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Inter-individual differences in the metabolism of environmental toxicants: cytochrome *P*450 1A2 as a prototype

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Abstract

Cytochrome *P*450 (*P*450) 1A2 provides an interesting paradigm for inter-individual differences in the metabolism of pro-carcinogens. The enzyme is known to vary 40-fold among individuals and may contribute to cancers caused by heterocyclic amines and other chemicals. Rat and human *P*450 1A2 are known to be 75% identical and were compared for several catalytic activities. The human enzyme was an order of magnitude more efficient in the *N*-hydroxylation of two heterocyclic amines. Further, the levels of *P*450 1A2 expressed in human livers show a 40-fold variation, with some as high as 0.25 nmol *P*450 1A2 per milligram microsomal protein. Some human liver samples are more active (than those isolated from polychlorinated biphenyl-treated rats) in the activation of heterocyclic amines. A bacterial genotoxicity assay has been developed in which human *P*450 1A2 and NADPH-*P*450 reductase are expressed within *Escherichia coli* and bacterial mutants can be assayed using reversion to *lac* prototrophy. A random mutagenesis strategy for human *P*450 1A2 has been developed and used to examine the changes in catalytic activity seen with many single-amino acid substitutions. These results may be of relevance in consideration of genetic polymorphisms. Further, the findings pose a challenge to molecular epidemiology effort in that results with one substrate do not necessarily predict those for others. Some dinitropyrenes are *P*450 1A2 substrates but others are not. 6-Nitrochrysene can be activated by human *P*450 1A2 but the (mono) nitropyrenes examined were not; these were oxidized by *P*450 3A4 instead.

Keywords: Cytochrome *P*450, 1A2; Cytochrome *P*450, human; Dinitropyrene; *Escherichia coli*, expression of proteins in; Genetoxicity assay; Mutagenesis, random; Heterocyclic amine

1. Introduction

The general issue of interest here is inter-individual variation in metabolism of potentially toxic and carcinogenic xenobiotics. A single enzyme, cytochrome *P*450 (*P*450) 1A2, will be used as a paradigm to illustrate both the progress that has been made and the complexity that still exists.

Human *P*450 1A2 was first purified on the basis of its ability to catalyze phenacetin *O*-deethylation activity [1] and was found to be distinct from *P*450

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2D6 (the debrisoquine 4-hydroxylase), contrary to expectations from the literature [2,3]. The purified enzyme was termed '$P450_{PA}$' because of the catalytic activity towards phenacetin. Immunochemical and spectral comparisons with previously characterized rat and rabbit liver proteins revealed many similarities, and subsequently all of these *P*450 proteins were given the classification '*P*450 1A2' regardless of species, when the systematic nomenclature system was developed [4]. The different *P*450 1A2 proteins (from rat, rabbit, and human) have many similar properties and share ~75% sequence identity; however, notable catalytic differences are observed (vide infra).

Pantuck et al. [5] had previously demonstrated that a number of dietary alterations influence in vivo phenacetin pharmacokinetics, e.g., smoking and ingestion of char-broiled meat accelerated phenacetin clearance. However, phenacetin can no longer be used in human studies in the United States because it was found to cause rodent tumors. An interesting development was the demonstration of the caffeine *N*-demethylation activity of human *P*450 1A2 [6]. This result provided a basis for a safe and reliable means of in vivo phenotyping of individual humans for hepatic *P*450 1A2 activity (*P*450 1A2 is expressed essentially only in the liver [7,8]).

Another interesting finding was that human *P*450 1A2 is, in general, the major enzyme involved in the activation of aryl and heterocyclic amines [6,8–11], which are of concern because of the presence of these carcinogens in industrial settings (aryl amines) and pyrolyzed food (heterocyclic amines) [12,13]. Subsequently, methods were developed for the heterologous expression of human *P*450 1A2 in cultured mammalian cells [9,10] and bacteria [14]. With the use of the tight-binding ligand α-naphthoflavone, recombinant *P*450 1A2 could be readily purified [14]. One example of the use of large amounts of purified human *P*450 1A2 was in the synthesis of bufuralol products for nuclear magnetic resonance (NMR) spectroscopy [15].

