement of cytochrome P-450 3A4 catalytic activities by cytochrome b_5 in bacterial membranes, *Drug Metab. Dispos. 27*, 999−1004.
21. Murakami, H., Yabusaki, Y., Sakaki, T., Shibata, M., and Ohkawa, H. (1987) A genetically engineered P450 monooxygenase: construction of the functional fused enzyme between rat cytochrome P450c and NADPH-cytochrome P450 reductase, *DNA 6*, 189−197.
22. Gilep, A. A., Guryev, O. L., Usanov, S. A., and Estabrook, R. W. (2001) Reconstitution of the enzymatic activities of cytochrome P450s using recombinant flavocytochromes containing rat cytochrome b_5 fused to NADPH-cytochrome P450 reductase with various membrane-binding segments, *Arch. Biochem. Biophys. 390*, 215−221.

23. Gilep, A. A., Guryev, O. L., Usanov, S. A., and Estabrook, R. W. (2001) Expression, purification, and physical properties of recombinant flavocytochrome fusion proteins containing rat cytochrome b_5 linked to NADPH-cytochrome P450 reductase by different membrane-binding segments, *Arch. Biochem. Biophys. 390*, 222−234.
24. Crawford, N. M., Smith, M., Bellissimo, D., and Davis, R. W. (1988) Sequence and nitrate regulation of the *Arabidopsis thaliana* mRNA encoding nitrate reductase, a metalloflavoprotein with three functional domains, *Proc. Natl. Acad. Sci. U.S.A. 85*, 5006−5010.
25. Johnson, J. L., and Rajagopalan, K. V. (1976) Purification and properties of sulfite oxidase from human liver, *J. Clin. Invest. 58*, 543−550.
26. Mitchell, A. G., and Martin, C. E. (1995) A novel cytochrome b_5-like domain is linked to the carboxyl terminus of the *Saccharomyces cerevisiae* delta-9 fatty acid desaturase, *J. Biol. Chem. 270*, 29766−29772.
27. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) Transformation of intact yeast cells treated with alkali cations, *J. Bacteriol. 153*, 163−168.
28. Sakaki, T., Shibata, M., Yabusaki, Y., Murakami, H., and Ohkawa, H. (1990) Expression of bovine cytochrome P450c21 and its fused enzymes with yeast NADPH-cytochrome P450 reductase in *Saccharomyces cerevisiae*, *DNA Cell Biol. 9*, 603−614.
29. Oeda, K., Sakaki, T., and Ohkawa, H. (1985) Expression of rat liver cytochrome P-450MC cDNA in *Saccharomyces cerevisiae*, *DNA 4*, 203−210.
30. Lowry, O. H., Roseebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol regent, *J. Biol. Chem. 193*, 265−275.
31. Omura, T., and Sato, R. (1964) The carbon monoxide-binding pigment of liver microsomes 2, *J. Biol. Chem. 239*, 2379−2385.
32. Omura, T., and Sato, R. (1964) The carbon monoxide-binding pigment of liver microsomes 1, *J. Biol. Chem. 239*, 2370−2378.
33. Estabrook, R. W., and Werringloer, J. (1978) The measurement of difference spectra: application to the cytochromes of microsomes, *Methods Enzymol. 52*, 212−220.
34. Williams, C. H., Jr., and Kamin, H. (1962) Microsomal triphosphopyridine nucleotide-cytochrome c reductase of liver, *J. Biol. Chem. 237*, 587−595.
35. Guengerich, F. P., and Johnson, W. W. (1997) Kinetics of ferric cytochrome P450 reduction by NADPH-cytochrome P450 reductase: rapid reduction in the absence of substrate and variations among cytochrome P450 systems, *Biochemistry 36*, 14741−14750.
36. Shibata, M., Sakaki, T., Yabusaki, Y., Murakami, H., and Ohkawa, H. (1990) Genetically engineered P450 monooxygenases: construction of bovine P450c17/yeast reductase fused enzymes, *DNA Cell Biol. 9*, 27−36.
37. Vergeres, G., and Waskell, L. (1992) Expression of cytochrome b_5 in yeast and characterization of mutants of the membrane-anchoring domain, *J. Biol. Chem. 267*, 12583−12591.
38. Vergeres, G., Ramsden, J., and Waskell, L. (1995) The carboxyl terminus of the membrane-binding domain of cytochrome b_5 spans the bilayer of the endoplasmic reticulum, *J. Biol. Chem. 270*, 3414−3422.
39. Hayashi, K., Sakaki, T., Kominami, S., Inouye, K., and Yabusaki, Y. (2000) Coexpression of genetically engineered fused enzyme between yeast NADPH-P450 reductase and human cytochrome P450 3A4 and human cytochrome b_5 in yeast, *Arch. Biochem. Biophys. 381*, 164−170.
40. Imaoka, S., Terano, Y., and Funae, Y. (1990) Changes in the amount of cytochrome P450s in rat hepatic microsomes with starvation, *Arch. Biochem. Biophys. 278*, 168−178.
41. Shet, M. S., Fisher, C. W., Holmans, P. L., and Estabrook, R. W. (1993) Human cytochrome P450 3A4: enzymatic properties of a purified recombinant fusion protein containing NADPH-P450 reductase, *Proc. Natl. Acad. Sci. U.S.A. 90*, 11748−11752.
42. Sibbesen, O., De Voss, J. J., and Montellano, P. R. (1996) Putidaredoxin reductase-putidaredoxin-cytochrome p450cam triple fusion protein. Construction of a self-sufficient *Escherichia coli* catalytic system, *J. Biol. Chem. 271*, 22462−22469.
43. Lacour, T., and Ohkawa, H. (1999) Engineering and biochemical characterization of the rat microsomal cytochrome P4501A1 fused to ferredoxin and ferredoxin-NADP(+) reductase from plant chloroplasts, *Biochim. Biophys. Acta 1433*, 87−102.
44. Roome, P. W., Jr., Philley, J. C., and Peterson, J. A. (1983) Purification and properties of putidaredoxin reductase, *J. Biol. Chem. 258*, 2593−2598.
45. Reipa, V., Holden, M. J., Mayhew, M. P., and Vilker, V. L. (2000) Temperature dependence of the formal reduction potential of putidaredoxin, *Biochim. Biophys. Acta 1459*, 1−9.
46. Keirns, J. J., and Wang, J. H. (1972) Studies on nicotinamide adenine dinucleotide phosphate reductase of spinach chloroplasts, *J. Biol. Chem. 247*, 7374−7382.
47. Tagawa, K., and Arnon, D. I. (1968) Oxidation-reduction potentials and stoichiometry of electron transfer in ferredoxins, *Biochim. Biophys. Acta 153*, 602−613.
48. Lewis, D. F. V. (1996) The Catalytic Cycle and Oxygenation Mechanism, in *Cytochrome P450-Structure, Function and Mechanism* (Lewis, D. F. V., Ed.), pp 79−113, Taylor and Francis Ltd., London.
49. Yamazaki, H., Johnson, W. W., Ueng, Y. F., Shimada, T., and Guengerich, F. P. (1996) Lack of electron transfer from cytochrome b_5 in stimulation of catalytic activities of cytochrome P450 3A4. Characterization of a reconstituted cytochrome P450 3A4/NADPH-cytochrome P450 reductase system and studies with apocytochrome b_5, *J. Biol. Chem. 271*, 27438−27444.

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