Functional analysis of three CYP1A2 variants found in a Japanese population

YOSHIRO SAITO, NOBUMITSU HANIOKA, KEIKO MAEKAWA, TAKASHI ISOBE, YUMI TSUNETO, RYOSUKE NAKAMURA, AKIKO SOYAMA, SHOGO OZAWA, TOSHIKO TANAKA-KAGAWA, HIDETO JINNO, SHIZUO NARIMATSU, and JUN-ICHI SAWADA

Project Team for Pharmacogenetics (Y.S., K.M., R.N., A.S., S.O., H.J., J.S.), Division of Biochemistry and Immunochemistry (Y.S., K.M., R.N., J.S.), Division of Pharmacology (S.O.), Division of Environmental Chemistry (T.T.-K., H.J.), National Institute of Health Sciences, Tokyo, Japan; Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, Japan (N.H., T.I., Y.T., S.N.).
Running title: Functional analysis of human CYP1A2 variants

Address correspondence to: Yoshiro Saito, Ph.D., Division of Biochemistry and Immunochemistry, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
Tel: +81-3-3700-9453
Fax: +81-3-3707-6950
E-mail: yoshiro@nihs.go.jp

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ABBREVIATIONS: CYP, cytochrome P450; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction; RT, reverse-transcription; SNP, single nucleotide polymorphism; WT, wild-type.
Abstract

Human cytochrome P450 1A2 (CYP1A2) catalyzes the metabolism of many important drugs and environmental chemicals. We previously reported three naturally-occurring genetic polymorphisms (125C>G, Pro42Arg, CYP1A2*15; 1130G>A, Arg377Gln, *16; 1367G>A, Arg456His, *8) found in a Japanese population. In this study, these variant enzymes were expressed in Chinese hamster V79 cells, and their mRNA and protein expression levels as well as catalytic activities were determined. All three variant enzymes showed reduced protein expression levels (66% for Pro42Arg and approximately 30% for Arg377Gln and Arg456His) compared to that of the wild-type (WT) without any change in mRNA expression levels. Kinetic analysis for 7-ethoxyresorufin O-deethylation revealed that $V_{\text{max}}$ and $V_{\text{max}}/K_{\text{m}}$ of all three variants were less than 3% and 1% of the WT, respectively, though the $K_{\text{m}}$ value was significantly increased only in the Arg377Gln variant (approximately a 9-fold increase). Markedly reduced activities of the three variants were also observed for phenacetin O-deethylation. In the reduced CO-difference spectral analysis using recombinant proteins produced in the Sf21/baculovirus system, the peak at 450 nm seen in the WT protein was hardly observed in the three variants, suggesting marked reductions in their hemoprotein formation. These results suggest that Pro42, Arg377, and Arg456 are critical amino acids for the production of catalytically active CYP1A2 holoenzyme.
Human cytochrome P450 (CYP) enzymes catalyze the metabolism of a wide variety of clinically, physiologically, and toxicologically important compounds (Lewis, 2001). The human CYP1A subfamily consists of CYP1A1 and CYP1A2. The former is expressed mainly in extrahepatic tissues, and the latter is almost exclusively expressed in the liver. CYP1A2 is responsible for the oxidative metabolism of drugs such as theophylline, mexiletine, and phenacetin (Distlerath et al., 1985; Sarkar and Jackson, 1994; Nakajima et al., 1998). This enzyme has also been shown to be involved in the metabolic activation of carcinogenic arylamines to produce reactive intermediates (Eaton et al., 1995).

Up to 60-fold interindividual variation in the CYP1A2 activity has been reported (Shimada et al., 1994; Saruwatari et al., 2002). Also, approximately 15- and 40-fold interindividual variations in CYP1A2 mRNA and protein expression levels have been observed in the human liver (Ikeya et al., 1989; Guengerich et al., 1999). These interindividual differences are likely to influence the drug metabolism and to be associated with drug efficacy and safety, and cancer susceptibility caused by procarcinogens. Environmental factors have been thought to influence the interindividual differences. Cigarette smoking and intake of oral contraceptive steroids are well established modifiers of CYP1A2 activity (Rasmussen et al., 2002). However, it has been suggested that approximately 35-75% of the interindividual variability in CYP1A2 activity is due to genetic factors (Kendler and Prescott, 1999; Rasmussen et al., 2002). Thus, several researchers have focused their efforts on the identification of CYP1A2 genetic variants.

