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Effects of cytochrome b_5 on drug oxidation activities of human cytochrome P450 (CYP) 3As: similarity of CYP3A5 with CYP3A4 but not CYP3A7

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Abstract

Effects of cytochrome b_5 (b_5) on catalytic activities of human cytochrome P450 (CYP) 3A5, CYP3A4, and CYP3A7 coexpressed with human NADPH-cytochrome P450 reductase in *Escherichia coli* membranes were investigated using 14 substrates. The activities of CYP3A5 were enhanced by addition of b_5 in approximately one third of the substrates employed in this study. Such enhancement by b_5 was roughly similar to that of CYP3A4, while the activities of CYP3A7 were not enhanced by b_5 with any substrates employed. V_{max} values for midazolam 1′-hydroxylation and amitriptyline *N*-demethylation by CYP3A5 were increased about twice by addition of b_5, which was also seen with CYP3A4, although the extent of the effects of b_5 on S_{50} (K_m) and Hill coefficient differed dependent on substrates used. In contrast, b_5 did not alter any of these kinetic parameters of CYP3A7. The effects of b_5 on kinetic parameters of CYP3A5 were similar to those of CYP3A4 but not CYP3A7. These results suggest that roles of b_5 in drug oxidation activities of CYP3A5 and CYP3A4 are different from those of CYP3A7.

Keywords: CYP3A5; CYP3A4; CYP3A7; Cytochrome b_5; Midazolam; Amitriptyline

1. Introduction

CYP comprises a superfamily of enzymes responsible for the oxidations of various endogenous and exogenous compounds, including drugs, toxicants, and procarcinogens [1]. CYP3A is one of the major subfamilies of CYP expressed in human livers [2]. This subfamily has been known to be involved in the metabolism of more than 50% of clinically used drugs, such as benzodiazepines, dihydropyridines, macrolides, and steroids [3]. This subfamily is also responsible for the bioactivation of many procarcinogens [4,5].

The human CYP3A subfamily expressed in human livers consists of CYP3A4 [6,7], CYP3A5 [8–10], and CYP3A7 [11]. CYP3A4 is the most abundant form of CYP3A (~30% of total CYP) expressed in adult human livers [2]. CYP3A5 is known to be polymorphically expressed in adult human livers [12]. The content of CYP3A5 accounts for approximately 20% of CYP3A contents in adult livers of Caucasians and Japanese [12,13]. A recent report has shown that CYP3A5 is expressed at an equivalent level of CYP3A4 in the livers of Caucasians and African-Americans [14]. CYP3A7 was originally isolated from fetal human livers [11] and was demonstrated as a fetal-specific form of CYP3A [15]. However, recent reports have demonstrated that CYP3A7 was expressed in some adult livers from normal Caucasian subjects and Japanese patients suffering from hepatocellular carcinoma [16,17].

CYP requires the reductase to function as a monooxygenase. In some oxidation reactions catalyzed by CYP3As, b_5 has been known to support the electron transfer from NADPH to CYP3As *via* the reductase [18]. The requirement of b_5 for oxidations by CYP3A4

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Abbreviations: CYP, cytochrome P450; reductase, NADPH-cytochrome P450 reductase; b_5, cytochrome b_5; DHEA, dehydroepiandrosterone; K_m, Michaelis constant.

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depends on substrates employed [19]. However, so far, studies on the roles of b_5 in drug oxidations by CYP3A5 and CYP3A7 have not been well understood.

In the present study, we compared the effects of b_5 on catalytic activities of CYP3A5, CYP3A4, and CYP3A7 expressed in *Escherichia coli* membranes using 14 substrates. We report herein that b_5 enhances the oxidation rates of CYP3A5 in about one third of the substrates employed, which is roughly similar to CYP3A4. In contrast, CYP3A7 activities are not affected by b_5.

