Biochemistry

 Volume 38, Number 17 April 27, 1999

Accelerated Publications

Selection and Characterization of Human Cytochrome P450 1A2 Mutants with Altered Catalytic Properties†

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Received January 21, 1999; Revised Manuscript Received March 2, 1999

ABSTRACT: Random mutagenesis is an approach that has the potential to provide useful information about cytochrome P450 (P450) enzymes but has not been extensively used to date. We applied our previously developed systems for generation of random libraries of human P450 1A2 with the putative substrate recognition sequences mutated (individual residues) and an *Escherichia coli* genotoxity assay involving reversion to *lac* prototrophy as a response to activation of the heterocyclic amine 2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline (MeIQ). A total of 27 mutants were screened from 6000 clones, a small portion of the library. The sequence changes were identified, and *E. coli* membranes containing each P450 (with NADPH−P450 reductase expressed using a bicistronic vector) were used to determine k_{cat} and K_m values for 7-ethoxyresorufin and phenacetin *O*-deethylation and the (in vitro) activation of MeIQ with another bacterial genotoxicity system (*Salmonella typhimurium umu*). Within each assay, the values of k_{cat}/K_m varied by 2 orders of magnitude, and in some cases these parameters were 3−4-fold higher than for the native enzyme. The profiles of the mutants varied considerably for the three different reactions. Some of the mutants in the Asp-320 region may be compared with site-directed mutants of rat P450 1A2 already reported, with several differences noted. Other mutants have not been studied before in human P450 1A2 or homologues and are of interest; i.e., all Phe-226 mutants showed considerably reduced activity but Glu-225 mutants had increased catalytic activities. In principle, this approach may be applied to random mutagenesis of any enzyme that converts chemicals to mutagens and can be expressed in bacteria.

The majority of microsomal cytochrome P450[1] enzymes display broad substrate specificity, allowing oxidation of a multitude of foreign compounds that possess vastly different structural characteristics. The tradeoff for this promiscuity is generally low catalytic turnover, with k_{cat} often in the magnitude of 1 min^{-1} (*1*). The potential for creating more efficient P450 enzymes by mutagenizing the active site has,

† This research was supported in part by U.S. Public Health Service (USPHS) Grants R35 CA44353, P30 ES00267 (F.P.G.), and T32 GM07347 (A.P., F.P.G.) and by the Natural Sciences and Engineering Research Council of Canada and the International Foundation for Ethical Research (P.D.J.). Nucleotide sequence analysis was supported in part by the Vanderbilt Cancer Center and USPHS Grant P30 CA68485.

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10.1021/bi990142+ CCC: $18.00
Published on Web 04/06/1999

therefore, been postulated. Such mutants might be expected to provide valuable information regarding P450 structure and function.

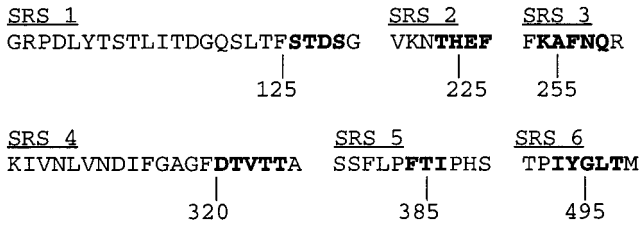
P450 1A2 has long been of interest because of the diversity of reactions it catalyzes. In addition to its participation in the metabolism of caffeine, phenacetin, and acetaminophen, it catalyzes the N-hydroxylation of aromatic and heterocyclic amines, such as those found in cigarette smoke and charbroiled meats (e.g., MeIQ), the first step in their conversion to potent DNA-damaging electrophiles (*2*). P450 1A2 appears to be subject to genetic polymorphism and thus may play a role in determining cancer susceptibility (*3*). In a series of site-directed mutagenesis studies of rat P450 1A2 by Shimizu and his associates, distal residues to the heme iron were identified and subsequently studied in detail by optical spectroscopy for perturbations in interactions with substrates (*4*), inhibitors (*5*), and CO (*6*).

In the absence of crystal structures, random mutagenesis presents an efficient alternative means of studying mammalian P450 structure and function. The approach has the advantages over site-directed systems that design bias is reduced and that mutants are selected on the basis of observed function, not predictions. Low levels of enzyme expression in vector systems, the need for accessory enzymes, and the slow reaction rates with typical P450 substrates have thus far presented major obstacles in developing suitable screening methods for random mutagenesis. To address these problems, a bicistronic expression system for P450s and NADPH−P450 reductase, a 78 kDa flavoprotein required for P450-mediated catalysis, was used to screen randomized P450 expression libraries in a mutagenicity tester strain sensitive to aryl and heterocyclic amines (*7*). Functional implications of the reported structural perturbations on kinetic parameters and substrate selectivity of 27 mutants are discussed.