To date, 23 CYP1A2 haplotypes, including 9 subtypes, have been publicized on the Human Cytochrome P450 Allele Nomenclature Committee homepage (www.imm.ki.se/CYPalleles/cyp1a2.htm). Since CYP1A2 is inducible by environmental factors, many investigators have tried to identify single nucleotide polymorphisms (SNPs) in the transcriptional regulatory regions: the distal enhancer region, the promoter region, non-coding exon 1, and intron 1. CYP1A2*1C (-3860G>A) was reported to be associated with decreased enzyme inducibility in Japanese smokers (Nakajima et al., 1999). CYP1A2*1F (-163C>A), located in intron 1, has been suggested to be linked with a higher enzyme inducibility in Caucasian smokers (Sachse et al., 1999). Recently, Aklillu et al. reported that the CYP1A2*1K haplotype
(-739T>G, -729C>T, and -163C>A; all in intron 1) was associated with decreased enzyme activity in Ethiopian non-smokers (Aklillu et al., 2003). As for the coding exons, Phe21Leu (CYP1A2*2) was first reported from the direct sequencing of DNA from one of eight Chinese subjects (Huang et al., 1999). The other 4 non-synonymous SNPs (Asp348Asn, CYP1A2*3; Ile386Phe, *4; Cys406Tyr, *5; Arg431Trp, *6) found in French Caucasians were reported to alter protein expression levels and/or alter enzymatic activities depending on the substrates (Zhou et al., 2004). An SNP at the splice donor site of intron 6 was also reported, which probably results in a splicing defect (CYP1A2*7) (Allorge et al., 2003). Recently, we analyzed the catalytic activities of 6 non-synonymous nucleotide polymorphisms (CYP1A2*9-14) found in a Japanese population and showed that one of the SNPs, Phe186Leu (*11), had a markedly reduced enzymatic activity in vitro for O-deethylation of both 7-ethoxyresorufin and phenacetin (Murayama et al., 2004). Recently, another nonsynonymous alteration, Gln478His, was also reported in a Japanese population, but did not change the catalytic activity for 7-ethoxyresorufin (Iwasaki et al., 2004).

In the previous study, we further searched all the CYP1A2 exons and their surrounding intronic regions in Japanese individuals, and found 3 non-synonymous polymorphisms (125C>G, Pro42Arg, *15; 1130G>A, Arg377Gln, *16; 1367G>A, Arg456His, *8) at frequencies of 0.002, 0.002, and 0.004, respectively (Soyama et al., 2005). In the present study, these three SNPs were characterized in a cDNA expression system using Chinese hamster V79 cells to examine whether these variations affect the expression and catalytic activity. Reduced CO-difference spectral analysis was also performed for spectral characterization using recombinant CYP1A2 proteins produced in the Sf21/baculovirus system.
Materials and Methods

Site-Directed Mutagenesis of CYP1A2 cDNA.

Mutations were introduced into the transfection plasmid carrying a CYP1A2 cDNA (pcDNA3.1/CYP1A2 wild-type, WT) by a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The mutant plasmids were sequenced on both strands for the entire cDNA regions to confirm the introduction of the mutation only at the target sites.

Transient transfection and preparation of microsomal fraction from V79 cells.

