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Heterologous expression and characterization of wild-type human cytochrome P450 1A2 without conventional N-terminal modification in *Escherichia coli*

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Abstract

In this study, wild-type human CYP1A2 without the conventional N-terminal modification (second codon GCT) or the truncation of the N-terminal hydrophobic region was functionally expressed in *Escherichia coli*. Its enzymatic properties were compared with N-terminally modified CYP1A2. Although modified CYP1A2 is almost all high-spin, some wild-type CYP1A2 shifted to low-spin. Spectral binding titrations with several ligands could be performed with wild-type enzyme, but not with modified enzyme. Kinetic parameters for several substrates were similar for the two CYP1A2 enzymes. However, the oxidation rates of phenacetin by modified enzyme were ~2-fold higher than those by wild-type enzyme. The intermolecular isotope effects were ~2 for phenacetin *O*-deethylation catalyzed by both enzymes. However, the wild-type enzyme, but not the modified enzyme, increased *C*-hydroxylation when *O*-deethylation rates were lowered by deuterium substitution. Molecular switching indicates that phenacetin rotates within the active site of wild-type enzyme and suggests a looser conformation in the active site of the wild-type enzyme than of the modified enzyme. These results reveal that the overall enzymatic properties of wild-type CYP1A2 enzyme are quite similar to those of modified CYP1A2, although its active site environment seems to differ from that of the modified enzyme.

Keywords: Human cytochrome P450 1A2; N-terminal membrane anchor domain; Catalytic function; Spin equilibrium; Heterologous expression; N-terminal modification

Cytochromes P450 (CYP[1] or P450) are the major enzymes involved in the oxidation of xenobiotic chemicals and endogenous substrates [1,2], and 57 CYP genes are present in humans [1,3]. Human CYP1A2, located predominantly in the liver, participates in the metabolism of a variety of drugs [4] and many carcinogens, particularly aromatic and heterocyclic amines [5]. This enzyme is also of considerable interest due to the diversity of reactions it metabolizes [6] and its relevance to cancer risk [7]. Among drug-metabolizing P450 enzymes in liver, CYP1A2 plays a predominant role in the metabolic clearance of caffeine and melatonin, as well as of marketed drugs such as flutamide, lidocaine, olanzapine, tacrine, theophylline, triamterene, and zolmitriptan [8]. Human CYP1A2 has been heterologously expressed in a number of systems and is used in

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[1] *Abbreviations used:* CYP or P450, cytochrome P450; CPR, NADPH-cytochrome P450 reductase; DLPC, L-α-dilauroyl-*sn*-glycero-3-phosphocholine; modified CYP1A2, human CYP1A2 with N-terminal modification; wild-type CYP1A2, human CYP1A2 without N-terminal modification; CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate; δ-ALA, δ-aminolevulinic acid; IPTG, isopropyl-1-thio-D-galacto-pyranoside; αNF, α-naphthoflavone.

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analyses of catalytic selectivity. Of the mammalian P450s, CYP1A2 has one of the highest expression levels in bacterial systems [9,10].

Common strategies for functional expression of mammalian P450s, including human CYP1A2, in *Escherichia coli* include N-terminal sequence modification (second codon GCT), truncation of the N-terminal hydrophobic region responsible for the interaction of P450s with membrane, and use of lower temperatures (usually 20–28 °C) for expression [3,10,11]. To achieve the maximal yield of catalytically active hemeprotein, various approaches have been used such as fusion proteins and signal peptides, and expression of P450s in the presence of molecular chaperones [3].

Considerable work has been done with recombinant human CYP1A2 and its mutants to study the reaction mechanism [12,13] and the interaction with membranes [14]. Very recently, the crystal structure of human CYP1A2 was reported [8]. For those studies, CYP1A2 enzymes with modified N-terminal segment were used instead of *per se* wild-type N-terminal segment.

It is generally accepted that alterations in the sequence of the membrane anchor domain do not affect the catalytic properties of P450s [3,11]. Today, it is possible to express and purify recombinant human P450s without the modified N-terminal segment using the baculovirus system, although this costs much more than using a bacterial system [3]. However, no systematic approaches have been used to study the effect of N-terminal modification on the enzymatic properties of human P450s, such as substrate binding, spin-state, kinetic isotope effects, and reduction kinetics.

To our knowledge, there are no reports of human P450 enzyme expression in bacteria without the conventional modification at the N-terminus. Here, human CYP1A2 without N-terminal modification (called 'wild-type CYP1A2') was cloned and functionally expressed in *E. coli* under the control of an inducible *tac* promoter in monocistronic format. The enzymatic properties of purified wild-type CYP1A2 were compared to those of N-terminally modified CYP1A2 (called 'modified CYP1A2') using various substrates. The roles of the CYP1A2 N-terminal segment on the enzymatic properties were then discussed.

Materials and methods

Materials

Phenacetin, 7-OH coumarin, 7-ethoxycoumarin, flutamide, and acetaminophen were purchased from Sigma–Aldrich (Milwaukee, WI). 7-Ethoxyresorufin, 7-methoxyresorufin, and 7-OH-resorufin were obtained from Molecular Probes (Eugene, OR). Substrates and products for the isotope effect experiments were prepared as previously described [12,15,16]. Recombinant rat NADPH–cytochrome P450 reductase (CPR) was expressed in *E. coli* and purified as described [17,18].

Construction of wild-type CYP1A2 expression plasmid

The cDNA for wild-type human CYP1A2 without the conventional N-terminal modification, which was cloned into the EcoRI and NotI sites of the pCNS-D2 plasmid, was obtained from The Center for Functional Analysis of Human Genome, KRIBB (Daejeon, Korea). The cDNA sequence was modified to include an NdeI site (5′-agc*catat gg*cattgtcccagtc) and a XbaI $(His)_5$ site (5′-tcg*tctaga* tcaatg atgatgatgatgattgatggagaagc) at the 5′ and 3′ ends, respectively. The PCRs were performed with *pfu* DNA polymerase as described by the manufacturer (Solgent, Daejeon, Korea) using an MJ Research PTC-200 Thermal Cycler (Reno, NV). The 1563-bp PCR product was resolved on a 1% (w/v) agarose gel, purified, digested with NdeI and XbaI, and ligated into pCW *Ori* plasmid that had been digested with the same endonucleases.

To introduce the modified codon of the second N-terminal amino acid (Ala) into wild-type CYP1A2, three kinds of 5′-oligomers were designed. Each of the oligos (5′-GG TCATATGGC*C*TTGTCCCAGT, 5′-GGTCATATGGC*G* TTGTCCCAGT, 5′-GGTCATATGGC*T*TTGTCCCAGT) was used to introduce a codon exchange into the "GCA" region (Fig. 1).

Expression and purification of recombinant CYP1A2 with or without N-terminal modification

Plasmids were transformed into *E. coli* DH5α F′-IQ cells. Prior to starting the expression culture, the transformed cells were grown overnight in Luria–Bertani (LB) broth with ampicillin (100 μg/ml) selection at 37 °C. The overnight cultures (20 ml) grown were used to inoculate a 250-ml culture of Terrific broth (TB) containing 100 μg/ml ampicillin, 1.0 mM thiamine, trace elements, 50 μM $FeCl_3$, 1 mM $MgCl_2$, and 2.5 mM $(NH_4)_2SO_4$. Cultures were grown at 37 °C and 250 rpm to an OD_{600} between 0.4 and 0.6. Following the addition of isopropyl-1-thio-D-galacto-pyranoside (IPTG) (1 mM) and δ-aminolevulinic acid (δ-ALA) (0.5 mM), cultures were grown at 28, 32, 37, or 40 °C at 180 or 250 rpm.

