Selection of Human Cytochrome P450 1A2 Mutants with Enhanced Catalytic Activity for Heterocyclic Amine N-Hydroxylation†

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ABSTRACT: Cytochrome P450 (P450) 1A2 is the major enzyme involved in the metabolism of 2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline (MeIQ) and other heterocyclic arylamines and their bioactivation to mutagens. Random mutant libraries of human P450 1A2, in which mutations were made throughout the entire open reading frame, were screened with *Escherichia coli* DJ3109pNM12, a strain designed to bioactivate MeIQ and detect mutagenicity of the products. Mutant clones with enhanced activity were confirmed using quantitative measurement of MeIQ N-hydroxylation. Three consecutive rounds of random mutagenesis and screening were performed and yielded a highly improved P450 1A2 mutant, SF513 (E225N/Q258H/G437D), with >10-fold increased MeIQ activation based on the *E. coli* genotoxicity assay and 12-fold enhanced catalytic efficiency (k_{cat}/K_m) in steady-state N-hydroxylation assays done with isolated membrane fractions. SF513 displayed selectively enhanced activity for MeIQ compared to other heterocyclic arylamines. The enhanced catalytic activity was not attributed to changes in any of several individual steps examined, including substrate binding, total NADPH oxidation, or H_2O_2 formation. Homology modeling based on an X-ray structure of rabbit P450 2C5 suggested that the E225N and Q258H mutations are located in the F-helix and G-helix, respectively, and that the G437D mutation is in the "meander" region, apparently rather distant from the substrate. In summary, the approach generated a mutant enzyme with selectively elevated activity for a single substrate, even to the extent of a difference of a single methyl group, and several mutations had interacting roles in the development of the selected mutant protein.

Microsomal P450[1] enzymes are involved in the oxidation of the majority of drugs, pollutants, and carcinogens and also many endogenous compounds including fatty acids, steroids, fat-soluble vitamins, and eicosanoids (*3*−*6*). The P450s with well-defined roles in physiological processes have limited selectivity, while the P450s that oxidize xenobiotics generally have rather broad catalytic specificity. Understanding and, if possible, predicting catalytic selectivity of these P450s is of considerable importance with practical applications in areas such as drug development.

Two main limitations have hindered analysis of structure−function relationships in P450s. One is that few three-dimensional structures of mammalian P450s are currently available (*7*−*10*). The other limitation is that the P450 catalytic mechanism is multistep and complex, and several individual steps may be subject to influence by different amino acids (*11*, *12*). To resolve the problem of structure−function relationships in mammalian P450 enzymes, many site-directed mutagenesis approaches have been applied. Early work on the mouse 2A and rabbit 2C subfamily P450s using chimeras identifed locations of residues that exert major influences on catalytic activity (*13*−*16*). In some cases, quite dramatic changes in catalytic selectivity could be achieved by changes at a single site (*13*, *14*). Since that time, more site-directed mutagenesis studies have been done on a variety of mammalian P450s in a number of laboratories, including our own (*17*−*24*). In almost all cases, the observed end point has been attenuated catalytic activity, with a gain of an alternate activity observed in some cases (*25*). Diminished catalytic activity is often considered to be an end point in site-directed mutagenesis work, but there is potential for misinterpretation of the basis for a loss of activity (*24*).

In recent years random mutagenesis and subsequent screening, so-called "directed evolution" or "molecular breeding", has become a widely used tool in protein engineering, which is now also increasingly applied to investigate the structure−function relationships of proteins (*26*, *27*). Several efforts to generate P450s with desired catalytic functions by random mutagenesis have been reported (*28*−*32*). The inherently low catalytic activities of many mammalian P450s and the flexibility of the active site provide considerable opportunities for improvement. Some studies in this area are oriented toward practical end points, such as production of fine chemicals (*33*). The catalytic mechanism and selectivity of P450s may be better understood through analysis of mutated P450s with both decreased and increased activity.

P450 1A2 is the major enzyme involved in the bioactivation of HAAs (*34*, *35*). HAAs are potent bacterial mutagens and potential human carcinogens that are formed

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[1] Abbreviations: P450, cytochrome P450 (*1*); ORF, open reading frame; SRS, substrate recognition site (*2*); IPTG, isopropyl β-D-thiogalactopyranoside; ML, minimal lactose; HAA, heterocyclic aromatic amine; MeIQ, 2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; PhIP, 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine; Glu-P-1, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]-imidazole.