Human *P*450 1A2 has been implicated in the metabolism of some drugs (e.g., theophylline, tacrine, acetaminophen, antipyrine, bufuralol, ondansetron, phenacetin, tamoxifen, warfarin) [8]. The level of the enzyme varies 40-fold among individual humans [6,7] and differences can influence the risk of drug interactions. Of particular interest is the possibility that inter-individual differences in *P*450 1A2 levels may contribute to cancer risk. Lang et al. [16] did not detect a significant contribution of the level of *P*450 1A2 activity (as judged by in vivo caffeine clearance) to colorectal cancer; however, high *P*450 activity was found to contribute when multivariate analysis included cooked meat preference and *N*-acetyltransferase activity.

Issues of interest in the remainder of this section include development of assays for detecting activation of heterocyclic amines to genotoxins by human *P*450 1A2, catalytic differences among *P*450 1A2 enzymes found in rats and humans, the effect of single amino acid changes on function of human *P*450 1A2, and the activities of human *P*450 1A2 towards nitropyrenes.

2. Development of new genotoxicity assays

Human *P*450 1A2 is known to activate many chemical carcinogens and a need exists to be able to assess more chemicals for their potential to cause cancer. Bacterial systems have great advantages in this regard because of their speed, low cost, and ease of manipulation. During the past decade, we have made considerable use of an *umu* system in which a reporter gene is used to quantify DNA damage [17]. However, this system relies on the external activation of chemicals by *P*450 and other enzymes, and we have been interested in developing systems capable of activating chemicals within the bacterial cells.

In 1995, we reported the expression of human *P*450 1A2 in *Salmonella typhimurium* TA1538, a classic Ames tester strain [18]. The approach involved the use of the same pCW vector we had been using for expression in *Escherichia coli* [14,19,20]. The system yielded mutants when aryl and heterocyclic amines were added [18]. However, the system was not as sensitive as traditional ones using rat liver $9000 \times g$ supernatant, primarily because electron transport from NADPH to *P*450 was totally dependent on bacterial flavodoxins, which are much less efficient than NADPH-*P*450 reductase [21,22].

A new system has been developed that overcomes this deficiency (Fig. 1). Human *P*450s can be co-expressed with NADPH-*P*450 reductase using a bi-

cistronic system (pCW′1A2:NPR), in which a single RNA is produced and is translated into two proteins [23]. The two enzymes are integrated into the bacterial membrane and are functionally active, both in the cells and within isolated bacterial membranes. Another plasmid (pNM12) codes for *Salmonella N*-acetyltransferase, which renders the hydroxylamine products of the *P*450 reaction more reactive due to acetylation. The bacterial genome has the *lacZ* gene deleted and a modified *lacZ* gene is introduced via an F′ episomal element. Thus, the bacteria do not make β-galactosidase and cannot grow on lactose media unless an appropriate mutation occurs in the *lacZ* gene of the F′ episome (Fig. 1).

The system responds to aryl and heterocyclic amines (Fig. 2). In contrast to previous efforts with *S. typhimurium* [18], this system is more sensitive with *P*450 1A2 (and reductase) expression than with added rat liver supernatant with some chemicals (Fig. 2). There are two advantages of this *E. coli* system over *S. typhimurium* systems. The first is that levels of enzyme expression are higher in *E. coli* (*Salmonella* are also notorious for their lack of tight *lac* control of vector expression). The other advantage is that the mutational target can easily be varied by changing the F′ element. The system used thus far responds to a −2 frameshift [24] but Cupples et al. [25] have developed a set of F′ episomes that can detect specific frameshift and base pair mutations.