Expression of CYP1A2 enzymes in *E. coli* DH5α-F′IQ was accomplished essentially as described [10]. Recombinant wild-type and N-terminally modified CYP1A2 enzymes containing a C-terminal $(His)_5$ tag were prepared from the corresponding CYP1A2 as described [12]. Membranes were solubilized for 3 h at 4 °C at a final concentration of 2 mg protein per ml in 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v), 1.0 mM EDTA, 30 μM αNF, 10 mM β-mercaptoethanol, and 1.0% 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS) (w/v). The solubilized material was then loaded onto a 2.5 × 10 cm Ni^{2+} Sepharose High Performance column (Amersham Biosciences, Piscataway, NJ) that had been pre-equilibrated with 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v), 0.5 M NaCl, 5 mM imidazole, and 0.5% CHAPS (v/v).

Fig. 1. Nucleotide (A) and amino acid (B) sequence comparisons of the N-terminal sequence of wild-type (called "wild-type") and N-terminally modified human CYP1A2 (called "modified"). Substitution of the second codon (GCA) to GCT, GCC, or GCG, in wild-type CYP1A2 is shown; all four (4) codons encode Ala.

Contaminating proteins were removed by extensive washing with equilibration buffer containing 20 mM imidazole. Recombinant CYP1A2 enzymes were eluted from the column with 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v), 300 mM imidazole, 0.5 M NaCl, and 0.5% CHAPS (w/v). Fractions containing P450 were pooled and dialyzed at 4 °C for 4 h against a 200-fold volume of 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v), 1.0 mM EDTA, and 0.1 mM dithiothreitol, followed by two more changes of the same buffer without dithiothreitol. SDS–PAGE was used to assess final protein purity, and P450 concentrations were determined by Fe^{2+}–CO versus Fe^{2+} difference spectroscopy [19].

Spectroscopy

UV–visible spectra were recorded on a Shimadzu UV-1601 (Shimadzu, Kyoto, Japan). The high-spin contents of CYP1A2 enzymes were estimated from the second-derivative spectra of the ferric enzymes as described [20].

All fluorescence experiments were performed at 25 °C, maintained using a circulating water bath. Fluorescence emission spectra were recorded on a Shimadzu RF-5301 PC spectrofluorometer equipped with a thermostated cuvette compartment. In all fluorescence experiments, each measurement under experimental conditions was corrected as described elsewhere for the inner filter effect due to light scattering and absorption [21].

Spectral binding titrations

Spectral binding titrations were used to determine dissociation constants (K_s) for substrates, inhibitor (α-naphthoflavone, αNF), and products as described [22]. Binding affinities of ligands to wild-type CYP1A2 were determined (at 23 °C) by titrating 1 μM enzyme with the ligand, in a total volume of 1.0 ml of 100 mM potassium phosphate buffer (pH 7.4). Final CH_3OH concentrations were <2% (v/v). Spectral dissociation constants (K_s) were estimated using GraphPad Prism software (GraphPad Software, San Diego, CA). Unless the estimated K_s was within 5-fold of the P450 concentration, nonlinear regression analysis was applied using the hyperbolic equation. For the high-affinity ligand, αNF, a quadratic equation was used to correct for the bound enzyme concentration [23].

Reduction kinetics

The reduction rate from ferric (Fe^{3+}) to ferrous (Fe^{2+}) P450 was measured at 23 °C by the increase in absorbance at 450 nm or the decrease at 390 nm [23]. Glass tonometers (under a positive CO atmosphere and containing the O_2 scrubbing system; see above) were used to fill the drive syringes of the stopped-flow instrument, with minimum exposure to the atmosphere. One of the syringes contained an anaerobic solution of CYP1A2 (0.5 μM, reconstituted as described above), and the second syringe contained 0.4 mM NADPH. UV–visible spectra (355–580 nm) were collected, and kinetic traces were extracted at the wavelengths of interest and further analyzed using GraphPad Prism. As in the binding kinetics experiments, between 10 and 1000 scans/s were acquired, depending on the data collection time, and averages of four experiments were generally used in the subsequent data analyses.

P450 catalytic activity assays

Several typical substrates of CYP1A2 were used to compare the catalytic activities of wild-type and modified CYP1A2s. Kinetic parameters (K_m and k_{cat}) were determined using nonlinear regression analysis with GraphPad Prism software.

EROD and MROD

Kinetic parameters (K_m and k_{cat}) were initially investigated using fluorescence assays for 7-methoxyresorufin *O*-demethylation and 7-ethoxyresorufin *O*-deethylation [24]. The reaction mixtures contained 50 pmol CYP1A2, 100 pmol CPR, L-α-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) (45 μM), 100 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system (0.5 mM $NADP^+$, 10 mM glucose-6-phosphate, and 1.0 IU glucose-6-phosphate dehydrogenase ml^{-1}), and varying concentrations of substrate (0.02–5 μM) in a total volume of 0.5 ml. The reaction mixtures were generally pre-incubated for 10 min at

37 °C. The reaction was initiated by the addition of the NADPH-generating system, incubated for 10 min at 37 °C, and terminated with 1 ml of CH_3OH. Metabolites were quantitated using fluorescence and a resorufin standard [25].

Phenacetin O-deethylation

The reaction mixtures consisted of 25 pmol CYP1A2, 50 pmol CPR, DLPC (45 μM), 100 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system and varying concentrations of substrate (0.5–100 μM phenacetin) in a total volume of 0.25 ml. Phenacetin *O*-dealkylation activity was determined by HPLC as described [12]. Reaction mixtures were generally incubated for 20 min at 37 °C, terminated with 0.5 ml of 17% $HClO_4$, and centrifuged (10^3g, 10 min); 0.5 ml of a mixture of $CHCl_3$ and 2-propanol (6:4, v/v) was added to the supernatant to extract the products, followed by centrifugation (twice at 10^3g). The organic layers were combined and solvent was removed under N_2. The products (acetaminophen and the acetol) were analyzed by HPLC using a Gemini C_{18} column (4.6 × 150 mm, 5 μm, Phenomenex) with the mobile phase $H_2O/CH_3OH/CH_3CO_2H$ (65:35:0.1, v/v/v; flow rate 1.0 ml min^{-1}) and monitoring at A_{254}.

7-Ethoxycoumarin O-deethylation

Reactions were incubated for 5–10 min at 37 °C, terminated with 0.10 ml of 17% $HClO_4$, and centrifuged (10^3g, 10 min). Products were extracted from the supernatant with CH_2Cl_2 (1.0 ml) followed by centrifugation at 10^3g. The organic layers were combined, and the CH_2Cl_2 was removed under a N_2 stream. The products, 7-hydroxy coumarin and 3-hydroxy-7-OR-coumarin, were analyzed by HPLC using a Toso ODS-80_{TM} octadecylsilane (C_{18}) column (4.6 × 150 mm, 5 μm) with a mobile phase of H_2O:CH_3CN (55:45, v/v) containing 10 mm $HClO_4$, a flow rate of 1.0 ml min^{-1}, and monitoring at A_{330} [15].

