0090-9556/05/3312-1837–1844$20.00
DRUG METABOLISM AND DISPOSITION
DMD 33:1837–1844, 2005

Vol. 33, No. 12
6031/3063281
Printed in U.S.A.

OXIDATION OF CAFFEINE BY CYP1A2: ISOTOPE EFFECTS AND METABOLIC SWITCHING

Kelly A. Regal,[1] Kent L. Kunze, Raimund M. Peter,[2] and Sidney D. Nelson

Department of Medicinal Chemistry, University of Washington, Seattle, Washington

Received June 14, 2005; accepted August 31, 2005

ABSTRACT:

Caffeine (1,3,7-trimethylxanthine) has previously been shown to undergo metabolic switching in vivo when the N-1 or the N-7 methyl groups were trideuteromethylated [Horning et al. (1976) *Proceedings of the Second International Conference on Stable Isotopes*, pp 41–54]. We have examined the effect of replacing the N-3 methyl group with a trideuteromethyl group. The corresponding isotope effects can then be used to distinguish the kinetic mechanism by which four primary metabolites can be formed from one substrate by one cytochrome P450 (P450). We have synthesized 3-CD_3-caffeine and 3-CD_3-7-CD_3-caffeine as well as trideuteromethylated analogs of each of the in vitro metabolites formed by cytochrome P4501A2. The observed competitive isotope effects for the metabolites, which do not result from deuterium abstraction (theobromine, theophylline), demonstrate that the nondissociative mechanism applies to caffeine metabolism by cytochrome P4501A2. Thus, there must be equilibration of the kinetically distinguishable activated P450-substrate complexes at rates competitive with hydrogen abstraction. The true isotope effects for the N-3 demethylation of caffeine were derived from the ratios of the amount of paraxanthine relative to the amount of theobromine or theophylline. The resultant ratios indicate that these isotope effects are essentially intrinsic. Observation of the isotope effects on N-3 demethylation was facilitated by branching to the minor in vitro metabolites as well as water formation. Product release is not rate-limiting for this system.

Cytochrome P4501A2 (CYP1A2) is estimated to be responsible for 90% of the primary metabolism of caffeine in humans (Tassaneeyakul et al., 1994) and is believed to be largely responsible for formation of the major metabolite, paraxanthine (PX), in vivo. The four in vitro metabolites formed by human CYP1A2 are PX (80%), theobromine (TB; 11%), theophylline (TP; 4%) and 1,3,7-trimethyluric acid (TMU; 1%) (Fig. 1; Gu et al., 1992). Presumably, multiple binding orientations exist within the active site of CYP1A2 since the entire periphery of caffeine is available for metabolism.

Experimental evidence suggests that when metabolically susceptible hydrogen(s) are replaced with deuterium atoms, there can be a decrease in the oxidation rate at the labeled site with no concomitant change in the overall extent of metabolism (Harada et al., 1984; Atkins and Sligar, 1986; Jones et al., 1986). Thus, the net effect of deuteration is an increased rate of formation of one or all of the alternate metabolites, a phenomenon known as "isotopically sensitive branching" or "metabolic switching." On the basis of studies that exhibited classical metabolic switching (Harada et al., 1984), it was hypothesized that switching occurs at the level of the activated P450 or "EOS complex," and not earlier in the catalytic cycle. Theoretical studies have supported this concept (Korzekwa et al., 1989; Nelson and Trager, 2003). Hence, deuterium-induced metabolic switching indicates that isomeric EOS complexes can interchange at rates competitive with product formation.

There are three mechanisms that can account for formation of multiple metabolites from one P450 (Gillette et al., 1994). The three mechanisms are distinguished on the basis of the fate of the activated enzyme-substrate complexes. In the "nondissociative mechanism," the substrate adopts multiple conformations within the active site after formation of the perferryl species. Interconversion rates of the EOS complexes are competitive with the rates of hydrogen abstraction and breakdown to the respective ES complexes and water. In the "dissociative mechanism," the substrate dissociates from the activated enzyme, reassociates in either a new orientation or the original one, and is then oxidized by the original perferryl species. In this scenario, the

This research was supported by National Institute of Health Grants R01 GM25418 and P01 GM32165 (S.D.N. and K.L.K.), the University of Washington-National Institute of Environmental Health Sciences (NIEHS)-sponsored Center for Ecogenetics and Environmental Health: NIEHS P30ES07033, National Research Award in Pharmacological Sciences GM07750, and a Hope Barnes Fellowship (K.A.R.).

[1] Current affiliation: Amgen, Thousand Oaks, California.

[2] Current affiliation: AstraZeneca, Department of Drug Metabolism and Pharmacokinetics, Macclesfield, United Kingdom.

Article, publication date, and citation information can be found at http://dmd.aspetjournals.org.

doi:10.1124/dmd.105.006031.