Chinese hamster lung fibroblast V79 cells were utilized for CYP1A2 transfection since these cells have been shown to lack the expression of the CYP1A enzyme, but express NADPH cytochrome P450 reductase, an enzyme required for P450 monooxygenase activity (Glatt et al., 1990; Fuhr et al., 1992). V79 cells were cultured and transfected with either empty, WT (pcDNA3.1/CYP1A2 WT) or one of the 3 variant plasmids (pcDNA3.1/Pro42Arg, pcDNA3.1/Arg377Gln or pcDNA3.1/Arg456His) as described previously (Murayama et al., 2004). At 28 h after transfection, the cells were rinsed with ice-cold 10 mM phosphate buffered saline (pH 7.4) and scraped in 50 mM potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose, 25 mM KCl, and 0.5 mM EDTA. After homogenization, the homogenates were spun at 9,000 x g for 10 min at 4°C, and the supernatants were further subjected to centrifugation at 105,000 x g for 1 h at 4°C. The resulting pellets were resuspended in 50 mM potassium phosphate buffer (pH 7.4) containing 150 mM KCl, and spun again at 105,000 x g for 50 min at 4°C. The pellets were resuspended in 50 mM potassium phosphate buffer (pH 7.4) with 10% (v/v) glycerol and kept frozen at -90°C until used. The protein concentrations of the microsomal fractions were assayed with the Protein Assay Kit (BioRad Laboratories, Hercules, CA) using bovine γ-globulin as a standard.

Expression of CYP1A2s in Spodoptera frugiperda (Sf21) cells.

The cDNA plasmids of the WT and three variant CYP1A2s (pcDNA3.1/CYP1A2s) were digested with BamH I and Not I. The inserts were purified and recloned into the pENTR4 vector (pENTR/CYP1A2s) (Invitrogen). Baculovirus construction and protein expression in Sf21 cells were performed using the BaculoDirect Baculovirus
Expression Systems according to the manufacturer’s protocol (Invitrogen). Briefly, Sf21 cells were directly transfected with the recombinant baculovirus DNA, which was generated by the LR reaction between entry clone (pENTR/CYP1A2s) and the BaculoDirect linear DNA. The cells were selected with 100 µM ganciclovir for 4 days, and the resulting viral stock was amplified twice by infecting the Sf21 cells. Finally, a high-titer viral stock (≥8 x 10^6 pfu/ml) was obtained.

Sf21 cells were infected with BaculoDirect/CYP1A2s at a multiplicity of infection of 1. After 24 h postinfection, δ-aminolevulinic acid (0.1 mM) and ferric citrate (0.1 mM) were added to the culture medium to compensate for a deficiency in heme biosynthesis. The cells were harvested 72 h after infection. The cells were lysed in 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 0.4% Emulgen 911 (kindly provided by KAO, Tokyo, Japan) by incubation for 30 min on ice with occasional agitation. After centrifugation at 9,000 x g for 10 min at 4°C, the resulting whole-cell lysate was diluted to protein concentration of 8 mg/ml. The reduced CO-difference spectra were recorded as described previously (Gonzalez et al., 1991). Human CYP1A2 Supersomes (BD Gentest, Woburn, MA) produced in insect cells by a baculovirus expression system were utilized as a positive control (2.5 mg/ml).

**Determination of protein expression levels by immunoblotting.**

Thirty micrograms of the microsomal fractions from V79 cells or 4 µg of the whole-cell lysates of Sf21 cells were dissolved in sodium dodecyl sulfate-sample buffer. After boiling for 5 min, the microsomal proteins were separated on 8% sodium dodecyl sulfate-polyacrylamide gels, and transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). For immunostaining of CYP1A2 or an endoplasmic reticulum-resident protein calnexin as a control, a goat anti-CYP1A1/1A2 antiserum (1:1,000 dilution, Daiichi Pure Chemical Co., Tokyo, Japan) or a rabbit anti-calnexin antiserum (1:2,000 dilution, StressGen Biotechnologies Corp., Victoria, BC, Canada) was used as the first antibody. As the second antibody, horseradish peroxidase-conjugated rabbit anti-goat IgG (1:1,000 dilution, ICN Pharmaceuticals Inc., Aurora, OH) or donkey anti-rabbit IgG (1:4,000 dilution, Amersham Biosciences, Piscataway, NJ) was utilized. The antibody-bound proteins were visualized with the
Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston, MA), and the band densities were quantified with Diana III and the Zero-Dscan software (Raytest, Straubenhardt, Germany). The expression levels were shown as the mean ± SD of three separate transfection experiments. The expression levels in V79 cells were determined by comparisons with the band intensities of known CYP1A2 levels in human liver microsomes (HG56, BD Gentest).