EXPERIMENTAL PROCEDURES

Chemicals. 7-Ethoxyresorufin was obtained from Sigma Chemical Co. (St. Louis, MO). [*Ring*-^{3}H]phenacetin was the generous gift of Dr. F. F. Kadlubar (National Center for Toxicology Research, Jefferson, AR). MeIQ was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada).

P450 1A2 Mutant Library Construction. A bicistronic plasmid expressing both human P450 1A2 and NPR has been described (*8*). pBluescriptKS$^-$ was purchased from Stratagene (La Jolla, CA). The procedure used for targeted random mutagenesis by whole plasmid PCR amplification is described in detail elsewhere (*9*). This protocol was adapted for the construction of six DNA libraries, corresponding to the six SRS regions defined by Gotoh (*10*), theoretically saturated with single point mutations at any of three to five consecutive amino acid residues. Individual residues in human P450 1A2 were chosen by alignment with bacterial P450 102 and rat P450 1A2 (*11*). Residues dispersed throughout the protein sequence (25 total) were targeted for mutagenesis (shown in boldface print below, with SRS regions and codon numbers indicated).



In brief, the wild-type human P450 1A2 gene sequence (subcloned into a pBluescript vector) was used as a template for PCR-based mutagenesis involving a mixture of mutagenic 5′ primers (primers encoding 5′ NNS 3′ at the positions to be randomized were synthesized in the Vanderbilt Diabetes Center Core Facility; primers were grouped by target SRS and then mixed in equal proportions) and a single 3′ primer, or vice versa. A unique restriction enzyme recognition sequence either already existed within the primer sequence 5′ to the mutagenized sequences or, preferably, was introduced by silent mutagenesis during primer design. The PCR was conducted and the products treated as described (*9*). Each DNA library was transformed (in three separate aliquots) into ultracompetent *Escherichia coli* DH10b cells (Life Technologies, Gaithersburg, MD) and plated on two large (15 cm) Petri dishes. The colonies were diluted into LB/ampicillin (100 μg mL^{-1}) medium and grown to saturation. Plasmid DNA was prepared by alkaline lysis (*12*). The entire P450-encoding cassette for each library was excised by digestion with *Nde*I and *Xba*I and gel-purified. These heterogeneous fragments were ligated overnight into the P450 1A2 bicistronic expression vector (*8*) and digested at these same restriction sites, in place of the wild-type P450 1A2. The ligation mix was transformed into DH10b cells, which were plated, pooled, and lysed for DNA preparation, exactly as before. This DNA solution (50 μL final volume) was stored at −20 °C until needed for further experiments. At appropriate times, 0.5 μL of this sample was used to transform electrocompetent DJ3109 pNM12 for mutagenicity testing.

Bacterial Strains. E. coli strains DH10b, used for library preparation, and DH5αF′IQ were purchased from Gibco BRL (Gaithersburg, MD). *Salmonella typhimurium* strain NM2009, used for the *umu* test, was the kind gift of Dr. Y. Oda (Osaka Institute of Public Health, Osaka). *E. coli* strain DH5αF′, used for cloning, was obtained from Life Technologies. Construction of *E. coli* DH5α strains coexpressing a human P450 enzyme and NADPH−P450 reductase has also been reported (*8*). *E. coli* mutagenicity tester strain DJ3109 pNM12 expresses *N*-acetyltransferase, a key enzyme involved in the metabolic pathway of arylamine-mediated mutagenesis. Wild-type 1A2 expression in this background results in reconstruction of strain DJ4309, which has been characterized in detail (*7*). Isolated random mutants were treated identically to DJ4309, since they differ by only one codon within the P450-encoding cassette (discussed above under *P450 1A2 Mutant Library Construction*) which is translated as a single amino acid change in the corresponding protein.

Phenotypic Selection. Bicistronic library DNA was transformed into DJ3109 pNM12 and plated onto 15 cm of LB/

[1] Abbreviations: P450, microsomal cytochrome P450 (also termed heme−thiolate protein P450, EC 1.14.14.1); MeIQ, 2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline; NPR, NADPH−cytochrome P450 reductase; SRS, substrate recognition sequence; PCR, polymerase chain reaction.