2. Materials and methods

2.1. Materials

Benzphetamine was kindly donated by UpJohn. Other chemicals were obtained from the following sources: [^{14}C]dehydroepiandrosterone (DHEA) was from Du Pont/NEN Research Products; midazolam and 1′- and 4-hydroxymidazolam were from Daiichi Pure Chemicals; clonazepam was from Sumitomo Chemical; ethylmorphine was from Sankyo; aminopyrine, amitriptyline, chlorpromazine, and dextromethorphan were from Wako Pure Chemicals; acetopromazine, clomipramine, 16α-hydroxylated DHEA, promazine, propionylpromazine, and triflupromazine were from Sigma; nortriptyline and pyrilamine were from ICN Biochemicals; $NADP^+$, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were from Oriental Yeast; and monomethylol dimethyl hydantoin was from Tokyo Chemical Industry. All other chemicals were of the highest grade commercially available.

2.2. Construction of expression plasmids

The 5′-termini of CYP3A5, CYP3A4, and CYP3A7 cDNAs were modified as described previously [20–22]. A bicistronic plasmid carrying each CYP3A and the reductase cDNAs was constructed as described previously [23]. A monocistronic plasmid possessing CYP3A7 or the reductase cDNAs was constructed according to the method reported by Inoue *et al*. [24] and Iwata *et al*. [23], respectively. Human b_5 cDNA was introduced into a pCW plasmid as reported by Holmans *et al*. [25].

2.3. Enzyme preparations from Escherichia coli cells expressing CYP and/or reductase

Each isoform of CYP3A and the reductase in the genetically engineered *E. coli* cells were expressed according to the method reported by Iwata *et al*. [23]. The expression of CYP3A7 was performed by using *E. coli* cells transformed with CYP3A7 and GroEL expression plasmids as described previously [24]. The expression of b_5 in *E. coli* cells was performed as reported by Holmans *et al*. [25]. Membrane fraction was prepared from *E. coli* cells harboring CYP3A and/or the reductase as described previously [26]. The membrane fraction was suspended in 100 mM Tris–HCl buffer (pH 7.5) containing 20% (v/v) glycerol and kept at −80° until use. Recombinant human b_5 was purified as described previously [27].

2.4. Enzyme assays

All assays were carried out with the membrane fraction of *E. coli* cells expressing both CYP3A and the reductase. Unless otherwise stated, a typical incubation mixture consisted of 100 mM sodium potassium phosphate buffer (pH 7.4), 50 μM EDTA, a substrate, 5–30 pmol CYP3A, and 0–60 pmol b_5. After preincubation for 5 min at 37°, reactions were initiated by the addition of an NADPH-generating system (0.5 mM $NADP^+$, 5 mM glucose 6-phosphate, and 1 unit/mL glucose 6-phosphate dehydrogenase). To assay the activity of CYP3A7, the membranes expressing the reductase alone were added to the membranes expressing CYP3A7 to yield 4 molar ratio of the reductase to CYP3A7, since the ratios of the reductase to CYP3A5 or CYP3A4 expressed in the membranes together with the reductase were about 4. All determinations were performed at least twice in duplicate.

Amitriptyline *N*-demethylase activity was determined as described below. An incubation mixture consisted of 12.5–1000 μM amitriptyline, 5 pmol CYP3A, and 0–20 pmol b_5 in a final volume of 200 μL. Incubations were carried out at 37° for 20 min and terminated by adding 10 μL of 70% (w/v) perchloric acid. After removal of protein by centrifugation, 150 μL of the supernatant was subjected to high-performance liquid chromatography equipped with a Mightysil RP-18 GP column (4.6 mm × 150 mm, 5 μm, Kanto Chemical). The mobile phase was 50 mM potassium phosphate buffer (pH 7.0) containing 35% acetonitrile. Elution was performed at a flow rate of 1.5 mL/min. The formation of nortriptyline was monitored at a wavelength of 220 nm.

N-Demethylase activities for 11 substrates (acetopromazine, aminopyrine, benzphetamine, chlorpromazine, clomipramine, dextromethorphan, ethylmorphine, promazine, propionylpromazine, pyrilamine, and triflupromazine) were measured by determination of formaldehyde formed according to the method of Rapoport *et al*. [28] with modifications. Briefly, incubation mixtures consisted of a substrate ranging from 250 to 1000 μM, 30 pmol CYP3A, and 0 or 60 pmol b_5 in a final volume of 1 mL. Incubations were carried out at 37° for 20 min and terminated by adding 1 mL of 8.8% (w/v) perchloric acid. After removal of protein by centrifugation, 1 mL of the supernatant was transferred to a tube containing two volumes of the Nash reagent [29]. After incubation at 37° for 30 min, the formation of formaldehyde was determined fluorometrically with an excitation wavelength at 410 nm and an emission wavelength at 510 nm.