**Determination of mRNA expression levels by TaqMan real-time reverse transcription (RT)-polymerase chain reaction (PCR).**

Total cellular RNA was isolated from the transfected V79 cells using ISOGEN (Nippon Gene, Toyama, Japan). Five-hundred nanograms of total RNA were first treated with DNase (Invitrogen, Carlsbad, CA) to minimize plasmid DNA contamination in the samples. Then, first-stand cDNA was prepared using a High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) with random primers. Using the 1/1,000 volume of the reverse-transcribed reaction mixture, real-time PCR assays were performed with CYP1A2 specific primers and TaqMan probes (Assay ID: Hs01070371_g1, Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems) with an ABI7700 PCR thermal cycler. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression levels quantified by TaqMan Rodent GAPDH Control reagents (Applied Biosystems) were utilized as an internal control. All the CYP1A2 mRNA expression levels were normalized against the GAPDH mRNA levels, and were expressed as a ratio to the WT (100%). The expression levels were shown as the mean ± SD of three separate transfection experiments.

**Assay for CYP1A2-dependent enzymatic activities.**

O-Deethylation activities of 7-ethoxyresorufin and phenacetin were determined by high-performance liquid chromatography (HPLC) as described previously with some modifications (Hanioka et al., 2000; Hanioka et al., 2002). The incubation mixture contained 7-ethoxyresorufin or phenacetin as a substrate, the microsomal fraction of V79 cells (400 µg of protein), and 1 mM NADPH in 50 mM potassium phosphate buffer (pH 7.4) in a final volume of 200 µl. The substrate concentrations were 0.25–4.0 µM.
for 7-ethoxyresorufin \( O \)-deethylation, and 5 or 100 \( \mu \)M for phenacetin \( O \)-deethylation. 7-Ethoxyresorufin and phenacetin were dissolved in methanol-dimethyl sulfoxide (50:50, v/v) and methanol, respectively. The final concentration of organic solvent (methanol and dimethyl sulfoxide) in the incubation mixture was 1%. The reaction was initiated by the addition of NADPH after a 1 min preincubation at 37°C. After incubation within linearity for 30 min (7-ethoxyresorufin \( O \)-deethylation) or 40 min (phenacetin \( O \)-deethylation) at 37°C, the reaction was terminated by the addition of 200 \( \mu \)l of methanol (7-ethoxyresorufin \( O \)-deethylation) or 2.5 ml of ethyl acetate (phenacetin \( O \)-deethylation). Samples for the determination of 7-ethoxyresorufin \( O \)-deethylation activity were spun at 12,000 x \( g \) for 20 min. The supernatant was filtered with a polytetra-fluoroethylene membrane filter with a 0.45 \( \mu \)m pore size (Millipore, Bedford, MA) and analyzed by HPLC. Samples for the determination of phenacetin \( O \)-deethylation activity were spiked with an internal standard (500 pmol of 3-acetamidophenol) and vigorously vortexed for 2 min. After centrifugation at 2,000 x \( g \) for 10 min, the organic phase was evaporated to dryness under a gentle stream of nitrogen at 35°C. The residues were dissolved in 100 \( \mu \)l of methanol-water (50:50, v/v) and analyzed by HPLC.

HPLC analysis was performed using a Shimadzu SCL-6B system controller (Kyoto, Japan) consisting of an LC-9A pump, an SPD-6A UV spectrophotometric detector, an RF-10A fluorescence detector, and a C-R4A chromatopac integrator. The samples (50 \( \mu \)l) were injected into an Inertsil ODS-80A column (150 x 4.6 mm i.d.: GL Sciences, Tokyo, Japan). The column was kept at 30°C. The product (resorufin) in the assay for 7-ethoxyresorufin \( O \)-deethylation activity was eluted isocratically with 20 mM phosphate buffer (pH 6.8)-acetonitrile-methanol (52:4:44, v/v/v) at a flow rate of 0.7 ml/min. Fluorometric detection was performed with excitation at 566 nm and emission at 584 nm. The product (4-acetamidophenol) in the assay for phenacetin \( O \)-deethylation activity was eluted isocratically with 20 mM phosphate buffer (pH 5.4)-methanol (86:14, v/v) at a flow rate of 1.2 ml/min. UV detection was performed at 245 nm. To create the standard curve for both assays, samples were prepared in the same manner as the test samples.

