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Functional characterization of four allelic variants of human cytochrome P450 1A2

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Abstract

Human cytochrome P450 1A2 catalyzes important reactions in xenobiotic metabolism, including the N-hydroxylation of carcinogenic aromatic amines. In 2001, Chevalier et al. [Hum. Mutat. 17 (2001) 355] reported four new P450 1A2 sequence variants in the human population. We have now expressed these variants in *Escherichia coli* and measured protein expression (optical spectroscopy of holoenzyme and immunoblotting) and bioactivation of IQ (2-amino-3-methylimidazo[4,5-*f*]quinoline) and MeIQ (2-amino-2,4-dimethylimidazo[4,5-*f*]quinoline) in the *lacZ* reversion mutagenicity test. Enzyme kinetic analyses were performed for *N*-hydroxylation of five heterocyclic amine substrates and for O-deethylation of phenacetin. The most drastic effect was that of the R431W substitution: no holoenzyme was detectable. This residue is located in the ''meander'' peptide region and earlier site-directed mutagenesis studies demonstrated that it is critical for maintenance of protein tertiary structure. The other three variants had subtly different catalytic activities compared to the wild-type enzyme.
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P450[1] enzymes are catalysts of central importance in pharmacology and toxicology [1]. Although 57 functional P450 genes are encoded on the human genome, only a handful of P450 enzymes are expressed in liver and make significant contributions to the oxidative metabolism of xenobiotics [2,3]. Some drug metabolism reactions are strongly associated with a single P450 enzyme; in other cases, many P450s contribute to drug clearance in vivo.

Genetic polymorphisms in human P450s [2] can cause dramatic differences in the response to specific drugs; the P450 2D6 debrisoquine poor-metabolizer phenotype is a well-known example. For each of the P450 enzymes 2C9, 2C19, 2D6, and 3A4, between a dozen and almost a hundred alleles have been identified. Human P450 polymorphisms include mutations both to non-coding (promoter, intron, etc.) and protein-coding sequences. The former mutations may alter the level of expression of an otherwise wild-type enzyme; in the latter case, mutations might change the structure and catalytic activities of the enzyme protein. An example of such altered catalytic specificity is the P450 2C9 I359L polymorphism, which affects the 6-hydroxylation of *R*-warfarin, while 4-hydroxylation of diclofenac is unchanged [3,4].

The P450 1 family enzymes (1A1, 1A2, and 1B1) are important catalysts of carcinogen bioactivation reactions, such as polycyclic aromatic hydrocarbon epoxidation and aromatic/heterocyclic amine N-hydroxylation [5]. P450 1A2, the only member of the P450 1 family expressed at significant levels in human liver, shows large inter-individual variation in activity [6]. Environmental determinants such as diet [7] and smoking [8] affect P450

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[1] *Abbreviations used:* δ-ALA, δ-aminolevulinic acid; DMSO, dimethyl sulfoxide; EROD, 7-ethoxyresorufin *O*-deethylase; GluP-1, 2-amino-6-methyldipyrido[1,2-*a* :3′,2′-*d*]imidazole; IPTG, isopropyl-β-D-thiogalactopyranoside; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; MeIQ, 2-amino-2,4-dimethylimidazo[4,5-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; Nitro-IQ, 3-methyl-2-nitro-imidazo[4,5-*f*]quinoline; P450, cytochrome P450; PCR, polymerase chain reaction; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; SRS, substrate recognition sequence.

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1A2 expression. However, Rasmussen et al. [9], using caffeine metabolite analysis to evaluate P450 1A2 activity in mono- and dizygotic twins, concluded that as much as three-quarters of the inter-individual variation in P450 1A2 activity is due to genetic factors. Several polymorphisms in the P450 1A2 upstream sequence have been discovered and some of these may affect P450 1A2 protein expression [10]. The first, and, until recently, only, reported coding sequence variant of P450 1A2 is F21L [11]. This substitution occurs in the N-terminal hydrophobic region of the protein and is not expected to affect the active site of the enzyme. Is the rarity of human P450 1A2 coding sequence polymorphisms real or merely apparent? Clozapine [12] and theophylline [13] are among the few drugs which are metabolized principally via P450 1A2 activity. Consequently, polymorphisms of P450 1A2 activity may go unnoticed in clinical studies.

In a 2001 publication, four new human polymorphisms of the P450 1A2 coding sequence were described [14]. The frequencies of these variant alleles in the human population have not yet been determined. In view of the importance of P450 1A2 in the metabolism of many environmental carcinogens and several drugs, it is of great interest and the goal of our study to determine the functional significance of these sequence alterations.