ABBREVIATIONS: PX, paraxanthine or 1,7-dimethylxanthine; TB, theobromine or 3,7-dimethylxanthine; TP, theophylline or 1,3-dimethylxanthine; TMU, 1,3,7-trimethyluric acid; P450, cytochrome P450; d_0, commercially available d_0 compounds ($^2H_0$, nondeuterated); d_3, trideuteromethyl functionality (CD_3; $^2H_3$); d_6, two trideuteromethyl functionalities ($^2H_6$); EOS, the active oxygen intermediate of cytochrome P450 complexed to substrate; ES, P450-substrate complex at the beginning of the catalytic cycle; GC-MS, gas chromatography-mass spectrometry; HPLC, high-performance liquid chromatography; DMF, dimethylformamide; HL103, human liver microsomes high in CYP1A2 content; ^Dk, k_H/k_D or the intrinsic isotope effect; P, primary isotope effect; PS^2, product of one primary and two secondary isotope effects; S, secondary isotope effect; ^DV, $(V_{max})_H/(V_{max})_D$; $^DV/K$, $(V_{max}/K_m)_H/(V_{max}/K_m)_D$.

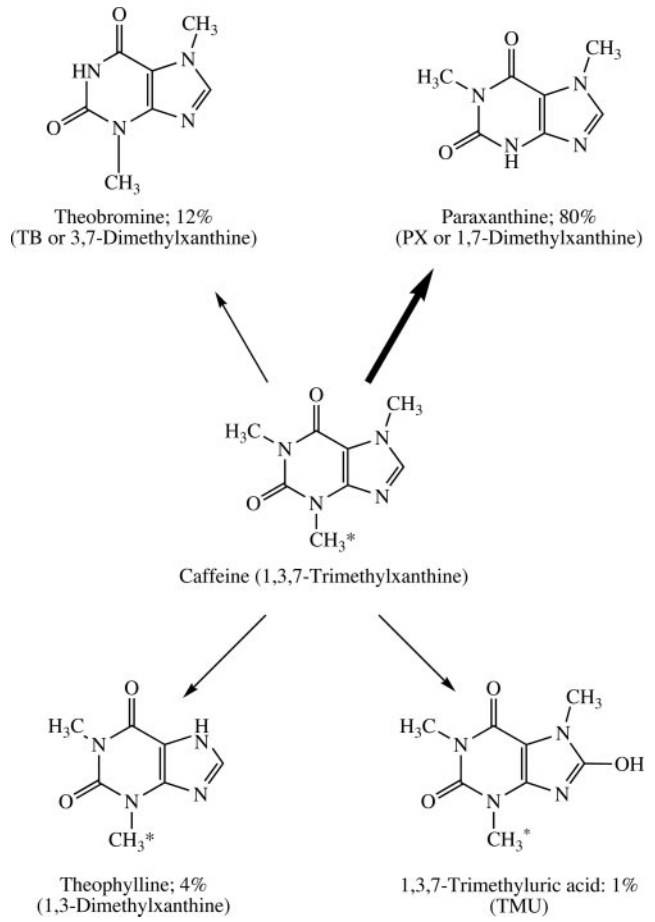


FIG. 1. In vitro metabolites of caffeine formed by human CYP1A2. * indicates site of deuterium label on the trideuteromethylated caffeine (3-CD_3-caffeine).

rates for dissociation and reassociation must be competitive with those for hydrogen atom abstraction and water formation. In the "parallel mechanism," the orientations of substrate within the active site are predetermined in the ES complexes and are fixed throughout the oxidation of the substrate; i.e., the rates for interconversion or dissociation/reassociation are zero. Figure 2 gives a simplified composite of the three potential kinetic mechanisms with caffeine as the substrate.

As shown in Table 1 and previously presented in the literature (Darbyshire et al., 1994; Gillette et al., 1994; Ebner et al., 1995), it is possible to use a series of competitive and noncompetitive isotope effect experiments to determine the presence or absence of metabolic switching as well as differentiate between the three mechanisms for multiple metabolite formation. In a competitive experiment, equimolar mixtures of both the unlabeled substrate and its isotopically labeled analog are used, whereas in a noncompetitive experiment, either the unlabeled substrate or an equimolar concentration of the isotopically labeled analog are incubated separately. Examples in the literature include the CYP2C11-catalyzed metabolism of testosterone and the CYP1A2-mediated catalysis of phenacetin, which follow the dissociative mechanism (Darbyshire et al., 1994; Yun et al., 2000), as well as the CYP2D6-catalyzed turnover of sparteine, which follows the nondissociative mechanism (Ebner et al., 1995). Metabolic switching with caffeine has been previously suggested in vivo (Horning et al., 1976). However, product ratios for only two of the four primary metabolites were published and there was no indication of metabolite formation rates. To facilitate the detection of small changes in the rates of caffeine metabolite formation, a stable isotope-dilution GC-MS assay was developed (Regal et al., 1998). The goal of these experiments was to look for evidence of metabolic switching to TB, TP, and TMU formation upon replacement of the N-3 methyl group of caffeine with a trideuteromethyl group. Competitive and noncompetitive experiments were carried out in an attempt to ascertain the mechanism leading to the formation of the four primary metabolites. Unfortunately, we were unable to quantitate TMU formation, but this proved to be unnecessary. We now present evidence that CYP1A2-catalyzed turnover of caffeine proceeds via a nondissociative mechanism and does not exhibit classical metabolic switching, in which a decrease in the rate of metabolism caused by deuterium substitution for hydrogen at a particular site leads to an equivalent increase in the rate of formation of other metabolites of the deuterium-labeled substrate.