Flutamide hydroxylation

The reaction mixtures consisted of 50 pmol CYP1A2, 100 pmol CPR, DLPC (45 μM), 100 mM potassium phosphate (pH 7.4), an NADPH-generating system, and varying concentrations of flutamide (0.5–100 μM) in a total volume of 0.5 ml. Following incubation for 15 min at 37 °C, each reaction was terminated by the addition of 100 μl of methanol:water (1:1, v/v). Incubates were vortex-mixed and centrifuged at 10^4g for 15 min at room temperature. Supernatants were analyzed by HPLC as previously described [26]. Separation was achieved by direct injection onto a Gemini C_{18} column (4.6 × 150 mm, 5 μm, Phenomenex). The mobile phase was delivered as 75% CH_3OH and 25% H_2O at a flow rate of 1 ml/min, and the eluate was monitored at A_{300}.

NADPH oxidation

CYP1A2s were reconstituted with CPR as described for steady-state phenacetin oxidation experiments. Reconstituted enzyme (950 μl) was pre-incubated for 3 min at 37 °C in the presence or absence of substrate (200 μM). Reactions were initiated with the addition of 50 μl of 4.0 mM NADPH, and the decrease in A_{340} was monitored for 1 min. Rates of NADPH oxidation were calculated using $\varepsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$ for NADPH [12].

H_2O_2 formation

Reaction systems were prepared as described above, except that the reaction volume was 0.25 ml. Reactions were initiated by addition of the NADPH-generating system. The concentration of H_2O_2 was determined spectrophotometrically using PeroXOquant according to the manufacturer's instructions (PIERCE, Rockford, IL).

Kinetic isotope effect determination

Deuterium isotope effects were determined by a noncompetitive method [27,28]. CYP1A2 was incubated with unlabeled (d_0) phenacetin or 7-ethoxycoumarin or with labeled (d_2) phenacetin or 7-ethoxycoumarin using a range of substrate concentrations (0.1–100 μM), and the products were analyzed by HPLC as described above. K_m and k_{cat} were determined by nonlinear regression [12,16].

Edman degradation

Wild-type CYP1A2 was separated by SDS–PAGE and electrophoretically transferred to PVDF membrane (Bio-Rad Laboratories, Hercules, CA) using the general protocols presented elsewhere [29]. Sheets were stained with Coomassie brilliant blue R-250 to locate polypeptides and then destained. Appropriate regions were cut out and subjected to sequence analysis at the Korea Basic Science Institute facilities (Seoul, Korea). In some cases, the polypeptides were treated with 3 N HCl for 24 h at ambient temperature before sequence analysis [30].

Results

Functional expression of wild-type CYP1A2 enzyme without conventional N-terminal modification

Since the initial functional expression of bovine CYP17A1 via modification of the N-terminal sequence, a series of human P450s, including CYP1A2, have been successfully expressed with this modification and having the expected catalytic activities [3,31].

The initial optimization of wild-type CYP1A2 expression in this work focused on the culture temperature of heterologous expression in *E. coli*. We examined the effect of culture temperature on the expression of wild-type CYP1A2 compared to that of modified CYP1A2. The induction time-dependent expression levels of wild-type and N-terminally modified CYP1A2 in *E. coli* cells were measured at several temperatures (28, 32, 37 and 40 °C) (Fig. 2). The production

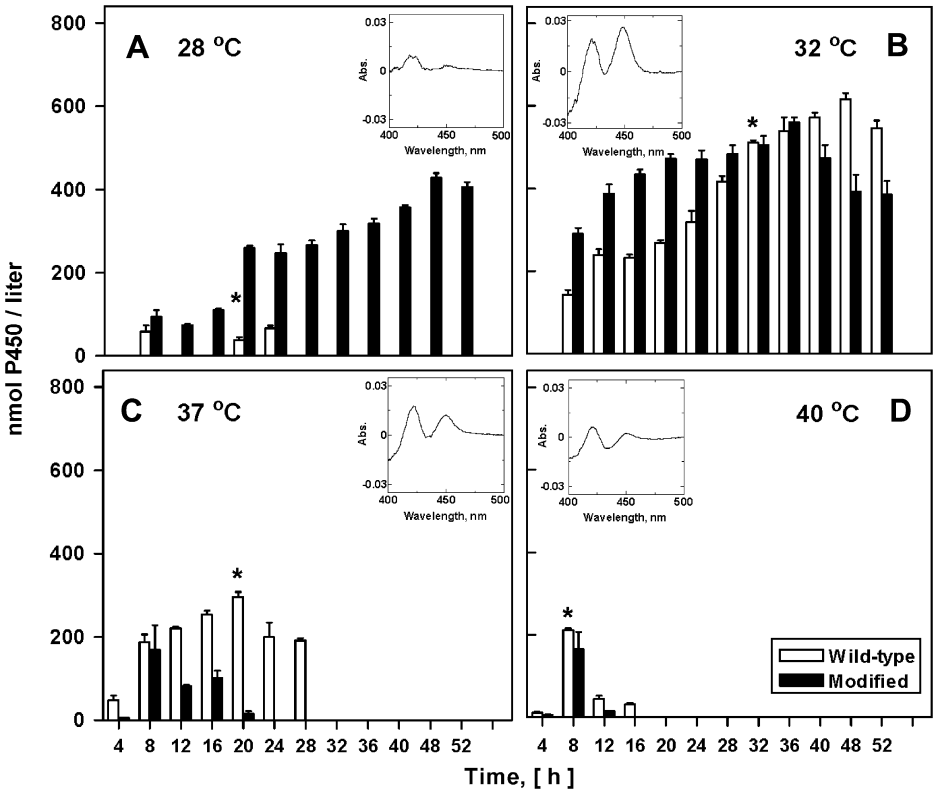

Fig. 2. Effects of culture temperature on CYP1A2 expression. Wild-type (open bar) and modified (closed bar) human CYP1A2 were heterologously expressed in *E. coli* at (A) 28 °C, (B) 32 °C, (C) 37 °C and (D) 40 °C for 52 h, and the levels of P450 expression were measured at the indicated time by CO difference spectra as described in "Materials and Methods". The insets show the CO difference spectra, which were obtained at the indicated time (*).

of wild-type human CYP1A2 was elevated with increasing induction time and maximal yield was obtained at around 48 h at 32 °C, as indicated by Fe^{2+}–CO versus Fe^{2+} difference spectra of whole *E. coli* cells. At 32 °C, the expression level of wild-type CYP1A2 reached 650 nmol (per liter culture) within 48 h, which is the optimal temperature condition for expression of wild-type CYP1A2 (Fig. 2B).

In the case of modified CYP1A2, the highest expression level was also observed at 32 °C with 36 h culture (Fig. 2B). The expression level of wild-type CYP1A2 at 28 °C was much lower than that of modified CYP1A2 (Fig. 2A). After 24 h culture at 28 °C, no apparent P450 peak was observed. However, the opposite phenomena were observed at 37 °C (Fig. 2C).

These results clearly indicate that wild-type CYP1A2 can be expressed in *E. coli* at 32 and 37 °C with expression levels of up to 650 nmol/l.

Effect of codon sequences on the expression of wild-type CYP1A2

Based on the first successes of mammalian P450 expression in *E. coli*, the second codon was modified to GCT to encode Ala for heterologous expression of several human P450 enzymes including CYP1A2 [10,31]. GCT is frequently found in the second position of *E. coli* genes [32].