Materials and methods

Chemicals

Glu-P-1, IQ, MeIQ, *N*-hydroxy-MeIQ, nitro-IQ, and PhIP were purchased from Toronto Research Chemicals (Toronto, ON). [*Ring* -^{3}H]-Phenacetin was a gift of Dr. F.F. Kadlubar (National Center for Toxicological Research, Jefferson, AR). IPTG, δ-ALA, and ampicillin were obtained from Sigma Chemical (St. Louis, MO). Other chemicals were of the highest grades commercially available.

Bacterial culture medium and molecular biology reagents

Bacto agar, bacto yeast extract, and peptone were purchased from Difco (Detroit, MI). Luria–Bertani medium (capsule formulation) was purchased from BIO 101 (Carlsbad, CA). QuikChange mutagenesis kit and *Pfu Turbo* DNA polymerase were purchased from Stratagene (La Jolla, CA). Plasmid DNA mini-prep kits were purchased from Clontech (BD Biosciences, Mississauga, ON). Oligonucleotide primers were purchased from Canadian Life Technologies (Burlington, ON).

Bacterial strains

Escherichia coli strain DH5αF′IQ and competent cells of strains DH10B (ElectroMAX) and DH5α were purchased from Invitrogen Life Technologies (Carlsbad, CA). *E. coli* strain DJ701, which bears a *lacZ* frameshift target carried on an F′ episome and a plasmid for expression of the *Salmonella typhimurium* acetyl CoA: arylamine *N*-acetyltransferase enzyme, has been described previously [15]. Mutagenicity test strain DJ702 and its isogenic strains were constructed by transforming strain DJ701 [15] with the bicistronic plasmid pCW1A2pcc [16] for expression of human P450 1A2 (GenBank Accession No. AH002667 or variants) and NADPH-P450 reductase.

Construction of P450 1A2 variants by PCR site-directed mutagenesis

Overlap-extension PCR-based site-directed mutagenesis was performed on plasmid pCW1A2pcc. Single-site mutants were generated using the pairs of forward and reverse primers shown below. Site-directed mutagenesis was conducted using the QuikChange mutagenesis kit, as per the supplier's protocol, with the following changes: DMSO concentration was 8%; cycle timing was modified as follows: initial denaturation, 95 °C, 2 min; followed by 12 cycles of: 95 °C, 1.5 min; 55 °C, 1 min; and 68 °C, 13 min. Following the PCR, reactions were treated with *Dpn*I at 37 °C for 1 h to digest methylated template DNA. *Dpn*I-treated reaction mixtures (2 μl) were electro-transformed (1.4 kV) into *E. coli* strain DH10B cells. Plasmid DNA was isolated with a miniprep kit and sequencing was performed at the Molecular Supercentre (Lab Services, University of Guelph). Each open reading frame was sequenced in its entirety and each mutation was confirmed by sequencing both strands; primers are shown below (altered bases for mutagenesis are indicated by underlining).

Primer pairs for site-directed mutagenesis

FWD348 5′ GAAGATCCAGAAGGAGCTG<u>A</u>ACACTGTG ATTGGC 3′
RVD348 5′ GCCAATCACAGTGT<u>T</u>CAGCTCCTTCTGG ATCTTC 3′
FWD386 5′ GCCCTTCACC<u>T</u>TCCCCCACAGC 3′
RVD386 5′ GCTGTGGGGGA<u>A</u>GGTGAAGGGC 3′
FWD406 5′ CCAAGAAATGCT<u>A</u>TGTCTTCG 3′
RVD406 5′ CGAAGACA<u>T</u>AGCATTTCTTGG 3′
FWD431 5′ GAGTTCCGGCCTGAG<u>T</u>GGTTCCTCACCGCC 3′
RVD431 5′ GGCGGTGAGGAACC<u>A</u>CTCAGGCCGGAACTC 3′

Sequencing primers

FWD259 5′ CTGGTGCGGCAGGGCGACGATTTCAAG 3′
RVD450 5′ CAGGTAGCAGGAGGATGAG 3′
RVD1146 5′ GTCCCTTGTTGTGCTGTG 3′
RVD1317 5′ CAGCATCATCTTCTCACTC 3′
RVD1690 5′ CCAGTAGGTTAGGAGACCCACGATGAGCG 3′

Optical spectroscopy

E. coli strain DH5α cells bearing the P450 1A2 expression constructs were grown in 25 ml Terrific broth supplemented with ampicillin (50 μg/ml), δ-ALA (0.5 mM), IPTG (0.2 mg/ml), and trace element mix [18], for 33 h at 30 °C in a water bath with vigorous shaking (280 rpm). The cells were harvested by centrifugation and the pellets were weighed and then resuspended in 6 ml sodium phosphate buffer (0.1 M, pH 7.4) containing 20% glycerol. P450 expression was determined using $Fe^{2+} \cdot CO$ versus Fe^{2+} difference spectroscopy [17].