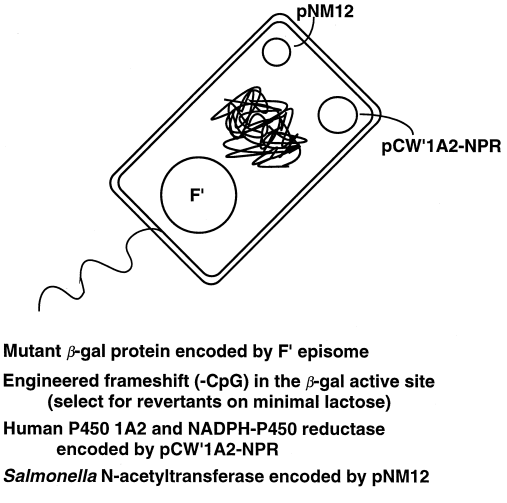

Fig. 1. Design of *E. coli* strain DJ4309 for analysis of mutagens with human *P*450 enzymes [23].

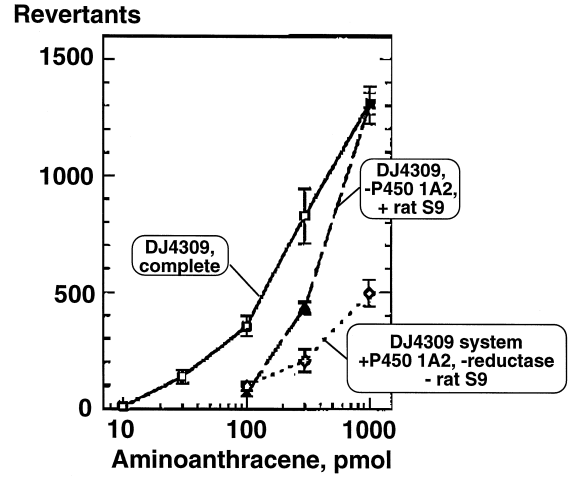

Fig. 2. Activation of 2-aminoanthracene in *E. coli* DJ4309 and derivatives. Complete DJ4309 system (□–□), system minus *P*450 1A2 but with rat liver 9000× *g* supernatant added (▲–▲), system with *P*450 1A2 but no reductase and no added rat liver supernatant (○–○) [24]. Results are presented as means (±SD) of triplicate assays.

The *P*450 can be changed by substitution in the pCW′1A2:NPR vector, and we have found that the systems can also be used with human *P*450s 1B1, 2C9, 3A4 (results not shown).

Similar systems for the use of recombinant human *P*450s have been developed by Suzuki et al. [26] (*S. typhimurium*) and Kranendonk et al. [27] (*E. coli*). Further improvement of these systems is still possible in order to make them more useful for genotoxicity screening and studies on the roles of *P*450s in bioactivation. One needed improvement is the incorporation of *umu* or *muc* genes to facilitate bypass replication around bulky lesions [28]. This is probably a critical feature with certain mutagens such as aflatoxin B_1 and polycyclic aromatic hydrocarbons [29]. Another improvement that is under investigation is the introduction of the bicistronic expression vector into the *umu* system, which has advantages over plate-counting methods in terms of speed and application to automated high-throughput methods [17].

3. Comparisons of catalytic activities of human and rat *P*450 1A2

The apparently orthologous proteins in different animal species are termed *P*450 1A2 and often

assumed to be very interchangeable in terms of their catalytic functions. There is support for this view in that some general functional similarities of these proteins have been shown. Even though they are ~75% identical in different species, all of the *P*450 1A2 enzymes do have a considerable high-spin iron component [14] and catalyze the reactions, 7-methoxyresorufin *O*-demethylation, phenacetin *O*-deethylation, and the activation of many aryl and heterocyclic amines. However, some differences have been detected. For instance, rat *P*450 1A2 apparently is much better at activating 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) than the human enzyme; both rat and human *P*450 1A2 activate 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole 1 (Trp-P-2) [11].