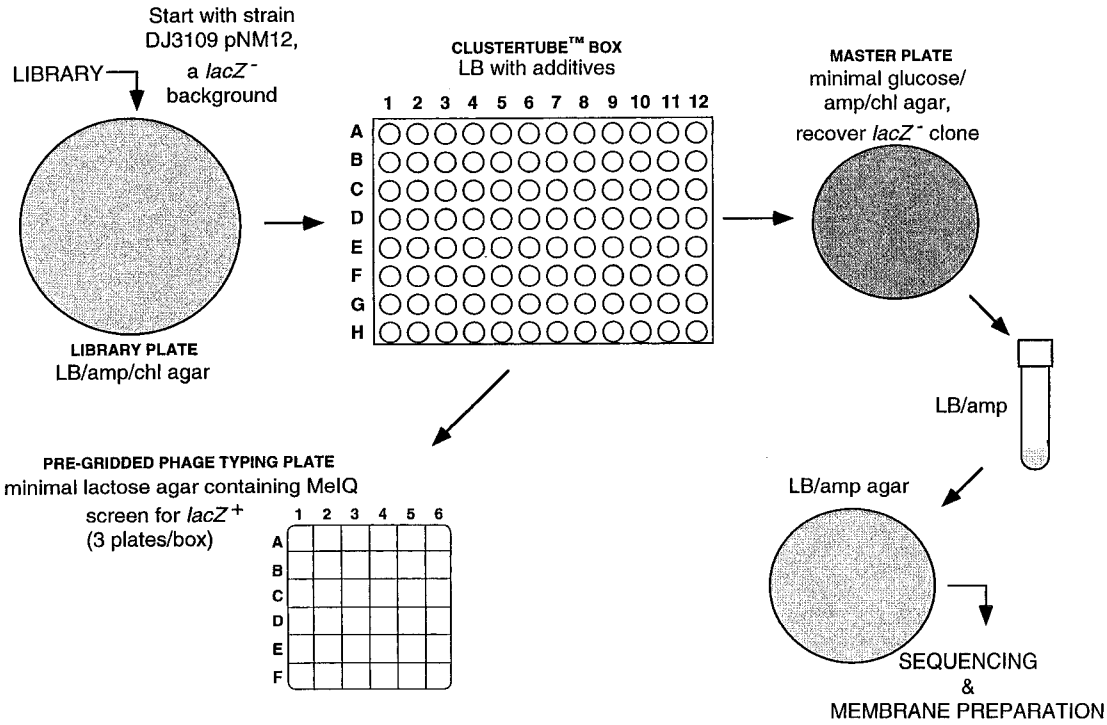


FIGURE 1: P450 1A2 single-codon randomized library screening by the isotyping method. See Experimental Procedures. Briefly, a randomized plasmid library is transformed into *E. coli* DJ3109 pNM12 and plated on LB agar with the appropriate antibiotics. Colonies are picked into single ClusterTube wells containing LB expression medium and grown. An aliquot from each well is spotted onto a single square of a pre-gridded minimal lactose plate and grown to test for MeIQ activation. Positive clones are picked from a master plate and the plasmid DNA is isolated and used to transform *E. coli* DH5α. Plasmid DNA is isolated from DH5α for nucleotide sequence analysis, and inner membranes are isolated for catalytic assays.

ampicillin (50 μg mL^{-1})/chloramphenicol (10 μg mL^{-1}) plates (Figure 1). Individual colonies were transferred to single wells of 96-well ClusterTube (Fisher Scientific, Pittsburgh, PA) boxes filled with 200 μL of LB expression medium. A flamed paper clip was used to pierce holes into the plastic tube to promote aeration. The boxes were then shaken at 30 °C and 200 rpm for 18 h to induce enzyme expression. All of the cultures were spotted onto a master plate using a Boekel 96-well replicator (Fisher Scientific). For mutagenicity testing, 7.5 μL of each culture was dispensed onto a single square of a 36-square Integrid plate (Fisher Scientific) containing minimal lactose selective medium in the bottom agar and MeIQ (10 pmol/plate) in 4 mL of top agar (i.e., nominal concentration 2.5 nM). The DJ4309 strain containing the wild-type P450 1A2 clone was spotted out identically as a positive control, and the null strain (DJ3109 pNM12) was spotted as a negative control. After 24 h, discrete small colonies of revertants could be observed for DJ4309 and some of the mutants. While this phase of screening was qualitative, all clones that appeared to possess higher-than-background reversion rates were carried on to the next step. These same clones were then picked from the master plate (which was not exposed to mutagen) and grown in 1.5 mL of LB/ampicillin (100 μg mL^{-1}). DNA was prepared from these cultures by alkaline lysis/2-propanol precipitation (*12*). This DNA was retransformed into *E. coli* DH5αF′IQ to eliminate the chloramphenicol-resistant *N*-acetyltransferase plasmid and for subsequent automated sequencing and protein expression. A single positive colony was transferred into 5 mL of TB/ampicillin (100 μg mL^{-1}) and grown overnight to saturation. DNA was prepared by alkaline lysis in combination with diatomaceous earth (*13*) using a Promega (Madison, WI) Vac-Man vacuum manifold. DNA was eluted from the diatomaceous earth in 50 μL of sterile H_2O. The DNA was then analyzed for nucleotide sequence with a specific primer corresponding to the SRS library from which the clone was isolated.