Preparation of membrane fraction and immunoblotting

Expression of P450 1A2 wild-type and variant enzymes in *E. coli* DH5αF′IQ and preparation of *E. coli* membranes containing recombinant P450 1A2 were accomplished as described [18,19]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis was carried out on 10% (w/v) acrylamide gels which were electroblotted onto a nitrocellulose membrane [20]. Immunoblotting was performed with polyclonal antibodies raised against recombinant human P450 1A2 in rabbits, after cross-reactive antibodies were removed by incubation with *E. coli* lysate, as described [20,21].

Catalytic activity assays

P450 1A2 enzyme activity was investigated with determination of kinetic parameters (K_M and k_{cat}) for N-hydroxylation of heterocyclic amines and O-deethylation of phenacetin. The reaction mixtures consisted of P450, 0.1 μM, in *E. coli* membranes (including NADPH-P450 reductase), potassium phosphate buffer (100 mM, pH 7.4), and an NADPH-generating system (0.5 mM $NADP^+$, 10 mM glucose 6-phosphate, and 1.0 IU yeast glucose 6-phosphate dehydrogenase per ml), and substrate. The N-hydroxylation of heterocyclic amines was measured by a modification of a method developed by Kadlubar et al. [22]. In brief, the reactions (500 μl) were incubated at 37 °C for 10 min and terminated with CH_2Cl_2 (2 volumes), followed by mixing with a vortex device and separation of the layers by centrifugation (3000*g*, 5 min). An aliquot (0.8 ml) of each CH_2Cl_2 extract was taken to dryness under N_2 and mixed with Batho-solution [40 mM sodium acetate, 60 mM acetic acid, 20% water-saturated amyl acetate (v/v), 1 mM of 4,7-diphenyl-1,10-phenanthroline, and 0.4 mM $FeCl_3$; 200 μl]. After 3 min, the color reaction was terminated by addition of 10 μl of 0.02 M H_3PO_4. The value of $\epsilon_{535} = 39{,}200\ M^{-1}\,cm^{-1}$ (two equivalents of Fe^{3+} reduced per equivalent of *N*-hydroxy metabolite) for *N*-hydroxyarylamines was validated with standard *N*-hydroxy-MeIQ and used for all systems. Phenacetin O-deethylation was measured by radio-thin-layer chromatography [23]. Kinetic parameters (K_M and k_{cat}) were determined using nonlinear regression with Graph-Pad Prism software (San Diego, CA), with estimation of standard errors.

Mutagenicity assays

The standard *lacZ* reversion mutagenicity assay was performed as described [15]. Briefly, cultures were grown to $OD_{600} = 1.0$ in 2.5 ml Luria–Bertani broth + IPTG + ampicillin + trace element mix (vide supra), 30 °C, with 250 rpm shaking for about 10 h. Mutagen (solvent, DMSO) and sodium phosphate buffer (100 mM, pH 7.4) were combined in a sterile snap-cap tube and an aliquot of cell suspension (100 μl) was added. Tubes were incubated at 30 °C for 30 min, molten top agar (2 ml) was added, and the mixture was overlaid on minimal lactose plates. Plates were incubated at 37 °C for 48–72 h and colonies were counted with the aid of a video analysis system.

Results and discussion

Site-directed mutagenesis

All four P450 1A2 variants were successfully constructed by PCR site-directed mutagenesis of the bicistronic plasmid expression vector. In each case, the complete P450 1A2 open reading frame and the *tac-tac* promoter regions were sequenced, to ensure that no additional mutations had been introduced.

Recombinant protein expression

Immunoblot analysis (Fig. 1) revealed that the levels of expression of P450 1A2 variants C406Y, D348N, and I386F were lower than that of the wild-type enzyme. Variant R431W, however, was undetectable, either as a full-length protein or as proteolytic fragment bands.