Kinetic parameters (\( K_m \) and \( V_{max} \)) for 7-ethoxyresorufin \( O \)-deethylation were estimated by analyzing Lineweaver-Burk plots using Prism v4.0 software (GraphPad
Software, San Diego, CA).

**Statistical analysis.**

Differences in the mRNA and protein expression levels and the enzyme activities among the WT and the three variants were analyzed by one-way analysis of variance, followed by Scheffe’s post hoc test using StatView software (SAS Institute Inc., Cary, NC). Differences were considered to be statistically significant when the $P$ value was <0.05.
Results

Expression levels of the three CYP1A2 variants in V79 cells.

To functionally characterize the three nonsynonymous CYP1A2 variants, the wild-type (WT) and variant (Pro42Arg, Arg377Gln, and Arg456His) CYP1A2 proteins were transiently expressed in V79 cells. In order to determine the expression levels of the variant CYP1A2 proteins, immunoblotting was performed on the microsomal fractions obtained from V79 cells transfected with WT or the variant CYP1A2 expression plasmids (n=3). One of the representative results is shown in Fig. 1. The band intensities of the endoplasmic reticulum-resident protein calnexin were comparable among the samples (P > 0.19), suggesting that the contents of the endoplasmic reticulum were similar throughout the microsomal fractions from the cells transfected with empty, WT, and the three variant plasmids. In contrast, the protein expression levels of Pro42Arg, Arg377Gln, and Arg456His variants were reduced to 66.0±13.4%, 29.8±3.5%, and 31.6±8.1%, respectively, when the WT expression level was defined as 100% (P < 0.001 for all). The expression level of WT was 11.2±1.4 pmol CYP1A2/mg protein. Thus, the expression levels of all three CYP1A2 variant proteins were significantly reduced when compared to that of the WT. It must be noted that there were no differences in the microsomal levels of NADPH-dependent P450 reductase among the WT and three variants (P > 0.98, data not shown).

To confirm that the expression plasmids were similarly transfected and the inserted cDNAs were comparably transcribed, the CYP1A2 mRNA expression levels were assessed using total RNA from the transfected V79 cells (n=3). The TaqMan real-time RT-PCR assay was performed for this purpose. CYP1A2 mRNA was not detected from cells transfected with the empty plasmid. As shown in Fig. 2, no significant differences were detected among the mRNA levels of the WT and the three variants (P > 0.85), suggesting that the transfection efficiency was similar among the WT and variants.

Taken together, these results suggest that the reduction in the protein levels of the three variants is likely to be derived from protein instability due to amino acid alterations or ineffective translation in V79 cells.

7-Ethoxyresorufin O-deethylation activities of the three CYP1A2 variants.
Metabolism of 7-ethoxyresorufin by the WT and three variants was compared using microsomal fractions from the transfected V79 cells (n=3). The kinetic parameters are summarized in Table 1. The $K_m$ value was significantly increased only in the Arg377Gln variant (approximately 9-fold increase) compared to the WT ($P<0.01$). In contrast, the $V_{max}$ and $V_{max}/K_m$ values of all three variants were markedly reduced, compared to those of the WT ($P<0.001$ for all comparisons). The $V_{max}$ values of the Pro42Arg, Arg377Gln, and Arg456His variants were 2.32±0.71%, 1.37±0.59%, and 2.09±0.36%, respectively, when the WT value was defined as 100%. As for $V_{max}/K_m$, the values of the Pro42Arg, Arg377Gln, and Arg456His variants were 0.85±0.19%, 0.15±0.04%, and 0.58±0.07% of the WT, respectively. Thus, these results suggest that all three amino acid alterations lead to reduced catalytic activity toward 7-ethoxyresorufin.