It is interesting to note that the second codon of wild-type CYP1A2 is GCA, not GCT. Furthermore, wild-type CYP1A2 was functionally expressed in *E. coli* without the GCT codon (Figs. 1 and 2). There are four (4) codons that encode the amino acid Ala: GCT, GCC, GCA, and GCG. We examined the effect of substitution of the second codon (GCA) to GCT, GCC, or GCG, on the expression of wild-type CYP1A2 in *E. coli* (Fig. 1). The CYP1A2 variants with GCT, GCC, and GCG as the second codon could be expressed as much as wild-type CYP1A2 (with GCA as the second codon) (Fig. 3). The expression level of wild-type CYP1A2 (with GCA) with 12 h culture at 32 °C was 250 nmol P450 (per liter culture); GCT variant, 270 nmol/l; GCC variant, 200 nmol/l; GCG, 170 nmol/l.

This result shows that substitution of GCT in the second position is not crucial for expression of CYP1A2, although the expression was enhanced somewhat by this substitution.

Properties of purified wild-type CYP1A2

We added a $(His)_5$ tag to the C-terminus of CYP1A2 proteins to expedite purification. The use of Ni^{2+}-nitrilotriacetate affinity chromatography reduced the time that the enzymes were subjected to chromatography [12]. The $(His)_5$ sequence did not influence the enzymatic properties of the wild-type enzyme (results not shown). A $(His)_5$ tag at the C-terminus of modified CYP1A2 also did not influence

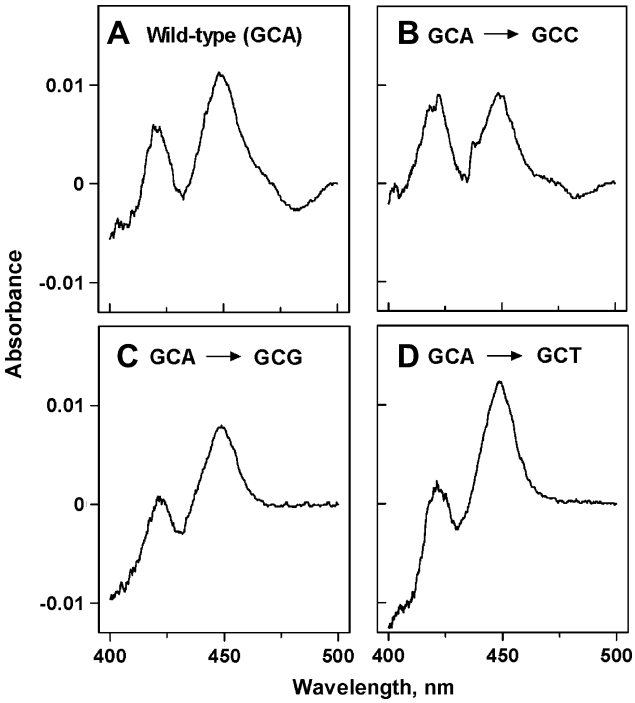

Fig. 3. Effect of silent mutation at the second amino acid on the expression of wild-type CYP1A2. The second amino acid (GCA) of wild-type CYP1A2 (A) was changed to (B) GCA → GCC, (C) GCA → GCG, and (D) GCA → GCT. See also Fig. 1. CO difference spectra were obtained after 12 h culture at 32 °C.

its enzymatic properties [12]. The purification process was confirmed through SDS–PAGE of purified samples.

The final specific contents of both purified wild-type and modified CYP1A2 were ~16 nmol of P450/mg of protein. The high-spin iron fractions of the CYP1A2 enzymes were estimated by second-derivative analysis of the absorption spectra [20]. Modified CYP1A2 is almost high-spin (82% high-spin) [12]. But we found that purified wild-type CYP1A2 exhibited a shift to low-spin (71% high-spin) (Fig. 4). This result indicates that the difference in the N-terminal membrane anchor sequence can affect the heme environment, although spin-state is not correlated to enzyme activity for modified CYP1A2 mutants [12].

We confirmed the purified wild-type CYP1A2 by determining the N-terminal amino acid sequence (MALSQ...) with or without 3 N HCl treatment, as described in "Materials and Methods". Although acid treatment increased the yield of predicted amino acid residues by 2-fold, both samples gave the expected sequence (results not shown).

Kinetic parameters of purified CYP1A2 enzymes

Kinetic parameters (k_{cat} and K_m) of catalysis by wild-type and modified CYP1A2s were compared using several typical CYP1A2 substrates: 7-ethoxyresorufin, 7-methoxyresorufin, phenacetin, 7-ethoxycoumarin, and flutamide (Table 1). In general, the catalytic efficiencies (k_{cat}/K_m) for most of the reactions catalyzed by wild-type and modified enzymes were quite similar. However, the k_{cat} and K_m values for phenacetin oxidation by wild-type enzyme were quite different from those by modified enzyme. The k_{cat} values for *O*-deethylation and *C*-hydroxylation by wild-type enzyme were 50% and 22% of those by modified enzyme, respectively. Moreover, the K_m values of wild-type enzyme were higher than those of modified enzyme.

NADPH oxidation, H_2O_2 formation, and coupling of CYP1A2 reactions

The difference in the N-terminal segment affected NADPH oxidation rates in the presence of several substrates (Table 2). However, no correlation was found among the rates of NADPH oxidation, product formation, and H_2O_2 formation. When the rates of product formation and NADPH oxidation were compared, the wild-type enzyme (0.93–4.2%) showed lower coupling efficiency than modified enzyme (2.0–5.5%) toward all substrates tested.

Spectral equilibrium binding titrations of wild-type CYP1A2 for substrates, products, and inhibitor

Ferric wild-type CYP1A2 exhibited some low-spin-state (71% high-spin), and the addition of substrates (phenacetin, caffeine), products (acetaminophen, acetol), and an inhibitor (αNF) produced a conversion in the spin-state depending on each chemical (Fig. 5). Affinities of various ligands with wild-type CYP1A2 were estimated spectrophotometrically by monitoring the heme spectral changes upon the addition of ligands. K_s values were obtained from the titration curves, as described under "Materials and Methods".

In the case of wild-type CYP1A2, phenacetin, methacetin, acetaminophen, and acetol products of phenacetin all resulted in a Type II shift in the heme Soret band, with a decrease in absorbance at 390 nm and an increase in absorbance at 420 nm, typical of a ligand (nitrogen atom) coordinating to the heme iron [33] (Fig. 5A–D). The binding affinities of wild-type CYP1A2 toward the substrates, phenacetin ($K_s = 14 \pm 2$ μM) and methacetin ($K_s = 11 \pm 5$ μM), were determined. The binding affinities of wild-type CYP1A2 with the products, acetaminophen ($K_s = 20 \pm 1$ μM) and acetol product ($K_s = 16 \pm 7$ μM), were also determined (Fig. 5C and D). In the case of modified enzyme, the addition of substrates and products tested here did not result in any apparent spectral changes.

αNF, the CYP1A2 inhibitor, had the highest apparent affinity ($K_s = 0.012 \pm 0.001$ μM). The titration of wild-type CYP1A2 with αNF produced a Type I shift, with an increase in absorbance at 390 nm and a decrease in absorbance at 420 nm (Fig. 5E), reflecting the displacement of H_2O as the sixth ligand and resulting in a low-to-high-spin change in the P450 iron. We also performed the binding titration of modified CYP1A2 for αNF and found that the modified enzyme has a high-affinity for the inhibitor, showing a Type I binding spectrum ($K_s = 0.010 \pm 0.001$ μM) (Fig. 5F).