DHEA 16α-hydroxylase activity was assayed according to the method described by Taylor and Hamilton [30] with

modifications. Briefly, incubation mixtures consisted of 100 mM Tris–HCl buffer (pH 7.5), 100 μM EDTA, 100 μM [^{14}C]DHEA, 30 pmol CYP3A, and 60 pmol b_5 in a final volume of 200 μL. Incubations were carried out at 37° for 60 min and terminated by adding 250 μL of diethyl ether and ethanol (3:1, v/v). The mixture was extracted with diethyl ether and ethanol (3:1, v/v) three times, and the solvent was evaporated. The residue was dissolved in diethyl ether and ethanol (3:1, v/v) and analyzed by thin-layer chromatography.

Midazolam 1′- and 4-hydroxylase activities at substrate concentrations ranging from 0.78 to 800 μM were determined as described previously [31].

2.5. Kinetic analysis

Data points were fitted to the Hill equation describing a sigmoidal relationship [32] by nonlinear least-squares regression analysis with Origin 6.1J software (OriginLab) to:

$$v = \frac{V_{max} S^n}{S_{50}^n + S^n}$$

where S_{50} was the substrate concentration at which reaction velocity was equivalent to 50% of V_{max}. n was equivalent to the Hill coefficient for cooperative substrate binding. When n corresponded to 1, the curve followed the Michaelis–Menten equation, and S_{50} the same as K_m value. Then, $V_{max}/S_{50}(K_m)$ value was represented as intrinsic clearance. When n exceeded 1, apparent maximal clearance, $v/[S]$, was calculated by the following formula [33]:

$$\frac{v}{[S]} = \frac{V_{max}}{S_{50}} \left(\frac{(n-1)}{n(n-1)^{1/n}} \right)$$

2.6. Other assays

The contents of CYP and b_5 were determined according to the method of Omura and Sato [34]. The reductase activity was determined as described previously [35]. The amounts of the reductase in *E. coli* membranes were estimated by assuming that 1 nmol of the reductase reduced 3.0 μmol of cytochrome *c* per min, based on the activity of purified human or rabbit reductase [36].

3. Results

3.1. Effects of b_5 on catalytic activities of CYP3A5, CYP3A4, and CYP3A7 expressed in E. coli membranes

Effects of b_5 on the rates of drug oxidations catalyzed by CYP3A5, CYP3A4, and CYP3A7 were compared (Fig. 1). The activities of CYP3A5 and CYP3A4 for midazolam 1′-hydroxylation were increased by b_5, while the activity of CYP3A7 was unaffected (Fig. 1A). Similar results were also obtained in amitriptyline *N*-demethylation (Fig. 1B). The rates of CYP3A5 and CYP3A4 reached a maximal level when a molar ratio of b_5 to CYP3A was about 2, although only the amitriptyline *N*-demethylase activity of CYP3A4 was increased 2.6-fold at the molar ratio of 4, compared with 2.0-fold at the ratio of 2.