**Phenacetin $O$-deethylation activities of the three CYP1A2 variants.**

The catalytic activities of the WT and three variants were also determined for another substrate phenacetin. Its $O$-deethylation by the WT and the three variant CYP1A2s was measured at 5 µM and 100 µM (n=3) (Table 2). Phenacetin $O$-deethylation activities of the three variants at a low (5 µM) substrate concentration were all below the detection limit (0.16 pmol/min/mg microsomal protein). The activities of the Pro42Arg and Arg456His variants at a high (100 µM) substrate concentration were 3.99±0.66% and 3.18±0.44%, respectively, when the WT value was defined as 100% ($P<0.001$ for both). The catalytic activity of the Arg377Gln variant was still below the detection limit with the high substrate concentration. Thus, remarkable reductions in the phenacetin $O$-deethylation activities were also observed for all three variants.

**Spectral analysis of the three CYP1A2 variants.**

The reduced CO-difference spectral analysis was carried out since the enzymatic activities of the three variants were dramatically reduced (Tables 1 and 2) compared to the relative expression levels of the immunoreactive proteins (Fig. 1). We produced recombinant WT and three variant CYP1A2 proteins in Sf21 cells for this purpose. In immunoblot analysis of the whole-cell lysates of Sf21 cells, the relative expression
levels of Pro42Arg, Arg377Gln, and Arg456His variants were 50.0±5.2%, 26.7±4.5%, and 39.0±3.7% of that of the WT (100%), respectively (Fig. 3A). These relative levels were similar to those observed in the V79 cells (Fig. 1). The reduced CO-difference spectrum of the WT enzyme showed a prominent peak at 450 nm, comparable to that of a positive control (Human CYP1A2 Supersomes) (Fig. 3B). From the absorbance at 450 nm, the expression level of the WT hemoprotein was calculated as 45 pmol/mg lysate protein. In contrast, the peak at 450 nm was hardly detected in all the three variant proteins, suggesting marked reductions in functional hemoprotein formation. The lysates of uninfected Sf21 cells gave only a peak at 420 nm. These findings well coincide with the dramatic reductions in the variant enzyme activities shown in the V79 cell microsomes.
Discussion

In this study, three CYP1A2 variants found in a Japanese population, Pro42Arg (125C>G, *15), Arg377Gln (1130G>A, *16), and Arg456His (1367G>A, *8), were characterized. Our data clearly indicate that all the Pro42Arg, Arg377Gln, and Arg456His variants had catalytic activities less than 1% and 4% of the WT for the O-deethylation of 7-ethoxyresorufin (Table 1) and phenacetin (Table 2), respectively. These reduction rates were striking as compared with those in the protein expression levels assessed by immunoblotting of the V79 cell microsomes (30% - 66% of the WT, Fig. 1). Reduced CO-difference spectral analysis using the variant proteins produced in the Sf21 cells revealed that almost no peak was observed at 450 nm in the three variants, suggesting their marked reductions of hemoprotein formation. Thus, most of the variant proteins detected by immunoblotting of Sf21 cell lysates (27% - 50% of the WT, Fig. 3A) are suggested to be their nonfunctional apoproteins. Also, the immunoreactive variant proteins in the V79 cell microsomes (Fig. 1) are assumed to be mostly apoproteins.

Pro42 of CYP1A2 is a well conserved amino acid residue and located 9 amino acids downstream of the membrane anchor region. This amino acid is the first proline residue in the N-terminal proline-rich motif (PPGPXPXPXXGN), which has been suggested to play an important role in the proper folding of the P450 enzyme (Kemper, 2004). Substitutions of the first proline residue in CYP2C2 resulted in reduced (substitutions with Ala, Ile, and Leu) or null (with Asp, Glu, Gly, and Val) enzymatic activities. When the CYP2C2 protein was expressed in insect cells, the Pro to Ala substitution at this site was reported to result in the reduced expression of functional cytochrome P450 hemoprotein and the increased expression of inactive cytochrome P420 (Chen et al., 1998). The amino acid alteration at this position was suggested to hamper the proper folding of CYP2C2 protein possibly by preventing interactions with other polypeptide regions (Kemper, 2004). A polymorphism of the first Pro site has also been reported in CYP2D6 as the *10 allele (Kagimoto et al., 1990). The Pro34Ser alteration in CYP2D6 produces the unstable and less active enzyme protein (Johansson et al., 1994; Nakamura et al., 2002). In our hands, the Pro42Arg variant of CYP1A2 also showed dramatically reduced enzymatic activities in V79 cells (less than 4% of the WT for 7-ethoxyresorufin and phenacetin, Tables 1 and 2). Also, the 450 nm peak was
hardly observed in the Pro42Arg variant produced in the baculovirus system (Fig. 3B). Improper folding of CYP1A2 protein might occur with this Pro to Arg substitution, resulting in nonfunctional apoprotein formation.