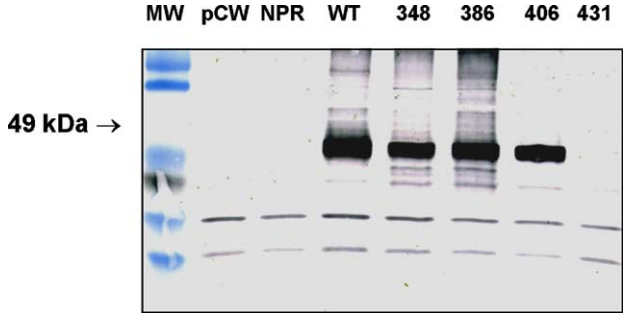

Fig. 1. Immunoblot analysis of P450 1A2 expression in *E. coli*. Protein (bacterial membrane fraction) loaded per lane, 1 μg. Lanes represent, from left: molecular weight markers; control strain (pCW vector only); control strain (reductase only); P450 1A2 wild-type; variants D348N, I386F, C406Y, and R431W.

Recombinant P450 holoenzyme can be assayed in *E. coli* whole cells by measurement of the $Fe^{2+} \cdot CO$ versus Fe^{2+} difference spectrum of the hemeprotein. Bacterial expression of the wild-type and variant P450 enzymes was examined in *E. coli* strains DH5α and DJ701. Results for strain DH5α are shown in Table 1; similar results were obtained in strain DJ701 (data not shown). The wild-type enzyme was expressed at the highest level. Expression of variant C406Y was consistently lower, although the difference was not statistically significant (Student's *t* test). Variants D348N and I386F were expressed at levels less than half of the wild-type and these differences (versus wild-type) were statistically significant ($p < 0.05$). The λ_{max} of the ferrous–CO complex was not altered for any variant. No holoenzyme spectrum was observed for variant R431W.

Enzyme assays

Enzyme kinetic assays with several P450 1A2 substrates were performed for the three variants for which expression was detectable (Fig. 2, Table 2). The source of enzyme was membrane fraction prepared from the bacterial cells. P450 content was measured by optical spectroscopy. Co-expressed NADPH-P450 reductase was assumed to be present in levels that were not rate-limiting, based upon our previous experience with the bicistronic expression system [16].

Table 1
Expression of recombinant P450 1A2 holoenzyme: *E. coli* strain DH5α; units: nmol P450 per gram wet weight cells; $n = 3$ (separate cultures on separate days)

Strain	Expression (mean ± SE)
wild-type	23.8 ± 3.9
D348N	7.2 ± 1.9
I386F	11.5 ± 1.9
C406Y	20.5 ± 0.9
R431W	<1

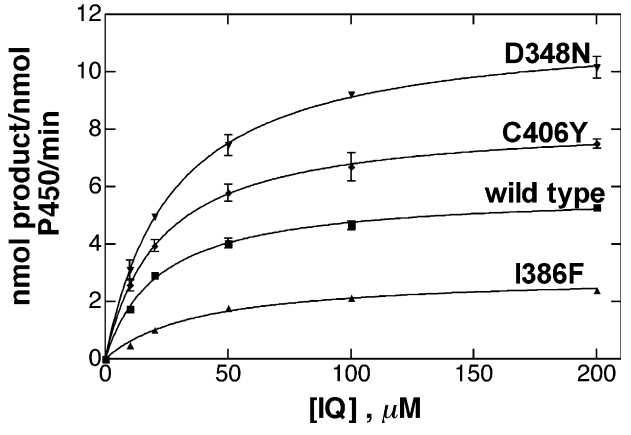

Fig. 2. Steady-state kinetics of IQ N-hydroxylation by P450 wild type and allelic variants. Each point represents mean ± SD of two triplicate assays. Calculated steady-state parameters are given in Table 2.

Table 2
Kinetic data for P450 1A2 variants

	k_{cat}	K_M	k_{cat}/K_M	Fold
MeIQ				
Wild-type	3.0 ± 0.2	26 ± 7	0.12	1
D348N	4.1 ± 0.2	17 ± 3	0.25	2.1
I386F	0.8 ± 0.1	4.5 ± 3	0.18	1.5
C406Y	4.5 ± 0.2	14 ± 2	0.31	2.6
MeIQx				
Wild-type	5.4 ± 0.4	27 ± 6	0.20	1
D348N	9.0 ± 0.4	33 ± 4	0.27	1.4
I386F	18 ± 1.7	46 ± 13	0.38	2.0
C406Y	7.6 ± 0.7	20 ± 6	0.38	1.9
IQ				
wild-type	5.8 ± 0.1	21 ± 2	0.27	1
D348N	11.6 ± 0.3	27 ± 2	0.43	1.6
I386F	2.9 ± 0.1	37 ± 4	0.08	0.3
C406Y	8.3 ± 0.3	22 ± 2	0.38	1.4
PhIP				
Wild-type	11 ± 0.9	71 ± 13	0.16	1
D348N	29 ± 2	62 ± 12	0.46	3.0
I386F	20 ± 5	341 ± 111	0.06	0.4
C406Y	26 ± 0.7	66 ± 5	0.39	2.5
Glu-P-1				
Wild-type	5 ± 0.2	4 ± 1	1.2	1
D348N	13 ± 0.3	13 ± 1	1.0	0.9
I386F	14 ± 0.3	16 ± 1	0.83	0.7
C406Y	13 ± 0.5	11 ± 2	1.2	1.0
Phenacetin				
Wild-type	1.5 ± 0.1	25 ± 6	0.058	1
D348N	0.9 ± 0.1	18 ± 5	0.051	0.9
I386F	2.6 ± 1.0	320 ± 161	0.008	0.1
C406Y	1.2 ± 0.2	62 ± 22	0.019	0.3