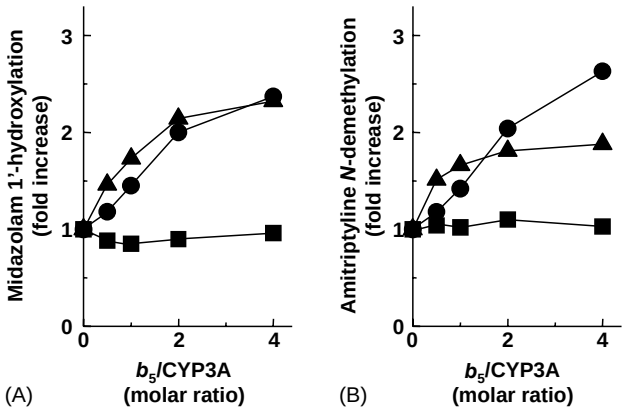

Fig. 1. Effects of b_5 on catalytic activities of CYP3A5 (▲), CYP3A4 (●), and CYP3A7 (■) expressed in *E. coli* membranes. (A) Midazolam (10 μM for CYP3A5 and CYP3A4; 100 μM for CYP3A7) was used as a substrate. The catalytic activities of CYP3A5, CYP3A4, and CYP3A7 without b_5 were 5.6, 2.1, and 1.3 nmol/min/nmol CYP3A, respectively. Since the catalytic activity of CYP3A7 was not detected with midazolam at a concentration of 10 μM, the activity was determined at the concentration of 100 μM. (B) Amitriptyline (200 μM) was used as a substrate. The catalytic activities of CYP3A5, CYP3A4, and CYP3A7 without b_5 were 2.1, 1.8, and 0.14 nmol/min/nmol CYP3A, respectively.

The effects of b_5 on activities of these three CYP3A enzymes were examined with the other 12 substrates at a b_5/CYP3A molar ratio of 2 (Table 1). The activities of CYP3A5 were increased 1.3–2.3 times by the addition of b_5 when ethylmorphine, aminopyrine, pyrilamine, amitriptyline, and midazolam were used as substrates. Similarly, the oxidation rates of CYP3A4 were enhanced 1.4–2.0 times by b_5 when chlorpromazine, pyrilamine, midazolam, and amitriptyline were employed. However, less or no effects of b_5 on the catalytic activities of CYP3A5 and CYP3A4 were seen when other substrates (benzphetamine, trifluprazine, DHEA, promazine, acetopromazine, propionylpromazine, clomipramine, and dextromethorphan for both of CYP3A5 and CYP3A4, chlorpromazine for CYP3A5, and aminopyrine and ethylmorphine for CYP3A4) were used. On the other hand, the activities of CYP3A7 were unaltered by the addition of b_5 under the present conditions. The observation that b_5 did not enhance the midazolam 1′-hydroxylase activity of CYP3A7 at a substrate concentration of 100 μM as shown in Fig. 1A and Table 1 was in contrast to stimulating effects of b_5 on the activities of CYP3A5 and CYP3A4 at the same substrate concentration (Table 1), indicating that such different effects of b_5 were not due to substrate concentrations examined.

Table 1
Effects of b_5 on catalytic activities of CYP3A5, CYP3A4, and CYP3A7

Reaction	Substrate concn. (μM)	Catalytic activity (nmol/min/nmol CYP3A)								
		CYP3A5			CYP3A4			CYP3A7		
		$-b_5$ (A)	$+b_5$ (B)[a]	B/A	$-b_5$ (A)	$+b_5$ (B)	B/A	$-b_5$ (A)	$+b_5$ (B)	B/A
Midazolam 1'-hydroxylation	100	8.3	19	2.3	5.3	9.8	1.8	1.3	1.1	0.8
Midazolam 4-hydroxylation	100	0.81	1.7	2.1	3.1	4.9	1.6	1.0	1.1	1.1
Amitriptyline *N*-demethylation	200	2.1	3.8	1.8	1.8	3.7	2.0	0.14	0.15	1.1
Pyrilamine *N*-demethylation	1,000	3.5	5.5	1.6	1.8	2.9	1.6	1.3	1.4	1.1
Aminopyrine *N*-demethylation	1,000	4.1	5.8	1.4	2.5	2.6	1.0	1.2	1.0	0.8
Ethylmorphine *N*-demethylation	1,000	5.9	7.7	1.3	9.7	9.0	0.9	N.D.[b]	N.D.	N.A.[c]
Dextromethorphan *N*-demethylation	1,000	9.8	12	1.2	5.0	4.1	0.8	2.0	1.7	0.9
Triflupromazine *N*-demethylation	250	3.1	3.6	1.2	1.7	2.0	1.2	3.5	3.1	0.9
Clomipramine *N*-demethylation	500	9.2	10	1.1	14	13	0.9	10	9.8	1.0
Acetopromazine *N*-demethylation	1,000	9.7	10	1.0	2.8	2.7	1.0	12	12	1.0
DHEA 16α-hydroxylation	100	0.24	0.25	1.0	0.32	0.35	1.1	0.49	0.57	1.2
Propionylpromazine *N*-demethylation	1,000	20	20	1.0	25	23	0.9	20	19	1.0
Benzphetamine *N*-demethylation	1,000	7.6	6.8	0.9	3.6	4.4	1.2	N.D.	N.D.	N.A.
Promazine *N*-demethylation	1,000	12	11	0.9	13	13	1.0	16	16	1.0
Chlorpromazine *N*-demethylation	500	4.9	4.0	0.8	4.7	6.6	1.4	18	17	0.9

All determinations were performed in duplicate.
[a] Molar ratio of b_5 to each CYP3A was 2.
[b] Not detectable (<1.0 nmol/min/nmol CYP3A).
[c] Not available.