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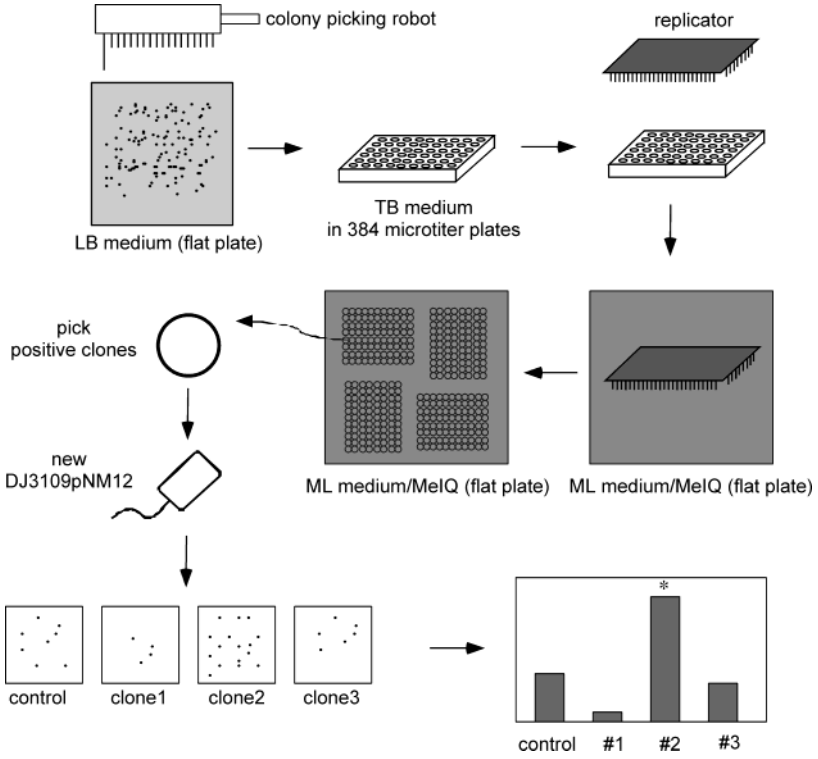

FIGURE 1: Scheme for screening P450 1A2 random mutant libraries. P450 1A2 random mutant libraries were transformed into *E. coli* strain DJ3109pNM12 and plated onto large bioassay LB/ampicillin flat plates (25 × 25 cm). Colonies (~1.2×10^4) were picked with a Qpix colony-picking robot. After plasmid DNA from putative positive clones was recovered, the phenotypes of selected clones were verified (following transformation of a new *E. coli* strain DJ3109pNM12) on the basis of the number of revertants in ML medium containing MeIQ.

in burned foods as a result of pyrolysis during cooking (*36*). The initial metabolic activation step is mediated primarily by P450 1A2 in all animal species examined to form *N*-hydroxy products which may react with DNA, either before or after acetylation (*37*). Human P450 1A2 is expressed almost exclusively in the liver (*5*). To date, 13 allelic variants have been identified, 5 of which are located in the coding region (*38*−*40*).[2] The catalytic activities of these variants had not been reported, and our own recent studies show relatively small effects toward several substrates (*42*). To our knowledge, no other systematic site-directed mutagenesis studies of human P450 1A2 have been reported. Systematic random mutagenesis of SRS regions of this enzyme in this laboratory has led to the identification of some residues that attenuate and enhance some catalytic activities. The effect appears to be at steps involved in the substrate oxygenation step, as opposed to earlier points (*31*, *43*).

We generated more human P450 1A2 mutants using random mutagenesis of the entire ORF and an expanded screening method. The work yielded a mutant with three mutations that has catalytic activity selectively enhanced toward the particular HAA (MeIQ) used in the screening. The mutations appear to act synergistically, and one is in a region considered to be remote from the substrate binding site.

EXPERIMENTAL PROCEDURES

Chemicals. MeIQ, *N*-hydroxy-MeIQ, MeIQx, IQ, PhIP, and Glu-P-1 were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Other chemicals were of the highest grade commercially available.

P450 1A2 Mutant Library Construction. A "bicistronic" plasmid (pCW1A2bc) expressing both human P450 1A2 and NADPH−P450 reductase has been described (*31*). The ORF region of the P450 1A2 gene (1.5 kb) was mutated using a low-fidelity polymerase PCR method and amplified in a 50 μL PCR reaction mixture including 50 ng of pCW1A2bc plasmid, 20 ng of forward primer (5′-GGAGGTCATATG-GCTCTGTTA-3′) and reverse primer (5′-AATTATTTCTA-GACCGGAAGC-3′), 2.5 units of Mutazyme DNA polymerase (Stratagene, La Jolla, CA), 0.2 mM each dNTP, and Stratagene 10× Mutazyme reaction buffer. The 1.5 kb amplified PCR library fragment was purified by agarose gel electrophoresis and cloned into the pCW1A2bc vector using the *Nde*I and *Xba*I restriction sites. The ligation mixture was transformed into *Escherichia coli* DH10b ultracompetent cells (Invitrogen, Carlsbad, CA), and then the library plamid DNA was purified using a Promega Wizard miniprep kit (Promega, Madison, WI).

Phenotypic Selection Based on Genotoxicity. The general concept has already been published (*31*). A bicistronic library DNA was transformed into *E. coli* DJ3109pNM12 by electrophoresis and then plated on LB ampicillin (100 μg mL^{-1})/chloramphenicol (34 μg mL^{-1}) Q tray plates (25 × 25 cm; Genetix, Hampshire, U.K.). Individual colonies were picked and transferred to single wells of 386-well plates filled with 50 μL of TB expression medium with the use of a Genetix colony-picking robot system (Genetix) (Figure 1). For each generation of library screening, ~1.2×10^4 colonies were picked. The 386-well plates were then incubated at 30 °C for 24 h to induce enzyme expression. For mutagenicity testing, all of the clones in the wells were spotted onto flat

[2] During the review of this paper, another six were reported, along with some activity measurements (*41*).