We carefully re-evaluated the expression levels of *P*450 1A2 in 51 different human liver samples (Fig. 3), taking care to use several antibodies and to quantify the antigen standard by quantitative amino acid analysis [30]. The results indicated that some samples had very high levels of expression, although not as high as those reported for *P*450 1A2 ($P450_{BNF/ISF\text{-}G}$) in livers of rats treated with high

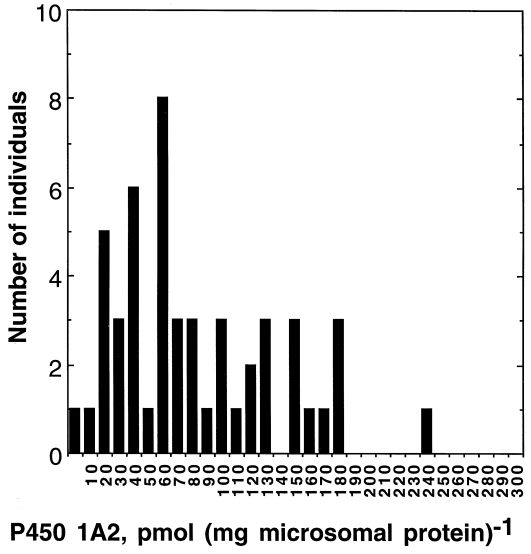

Fig. 3. Frequency distribution of *P*450 1A2 in 51 human liver microsomal samples. Each has indicated a value (determined by immunochemical assay) between that indicated and the next value, e.g., the bar '50' indicates a value in the range of 50–60 pmol *P*450 (mg protein)$^{-1}$ [30].

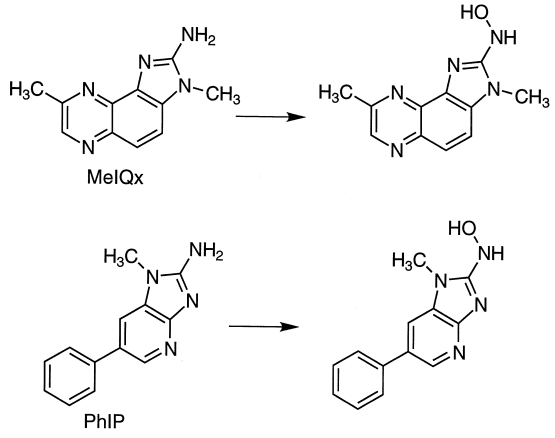

Fig. 4. *N*-Hydroxylation reactions of MeIQx and PhIP.

concentrations of inducers such as 3-methylcholanthrene or polychlorinated biphenyls [31,32]. The range of level of expression was ~40-fold among humans, similar to that found earlier in assays with liver samples [6] or using in vivo caffeine pharmacokinetics [7].

The intrinsic catalytic activities of purified rat liver *P*450 1A2 and human (recombinant) *P*450 1A2 were measured. Both enzymes yielded similar kinetic parameters for 7-methoxyresorufin *O*-demethylation (rat: k_{cat} 1.7 min^{-1}, K_m 0.21 μM, k_{cat}/K_m 8.2 min^{-1} μM^{-1}; human: k_{cat} 1.3 min^{-1}, K_m 0.22 μM, k_{cat}/K_m 6.0 min^{-1} μM^{-1}). The *N*-hydroxylation of 2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine (PhIP) (Fig. 4) was measured and human *P*450 1A2 was considerably more active than the rat enzyme-rat: k_{cat} 6.8 min^{-1}, K_m 160 μM, k_{cat}/K_m 0.043 min^{-1} μM^{-1}; human: k_{cat} 9.7 min^{-1}, K_m 12 μM, k_{cat}/K_m 0.80 min^{-1} μM^{-1}. The lower enzyme efficiency with the rat enzyme is due to the K_m effect. We also measured the *N*-hydroxylation of 2-amino-3,8-dimethylimidazo [4,5-*f*]quinoxaline (MeIQx) (Fig. 4) and found the following parameters—rat *P*450 1A2: k_{cat} 0.53 min^{-1}, K_m 14 μM, k_{cat}/K_m 0.039 min^{-1} μM^{-1}; human *P*450 1A2 k_{cat} 8.2 min^{-1}, K_m 19 μM, k_{cat}/K_m 0.44 min^{-1} μM^{-1}. In the case of MeIQx, the difference is in k_{cat}, not K_m. Thus, the intrinsic enzyme efficiency for *N*-hydroxylation is 10–20× in favor of human over rat *P*450 1A2 with both PhIP and MeIQx.