3.2. Kinetic analysis for drug oxidations catalyzed by CYP3A5, CYP3A4, and CYP3A7 in the presence or absence of b_5

Reaction velocity vs. substrate concentration was plotted in the presence or absence of b_5 to investigate the effects of b_5 on kinetic parameters for midazolam 1′-hydroxylation and amitriptyline *N*-demethylation catalyzed by CYP3A5, CYP3A4, and CYP3A7 (Fig. 2). These oxidation rates saturated within the substrate concentrations examined except for amitriptyline *N*-demethylation catalyzed by CYP3A7. Activities of CYP3A7 toward amitriptyline increased linearly up to 1000 μM of the substrate concentrations.

Kinetic parameters for each reaction were determined based on the best fit of the velocity vs. substrate concentration plots of Fig. 2 (Table 2). V_{max} values for midazolam 1′-hydroxylation catalyzed by CYP3A5 and CYP3A4 were enhanced 2.3 and 1.9 times by the addition of b_5, respectively, while S_{50} values were slightly decreased by b_5. The n values for the hydroxylation by CYP3A5 and CYP3A4, which showed a sigmoidicity of the curve for oxidation rate vs. substrate concentration, were decreased by the fortification of b_5. In particular, a nonlinear Eadie–Hofstee plot for the oxidation by CYP3A4 was changed to a linear plot by the addition of b_5 (Fig. 2). The $v/[S]$, apparent maximal clearance values, for CYP3A5 and CYP3A4 were increased 3.7 and 4.4 times by b_5, respectively.

Table 2
Kinetic parameters for midazolam 1'-hydroxylation and amitriptyline *N*-demethylation catalyzed by CYP3A5, CYP3A4, and CYP3A7

Substrate	b_5[a]	CYP3A5				CYP3A4				CYP3A7			
		S_{50}[b]	V_{max}[c]	n[d]	$v/[S]$[e]	S_{50}	V_{max}	n	$v/[S]$	S_{50}	V_{max}	n	$v/[S]$
Midazolam	−	6.6 ± 0.5	8.1 ± 0.2	1.6 ± 0.2	630	8.9 ± 0.6	5.4 ± 0.1	1.5 ± 0.1	320	310 ± 52	4.8 ± 0.3	1.0[f]	15
	+	4.8 ± 0.3	19 ± 0.3	1.3 ± 0.1	2,300	7.0 ± 0.9	10 ± 0.4	1.0 ± 0.1	1,400	330 ± 57	4.8 ± 0.4	1.0[f]	15
Amitriptyline	−	110 ± 9	2.7 ± 0.1	2.1 ± 0.3	12	50 ± 5	1.8 ± 0.1	2.2 ± 0.4	18	N.A.[g]	0.8[h]	N.A.	N.A.
	+	150 ± 8	5.9 ± 0.2	1.9 ± 0.1	20	74 ± 10	3.9 ± 0.3	2.0 ± 0.4	26	N.A.	0.7[h]	N.A.	N.A.

Values are mean ± S.E. of kinetic parameters.
[a] Molar ratio of b_5 to each CYP3A was 2.
[b] Substrate concentration to give 50% of V_{max} (μM).
[c] nmol/min/nmol CYP3A.
[d] Hill coefficient for cooperative substrate binding.
[e] Calculation methods of apparent maximal clearance ($v/[S]$, μl/min/nmol CYP3A) were described in MATERIALS AND METHODS.
[f] Determined by using Michaelis-Menten equation.
[g] Not available.
[h] Catalytic activity at 1,000 μM amitriptyline which was the highest concentration examined.