Q tray plates (25 × 25 cm) containing ML selective medium in the bottom agar and MeIQ (500 pmol per plate) in 30 mL of the top agar. The two DJ3109pNM12 strains containing the wild-type P450 1A2 clone and the library template clone were spotted as positive controls. The 386-well plates, including the library cultures, were kept as master stocks. After 40 h of incubation at 37 °C, discrete colonies of revertants could be observed for positive controls and some of the mutants. The clones that appeared to show higher reversion levels than control were identified, recovered from the master 386-well plates, and grown in 2 mL of LB ampicillin (100 μg mL^{-1}). Plasmid DNA was prepared from the cultures (Promega Wizard plasmid miniprep kit, vide supra).

Phenotypic Verification of Selected Clones. The recovered DNA for the selected clones was retransformed into fresh *E. coli* DJ3109pNM12 by a heat-shock method (*44*). The transformed clone (in DJ3109pNM12) strains were inoculated into 1 mL of TB expression medium in 24-well plates and grown at 30 °C for 24 h. A 500 μL aliquot of each individual culture was applied onto minimal lactose agar medium in 36-square flat Integrid plates (8 × 8 cm; Fisher Scientific, Pittsburgh, PA) using 5 mL of top agar, which included 100 pmol of MeIQ. After 40 h of incubation at 37 °C, the number of revertant colonies was counted (Figure 1).

Nucleotide Sequencing. Sequencing of the plasmid DNA in the clones that displayed increased numbers of revertant colonies (following verification) was performed in the Vanderbilt University DNA Sequencing Facility on a Model 3700 fluorescence sequencing unit using a Taq dye terminator kit (PE Applied Biosystems, Foster City, CA). The mutated sequences were verified by comparing the sequences of the antisense strand (entire ORF).

Preparation of Membrane Fractions. Bacterial inner membranes containing mutant P450 1A2 and NADPH−P450 reductase were isolated from 1 L TB/ampicillin (100 μg mL^{-1}) expression cultures of *E. coli* DH5αF′IQ (*45*). The 10^5g pellet was resuspended in a final volume of 10 mL of 200 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v) and homogenized with a Dounce homogenizer. The homogenized membrane fraction was stored on ice until analysis.

Enzymatic Assay for N-Hydroxylation of Heterocyclic Amines. The reaction mixtures consisted of 0.10 μM P450 in bicistronic membranes (including NADPH−P450 reductase at a similar concentration), 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 of glucose-6-phosphate dehydrogenase mL^{-1}), and varying concentrations of substrate in a total volume of 0.50 mL. The N-hydroxylation of heterocyclic amines was measured by a modification of a method developed by Kadlubar et al. (*46*). This method is quite sensitive and was used routinely because of the large numbers of assays required and the inherent instability of aryl *N*-hydroxylamines. In brief, the reactions (500 μL) were performed at 37 °C for 10 min and terminated with 2 volumes of CH_2Cl_2. The CH_2Cl_2 extract was reduced to dryness under N_2 and dissolved in 200 μL of Batho solution [40 mM sodium acetate, 60 mM acetic acid, 20% water-saturated amyl acetate (v/v), 1 mM 4,7-diphenyl-1,-10-phenanthroline, and 0.4 mM $FeCl_3$]. After 3 min, the color reaction was terminated by addition of 10 μL of 20 mM H_3PO_4. The extinction coefficient $\epsilon_{535} = 39200$ M^{-1} cm^{-1} (2 equivalents of Fe^{3+} reduced per equivalent of *N*-hydroxylamine) for *N*-hydroxyarylamines was validated with standard *N*-hydroxy-MeIQ and used for all systems. Kinetic parameters (K_m and k_{cat}) were estimated using nonlinear regression analysis with Graph-Pad Prism software (San Diego, CA).

Purification of Recombinant Wild-Type P450 1A2 and Mutants. Purification of P450 1A2 enzymes was as previously described (*43*). Briefly, membranes were solubilized at 4 °C in 100 mM phosphate buffer (pH 7.4) containing 20% glycerol (v/v), 30 μM α-naphthoflavone, 10 mM β-mercaptoethanol, and 1.5% CHAPS (w/v). The solubilized protein [C-terminal $(His)_5$ tag] was purified using a Ni^{2+} nitrilotriacetate column (Qiagen, Valencia, CA) and eluted with imidazole (*43*).

Estimation of Substrate Binding Affinity. Fluorescence quenching titrations were performed to determine the dissociation constants (K_d) as described previously (*43*).[3] P450 1A2 intrinsic fluorescence (tryptophan) was determined by exciting at 295 nm and detecting emission at 340 nm. The decrease of the fluorescence emission intensity at 340 nm was measured as a function of MeIQ concentration.

NADPH Oxidation and H_2O_2 Formation. Purified P450 1A2 enzymes were reconstituted with NADPH−P450 reductase for some steady-state kinetic experiments (*49*). Reconstituted enzyme was preincubated for 5 min at 37 °C in the presence or absence of MeIQ (100 μM). Reactions were initiated with the addition of 15 μL of 10 mM NADPH, and the decrease in A_{340} was monitored using a Cary14/OLIS spectrophotometer (On-Line Instrument Systems, Bogart, GA).

H_2O_2 formation was measured as described elsewhere (*43*). Reactions (500 μL) were initiated by adding the NADPH-generating system and terminated by adding 1 mL of cold Cl_3CCO_2H (3%, w/v) after 60 s. H_2O_2 was determined spectrophotometrically by reaction with ferroammonium sulfate and KSCN as described (*50*).

Construction of Site-Directed Mutants. Site-directed mutagenesis was conducted using the QuickChange mutagenesis kit and the supplier's protocol (Stratagene). Mutations for constructed site-directed mutant plasmids were verified by sequencing both strands of DNA (vide supra).