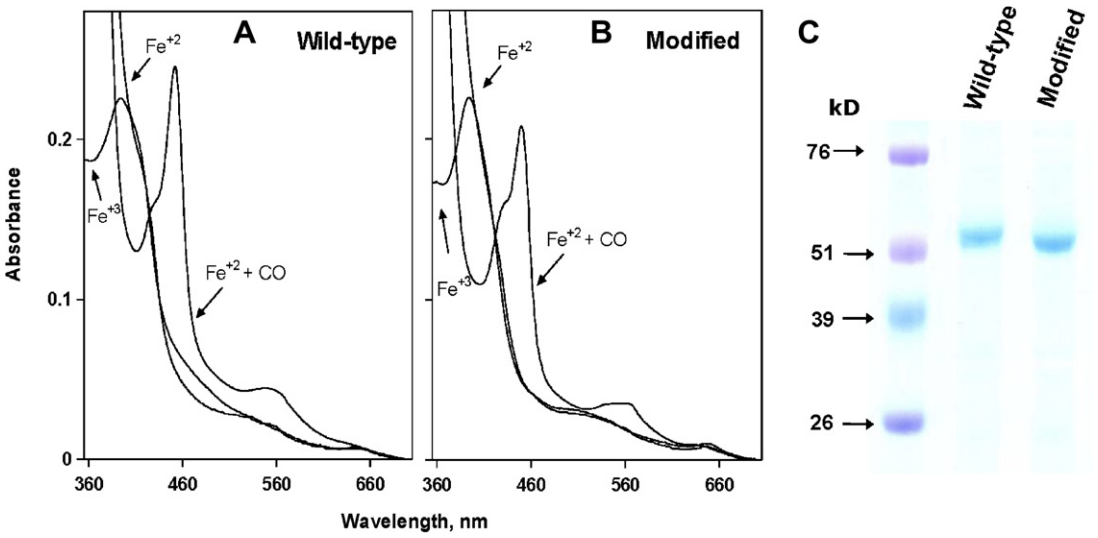

Fig. 4. Absorption spectra of wild-type and N-terminally modified human CYP1A2 enzymes. Absorption spectra of ferric, sodium dithionite-reduced ferrous, and ferrous–CO complexes of wild-type CYP1A2 (A) and modified CYP1A2 (B) in 100 mM potassium phosphate buffer (pH 7.4) are shown. After spectra of ferric P450s were measured, a few grains of sodium hydrosulfite were added to the P450 solution to obtain ferrous P450. Prior to each measurement, each P450 was diluted to 1.0 μM in potassium phosphate buffer (pH 7.4) in a final volume of 1.0 ml. (C) SDS-polyacrylamide gel electrophoresis of wild-type and N-terminally modified CYP1A2 enzymes. A 10% gel was stained with Coomassie Brilliant Blue R250. Purified CYP1A2 enzymes (each 2 μg) were loaded with protein molecular weight markers (8 μg).

Table 1
Kinetic parameters of oxidations of typical substrates by purified CYP1A2 with or without N-terminal modification

Substrates	Reaction	Wild-type P450 1A2			N-terminal modified P450 1A2		
		k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m
Ethoxyresorufin	*O*-deethylation	1.7 ± 0.1	0.25 ± 0.07	6.8 ± 2.0	2.3 ± 0.2	0.37 ± 0.10	6.2 ± 1.8
Methoxyresorufin	*O*-demethylation	2.4 ± 0.2	1.0 ± 0.3	2.4 ± 0.7	3.0 ± 0.4	1.4 ± 0.5	2.1 ± 0.8
Flutamide	2-Hydroxylation	2.1 ± 0.1	8.5 ± 1.5	0.24 ± 0.04	2.5 ± 0.1	12 ± 1	0.20 ± 0.02
Phenacetin	*O*-deethylation	1.0 ± 0.1	9.5 ± 1.3	0.10 ± 0.02	2.0 ± 0.1	16 ± 3	0.0.13 ± 0.03
	C-hydroxylation	0.060 ± 0.006	14 ± 4	0.0043 ± 0.0014	0.27 ± 0.02	27 ± 6	0.010 ± 0.002
7-Ethoxycoumarin	*O*-deethylation	3.2 ± 0.2	59 ± 10	0.054 ± 0.0098	3.3 ± 0.2	55 ± 6	0.060 ± 0.010
	C-hydroxylation	3.1 ± 0.2	50 ± 7	0.062 ± 0.010	3.8 ± 0.2	69 ± 8	0.055 ± 0.010

Reduction kinetics

The dependence of the catalytic activities of CYP1A2 enzymes on CPR concentration was examined (Fig. 6A and B). The activities of both CYP1A2 enzymes increased with CPR/P450 ratio and there were no apparent differences in the catalytic activities of either enzyme. The rates of reduction of ferric P450 enzymes were also measured in an anaerobic CO environment, with ferrous P450 trapped as the CO complex (Fig. 6C). The rate of binding of ferrous P450 and CO is much faster than the rate of reduction. The rate of reduction of ferric P450 was slow in the absence of phenacetin and fit to a single exponential of 3.0 s^{-1} for both enzymes. This result indicates that both CYP1A2 enzymes show very similar reduction kinetics.

Kinetic hydrogen isotope effects

The rate of C–H bond-breaking is a major factor in determining the rate of a reaction under any conditions. Previously, we extensively studied the kinetic isotope effects for the *O*-dealkylation of phenacetin [12] and 7-alkoxycoumarin [16] by modified CYP1A2.

In this work, we examined the rate contribution of C–H bond cleavage to the CYP1A2 catalytic cycle for wild-type CYP1A2 using deuterium-labeled substrates. The results were compared to those of modified CYP1A2. Noncompetitive kinetic isotope effects were measured by running assays with d_0 and d_2 substrates and comparing the kinetic parameters [^{D}V and $^{D}(V/K)$][2] [12] (Fig. 7 and Table 3).

The patterns were very similar for phenacetin and 7-ethoxycoumarin, with both wild-type and modified CYP1A2 enzymes showing low isotope effect for phenacetin *O*-deethylation [$^{D}V = 2.5$ and $^{D}(V/K) = 1.4$–1.9] and high isotope effect for 7-ethoxycoumarin *O*-deethylation [$^{D}V =$ 6.0–8.9 and $^{D}(V/K) = 6.9$–11] (Table 3).

Modified CYP1A2 apparently did not increase acetol formation (*C*-hydroxylation) when rates of *O*-deethylation were lowered by deuterium substitution [$^{D}V = 1.2$ and $^{D}(V/$

[2] The conventions used for kinetic hydrogen isotope effects are $^{D}V = {}^{H}k_{cat}/{}^{D}k_{cat}$ and $^{D}(V/K) = ({}^{H}k_{cat}/{}^{H}K_m)/({}^{D}k_{cat}/{}^{D}K_m)$.