The units are: k_{cat}: pmol per min per pmol P450; K_M: μM. The last column gives the fold change in k_{cat}/K_M, relative to wild-type. Duplicate assays were performed. A sample with no NADPH-generation system was used as blank, in each case. The error values are estimates from the numerical curve-fitting program (Prism); see Fig. 2.

Phenacetin O-deethylation, a characteristic P450 1A2 activity, was assayed by radio-thin-layer chromatography. Values of k_{cat}/K_M were lower for all of the variants, particularly I386F. The ability of P450 1A2 to activate aromatic and heterocyclic amines by N-oxidation is a well-known toxicological role of the enzyme. N-Hydroxylation assays were performed (colorimetric assay) for several heterocyclic amines. For Glu-P-1, each of the variants had somewhat higher values of both k_{cat} and K_M, while the ratio k_{cat}/K_M was little changed. Three aminoimidazoquinoline mutagens—IQ, MeIQ, and MeIQx—were examined. With IQ, k_{cat}/K_M was slightly increased for D348N and C406Y, and decreased for I386F. All three variants showed slightly increased k_{cat}/K_M with both MeIQ and MeIQx. With the aminoimidazopyridine mutagen PhIP, k_{cat}/K_M values were lower than wild-type for I386F and higher than wild-type for D348N and C406Y.

Mutagenicity assays

We have previously established an *E. coli* strain, DJ702, in which expression of recombinant P450 1A2 plus NADPH-P450 reductase carries out bioactivation of aromatic and heterocyclic compounds to mutagenic species. Mutagenicity is assayed by reversion of a *lacZ* frameshift target carried on an F′ episome. (The strain also carries a plasmid for expression of the *S. typhimurium* acetyl CoA: arylamine *N*-acetyltransferase enzyme, which enhances sensitivity to *N*-aryl mutagens.) This mutagenicity assay provides a sensitive read-out of the functional consequences of P450 enzyme activity.

We constructed a series of strains identical to DJ702 but expressing the variant forms of P450 1A2. Negative (spontaneous) and positive-control mutagenicity data are shown in Table 3. Introduction of the plasmid encoding recombinant P450 lowered the spontaneous revertant yield, even in the case of variant R431, which is not detectably expressed (Table 3). (Suppression of spontaneous revertant yield by introduction of plasmids for expression of P450 enzymes was observed in a previous study in our laboratory [5].) Furthermore, the response to a positive control (i.e., P450 activation-independent) mutagen, nitro-IQ, was also greatly reduced. The same effects were seen in a control strain carrying only pCW vector, with no insert (data not shown). In contrast, two other plasmids, pUC18 and pTYB1 (New England Biolabs, Beverly, MA), did not alter the spontaneous or positive-control responses (data not shown). These suppressive effects of pCW appear to be specific to that plasmid, but the mechanism is unknown.

We studied the responses to two imidazoquinoline mutagens MeIQ and IQ (Fig. 3). Results of mutagenicity are shown as dose–response curves and are not normalized for the level of expression of P450 holoenzyme, since the response is not necessarily a linear function of P450 expression level. No mutagenicity is seen in the background strain devoid of P450 activity. Both mutagens are effectively activated by the wild-type enzyme. MeIQ is about 10-fold more potent than IQ, on a dose basis. Variants D348N and I386F showed reduced bioactivation of both mutagens. Variant C406Y gave a different pattern of response. MeIQ activation was higher than wild-type at low doses of MeIQ, but the dose–response curve turned downward at higher doses, presumably due to the onset of toxicity. In the case of IQ, activation paralleled the wild-type at low doses, but then diverged downward Fig. 3.