Membrane Preparation. Bacterial inner membranes ("bicistronic membranes") containing a mutant P450 1A2 protein and NADPH−P450 reductase were isolated from 1 L DH5αF′IQ TB/ampicillin (100 μg mL^{-1}) expression cultures (*14*). The 10^5g pellet was completely resuspended in a final volume of 10 mL of 200 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol (v/v) with a dounce homogenizer and stored on ice until analysis.

P450 and NADPH−P450 Reductase Assays. P450 was quantitated by the spectral method of Omura and Sato (*15*). Whole bacterial cells were resuspended in an equal volume of spectral assay buffer (50 mM potassium *N*-morpholinopropanesulfonic acid buffer, pH 8.0, containing 100 mM KCl, 1.0 mM EDTA, and 1.0 mM dithiothreitol). Bacterial membranes were diluted 1:20 into 100 mM potassium phosphate buffer, pH 7.4. NADPH−P450 reductase activity was estimated by measuring cytochrome *c* reduction rates of bacterial membranes in the presence of KCN (1 mM, to block respiratory proteins that catalyze this activity) and assuming a turnover number of 3200 nmol of cytochrome *c* reduced min^{-1} (nmol of reductase)$^{-1}$ at 37 °C (*16*).

7-Ethoxyresorufin *O*-deethylation activity was measured by fluorescence assay (*17*). Phenacetin *O*-deethylation was measured by radio-TLC (*18,19*). A quantitative *umu* activation assay (*20, 21*) was used as a measure of MeIQ hydroxylation. Results from reduced-CO spectral measurements (*15*) were used to standardize for P450 content prior to activity assays on membrane preparations.