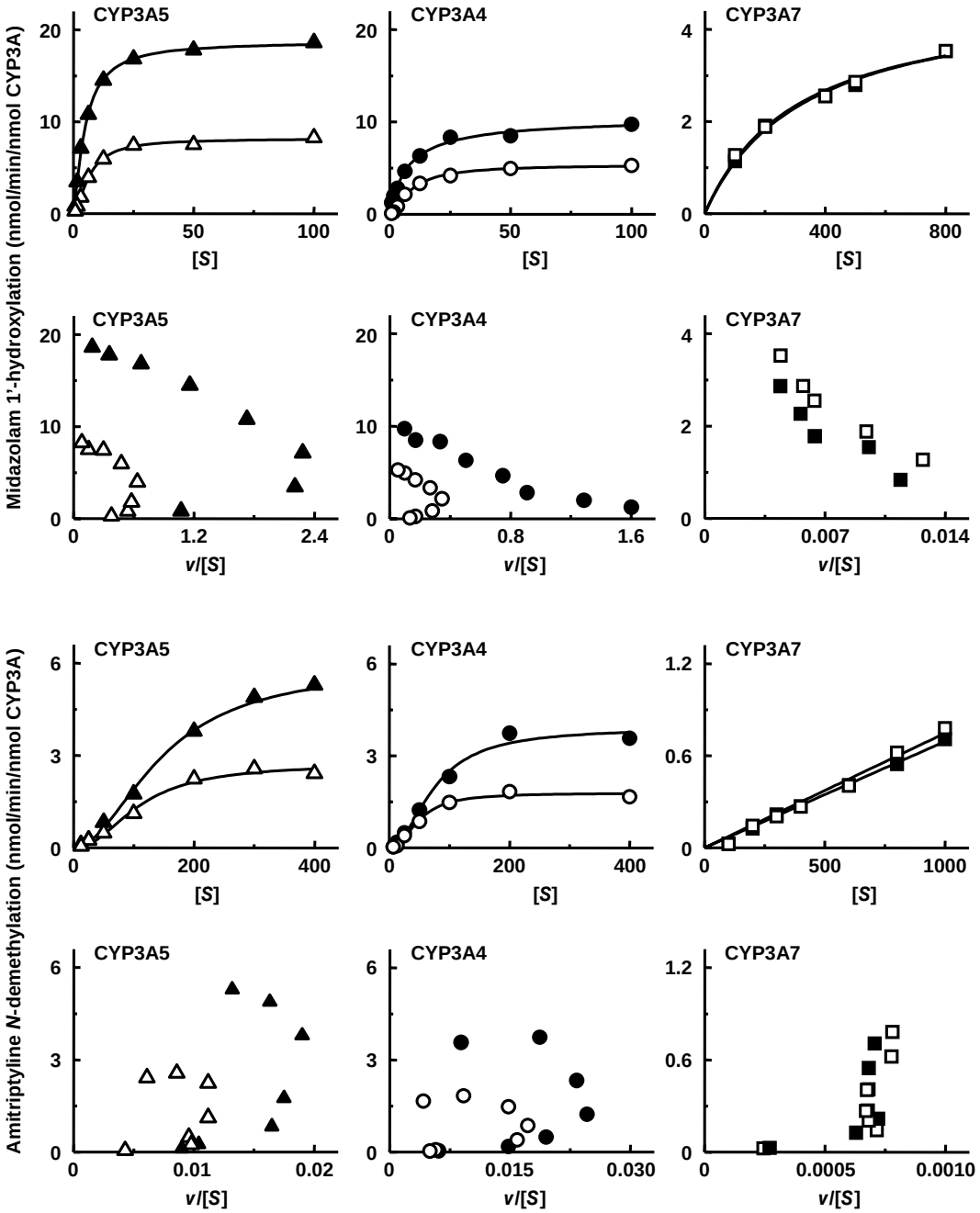

Fig. 2. Velocity vs. substrate concentration and Eadie–Hofstee plots for drug oxidations catalyzed by CYP3A5, CYP3A4, and CYP3A7. The membranes prepared from *E. coli* cells expressing each CYP3A were incubated with a drug in the absence (open symbols) or presence (closed symbols) of b_5.