Table 2
NADPH oxidation, H_2O_2 formation, and product formation by CYP1A2 with or without N-terminal modification

Substrates	CYP1A2	nmol of product min^{-1} (nmol of P450)$^{-1}$				
		NADPH oxidation (A)	Product formation (B)	H_2O_2 formation	H_2O formation[a]	Coupling efficiency (%) ($B/A \times 100$)
Ethoxyresorufin	Wild-type	160 ± 1	1.5 ± 0.5	18 ± 1	70 ± 1	0.93
	Modified	115 ± 1	2.3 ± 0.7	21 ± 1	46 ± 1	2.0
Methoxyresorufin	Wild-type	125 ± 1	2.0 ± 0.1	20 ± 1	52 ± 1	1.6
	Modified	101 ± 1	2.3 ± 0.1	24 ± 1	38 ± 1	2.3
Flutamide	Wild-type	43 ± 1	1.8 ± 0.2	15 ± 1	13 ± 1	4.2
	Modified	46 ± 1	2.2 ± 0.1	16 ± 1	14 ± 1	5.5
Phenacetin	Wild-type	26 ± 1	0.86 ± 0.01[b]	7.0 ± 0.1	9.0 ± 1	3.3
	Modified	39 ± 1	1.6 ± 0.1[b]	3.0 ± 0.1	17 ± 1	4.1
7-Ethoxycoumarin	Wild-type	nd[d]	4.6 ± 0.1[c]	6.0 ± 0.1	nd	nd
	Modified	nd[d]	4.9 ± 0.1[c]	8.0 ± 0.1	nd	nd

[a] H_2O formation was determined by calculating the difference between total NADPH utilized and the sum of H_2O_2 and products produced and then dividing by 2, because four electrons are required to reduce O_2 to H_2O [45]. No correction was made for any O_2, which was not measured.
[b] Sum of acetaminophen formation and acetol formation.
[c] Sum of *O*-deethylation and 3-hydroxylation activity.
[d] Not determined.

$K) = 0.77$], as shown previously [12]. However, wild-type CYP1A2 showed increased acetol formation with deuterated phenacetin [$^{D}V = 0.5$ and $^{D}(V/K) = 0.47$] (Fig. 7). This result is interpreted as evidence for "metabolic switching," in which the commitment of an enzyme intermediate to product formation allows some turning of the substrate for the iron–oxygen complex to encounter an alternate site when oxidation at the normal site becomes less favorable [13]. Apparently, binding of phenacetin to wild-type CYP1A2, but not to modified enzyme, is such that this movement is possible. In the *O*-deethylation reaction of 7-ethoxycoumarin, "metabolic switching" was not apparent for either enzyme [$^{D}V = 0.87$–1.1].

Discussion

Several strategies have been developed for the expression of recombinant human P450s in bacteria and are now widely used [3]. Since bovine recombinant CYP17 was first expressed with the N-terminal modification in *E. coli* at 28 °C, recombinant P450s have been expressed at lower temperature, usually lower than 30 °C. However, expression of human recombinant CYP1A2 and CYP3A4 with conventional N-terminal modification at higher temperature could produce active P450s within a shorter culture time (3 h) [34,35]. Although wild-type CYP1A2 was expressed at 80 nmol P450/l after 24 h culture at 28 °C, the optimal temperature was 32 °C and the maximal expression level was 650 nmol P450/l with 36 h culture (Fig. 2). At present, the distinct mechanism of dependence of wild-type and modified CYP1A2 enzymes on culture temperature is not known.

Initial optimization of P450 holoenzyme expression focused on altering N-terminal nucleotide sequences to facilitate translation by *E. coli*. Such modifications were based on the hypothesis that the N-terminal hydrophobic sequence of mammalian P450s functions as a membrane anchor rather than playing any direct role in the catalytic cycle, substrate binding, or coupling with accessory proteins [11]. Besides the conventional N-terminal modification having membrane anchor motif (MALLLAVL...) for the functional expression of several human P450 enzymes in *E. coli*, another point to be considered is the difference in codon usage between bacterial and mammalian systems [3]. High-level expression was achieved by replacing the second codon with GCT, which is frequently found in the second position of *E. coli* genes [32]. Other bases in the 5′ terminus were also modified to reduce the free energy of base pairing (i.e., hydrogen bonding) and the tendency for the mRNA to form secondary structures that could inhibit translation [36,37].

In the present study, we examined the effect of substitution of the second codon (GCA) with GCT, GCC, or GCG, on the expression of wild-type CYP1A2 in *E. coli* (Fig. 3). The substitution of GCT in the second position does not seem to be critical for the functional expression of wild-type CYP1A2 in *E. coli*. Taken together, we concluded that neither substitution of GCT in the second position nor truncation of the hydrophobic N-terminus served to consistently enhance expression of human P450 enzymes in *E. coli*. Our results suggest that optimal expression of human P450s in *E. coli* can be achieved without modification of the N-terminal domain. However, the concept of "codon bias" may be applicable to human P450 expression in *E. coli* [3,38,39].

P450s exist in two spin-states, high-spin and low-spin, the relative fractions of which reflect an intrinsic property of each P450. The spin state refers to the coordination of the heme iron, resulting in a λ_{max} at 420 nm. The absence of the water ligand results in a λ_{max} at 390 nm. In some cases, a binding event displaces the water molecule to shift

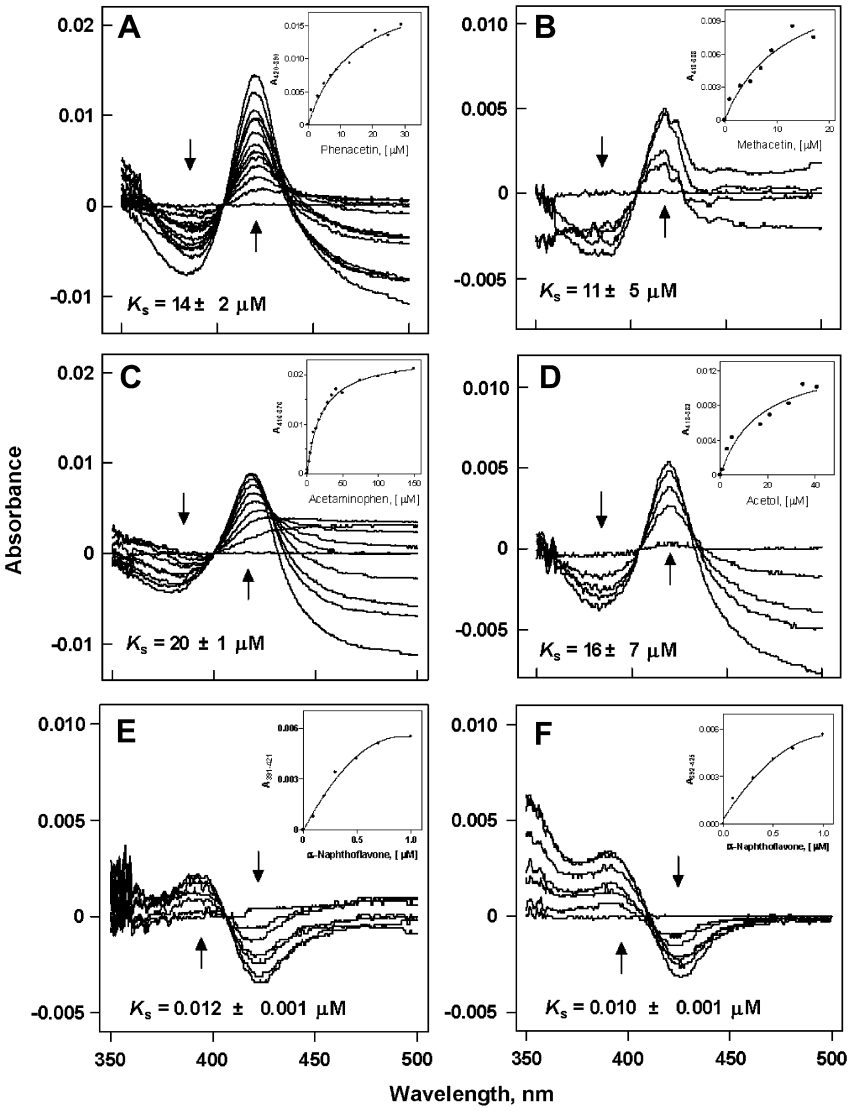

Fig. 5. Titration of ferric wild-type CYP1A2 with (A) phenacetin, (B) methacetin, (C) acetaminophen, (D) acetol product of phenacetin, and (E) α-naphthoflavone (αNF). Wild-type CYP1A2 (2 μM) was divided into each of two 1.0-ml glass cuvettes and baseline was set. Aliquots of substrates (phenacetin or methacetin), product (acetaminophen or acetol product), and inhibitor (αNF) in CH_3CN were added to the sample cuvette and equal volumes of CH_3CN were added to the reference cuvette. With increasing concentrations of chemicals, spectra were changed in the direction indicated by the arrows. The insets show the changes in absorbance, which were recorded using the dual wavelength mode, as a function of the indicated chemical concentration. (F) Titration of ferric modified CYP1A2 with αNF was also determined as described for the wild-type CYP1A2.