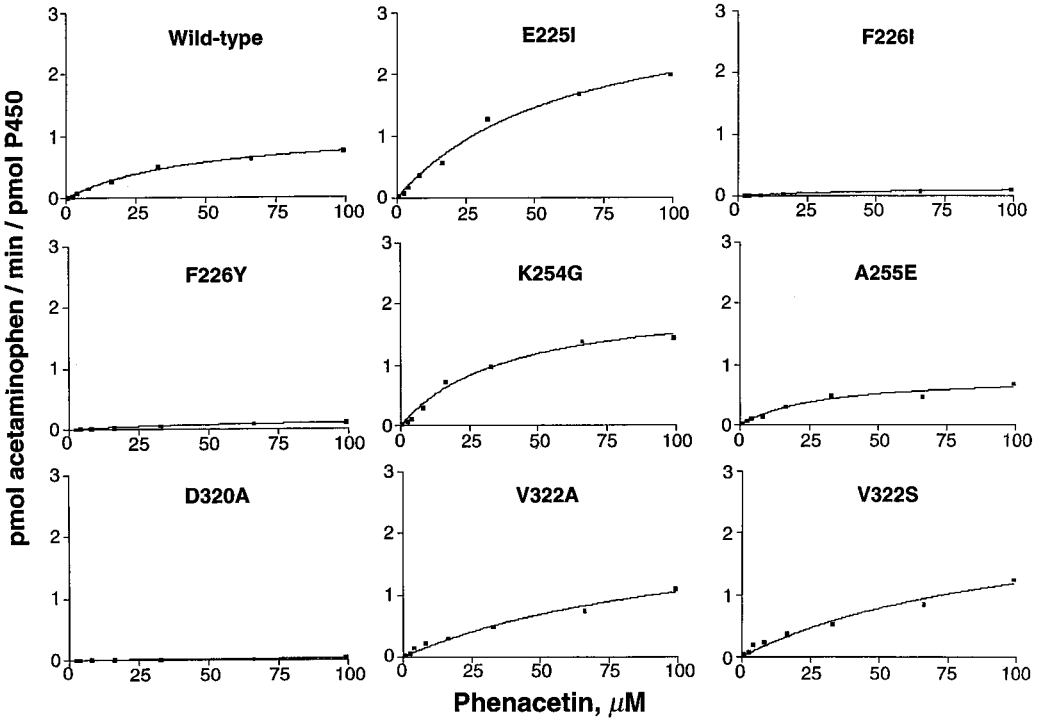


FIGURE 2: Michaelis−Menten (v vs S) plots for phenacetin *O*-deethylation by selected P450 1A2 mutants. The following parameters were estimated for wild-type P450 1A2 and the mutants [k_{cat} expressed as pmol of acetaminophen formed min^{-1} (pmol of P450)$^{-1}$ and K_m, in μM, with estimation of parameters and variance using ABI Prism for Macintosh (GraphPad Software, San Diego, CA)]. Wild-type: k_{cat} 1.1 ± 0.08, K_m 48 ± 7 ([compare with k_{cat} 3.9 and K_m 43 for the enzyme isolated from human liver (*19*)]; E225I: k_{cat} 3.3 ± 0.3, K_m 63 ± 13; F226I: k_{cat} 0.14 ± 0.01, K_m 42 ± 8; F226Y: k_{cat} 0.20 ± 0.02, K_m 80 ± 13; K254G: k_{cat} 2.1 ± 0.2, K_m 38 ± 8; A255E: k_{cat} 0.80 ± 0.09, K_m 28 ± 9; D320A: k_{cat} 0.07 ± 0.04, K_m 100 ± 99; V322A: k_{cat} 2.2 ± 0.5, K_m 109 ± 43; V322S: k_{cat} 2.4 ± 0.6, K_m 100 ± 43.

RESULTS

The regions targeted for mutagenesis were selected on the basis of published sequence alignments of P450 1A2 with bacterial P450s and are putative substrate binding regions, as predicted by that approach (*10*, *11*). Twenty-seven mutants representing 16 unique residues within the human P450 1A2 protein were isolated after screening ~6000 clones from 6 randomized single-codon libraries. Seven mutants localized to SRS region 5, six each to SRS 1, SRS 2, and SRS 4, two to SRS 3, and none to SRS 6. All were qualitatively positive for MeIQ activation when expressed in the DJ3109 pNM12 tester strain, the phenotype used for identifying active clones. All but three of the mutations (T321S, V322A, I386L) resulted in altered amino acid charges.

Characterization of mutants was performed at the membrane level. Reduced-CO spectroscopy indicated that expression levels of P450 1A2 varied between the mutants, ranging from 0.64 to 17 μM in the membrane preparations obtained under the single set of conditions used here. NADPH−P450 reductase expression, as judged by cytochrome *c* reduction, was considerably more consistent, displaying 2-fold variation (2.5−6.0 μM NADPH−P450 reductase in *E. coli* bicistronic membranes).

Bicistronic membranes from mutants were subjected to a panel of three marker P450 activity assays using a range of substrate concentrations, and the kinetic constants k_{cat} and K_m were calculated. Examples of results for the wild-type enzyme and some mutants of interest (phenacetin *O*-deethylation assays) are shown in Figure 2. (In the case of D320A, the k_{cat} estimate is approximate but the k_{cat}/K_m ratio is more reliable, being obtained from the slope, vide infra.)

Enzyme velocities (k_{cat}) for all mutants are presented for 7-ethoxyresorufin, phenacetin, and MeIQ oxidations (Figure 3A). All of the mutants displayed fairly similar enzymatic velocities toward MeIQ, the substrate used in the screening process, as judged by the *umu* assay. The greatest increases over wild-type P450 1A2 with any of these three reactions were 3-fold.

Catalytic efficiencies (k_{cat}/K_m) of mutants for 7-ethoxyresorufin, phenacetin, and MeIQ oxidations are shown in Figure 3B.