Homology Modeling. A molecular model of human P450 1A2 was constructed using the rabbit P450 2C5 crystal structure (*7*; PDB entry 1DT6) as a template. The P450 1A2 sequence was aligned with that of P450 2C5 using the SIM program (*51*), and the coordinates of the model were obtained using the software ProModII from the Swiss Institute of Bioinformatics (http://swissmodel.expasy.org). Energy minimization was performed using the GROMOS96 program (BIOMOS, Zurich, Switzerland). The P450 1A2 model and the location of mutation were built using PyMOL software (DeLano Scientific, South San Francisco, CA).

RESULTS

Library Screening and Evolutionary Process. The ORF of human P450 1A2 was mutagenized using error-prone PCR, and screening was done for clones with enhanced

[3] Human P450 1A2 is isolated in the high-spin state (*43*, *47*), and the usual method of estimating substrate binding affinity by Soret spin state change (*48*) could not be applied (*43*).

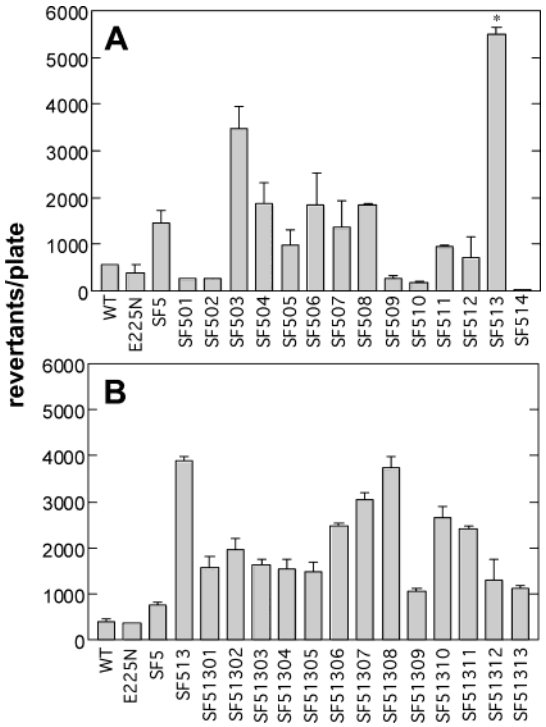

FIGURE 2: Comparison of numbers of MeIQ-induced revertants for wild-type P450 1A2 and selected mutants. (A) Third generation screening. Fourteen clones were selected, and the number of revertants in ML medium (containing 100 pmol of MeIQ per plate) is shown. (B) Fourth generation library screening. Thirteen clones were selected, and the number of revertants in ML medium containing 50 pmol of MeIQ per plate is shown. WT = wild type. Results are presented as means ± SD of triplicate assays.

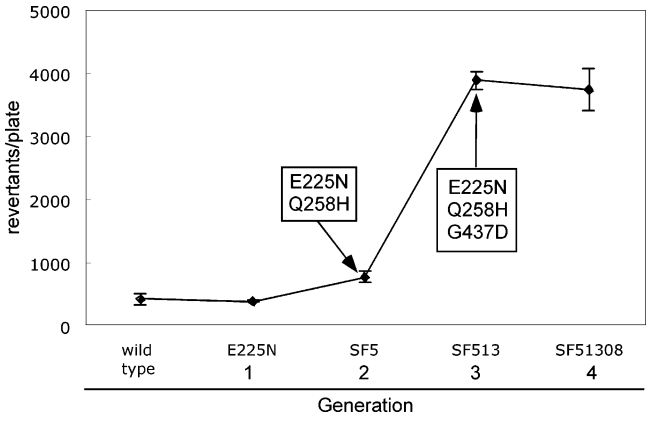

FIGURE 3: Evolutionary process of P450 1A2 mutants. The wild-type and mutant clones were assayed in ML medium containing 50 pmol of MeIQ per plate. Results are presented as means ± SD of triplicate assays.

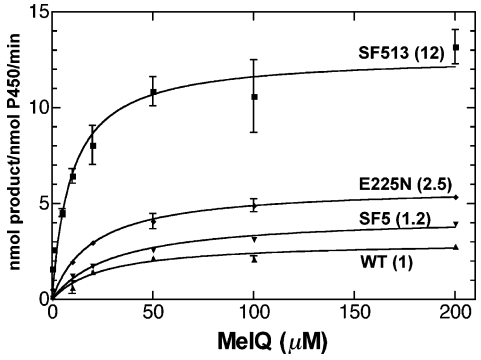

FIGURE 4: Steady-state kinetics of MeIQ N-hydroxylation by P450 mutants. Each point presented is a mean ± SD of triplicate assays. The parameters were estimated for wild-type 1A2 and the mutants (k_{cat} expressed as nmol of *N*-hydroxy-MeIQ formed min^{-1} $nmol^{-1}$ of P450 and K_m in μM): wild type, k_{cat} 3.0 ± 0.2, K_m 26 ± 7, k_{cat}/K_m 0.12 ± 0.03; E225N, k_{cat} 5.9 ± 0.3, K_m 20 ± 3, k_{cat}/K_m 0.30 ± 0.05; SF5 (E225N/Q258H), k_{cat} 4.3 ± 0.2, K_m 30 ± 4, k_{cat}/K_m 0.14 ± 0.02; SF513 (E225N/Q258H/G437D), k_{cat} 13.5 ± 0.8, K_m 10 ± 2, k_{cat}/K_m 1.35 ± 0.28. Numbers in parentheses indicate the fold increase of the catalytic efficiency (k_{cat}/K_m) compared to wild type.