Because a significant fraction of humans express relatively high hepatic levels of *P*450 1A2, and human *P*450 1A2 has intrinsically higher efficiency than rat *P*450 1A2 in the activation of some heterocyclic amines, we considered the relative amounts of activation of these chemicals in liver microsomes in a *S. typhimurium* (TA98) reversion assay (Fig. 5) [30]. In the case of MeIQx, some human samples (e.g., HL100) yielded results similar to those prepared from untreated rats (Fig. 5A). However, samples from other individuals were as active, on a milligram protein basis, as liver samples prepared from rats treated with polychlorinated biphenyls (PCBs), a classic method of inducing high levels of *P*450 1A2 (and 1A1) in rat liver [33]. One human sample (HL G) was even more active than the PCB-treated rat liver preparation. Similar patterns were seen with PhIP (Fig. 5B). Two differences were seen with regard to MeIQx. First, higher heterocyclic amine concentrations were needed, as expected [34]. Second, none of the human samples were as active as the liver microsomes preparations derived from PCB-treated rats. The explanation for the difference with the MeIQx results in that *P*450 1A1 is also induced (by PCBs) in rat liver [31,35] and is known to contribute to PhIP *N*-hydroxylation (but not appreciably to MeIQx *N*-hydroxylation) [30,34].

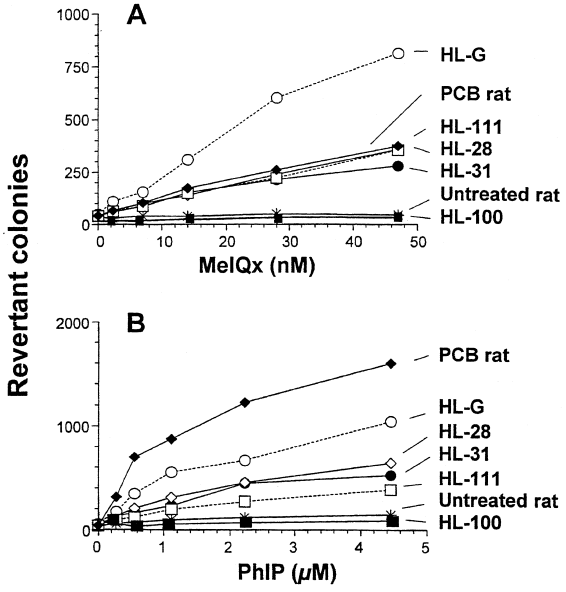

Fig. 5. Mutagenicity of MeIQx (A) and PhIP (B) in the Ames reversion assay using *S. typhimurium* TA98 with four active human liver samples (HL-28, 32, 111, and G containing 130–190 pmol *P*450 1A2 (mg microsomal protein)$^{-1}$), and a human liver sample with a low *P*450 1A2 level (HL-100, <10 pmol (mg microsomal protein)$^{-1}$), and liver samples from an untreated rat and a rat pretreated with the polychlorinated biphenyl (PCB) mixture Aroclor 1254 (500 mg kg^{-1}, i.p.) [30]. The concentrations of heterocyclic amines indicated are those used in the 30-min preincubation step.