a low-spin P450 to the high-spin-state, resulting in a Type I spectrum [33]. It is known that the spin-state does not correlate to enzyme activity for rat P450s [40] and human modified CYP1A2 and its mutants [12].

In the case of wild-type CYP1A2, phenacetin produced a Type II shift in the heme Soret band, with a decrease in absorbance at 390 nm and an increase in absorbance at 420 nm ($K_s = 14 \pm 2$ μM) (Fig. 5A). However, modified CYP1A2 D320A mutant, which was mainly low-spin, showed a typical low- to high-spin conversion (Type I difference spectrum) upon the addition of phenacetin with a value of $K_s = 13 \pm 1$ μM [12]. Interestingly, both enzymes showed similar binding affinities toward phenacetin, although they showed opposite spin-state conversions. The observed Type II binding spectrum is indicative of Fe–N binding to the only nitrogen atom among the ligands.

The titration of both CYP1A2 enzymes with αNF indicated Type I binding spectra with increase in absorbance at 390 nm and a decrease in absorbance at 420 nm (Fig. 5E and F), similar to CYP3A4. Both enzymes showed much higher affinity ($K_s = 0.010$–0.012 μM) than CYP3A4 ($K_s = 5.7$ μM) [23].

The dissociation constant is probably an apparent K_d value that reflects the contributions of two enzyme–substrate complexes. The two observed products, acetaminophen and the acetol product, require two binding orientations for phenacetin. If the K_d values of two CYP1A2 enzymes toward substrate and products could be directly compared, the inherent affinity for the substrate

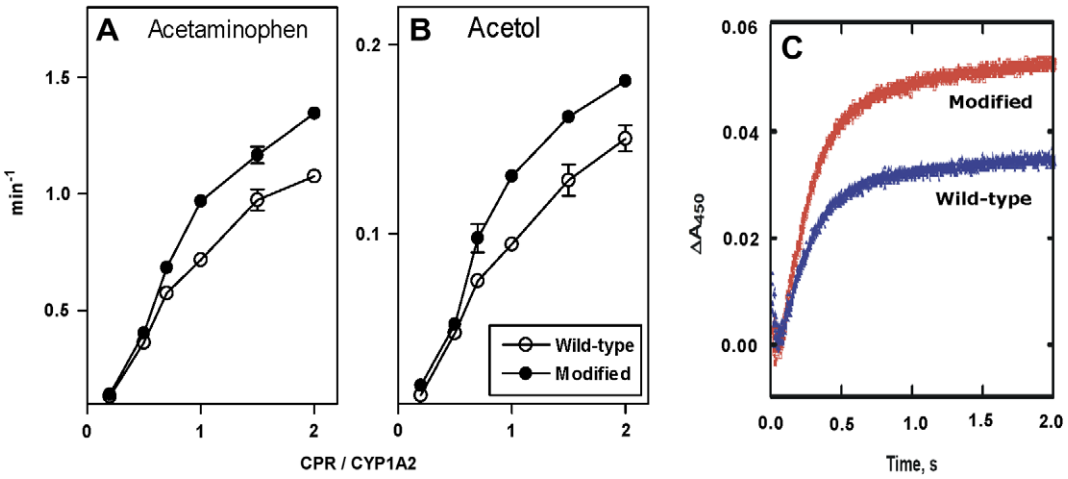

Fig. 6. Concentration dependence of phenacetin *O*-deethylation and acetyl hydroxylation by CYP1A2s on CPR concentration and reduction kinetics of the CYP1A2 enzymes. Wild-type CYP1A2 (○) or N-terminally modified CYP1A2 (●) (0.050 μM) was incubated with CPR (final molar ratio relative to P450) in the presence of phenacetin (100 μM) in 100 mM potassium phosphate buffer (pH 7.4) containing DLPC (40 μM). (C) Reduction kinetics of CYP1A2s by CPR under anaerobic conditions. Experiments were performed anaerobically under a CO atmosphere in an OLIS RSM-1000 stopped-flow spectrophotometer. One syringe contained NADPH (300 μM) in 100 mM potassium phosphate buffer (pH 7.4). The other tonometer contained a mixture of CYP1A2 (0.50 μM), CPR (1.0 μM), and DLPC (45 μM) in 100 mM potassium phosphate buffer (pH 7.4). Rates were fit to exponential plots using the manufacturer's software; without substrate, 3.0 s^{-1} (no correction for the apparent lag).

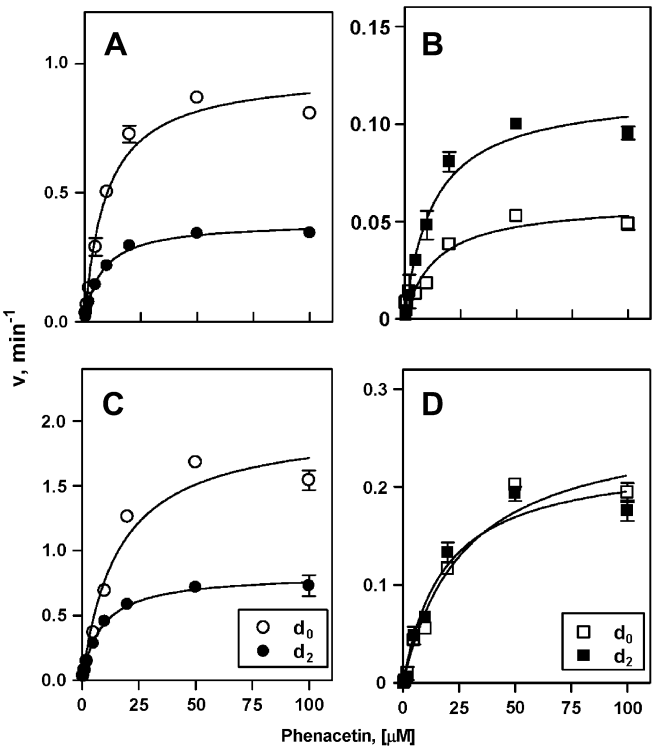

Fig. 7. Effect of deuterium substitution on *O*-deethylation and acetyl hydroxylation of phenacetin by CYP1A2s. Typical steady-state reaction conditions were used for d_0 (A) or *N*-(4-[1,1-^{2}H]ethoxyphenyl)acetamide (phenacetin-d_2) (B) by wild-type CYP1A2 and d_0 (C) or *N*-(4-[1,1-^{2}H]ethoxyphenyl)acetamide (phenacetin-d_2) (D) by modified CYP1A2. The products were analyzed by HPLC (100 μM substrate in each case). See Table 3 for kinetic parameters.

and product could be examined. The binding affinity of the substrate (phenacetin, $K_s = 14 \pm 2$ μM) was slightly higher than those of products (acetaminophen, $K_s = 20 \pm 1$ μM; acetol product, $K_s = 16 \pm 7$ μM) (Fig. 5). The modified enzyme could not be used for spectral titrations.