The previous reports defined six substrate recognition sites of CYP1A2 based on the amino acid sequence alignment of CYP2 family members (Gotoh, 1992; Lewis, 2001; Lewis et al., 2003). In this study, the Arg377Gln variant showed the most drastic reduction in the \( V_{\text{max}}/K_m \) value for 7-ethoxyresorufin O-deethylation, with an approximately 9-fold increase in the \( K_m \) value (Table 1 and 2). Arg377 is conserved within the human CYPs, and located in the K-helix and only 2 amino acids upstream of substrate recognition site 5 (Lewis, 2001; Lewis et al., 2003). Furthermore, it appears that Arg377 corresponds to the arginine residue in the E-X-X-R motif of the K-helix that forms a salt bridge with and arginine in the meander region of CYPs (Hasemann et al., 1995). This salt bridge is one of the constituents to retain highly conserved three-dimensional structure that is important for heme binding to CYPs. It is possible that the Arg-to-Gln substitution may disrupt this salt bridge, which leads to incapability to retain heme. This notion would be supported by the result from the reduced CO-difference spectral analysis of Arg377Gln variant (Fig. 3B).

As for Arg456His, an earlier studies on rat CYP1A2 showed that the region, including this amino acid, is suggested to be important for heme-binding and the interaction with NADPH-P450 reductase (Furuya et al., 1989a; 1989b). Indeed, the substitution of Lys453Glu (corresponding to Lys455 in human CYP1A2) abrogated its enzymatic activity, and the substitution of Arg455Gly (corresponding to Arg457 in human CYP1A2) also showed very low activities to benzphetamine, acetanilide, and 7-ethoxycoumarin. In the rat CYP1A2, Arg454 (corresponding to the Arg456 in human CYP1A2) is suggested to interact with the heme. The rat Arg454His substitution showed markedly reduced activities toward acetanilide and benzphetamine, but an increased activity to 7-ethoxycoumarin, without remarkable changes in \( K_m \) values (Furuya et al., 1989a; 1989b). In this study of the human Arg456His variant, we observed marked reductions of the catalytic activities for 7-ethoxyresorufin and phenacetin. Kinetic analysis revealed that the reduced activities to 7-ethoxyresorufin were attributed to the reduced \( V_{\text{max}} \) values, not to a change in the \( K_m \) values. Although both Arg and His have basic side chains, the pKa values for ionizable groups...
are different between guanidinium (for Arg) and imidazole (for His). The difference in their pKa values may affect the ionic interactions between heme and apoprotein. This hypothesis seems to be likely because the result in the reduced CO-difference spectra using the Arg456His variant protein expressed in the Sf21 cells showed the dramatic reduction of the peak at 450 nm (Fig. 3B).