ability to activate MeIQ (Figure 1). The error-prone PCR conditions were optimized to incorporate one- or two-residue changes in the P450 1A2 ORF for each generation library. More than 1.2×10^4 clones were screened in each generation of a library, and the putative positive clones were selected and verified by counting the revertants resulting from bioactivation of MeIQ to genotoxic products. In the third generation of screening, clone SF513 showed highly enhanced activity compared with wild-type P450 1A2 (Figure 2A). Mutant SF513 also displayed concentration-dependent MeIQ activation (data not shown). In further library generations, clones with greater activity than mutant SF513 were not found (Figure 2B). Sequence analysis showed that mutant SF5, the clone selected in the screening of the second generation library, had the mutations E225N and Q258H and that mutant SF513 contained the additional mutation G437D. Thus, the evolutionary process for MeIQ bioactivation activity of P450 1A2 mutants was observed through the consecutive library screening process (Figure 3).

Kinetic Parameters of Wild-Type P450 1A2 and Mutants. Rates of MeIQ N-hydroxylation by P450 1A2 and the mutants selected from each round of screening were measured. Steady-state kinetic analysis of mutant SF513 showed a 12-fold increase in catalytic efficiency (k_{cat}/K_m) (Figure 4). Rates were also compared for other HAAs (Table 1). Mutant SF513 showed selectively enhanced activity for MeIQ, which had been used in the screening, compared to various other HAAs.

Purification of Wild-Type P450 1A2 and Mutants. To analyze some possible differences for the reaction activity between wild-type P450 1A2 and the SF513 mutant, the enzymes were expressed in *E. coli* and purified using Ni^{2+} nitrilotriacetate affinity chromatography. All were apparently homogeneous as judged by sodium dodecyl sulfate−polyacrylamide gel electrophoresis and were devoid of cytochrome P420. The final specific content ranged from 6 to 16 nmol of P450/mg of protein (Table 2). The mutant enzymes all contained more apoprotein than the wild-type enzyme.

Binding Affinities, NADPH Oxidation, and H_2O_2 Formation. Binding affinities of wild-type P450 1A2 and mutants for MeIQ were determined using the change in intrinsic tryptophan fluorescence intensity of enzymes upon binding of substrate (*43*). None of the mutants exhibited significantly altered binding affinities for MeIQ (Table 3), suggesting that enhanced ground-state substrate binding is not the basis for the enhanced catalytic activity. As previously reported for phenacetin O-deethylation (*43*), wild-type P450 1A2 and these mutants also showed low coupling efficiency between NADPH oxidation and N-hydroxylation of MeIQ. Mutants did not affect the rate of H_2O_2 formation very much in the absence or presence of substrate (SF513 showed a small

Table 1: Kinetic Parameters of N-Hydroxylation of HAAs by P450 Mutants[a]

	MeIQ N-hydroxylation			MeIQx N-hydroxylation			IQ N-hydroxylation		
P450	k_{cat}	K_m	k_{cat}/K_m (rel increase)	k_{cat}	K_m	k_{cat}/K_m (rel increase)	k_{cat}	K_m	k_{cat}/K_m (rel increase)
wild type	3.0 ± 0.2	26 ± 7	0.12 ± 0.03 (1)	5.4 ± 0.4	27 ± 6	0.20 ± 0.05 (1)	5.8 ± 0.1	21 ± 2	0.27 ± 0.03 (1)
E225N	5.9 ± 0.3	20 ± 3	0.30 ± 0.05 (2.5)	14 ± 1	33 ± 4	0.41 ± 0.06 (2.1)	16 ± 1	24 ± 3	0.68 ± 0.10 (2.5)
SF5	4.3 ± 0.2	30 ± 4	0.14 ± 0.02 (1.2)	8.1 ± 0.4	30 ± 5	0.27 ± 0.05 (1.3)	7.3 ± 0.5	31 ± 6	0.24 ± 0.05 (0.9)
SF513	13.5 ± 0.8	10 ± 2	1.35 ± 0.28 (12)	9.9 ± 0.7	28 ± 6	0.35 ± 0.08 (1.8)	17.2 ± 0.5	34 ± 3	0.51 ± 0.05 (1.9)

	PhIP N-hydroxylation			Glu-P-1 N-hydroxylation		
P450	k_{cat}	K_m	k_{cat}/K_m (rel increase)	k_{cat}	K_m	k_{cat}/K_m (rel increase)
wild type	11.0 ± 0.9	71 ± 13	0.16 ± 0.03 (1)	5.0 ± 0.2	4 ± 1	1.2 ± 0.3 (1)
E225N	39 ± 4	68 ± 17	0.58 ± 0.16 (3.7)	20 ± 1	17 ± 1	1.1 ± 0.1 (1.0)
SF5	21 ± 1	67 ± 10	0.31 ± 0.05 (2.0)	9.3 ± 0.5	17 ± 3	0.54 ± 0.10 (0.5)
SF513	37 ± 2	82 ± 11	0.44 ± 0.06 (2.9)	20 ± 1	20 ± 2	1.2 ± 0.1 (0.9)

[a] SF5 = E225N/Q258H, SF513 = E225N/Q258H/G437D. Results were obtained using membrane preparations.