Such effects of b_5 were not seen in any of these kinetic parameters for CYP3A7. In the cases of amitriptyline *N*-demethylation by CYP3A5 and CYP3A4, V_{max} values were increased 2.2 times by the addition of b_5. In addition, S_{50} values were enhanced approximately 1.5 times by b_5. The n values (∼2) were unaffected, indicating sigmoidal profiles of velocity vs. substrate concentration and non-linear Eadie–Hofstee plots regardless of the presence or absence of b_5 (Fig. 2). The $v/[S]$ values for CYP3A5 and CYP3A4 were increased 1.7 and 1.4 times by the addition of b_5, respectively. In contrast, such stimulating effect of b_5 on the activity of CYP3A7 was not observed within the substrate concentrations examined.

4. Discussion

In general, effects of b_5 on the catalytic activities of CYP have been examined by reconstituting purified CYP, the reductase, and b_5 fortified with phospholipids and/or detergents, such as cholate. A recent report has shown that the activities of CYP3A4 are increased by facile insertion of

purified b_5 into the lipid bilayers of *E. coli* membranes expressing CYP3A4 and the reductase [37,38]. Therefore, we investigated the effects of b_5 on the oxidation rates of CYP3A5, CYP3A4, and CYP3A7 expressed in *E. coli* membranes under the same experimental conditions. It has been shown that 1 molar ratio of b_5 to CYP3A4 produces the highest rate of testosterone 6β-hydroxylation when a molar ratio of the reductase to CYP3A4 expressed in *E. coli* membranes was 2 [37]. In the present study, CYP3A5 and CYP3A4 required 2 molar excess amounts of b_5 for the maximal rates of midazolam 1′-hydroxylation and amitriptyline *N*-demethylation. It was thus assumed that 2 molar ratio of b_5 to CYP3A was needed for the maximal activities because the reductase was expressed in *E. coli* membranes at 4 molar equivalent of each CYP3A enzyme.

The enhancement of the activities of CYP3A5 and CYP3A4 by b_5 was seen in approximately one third of the substrates used in this study. However, we could not find any relationship between chemical structures and effects of b_5. The reason why the requirement of b_5 for the oxidations by CYP3A5 and CYP3A4 depends on substrates employed is unclear. Interestingly, the effects of b_5 on the activities of CYP3A5 were similar to those of CYP3A4 but not CYP3A7. Furthermore, the effects of b_5 on kinetic parameters for midazolam 1′-hydroxylation and amitriptyline *N*-demethylation by CYP3A5 were also similar to those of CYP3A4 but not CYP3A7. b_5 in the presence of two times molar excess of CYP3A7 did not affect the activities of CYP3A7 toward the substrates examined. However, 1.7-fold increase of DHEA 16α-hydroxylase activity of CYP3A7 by addition of b_5 was observed at 4 molar ratio of b_5 to CYP3A7, although no effects of b_5 were seen in the same reaction by CYP3A5 and CYP3A4; b_5 affected DHEA 7β-hydroxylation, which is a major metabolic pathway of DHEA in adult liver microsomes [39], by CYP3A5 or CYP3A4 and CYP3A7 *vice verse* (data not shown). Such stimulating effect of b_5 on the DHEA 16α-hydroxylase activity of CYP3A7 was consistent with the result reported by Kitada *et al.* [40], who indicated that b_5 enhances the activity of DHEA 3-sulfate 16α-hydroxylation by CYP3A7 in the reconstitution system. Similarly, Ohmori *et al.* [41] demonstrated that b_5 increased the DHEA 16α-hydroxylase activity of CYP3A7 expressed in the microsomes from insect cells infected with baculovirus systems. The fact that the effects of b_5 on the activities of CYP3A5 and CYP3A4 are distinct from those of CYP3A7 suggests that roles of b_5 in drug oxidation activities of CYP3A5 and CYP3A4 are different from those of CYP3A7.