In conclusion, the three CYP1A2 variants found in a Japanese population, Pro42Arg, Arg377Gln, and Arg456His, were functionally characterized. All three variants showed dramatically reduced O-deethylation activities for 7-ethoxyresorufin and phenacetin, in addition to decreased protein expression levels in the transfected V79 cells. The reduced CO-difference spectra using the infected Sf21 cell lysates suggested the markedly reduced hemoprotein levels of the three variant enzymes. Thus, these results suggest that Pro42, Arg377, and Arg456 are critical amino acids for the hemoprotein expression and thus catalytic activities of CYP1A2. Therefore, subjects with these amino acid variations might show altered metabolism for any prescribed CYP1A2-catalyzing drugs.
Acknowledgments

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References


Rasmussen BB, Brix TH, Kyvik KO, and Brosen K (2002) The interindividual differences in the 3-demethylation of caffeine alias CYP1A2 is determined by both


Footnotes
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Reprint requests to: Yoshiro Saito, Ph.D., Division of Biochemistry and Immunoochemistry, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. E-mail: yoshiro@nihs.go.jp
Figure legends

Fig. 1. Protein expression levels of wild-type and three variant CYP1A2s in V79 cells assessed by immunoblotting. Microsomal proteins (30 µg) from V79 cells transfected with wild-type and three variant CYP1A2 plasmids were electrophoresed, transferred, and immunoblotted with anti-CYP1A2 or anti-calnexin (control) antibody. One representative result from three separate experiments is shown.

Fig. 2. mRNA expression levels of wild-type and three variant CYP1A2s in V79 cells assessed by TaqMan real-time RT-PCR. The CYP1A2 mRNAs were reverse-transcribed from the total cellular RNA, and quantitatively measured by TaqMan real-time RT-PCR using CYP1A2-specific primers and a TaqMan probe. Each sample was normalized on the basis of the GAPDH mRNA content and expressed as a percentage of wild-type (100%). Each bar represents the mean ± SD of three separate experiments.

Fig. 3. Expression of wild-type and three variant CYP1A2s in Sf21 cells and their reduced CO-difference spectra. (A) Immunoblot analysis of whole-cell lysates from Sf21 cells (4 µg/lane). (B) Reduced CO-difference spectra of recombinant wild-type and three variant CYP1A2s (8 mg/ml). Those of uninfected Sf21 cell lysates (8 mg/ml) and a positive control (Human CYP1A2 Supersomes, 2.5 mg/ml) are also depicted. Representative spectra obtained from four separate infections are shown.
TABLE 1 Kinetic parameters of WT and variant CYP1A2s for 7-ethoxyresorufin O-deethylation

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<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol/min/mg microsomal protein)</th>
<th>$V_{max}/K_m$ (μl/min/mg microsomal protein)</th>
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<tr>
<td>Wild-type</td>
<td>0.339 ± 0.035</td>
<td>1.754 ± 0.159***</td>
<td>5.213 ± 0.654***</td>
</tr>
<tr>
<td>Pro42Arg</td>
<td>0.978 ± 0.430</td>
<td>0.040 ± 0.009***</td>
<td>0.044 ± 0.012***</td>
</tr>
<tr>
<td>Arg377Gln</td>
<td>3.025 ± 1.064**</td>
<td>0.024 ± 0.010***</td>
<td>0.008 ± 0.001***</td>
</tr>
<tr>
<td>Arg456His</td>
<td>1.221 ± 0.232</td>
<td>0.037 ± 0.008***</td>
<td>0.030 ± 0.004***</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SD of three separate experiments.

** $P<0.01$, *** $P<0.001$ compared to the wild-type.
<table>
<thead>
<tr>
<th></th>
<th>Activity (pmol/min/mg microsomal protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 μM</td>
</tr>
<tr>
<td>Wild-type</td>
<td>2.575 ± 0.205</td>
</tr>
<tr>
<td>Pro42Arg</td>
<td>ND***</td>
</tr>
<tr>
<td>Arg377Gln</td>
<td>ND***</td>
</tr>
<tr>
<td>Arg456His</td>
<td>ND***</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SD of three separate experiments. ND: Not detectable. Detection limit: 0.16 pmol/min/mg microsomal protein. ***P < 0.001 compared to the wild-type.
Fig. 1

CYP1A2

Empty plasmid  Wild-type  Pro42Arg  Arg377Gln  Arg456His

Calnexin
Fig. 3

A

B

CYP1A2

Wild-type  Pro42Arg  Arg377Gln  Arg456His  Uninfected

Absorbance

Wavelength (nm)

Wild-type  Pro42Arg  Arg377Gln  Arg456His  Uninfected  Positive control