To rule out any major kinetic effects caused by differential NPR expression following standardization for P450 content, we retested six freshly isolated membrane preparations. Although P450 expression was altered for several of the second preparations relative to the first, the observed kinetic constants were highly reproducible.[2]

The P450 to NADPH−P450 reductase ratio varied considerably between membrane preparations of different mutants and also between individual preparations of the same mutants, but replicates of each yielded virtually identical kinetic parameters.[3] Some of the mutants with low P450 activity exhibited high relative reductase content (as judged

[2] For example, for the P450s wild-type, S129H, E225I, F226I, K254G, and A255E, the values of k_{cat}/K_m for 7-ethoxyresorufin *O*-deethylation for two sets of preparations were, respectively, 1.0, 1.0, 0.6, 0.2, 0.7, and 0.8 in the first experiment and 1.0, 0.9, 0.5, 0.2, 0.9, and 0.7 in the second. (Values from the first preparation are represented in Figure 3B).

[3] For example, for the S129H mutant in the previous footnote (footnote 2), the P450 concentrations in the membranes prepared from separate bacterial cultures were 12.7 and 10.6 μM, giving k_{cat}/K_m values of 1.0 and 1.1 for 7-ethoxyresorufin *O*-deethylation in the two cases.

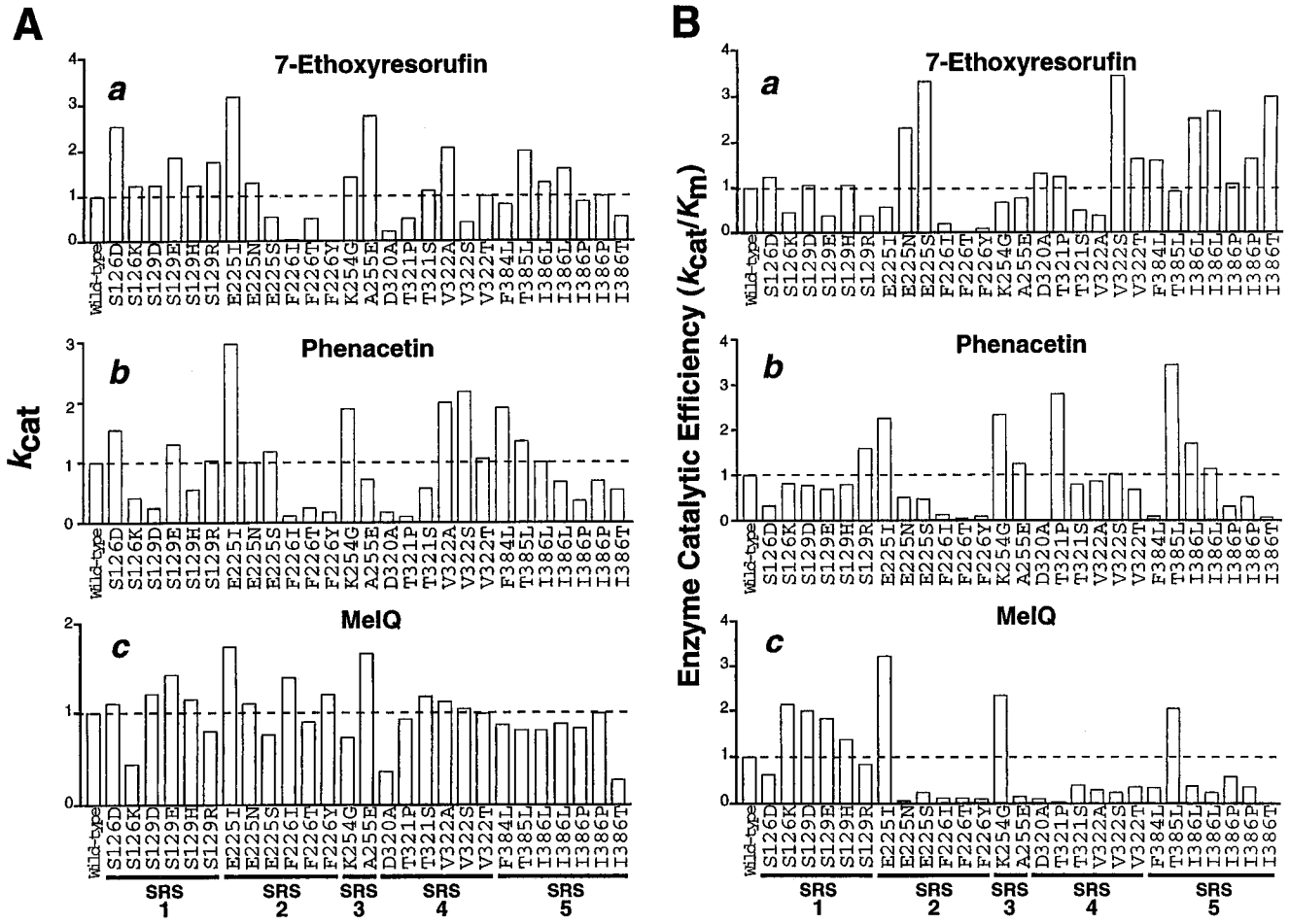


FIGURE 3: Comparison of k_{cat} and k_{cat}/K_m for wild-type P450 1A2 and 27 P450 1A2 mutants with regard to three marker reactions. Activities of the wild-type 1A2 construct have been normalized to unity. k_{cat} and K_m values for wild-type P450 1A2-catalyzed phenacetin *O*-deethylation are given in Figure 2. For 7-ethoxyresorufin *O*-deethylation by wild-type P450 1A2, $k_{cat} = 1.70 \pm 0.34$ pmol resorufin formed^{-1} min^{-1} pmol of P450^{-1} and $K_m = 1.7 \pm 0.8$ μM. For MeIQ activation by wild-type P450 1A2, $k_{cat} = 776 \pm 80$ umu units (nmol of P450)$^{-1}$ and $K_m = 13 \pm 5$ μM. The mutants I386L and I386P were each isolated twice in the process of screening.

by cytochrome *c* reduction assay) and vice versa. We therefore concluded that reductase concentrations were not rate-determining and did not appreciably influence the observed kinetics of the mutant enzymes. Further, when the wild type, E225I, F226Y, D320A, V322A, and I386L proteins were purified and reconstituted with NADPH−P450 reductase and L-α-dilauroyl-*sn*-glycero-3-phosphocholine in the usual manner (*22*, *23*), rates of 7-ethoxyresorufin *O*-deethylation were in accordance with those expected from Figure 3 (Yun, C-H., and Guengerich, F. P., results not presented).

DISCUSSION