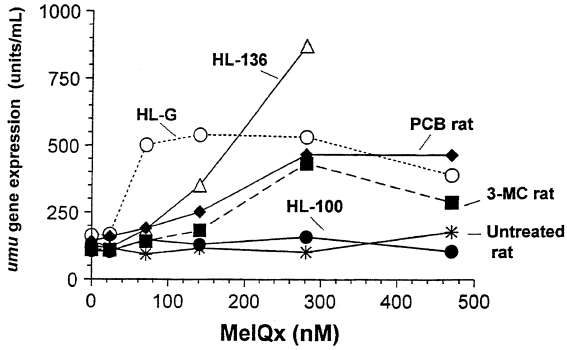

Fig. 6. Genotoxicity of MeIQx in the *umu* assay [17] with human and rat liver microsomes [30]. See legend to Fig. 6. '3MC' denotes liver microsomes from rats treated with 3-methylcholanthrene (40 mg kg^{-1}, i.p. $\times 3$ days).

Another genotoxicity assay, the *S. typhimurium umu* test [17], was also used with MeIQx activation (Fig. 6) and similar results to those found with the reversion test (Fig. 5) were observed. Two of the human liver samples had activities appreciably higher than the PCB-treated rat liver samples.

Our conclusion is that considerable variation exists among humans in their abilities to activate heterocyclic amines. The intrinsically greater ability of human *P*450 1A2 to activate some heterocyclic amines, as compared to rat *P*450 1A2, coupled with the high levels of hepatic human *P*450 1A2, argues that risk estimates of heterocyclic amine-induced cancer based only on work with rats may underestimate the potential danger of these compounds, which are abundant in pyrolyzed meat and are also in cigarette smoke [36].

We have now constructed a bacterial strain identical to *E. coli* DJ4309 (vide supra) but expressing rat (instead of human) *P*450 1A2, which will be used in further studies of species differences.

4. Effects of single amino acid replacements on human *P*450 1A2 catalytic activities

The work described above indicates that some humans express relatively high levels of *P*450 1A2 and that the 25% sequence difference between rat and human *P*450 1A2 can have a very significant influence on the catalytic activity (Figs. 3, 5 and 6). We are also interested in the effects of single amino acid changes in human *P*450 1A2. Evidence for genetic polymorphism in human *P*450 1A2 has been presented, based upon in vivo caffeine N^3-demethylation pharmacokinetics [16,37]. Some of the variations may be related to the regulation of expression levels. To our knowledge, there have been no systematic attempts to search for individual coding-region changes linked to altered enzyme activity.

The approach we have begun is part of a more general effort to use random mutagenesis to study structure–function relationships in human *P*450 proteins. The development of the methodology required solution of three major problems. The first was the production of catalytically active *P*450 systems in bacteria. This was accomplished with the use of the *E. coli* bicistronic vector system mentioned earlier, which was used to produce both *P*450 (1A2) and NADPH-*P*450 reductase together (in bacterial membranes). The second step was development of better methods of generating random libraries. This was accomplished using a modification of a commercially available system, and the new method yields libraries in which base changes are introduced into the so-called substrate recognition sequence (SRS) regions suggested to be important (by alignment and modeling studies) in *P*450s [38,39]. Details of this approach have been presented elsewhere [40]. The third aspect was the development of a suitable screening procedure for analyzing the effects of mutations. Resistance to antibiotics (due to inactivation by *P*450s) was attempted but without success. The approach that was successful involves the use of the genotoxicity screening system described earlier in this report (Fig. 1). Randomized libraries (single-codon) are introduced into *E. coli* and colonies are grown in small cultures (1–2 ml) on a large scale ($\sim$ 600/experiment). Aliquots are spotted onto agar grids and examined for their abilities to grow (in gridded plates) in lactose medium, in the presence of 2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline (MeIQ). Thus, *P*450s with low ability to *N*-hydroxylate MeIQ will yield no or few colonies, and *P*450s with high *P*450 *N*-hydroxylation activity will produce more colonies than the wild-type enzymes. When the qualitative results are obtained, the stocks (stored on a master plate) are recovered and used to produce either DNA (for nucleotide sequence analysis) or bacterial membranes. The membranes are analyzed for *P*450 expression (spectrally) and for steady-state kinetic parameters with model reactions.