Table 2: Specific P450 Content of Purified P450 1A2 Mutants[a]

P450	expression level (nmol/1 L culture)	specific content (nmol of P450/ mg of protein)	% P450 holoenzyme[b]
wild type	600	16.1	91
E225N	310	8.0	45
SF5	450	7.7	44
SF513	100	6.5	37

[a] All purified enzymes were apparently homogeneous as judged by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (data not presented). [b] Percent P450 containing heme based on a calculated value (from M_r) of 17.7 nmol/mg of protein. None of the purified enzymes contained detectable P420 (i.e., <5% of P450).

increase compared to wild type). The coupling efficiency for the SF513 mutant was ~5-fold better than for the wild-type enzyme, but the enhanced catalytic activity cannot be attributed solely to this change.

Kinetic Parameters of Site-Directed Mutants. Four site-directed mutants related to the SF513 mutant were constructed, corresponding to individual amino acids changed in the mutants but not detected in the absence of other substitutions. Three other changes were made at Gly437. No major increase or decrease of MeIQ N-hydroxylation activity was observed, although most mutants showed some slightly increased activities (Table 4). Relative catalytic efficiencies of the site-directed mutants ranged from 2- to 4-fold (compared to wild type) for N-hydroxylation of MeIQ and from 1- to 2-fold for N-hydroxylation of IQ. Mutants containing the G437D mutation appeared to have decreased K_m values for N-hydroxylation of MeIQ. This result may suggest that the G437D mutation influences the K_m parameter, though the meaning of K_m is not clear with P450 1A2 (*43*). Mutations in SF513 may collaborate globally to specifically increase the N-hydroxylation activity with MeIQ.

Homology Modeling with P450 2C5. The sequences of P450 1A2 and P450 2C5 were aligned as shown in Figure 5A. The sequence identity between P450 1A2 and P450 2C5 is 31%. On the basis of this sequence alignment, a homology model was constructed, and the mutated residues were positioned (Figure 5B). E225N and Q258H mutations are located in the F-helix and G-helix, respectively. G437D mutation is located in the "meander" region. Although the construction of the model of P450 1A2 from the P450 2C5 template has limitations, it suggests that the structural elements outside of what appears to be the formal active site may play a role in changing the enzymatic activity.

DISCUSSION

In the absence of crystal structures, the coupling of random mutagenesis/screening with kinetic characterization of respective mutants provides a potentially useful approach to understanding structure−function relationships of mammalian P450s. An advantage of random mutagenesis is that a large number of modified potential proteins can be selected on the basis of altered function, without prior bias in hypotheses. However, screening of mutants has typically been limited to a relatively small number in most studies of P450s. Our laboratory, in collaboration with Josephy, developed a bacterial tester strain (*E. coli* DJ3109pNM12)

Table 3: Substrate Binding Affinities, NADPH Oxidation, and H_2O_2 Formation with P450 1A2 Mutants

			nmol of product min^{-1} (nmol of P450)$^{-1}$				
P450 1A2[a]	K_d[a] (μM)	MeIQ (100 μM)	NADPH oxidation	MeIQ hydroxylation	H_2O_2 formation	H_2O formation[b]	coupling efficiency (%)
wild type	67	+	53 ± 6	2.1 ± 0.2	17 ± 1	17	4.1
		−	43 ± 2		11 ± 4	16	
E225N	75	+	33 ± 6	4.9 ± 0.5	14 ± 1	7	15
		−	39 ± 2		14 ± 2	13	
SF5	76	+	42 ± 6	3.1 ± 0.1	17 ± 1	11	7.5
		−	41 ± 1		16 ± 1	12	
SF513	65	+	56 ± 2	10.6 ± 2.7	27 ± 1	9	19
		−	45 ± 1		24 ± 2	10	

[a] Assays were done with purified P450s and NADPH−P450 reductase. [b] K_d values were determined by measuring changes in intrinsic enzyme fluorescence intensity upon binding of MeIQ. [c] H_2O formation was determined by calculating the difference between total NADPH utilized and the sum of H_2O_2 formation and products produced and then dividing by 2 (*52*).

Table 4: Kinetic Parameters of MeIQ and IQ N-Hydroxylation for Site-Directed Mutants

site-directed P450 mutants	MeIQ N-hydroxylation			IQ N-hydroxylation		
	k_{cat}	K_m	k_{cat}/K_m (fold increase)[a]	k_{cat}	K_m	k_{cat}/K_m (fold increase)[a]
Q258H	7.0 ± 0.3	27 ± 4	0.26 ± 0.04 (2.2)	14.2 ± 0.5	23 ± 3	0.61 ± 0.08 (2.3)
G437D	6.7 ± 0.2	19 ± 2	0.35 ± 0.04 (3.0)	13.7 ± 0.3	30 ± 2	0.47 ± 0.03 (1.7)
E225N/G437D	6.0 ± 0.2	18 ± 2	0.34 ± 0.04 (2.9)	12.9 ± 0.4	28 ± 3	0.46 ± 0.05 (1.7)
E225N/Q258K/G437D	5.2 ± 0.3	16 ± 4	0.33 ± 0.08 (2.8)	8.4 ± 0.4	14 ± 3	0.62 ± 0.14 (2.3)
E225N/Q258H/G437N	6.5 ± 0.3	22 ± 3	0.30 ± 0.04 (2.5)	10.4 ± 0.4	23 ± 3	0.44 ± 0.06 (1.6)
E225N/Q258H/G437K	8.2 ± 0.2	24 ± 2	0.34 ± 0.03 (2.9)	15.8 ± 0.4	28 ± 2	0.56 ± 0.04 (2.1)
E225N/Q258H/G437E	9.7 ± 0.5	22 ± 4	0.45 ± 0.09 (3.8)	8.0 ± 0.4	38 ± 5	0.23 ± 0.03 (0.9)

[a] Catalytic efficiency of the mutants was compared to the wild-type enzyme. Results were obtained using membrane preparations.