We next compared the catalytic activities of wild-type and modified CYP1A2 using five (5) typical CYP1A2 substrates (Table 1). Four of these substrates exhibited the same kinetic parameters. In general, the catalytic efficiencies (k_{cat}/K_m) of reactions catalyzed by wild-type and modified enzymes were similar. However, the k_{cat} and K_m values for phenacetin oxidation by wild-type enzyme were quite different from those of modified enzyme. The k_{cat} values for *O*-deethylation and *C*-hydroxylation by wild-type enzyme were 50% and 22% that of modified enzyme, respectively. Also, the K_m values of wild-type enzyme were higher than those of the modified enzyme.

A goal of this work was to assess the nature of the effect of the N-terminal segment substitution on catalytic activity toward typical CYP1A2 substrates. Although both CYP1A2 enzymes showed very similar catalytic activities toward most substrates, they exhibited significantly different activity towards phenacetin, a typical CYP1A2 substrate.

The intermolecular isotope effects were ~2 for *O*-deethylation of phenacetin catalyzed by wild-type and modified enzymes (Table 2) [12]. Modified CYP1A2 did not increase acetol formation (*C*-hydroxylation) when rates of *O*-deethylation were lowered by deuterium substitution [$^DV = 1.2$ and $^D(V/K) = 0.8$], as shown previously [12]. However, wild-type CYP1A2 showed increased acetol formation with deuterated phenacetin [$^DV = 0.5$ and $^D(V/K) = 0.47$] (Fig. 7), as shown in our previous work with methacetin [13]. This result is interpreted as evidence of "metabolic switching", in which the commitment of an enzyme intermediate to product formation allows some turning of the substrate for the iron–oxygen complex to

Table 3
Intermolecular Isotope Effects on *O*-deethylation and hydroxylation of phenacetin and 7-ethoxycoumarin by CYP1A2 with or without N-terminal modification

CYP 1A2	Phenacetin	*O*-deethylation (acetaminophen formation)					Acetyl hydroxylation (acetol formation)				
	d_n	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m	^DV	$^D(V/K)$	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m	^DV	$^D(V/K)$
Wild-type	d_0	1.0 ± 0.1	9.5 ± 1.3	0.10 ± 0.02	2.5 ± 0.3	1.9 ± 0.5	0.060 ± 0.01	14 ± 4	0.0043 ± 0.0014	0.50 ± 0.09	0.47 ± 0.17
	d_2	0.40 ± 0.01	7.7 ± 0.6	0.052 ± 0.010			0.12 ± 0.01	13 ± 2	0.0092 ± 0.0016		
Modified	d_0	2.0 ± 0.1	16 ± 3	0.13 ± 0.03	2.5 ± 0.2	1.4 ± 0.4	0.27 ± 0.02	27 ± 6	0.010 ± 0.002	1.2 ± 0.1	0.77 ± 0.24
	d_2	0.80 ± 0.03	8.6 ± 0.9	0.093 ± 0.010			0.23 ± 0.02	18 ± 5	0.013 ± 0.003		
	7-OEt coumarin	7-Hydroxycoumarin					3-Hydroxy-7-ethoxycoumarin				
	d_n	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m	^DV	$^D(V/K)$	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m	^DV	$^D(V/K)$
Wild-type	d_0	3.2 ± 0.2	59 ± 10	0.054 ± 0.098	6.0 ± 1.2	11 ± 4	3.3 ± 0.2	55 ± 7	0.060 ± 0.010	0.87 ± 0.06	0.43 ± 0.09
	d_2	0.54 ± 0.09	107 ± 32	0.0050 ± 0.0017			3.8 ± 0.1	28 ± 3	0.14 ± 0.02		
Modified	d_0	3.1 ± 0.2	50 ± 7	0.062 ± 0.010	8.9 ± 1.0	6.9 ± 2	3.8 ± 0.2	69 ± 8	0.055 ± 0.010	1.1 ± 0.1	0.37 ± 0.10
	d_2	0.35 ± 0.03	39 ± 11	0.0090 ± 0.0027			3.4 ± 0.2	23 ± 5	0.15 ± 0.03		

encounter an alternative site when oxidation of the normal site becomes less favorable [13]. Interestingly, the modified enzyme did not exhibit any apparent "switching". Apparently, the binding of phenacetin to wild-type CYP1A2, but not to modified enzyme, is such that this movement is possible. This distinction might be a result of different active site environments in the two CYP1A2 enzymes. The alteration of the active site suggested by the shifts in the iron spin-state were also evident from the steady-state studies of phenacetin *O*-deethylation catalyzed by both CYP1A2 enzymes (Tables 1 and 2).

For the modified CYP1A2, the absence of molecular switching with deuterated phenacetin argues against rotation within the active site, thus the adoption of either binding mode requires phenacetin release and rebinding. However, the wild-type enzyme underwent molecular switching, which indicates that phenacetin rotates within the active site. The switching may explain the lower activity of wild-type enzyme compared to the modified enzyme.

For 7-ethoxycoumarin *O*-deethylation catalyzed by both CYP1A2 enzymes, we found very high kinetic deuterium isotope effects (6–10) as in our previous work (Table 3) [16]. In the *O*-deethylation reaction of 7-ethoxycoumarin, "metabolic switching" was not apparent for either enzyme [$^DV = 0.87$–1.1].

At present, there is no direct information on the membrane topology of either wild-type or modified CYP1A2s because no crystal structure is yet available for the membrane-bound form. Although the crystal structure of human CYP1A2 was recently elucidated, it is the structure of only the soluble protein without the N-terminal membrane anchor region, which was deleted and modified for efficient expression and solubility. Microsomal CYP1A2 is believed to be anchored to the ER membrane by a hydrophobic amino-terminal region, which is inserted into the membrane in a vectorial fashion [41,42], and this interaction with the membrane is thought to be important for proper protein function [14,43,44].

The only difference in the amino acid sequences of wild-type and modified CYP1A2 is the N-terminal membrane anchor domain (Fig. 1). In the modified enzyme, amino acids 3–13 are deleted (LSQSVPFSATE) and amino acids 18–20 are changed (from SAI to VFL) compared to wild-type enzyme. The N-terminal domain is membrane-anchored and can interact with phospholipids. The N-terminal domain may be important for modulating the overall conformation and the environment of the active site.

In summary, we functionally expressed and purified wild-type human CYP1A2 without conventional N-terminal modification in *E. coli*. We studied the binding titration of wild-type CYP1A2 to substrates, products, and an inhibitor due to its lower high-spin content. The kinetic parameters of the two types of enzymes toward several substrates were quite similar. However, the oxidation rates of phenacetin by modified enzyme were ~2-fold higher than those of wild-type enzyme. We observed metabolic switching of deuterated phenacetin only in the oxidation reaction catalyzed

by wild-type CYP1A2 enzyme, but not by modified enzyme. Molecular switching, which was apparent only in the wild-type enzyme, indicates the rotation of phenacetin within the active site of the wild-type enzyme. The switching may also suggest a looser conformation in the active site of the wild-type enzyme than in the modified enzyme.

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