The results of the analysis of a set of single-amino acid mutants are shown for 7-ethoxyresorufin and phenacetin *O*-deethylation and *umu* responses to MeIQ (Fig. 7), both in terms of k_{cat} and the ratio k_{cat}/K_m, the best measure of enzyme efficiency. Several points should be made. First, K_m values are altered so that different plots are seen for k_{cat} and K_m. For instance, the k_{cat} plot with MeIQ activation shows less variability than the k_{cat}/K_m plot, possibly because the mutants were selected at a relatively high MeIQ concentration. Second, some of the single-residue mutants have enzyme efficiencies 3- to 4-fold greater than wild-type *P*450 1A2. Third, single residue mutants vary 100-fold in terms of their enzyme efficiencies with a single reaction. Finally, the patterns (k_{cat}/K_m) of the mutants vary for the three reactions.

We plan to use this approach to find additional interesting mutants of human *P*450 1A2 and other *P*450s that can be utilized in studies of *P*450 structure–function relationships. However, the available results are also of use in consideration of the significance of amino acid substitutions and genetic polymorphism. Single base changes in the protein coding region can have dramatic effects on catalytic activities. Further, the effect on one reaction may or may not provide insight into the effect on another reaction. For example, the change of Ser-126 to Lys decreased activity towards MeIQ (Fig. 7B). The catalytic effects of such substitutions may have more significance as more information about genetic polymorphism is developed.

5. Nitropyrene metabolism

Nitropolycyclic hydrocarbons are of interest because of their presence in urban air and particularly