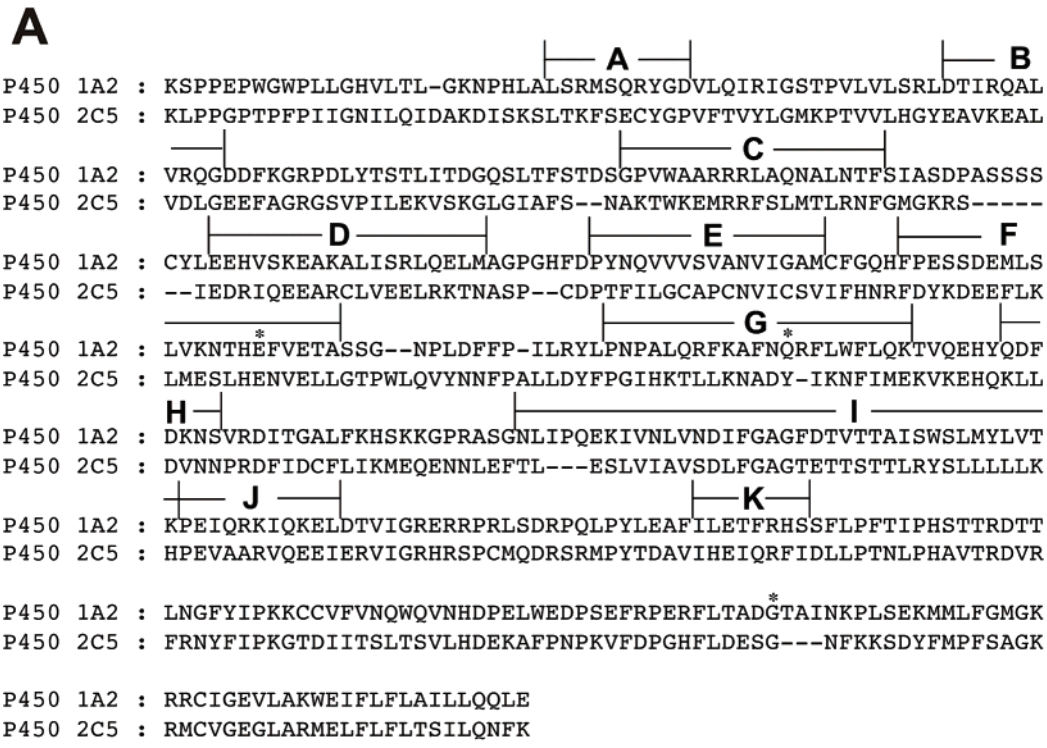

FIGURE 5: Homology modeling of P450 1A2. (A) Sequence alignment of human P450 1A2 and rabbit P450 2C5. An asterisk indicates the mutated residues of SF513. The putative helices A−K are labeled with block capital letters. (B) Model of P450 1A2 based on the structure of P450 2C5.

to screen the ability of P450 to activate HAAs (*53*), which was utilized in the initial screening (*31*). Redesigning P450 enzyme activity by mutating only SRS residues has provided limited success (*28*, *31*). It may be more common rather than exceptional that improving enzyme activity requires remodeling throughout the protein with changes in the backbone flexibility, domain motion, or subunit arrangement, as observed with other enzymes (*54*−*56*). We utilized a random mutagenesis method in this work, using error-prone PCR reaction, to produce the mutations throughout all of the P450

ORF and coupled this with a higher throughput screening method than employed previously (*31*).

SF513, the mutant with highly increased activity for activating MeIQ, was isolated by this method at the third generation library screen and contained the mutations E225N/Q258H/G437D. Steady-state kinetic analysis showed that this mutant displayed a 12-fold increase of catalytic efficiency (k_{cat}/K_m for N-hydroxylation of MeIQ). The enhancement of catalytic activity of SF513 was selective for MeIQ compared to other HAA substrates. Although MeIQ is an imidazoquinoline structure and has very close structural similarity to IQ (only an additional methyl group), hydroxylation of IQ was not improved in SF513. Differences in microsomal catalytic activity among HAAs of similar chemical structure have been observed in previous studies (*37*, *57*). Previously isolated random mutants of P450 1A2 also suggested that the mutants showed different changes in activity for different substrates, although the substrates were not all HAAs (*31*). This finding suggests that the effects of any particular mutation are relatively unique to enzyme−substrate complementarity and may diverge with different substrates.