Table 3
Negative and positive control mutagenicity data for *E. coli lacZ* strains expressing P450 1A2 variants

Strain (P450 1A2 variant)	Revertants per plate			
	Spontaneous		nitro-IQ, 10 pmol	
	Mean ± SE	*n*	Mean ± SE	*n*
DJ701	98 ± 5	24	763 ± 108	12
wild-type	60 ± 4	21	121 ± 20	12
D348N	28 ± 4	18	198 ± 31	12
I386F	64 ± 5	18	286 ± 44	12
C406Y	59 ± 7	21	144 ± 20	12
R431W	23 ± 7	18	159 ± 27	12

Two mammalian P450 enzyme crystal structures have been published recently, rabbit P450 2C5 [24] and human P450 2C9 [25], but high-resolution structural information is not yet available for any member of the P450 1 family. Nevertheless, the roles of the four residues altered in the variant enzymes studied here can be analyzed on the basis of primary sequence alignments, reference to known structures of other P450 enzymes, and examination of computer-generated structural models. Each of the four residues (Table 4) is conserved among all the mammalian P450 family 1 sequences available on GenBank, with the exception of C406, which is replaced by V in P450 1B1 enzymes.

D348N

This residue is expected to occur in helix J, far from the putative active site. The model of Lozano et al. [26] places D348 on the surface of the protein. The substitution D348N replaces a weak alpha-former (Asp) by a weak helix-breaker (Asn). The level of holoenzyme expression in *E. coli* was lower than for the wild-type and the activation of mutagens was also reduced. Kinetic results were generally similar to the wild-type enzyme, although k_{cat} for PhIP was more than doubled.

I386F

This residue is located in SRS 5. In a previous study [27], we conducted random mutagenesis of short sequences within each of the six SRS regions, including the sequence 384 FTI 386 in SRS 5, screening for mutagenic activation of MeIQ. We identified three mutants at position 386, namely I386L, I386P, and I386T. Each of these variants retained catalytic activity for marker reactions. Variant I386T, the least conservative of these three substitutions, showed a marked shift in catalytic specificity (k_{cat}/K_M), with much-reduced phenacetin O-deethylation and MeIQ N-hydroxylation activities but increased EROD activity. The substitution I386F replaces a large aliphatic side-chain with an aromatic side-chain. In the present study, this resulted in small shifts in catalytic specificity, with k_{cat}/K_M increased for MeIQx but reduced for phenacetin, for example (Table 2). As discussed previously [28], substitutions at this residue might be anticipated to alter substrate specificity, in view of the proximity of the residue to the substrate binding site (near heme ring D-propionate), as suggested by comparison with the rabbit P450 2C5 structure.