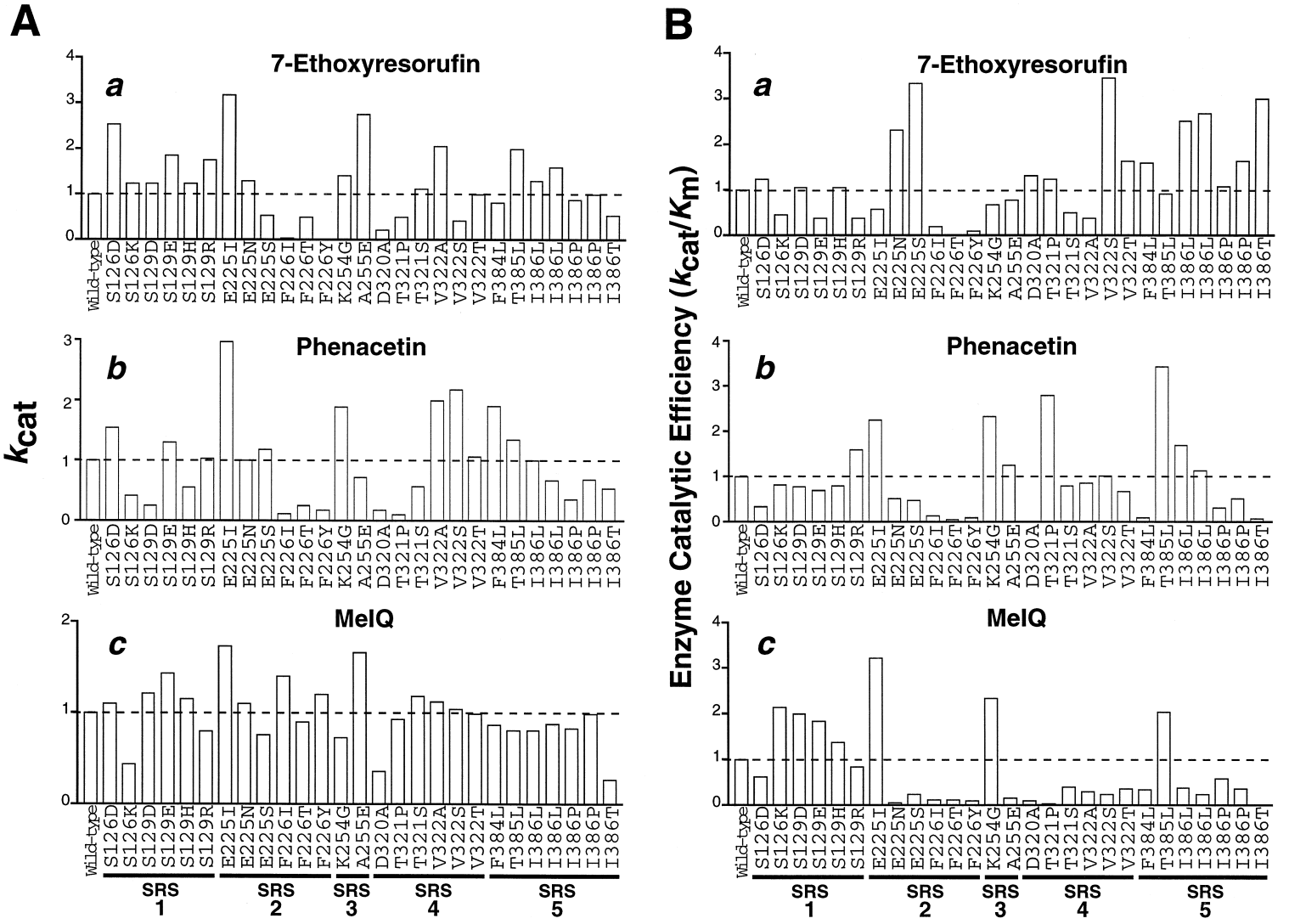

Fig. 7. Comparison of (A) k_{cat} and (B) k_{cat}/K_m for wild-type human *P*450 1A2 and 27 single-amino acid *P*450 1A2 mutants for three marker reactions: (a) 7-ethoxyresorufin and (b) phenacetin *O*-deethylation and (c) MeIQ activation as measured using the *umu* assay [17]. Assays were done with membranes of *E. coli* in which both *P*450 and NADPH-*P*450 reductase had been co-expressed [23]. Results are shown for independent I386L and I386P mutants.

diesel emissions. These combustion products are important because of their genotoxicity and their demonstrated carcinogenicity in rodent models [41]. In addition, we and others have shown directly that motorcycle exhaust can induce levels of *P*450s in rat tissues [42]. Nitropolycyclic hydrocarbons are often considered as a single group of compounds in terms of health effects and risk assessment. However, they differ considerably in their routes of metabolism.

6-Nitrochrysene is a strong liver and lung carcinogen in mice. Activation proceeds via two independent pathways: oxidation to *trans*-1,2-dihydro-1,2-dihydroxy-6-nitrochrysene is catalyzed by *P*450 1A2 in human liver and *P*450 1A1 in human lung; reduction to 6-aminochrysene is catalyzed by *P*450 3A4. Further oxidation of the diol requires *P*450 3A4 [43].

Dinitropyrenes are directly mutagenic in bacterial tester strains, probably due to the facile nitroreduction systems present (which form hydroxylamines). These compounds are inactivated by *P*450 enzymes via further reduction, apparently to amines. 1,3-Dinitropyrene is selectively inactivated by human *P*450 1A2 and 1,6-dinitropyrene is inactivated by *P*450 3A4 [43]. 1,8-Dinitropyrene is inactivated by another *P*450 that has not been identified [44].

Nitropyrenes have also been investigated. 1-Nitropyrene and especially 4-nitropyrene are abundant in diesel exhaust. These compounds were found to be oxidized largely by *P*450 3A4, and *P*450 1A2 made little contribution [45].

Collectively, these studies show that nitropolycyclic hydrocarbons constitute a complex group of chemicals with very different metabolisms, particularly with regard to individual enzymes involved. To a large extent, these compounds must be considered individually and with rather empirical approaches. However, many of the most critical questions about the health effects of these compounds involve issues of mixtures and a need exists for development of rational scientific strategies for dealing with the materials.

Acknowledgements

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