For reasons that are not clear, the level of expression of the mutant P450s was decreased relative to the wild-type enzymes (Table 2). The holoprotein content also decreased in the (purified) mutants. A possible explanation for the increased apoprotein in the purified mutants may be instability of the protein through the accumulation of mutations, due to changes in the overall structure. These results indicate that the increased *lac* reversion activities (Figures 2 and 3) cannot be attributed to increased enzyme expression, a finding confirmed by the MeIQ N-hydroxylation results with the mutant P450s normalized for P450 content (Figure 4). We would have expected that the enhanced in vivo reversion levels (Figure 3) obtained with lower expression levels (Table 2) would have been associated with an even greater improvement in the inherent catalytic activity of the enzyme in vitro (in the MeIQ N-hydroxylation assay) (Figure 4). One caveat in this analysis is that the enzyme expression levels in the large-scale studies may not affect the levels in the reversion assays (Figures 1−3).

The mutations in SF513 affected both k_{cat} and K_m values. A G437D mutation in the third generation lowered the K_m value (Figure 4). Because the K_d values for MeIQ were not altered by the mutations (Table 3), the respective mutant K_m values apparently include more than the rate constants describing MeIQ binding and dissociation.

H_2O_2 and H_2O are produced by the uncoupling of catalytic function (*50*, *52*). No large change of the NADPH oxidation rate was observed for the mutants compared to wild type. The overall coupling efficiency of SF513 was increased 5-fold compared to wild type (Table 3), mainly due to an increased MeIQ hydroxylation rate of SF513 rather than decreased NADPH oxidation or production of H_2O_2 and H_2O. As previously reported (*43*), all of the P450s demonstrated relatively low coupling for MeIQ hydroxylation. Thus, this portion of the catalytic cycle could not fully explain the enhanced activity of mutants for MeIQ hydroxylation.

By alignment with the modified rabbit P450 2C5, Glu225 and Gln258 are predicted to lie in helices F and G (Figure 5). It is unlikely that these residues have direct contact with substrates, if these models have validity. However, helices F and G have been proposed as a flexible lid to move helix I when the substrate binds in the active site through a substrate access channel in rabbit P450 2C5 (*9*), and an influence may be exerted in this manner. Gly437 is located in the so-called "meander region". The meander region appears to be critical for enzymatic activity in P450 4B1 (*58*, *59*) and possibly P450 1A2 (*42*). Though the alignment study with P450 2C5 provides limited information due to the limited sequence similarity, it suggests that structural elements outside of the SRS regions may play roles in changing the catalytic activity, as seen in another study with rabbit P450 2B5 (*60*), where two non-SRS mutants of P450 2B5, H120R and P221S, showed significant alternation of progesterone hydroxylation activities.

On the basis of the modeling, the G437D mutation in SF 513 may be involved in an ionic interaction with the basic functional group of enzyme structure to improve the catalytic efficiency (Figure 5B). Replacement of the Asp437 of SF513 by Asn (neutral) or Lys (basic) did not yield a major change in catalytic efficiency compared to the wild-type enzyme (Table 4). Interestingly, substitution with the acidic residue Glu did not alter the activity of SF513 either (Table 4). A similar result was observed in the site-directed mutagenesis study of rat P450 1A2 in which Glu and Asp changes at residue 318 yielded different catalytic activities (*61*). The present result may exclude the possibility that the Asp437 residue exerts a role through the ionic interaction in the structural moiety, although the distance for binding may be an issue. In addition, none of the individual site-directed mutants altered the catalytic discrimination between MeIQ and IQ. We conclude that the mutated residues in SF513 exert their functions through what are probably rather synergistic interactions.

Why do the mutants, particularly the triple mutant SF513, show enhanced catalytic activity? As we consider the generalized P450 cycle (*11*), we can rule out several possibilities. Wild-type human P450 1A2 already shows rapid reduction of ferric iron (even in the absence of substrate) (*43*). The wild-type enzyme also shows an absence of burst kinetics (*43*), indicating that the rate-limiting step must precede product formation. We have shown here that ground-state substrate binding is not improved in the mutant (Table 3), nor are NADPH oxidation and H_2O_2 production (and apparently H_2O production) altered. HPLC analysis indicated that no other products are detected, even with the wild-type enzyme, so shifting the regioselectivity is not a factor. Thus, several of the steps in the cycle can be dismissed. Our best present explanation is simply that a transition state for N-hydroxylation is more favorable, probably due to proximity effects and also inducing more selectivity. In principle, one could use an approach such as kinetic hydrogen isotope effect analysis here, but the site of hydroxylation ($-NH_2$) is not very amenable to such studies.

In conclusion, we have improved the MeIQ N-hydroxylation activity of P450 1A2 through a process of random mutagenesis and screening. The three-dimensional model suggests that none of the mutated residues contact the substrate directly and that more global conformational changes in the enzyme are responsible for the enhanced activity. Further investigation of the selected P450 1A2 mutants may provide insight into mechanistic and functional relationships of P450 1A2 enzymes. This specific approach can be also applied to high-throughput mutant library

screening of P450s 1A1 and 1B1, which are also known to bioactivate HAAs (*62*−*64*). With modification of the *lac* target plasmid, the method could also be applied to other promutagens and P450s (e.g., with aflatoxins) (*53*). The general approach can also be extended to other enzymes that activate promutagens. In principle, an opposite approach could be used to screen for enzyme mutants with enhanced ability to deactivate antibiotics or other chemicals toxic or mutagenic to bacteria, but our efforts in this area have been less successful, probably due to the need to detoxicate a large amount of substrate with a limited amount of enzyme.

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