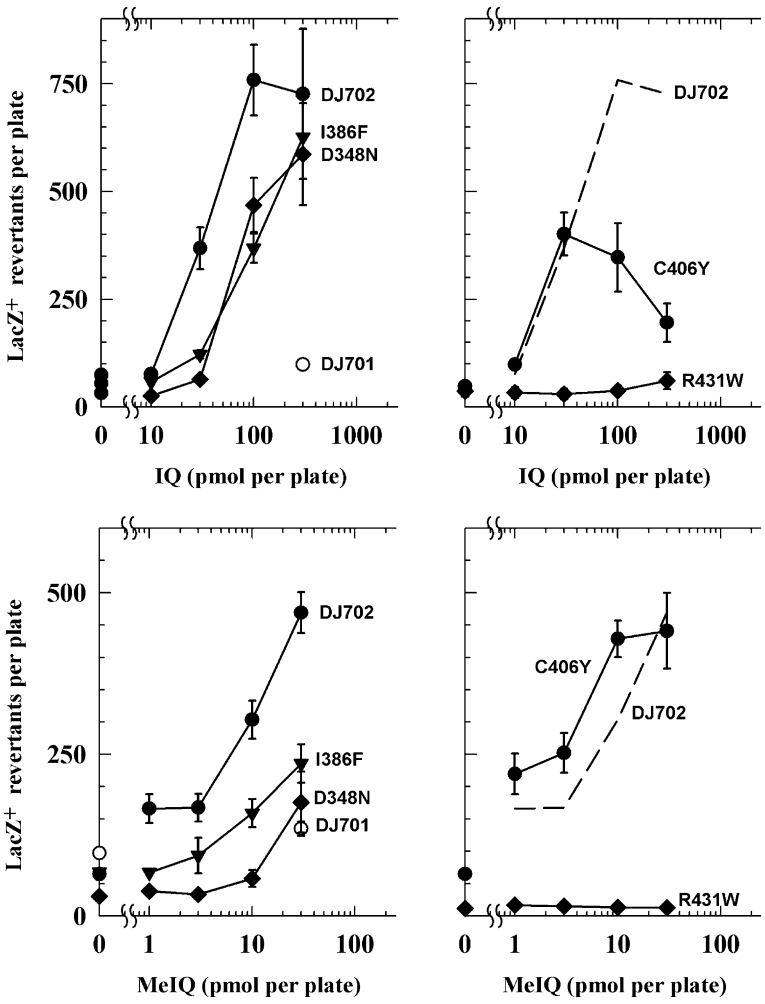

Fig. 3. Mutagenicity of IQ and MeIQ; *E. coli lacZ* assay. Data represent means ± SE of plate counts from at least three triplicate experiments performed on separate days. For each compound, data sets are split between left and right panels, for clarity. Control strain DJ701 (no P450 expression): open circles with no connecting line; data shown for zero dose and highest dose only. Strain DJ702 (expressing recombinant wild-type P450 1A2): filled circles; (on the right panel, the same data are shown, for comparison purposes, as a dashed line with no data points). P450 1A2 variants: I386F, left panels, triangles; D348N, left panels, diamonds; C406Y: right panels, filled circles; and R431W: right panels, diamonds.

C406Y

Cys406 is expected to be located at the beginning of the third strand of the four-strand β1 sheet. The variant replaces Cys, a weak beta-forming residue, with Tyr, a strong beta-forming residue, so the substitution might be predicted to be compatible with retention of the overall β1 sheet structure. The effects of the substitution on protein expression, enzyme activities, and mutagen activation were not great, although the catalytic specificity (k_{cat}/K_M) was increased about 2-fold for IQ, MeIQ, MeIQx, and PhIP. An unusual dose–response was obtained in the mutagenicity assays with MeIQ and IQ, showing an early onset of toxicity. The basis of this effect is unknown. However, it should be noted that N-hydroxylation (the presumed pathway of mutagenic activation) is not the only possible outcome of P450 1A2-catalyzed heterocyclic amine metabolism. For example, MeIQx is also converted to a detoxication product by P450 1A2-catalyzed oxidation of the 8-methyl group to a carboxylic acid [29]. The altered shape of the dose–responses obtained with this variant could be due to an altered distribution of metabolites, although we have not yet examined this possibility in our experiments.

R431W

Variant R431W did not yield detectable P450 holoenzyme. Arg431 and its neighboring residues (PERF) are conserved in all P450 family 1 sequences; Arg is replaced by another basic residue, His, in P450 2 enzymes (PGHF). In one of the first site-directed mutagenesis studies of P450 1A2, Furuya et al. [30] constructed the mutation R429L in rat P450 1A2; rat P450 1A2 residue 429 corresponds to human P450 1A2

Table 4
Positions of the P450 1A2 variants: comparisons with other P450 enzyme sequences

Variant	Sequence context	Corresponding sequences:		
		P450BM3	Human 2C9	Structural context
D348N	KELDTV	EEAARV	EEIERV	Helix J
I386F	FTIPH	-AFSL	TSLPH	Strand 4 of β 1
C406Y	KKCCVF	KGDELM	KGTTIL	Strand 3 of β 1
R431W	FRPERF	FRPERF	FDPHHF	Meander

Table 5
Partial sequences of the K helix and meander regions of selected P450 enzymes

K helix	
P450 BM3	310 QLKYVGMVLNEALRL
human P450 1A2	364 QLPYLEAFILETFRH
rat P450 1A2	362 QLPYLEAFILEIYRY
human P450 2C9	344 HMPYTDAVVHEVQRY
Meander	
P450 BM3	369 DDVEEFRPERFE
human P450 1A2	422 EDPSEFRPERFL
rat P450 1A2	420 KDPFVFRPERFL
human P450 2C9	402 PNPEMFDPHHFL

The putative salt-bridged acid–base pair (see text) is underlined.

residue R431 (alignments of relevant sequences are shown in Table 5). The mutated protein R429L, expressed in yeast cells, did not bind heme to form holoenzyme. Recently, Zheng et al. [31], in a study of human P450 4B1, constructed the corresponding mutations R424H and R424L; the former (conservative) substitution maintained holoenzyme formation and catalytic activity, but the latter (non-conservative) substitution gave no detectable holoenzyme expression (baculovirus/insect cell expression system). In summary of the site-directed mutagenesis results, it appears that mutations which eliminate the positive charge of residue 431 (or the corresponding residue in other P450 enzymes) are highly destabilizing; the resulting proteins do not bind heme and are inactive.

Arg431 is the last residue of the highly conserved P450 structural element known as the "meander" [31] [32]. The meander is a loop, about ten residues long, which lies on the surface of the P450 enzyme and covers the K helix. Multiple interactions bind the residues of the meander and the K helix, and disruption of these interactions is highly deleterious. For example, in studies of human aromatase (P450 19), mutations of K-helix residue Arg365 (even to another basic residue, R365K) yielded inactive protein [33]. The corresponding residue in P450 2C9 [25], Arg357, forms multiple H-bonds to meander region residues, including the carbonyl O atoms of Met406 and Pro404.