Random mutagenesis approaches should, in principle, have considerable potential for enhancing understanding of P450 structure/function relationships. However, previous efforts in our laboratory and others (*24, 25*) have been limited by low rates of catalysis and the lack of availability of screening methods appropriate for the analysis of large numbers of mutants. We utilized the *E. coli* mutagenicity tester strain DJ3109 pNM12, which expresses *Salmonella N*-acetyltransferase and has a *lacZ* reporter allele, to screen randomized P450 plasmid libraries for mutant P450 1A2 enzymes. Only mutagenesis of the reporter gene resulting from the enzymatic activity of a functional P450 enzyme permits a high degree of growth on lactose-selective medium in this host background. (In these experiments, only −2 frameshifts are detected (*7, 26*), but the system could potentially be varied by substitution of an alternate F′ element (*27*).]

Twenty-seven mutants in the putative substrate binding regions of the protein were isolated by this procedure and subjected to k_{cat} and K_m determinations for three P450 1A2 substrates: 7-ethoxyresorufin, phenacetin, and MeIQ. These three substrates are quite structurally unrelated. Interestingly, the mutants displayed a 30-fold variation in k_{cat} toward 7-ethoxyresorufin and phenacetin. Only the mutants S126D, S129E, E225I, V322A, and T385L had significantly elevated activities toward both of these substrates, emphasizing that the effects of any particular mutation are unique to the enzyme−substrate (or enzyme-transition state) fit and may diverge with different substrates. Although phenacetin is a significantly smaller compound than either 7-ethoxyresorufin or MeIQ, its propensity for *O*-deethylation by P450 1A2 is similar to that of 7-ethoxyresorufin for many of the mutants. Very little is known about the three-dimensional localization of these residues. Most P450s display a cluster of three Thr residues and a Glu or Asp residue in the SRS 4 region. Val-322, which has the rat P450 1A2 counterpart Val-320, has been suggested by Shimizu and his associates to be located in the Thr cluster distal to the heme in the I-helix (*28*). By

alignment with P450 102 (*11*), Ser-129 would fall in the region between helices B′ and C, and Lys-254 would lie in poorly conserved helix G. It is unlikely that these residues have a consistent effect on all reactions as evidenced by the fact both phenacetin and 7-ethoxyresorufin O-deethylation rates (k_{cat}) change in a parallel fashion for some other mutations at these same positions (S129E, V322A). Mutating Glu-225 to Ile increased the rates of catalysis with all three substrates, but mutation to Asn had no effect on k_{cat} in any case and mutation to Ser led to decreased activity with 7-ethoxyresorufin and MeIQ. This observation is in contrast to the low rates exhibited by mutations at Phe-226. Three mutants at this position (F226I, F226T, F226Y) displayed very low k_{cat} values for both 7-ethoxyresorufin and phenacetin oxidations (but not MeIQ). One might theorize that the active site of P450 1A2, as well as other microsomal P450s, constitutes a hydrophobic binding pocket that orients substrate for attack by activated oxygen in a regio- and stereoselective fasion. Bulky aromatic residues such as Phe would be expected to play a major role in substrate docking. In fact, there is at least one Phe residue present in each of the SRS regions of 1A2. Removal of the charge at the adjoining Glu-225 may therefore have resulted in the opposite effect, increasing rates.