The mechanisms involved in the enhancement of the activities of CYP by b_5 have been investigated extensively [42–44]. Hildebrandt and Estabrook [45] first demonstrated the donation of the second of two electrons from b_5 to CYP. This has been supported later using the reconstituted systems [46,47]. In addition, more detailed studies have provided evidence that the second electron supply for the oxygen activation coming from b_5 prevents the oxygenated CYP complex from decomposition [48,49]. We also previously proposed three mechanisms on stimulating effects of b_5 on the activities of CYP: the increased affinity of CYP to substrates, resulting in the enhancement of affinity between CYP and the reductase, and facilitating the donation of the first of two electrons from b_5 to CYP [50]. The varying degrees of the stimulating effects of b_5 on the activities of CYP3A5 and CYP3A4, as shown in Table 1, may be explained by the combination of several mechanisms described above.

Furthermore, in order to propose the putative mechanism that the roles of b_5 in the catalytic function of CYP3A5 and CYP3A4 are distinct from those of CYP3A7, we compared the amino acid sequence of CYP3A5, CYP3A4, and CYP3A7. It has been reported that positively charged amino acid residues in CYP proteins interact electrostatically with negatively charged ones in b_5 protein [51]. We found 28 amino acid residues seen in common in both CYP3A5 and CYP3A4 but not in CYP3A7. Among these amino acid residues, a positively charged amino acid in CYP3A5 and CYP3A4 molecules was Arg at the site of 255 (Fig. 3A), while the corresponding residue of CYP3A7 was Gln, neutral amino acid. This residue is located in helix G of CYP3A4 [52], facing helix F, which appeared to be close to the heme group in rabbit CYP2C5 [53]. This residue is predicted to be exposed to the surface of the CYP3A molecules, based on the crystal structure of CYP2C5 [54]. One possibility is that Arg at 255 in CYP3A5 and CYP3A4 might contribute to the stimulating effects of b_5 on these activities. However, there is no direct evidence that this residue is involved in the interaction with b_5. Further studies on the binding sites of CYP3A to b_5 are needed.

A previous report has proposed that a motif of Arg-Arg-Phe-Ser (residues 128–131) in mouse CYP2A5 plays a role in the binding of this enzyme to b_5 [55]. This hypothesis has been supported by Juvonen *et al.* [56], demonstrating that the substitution of Arg to Ser at the site of 129 in CYP2A5 eliminated the stimulating effect of b_5 on the coumarin 7-hydroxylation and decreased the binding affinity to b_5-conjugated Sepharose 4B. The sequence of Arg-Arg-Phe-Ser is highly conserved among the CYP2 family [57]. However, CYP isoforms other than the CYP2 family do not have the corresponding motif, whereas the first Arg in the motif is highly conserved throughout the CYP superfamily [52]. In fact, this motif did not exist in all of CYP3A5, CYP3A4, and CYP3A7 (Fig. 3B). The sites of these CYP3A isoforms other than the motif of Arg-Arg-Phe-Ser might contribute to the interaction with b_5, although we cannot exclude a possibility that the first Arg in the motif is involved in the binding of CYP3As to b_5.

In conclusion, we found that the effects of b_5 on the drug oxidations by CYP3A5 were similar to those of CYP3A4 but not CYP3A7.

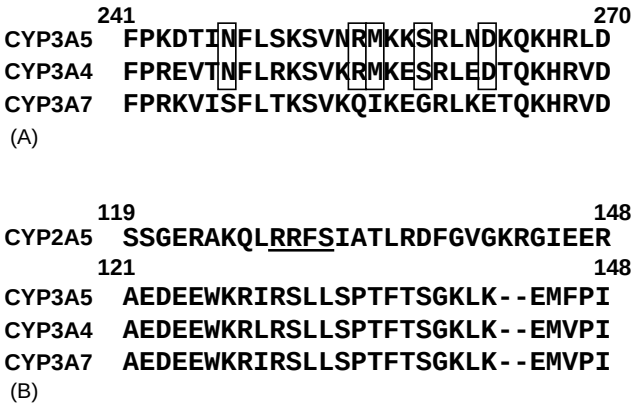

Fig. 3. Comparison for the amino acid sequence of CYP3A5, CYP3A4, and CYP3A7. (A) Open boxes show the residues seen in common in both CYP3A5 and CYP3A4 but not in CYP3A7. (B) Alignment of mouse CYP2A5 and human CYP3A proteins was performed based on the previous report [52]. An underline shows the proposed region of b_5 binding in mouse CYP2A5.

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