Examination of the crystal structure of bacterial P450 BM3 is instructive, since the meander region sequence EFRPERF occurs identically in P450 BM3 and human P450 1A2 (Tables 4 and 5 and Fig. 4). A salt bridge is formed between the charged residues R378 (meander) and E320 (K helix) in P450 BM3. One expects that a salt bridge also exists between the corresponding residues of human P450 1A2 (R431 and E374). In the case of human P450 2C9, the corresponding residues are H411 and

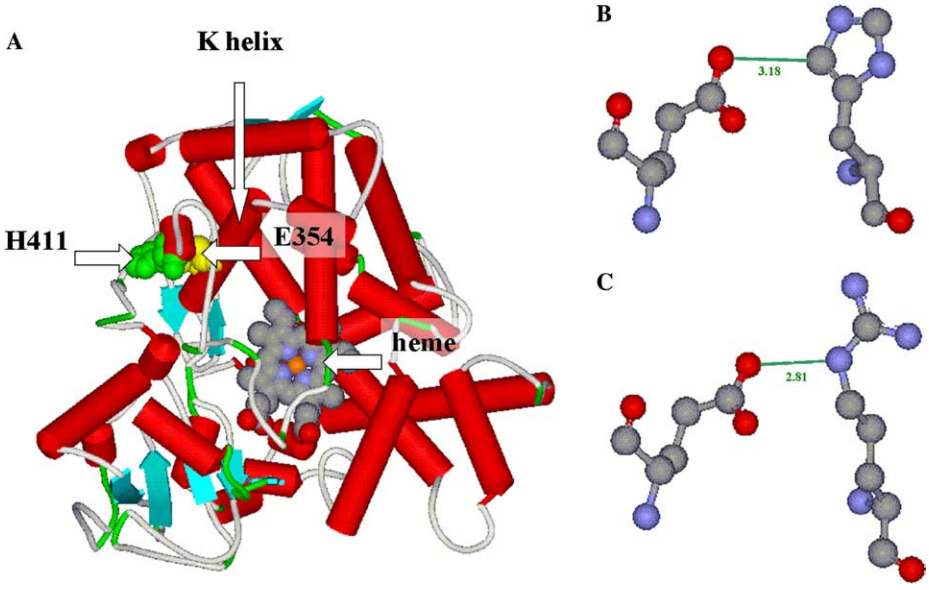

Fig. 4. (A) P450 2C9 (PDB coordinate file 1OG5.pdb rendered with DS Viewer Pro, Accelrys Software). The polypeptide is shown as red tubes (α helices) and cyan ribbons (β strands); the heme group is shown as a space-filling model; and residues E354 and H411 are shown as yellow and green space-filling models, respectively. (B) Residues E354 and H411 of P450 2C9 are shown as ball-and-stick models. (C) Residues E320 and R378 of P450 BM3 (PDB coordinate file 1BU7.pdb) are shown as ball-and-stick models.

E354. Careful examination of the recently published structure of P450 2C9 (and also rabbit P450 2C5) shows an anomaly here: the imidazole ring of H411 presents a carbon atom, rather than a nitrogen atom, to a carboxylate oxygen atom of E354. We believe that this represents an error in the interpretation of the electron-density map. The imidazole ring of H411 should be flipped about the beta carbon, with a bridge between the imidazole N and carboxylate O atoms. The consistent interpretation of the data in our study, the previous site-directed mutagenesis studies, and the X-ray structural studies is that a salt bridge between residues R431 and E374 (human P450 1A2), or between the corresponding residues in other P450s, is essential for stabilizing the K helix and, consequently, the entire heme-binding alpha-helical framework of the P450 protein. Replacement of the arginine by a neutral hydrophobic leucine residue is incompatible with the tertiary structure in rat P450 1A2, and the bulky neutral hydrophobic tryptophan in human P450 1A2 variant R431W would likely be even more disruptive.

In summary, we have expressed four allelic variants of human P450 1A2 and measured some of their catalytic activities. One of these variants, R431W, was enzymatically inactive in the expression system we used and the remaining variants showed changes in catalytic activity towards heterocyclic amines and substrate phenacetin. Since P450 1A2 is an important enzyme for the bioactivation of carcinogenic aromatic and heterocyclic amines, the prevalence of these polymorphisms and their possible effects on sensitivity to drugs and xenobiotics should be examined in future research.

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