A better indicator of the catalytic efficiency of an enzyme is the ratio k_{cat}/K_m (Figure 3B). Moreover, this ratio (the slope of the v vs S plots) is subject to less error in high K_m situations. The k_{cat}/K_m profiles show more variation than the k_{cat} plots when the MeIQ results are included. The profiles of enzyme efficiency vary for all three reactions. k_{cat}/K_m was very low for all three of the Phe-226 mutants. One might have predicted that F226Y would not have been as poor a catalyst as was experimentally determined, in consideration of the relatively small modification. In lieu of the findings, however, it should be suggested that the P450 1A2 active site structure may in fact be more constrained than previously imagined.

The human P450 1A2 D320A mutant corresponds to the rat P450 1A2 E318A mutant constructed and extensively studied by Shimizu and co-workers (*29*). The view that this acidic residue stabilizes the I-helix in the heme distal region [based on P450 101 crystal structure (*30*)] and is always required for oxygen activation was challenged by the finding that oxygen incorporation into substrate was actually more efficient for the rat P450 1A2 T319A mutant than for the wild-type enzyme, although the k_{cat} was reduced by about 3-fold. D320A was one of two mutants not recovered during the isotyping and sequencing process (along with T385F), but we reconstructed it in a site-directed manner.[4] This enzyme is relatively inefficient with the substrates phenacetin and MeIQ (Figure 3B); the low k_{cat} for 7-ethoxyresorufin *O*-deethylation is compensated in part by a lower K_m to preserve the enzyme efficiency.

The previous alignments place Thr-321 at the site corresponding to Thr-319 in rat P450 1A2 (and possibly involved in oxygen activation). Mutants at Thr-321 (T321S, T321P) varied in activity, with less efficiency in some cases but more in others (Figure 3B). Shimizu also reported that the rat P450 1A2 T319A mutant had low activity in some reactions but elevated activity in others (*28*, *31*, *32*). These shifts in activity were typically accompanied by increases in the respective K_m. The rat P450 1A2 mutants also yielded shifts in heme alkylation patterns with phenyldiazene, indicating an opening of the substrate binding site (*33*).

Other residues at which mutations show very contrasting effects among the three reactions are Val-322 and Ile-386 (Figure 3B). At this point we could speculate on the effects of the mutations on catalytic selectivity or even attempt docking molecular models. However, such efforts would have two serious caveats: (i) the protein structure is unknown, even in the absence of ligand; and (ii) the multistep catalytic mechanism (*1*) is complex, and there is still little information about what the parameters k_{cat} and K_m really represent in the case of this P450 (and different mutations may affect different steps). Our goal is to characterize several of these mutants to determine which catalytic steps have been modified with respect to the wild-type human P450 1A2.

The basic screening system developed here can be used with at least five human P450s (1A1, 1A2, 1B1, 2C9, and 3A4), based upon current experience (Yoshihara, S., Josephy, P. D., and Guengerich, F. P., unpublished results). Although P450 1A2 is much more effective in activating heterocyclic amines (*34*), these other P450s can be screened for activity with higher plate concentrations of the promutagens. In principle, the basic system can be utilized for random mutagenesis with any enzyme that activates a promutagen and can be expressed in bacteria, and the genetic readout can be changed by substituting the appropriate episomal element (*27*).

ACKNOWLEDGMENT

We acknowledge the contribution of Dr. David H. Evans of the Department of Molecular Biology and Genetics, University of Guelph, who suggested the mutagenesis-screening approach used in this paper, in discussions with one of us (P.D.J.) in 1997. We also thank Drs. R. Mernaugh, T. Omura, and S. Yoshihara for their suggestions and L. M. Guengerich for her excellent technical assistance.

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[4] The nucleotide sequence for D320A was obtained by analysis of a positive clone, but the plasmid was not recovered from serial transformations and minipreps during the isolation process. The D320A mutant was reconstructed using the same approach described for random library construction (but with a pair of site-directed primers) and verified by nucleotide sequence analysis.

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BI990142+