Materials and Methods

Chemicals. Caffeine was purchased from Sigma-Aldrich (St. Louis, MO) and further purified by preparatory HPLC to remove trace amounts of contaminating xanthines. 2-^{13}C-1,3-$^{15}N_2$-caffeine (Cambridge Isotope Laboratories, Andover, MA) was purified in a similar manner. The components for the NADPH regeneration system ($NADP^+$, glucose 6-phosphate, glucose-6-phosphate dehydrogenase) were obtained from Roche Diagnostics (Indianapolis, IN). HyQCCM-3 medium was purchased from HyClone Laboratories (Logan, UT). Microsomal preparations of human lymphoblast-expressed CYP1A2 were obtained from BD Gentest (Woburn, MA). Deuteromethyliodide (C^2H_3I or CD_3I; % d_3 = 99%; Aldrich Chemical Co., Milwaukee, WI) was stored at −20°C, to avoid excessive evaporation. PX and sodium cholate were obtained from Fluka (Buchs, Switzerland). Dimethylformamide (DMF) was stirred with potassium hydroxide, vacuum distilled from calcium oxide, and stored over molecular sieves (4 Å). Reagent-grade potassium carbonate (K_2CO_3) was dried for 16 h in a vacuum oven (>100°C). All other chemicals and solvents were reagent grade.

Instrumentation. Reaction monitoring and reverse phase preparative purifications were performed on an HP 1090 Series IIL liquid chromatograph equipped with a DR5 ternary solvent delivery system, a temperature-controlled autoinjector and column compartment, a built-in diode array detector Series II, and a DOS system control unit (Hewlett Packard, Palo Alto, CA). Normal phase chromatography was performed on an LKB model 2152-2SD dual-pump instrument equipped with an LKB model 2151 variable-wavelength detector. NMR measurements were performed on a Varian VXR-300 FT-NMR (Varian, Inc., Palo Alto, CA).

Deuterium incorporation measurements were carried out on a Micromass 7070H GC-MS apparatus (Waters, Milford, MA). Other GC-MS analyses were performed on a Micromass Trio 2000 quadrupole mass spectrometer, fitted with a Hewlett-Packard 5890 Series II gas chromatograph. A BPX5 fused-silica capillary GC column [30 m × 0.32 mm i.d., 0.25-μm film thickness (5% phenyl polysilphenylene-siloxane); SGE, Austin, TX] was used to separate metabolites. The HPLC, GC, and MS conditions have been published previously (Regal et al., 1998).

Synthesis. 3-CD_3-Caffeine (d_3-caffeine) was synthesized by alkylating PX with CD_3I, in the presence of K_2CO_3 and DMF, as previously described for 3-CD_3-TP (Ebner et al., 1995). 3-CD_3-7-CD_3-Caffeine (d_6-caffeine) was prepared by exposing 1-methylxanthine in a similar manner. After evaporation of the DMF, preparative normal-phase HPLC [Whatman Partsil 10 column (Fisher Scientific Co., Pittsburgh, PA); 500 × 9.4 mm; 5 μm] was used to purify both products. Labeled substrate, purified from contaminant xanthines in a mobile phase of 5% isopropanol/95% chloroform, eluted at approximately 22 min. Products were detected by monitoring at 272 nm, and both labeled compounds were shown to have the same retention time and UV spectrum as commercial caffeine (d_0). The appropriate methyl resonances were shown to be missing by NMR (Berlioz et al., 1987). Commercial, unlabeled caffeine and 2-^{13}C-1,3-$^{15}N_2$-caffeine were purified in a similar fashion. Based on mass spectral analysis, the $^2H_3$ compound was 99.0% of the $^2H_3$ isotopomer, 0.7% $^2H_2$, 0% $^2H_1$, and 0.3% $^2H_0$. The $^2H_6$ compound was 98.0% of the $^2H_6$ isotopomer, 1.2% $^2H_5$, 0% $^2H_4$, 0.6% $^2H_3$, 0% $^2H_2$, 0% $^2H_1$, and 0.2% $^2H_0$.

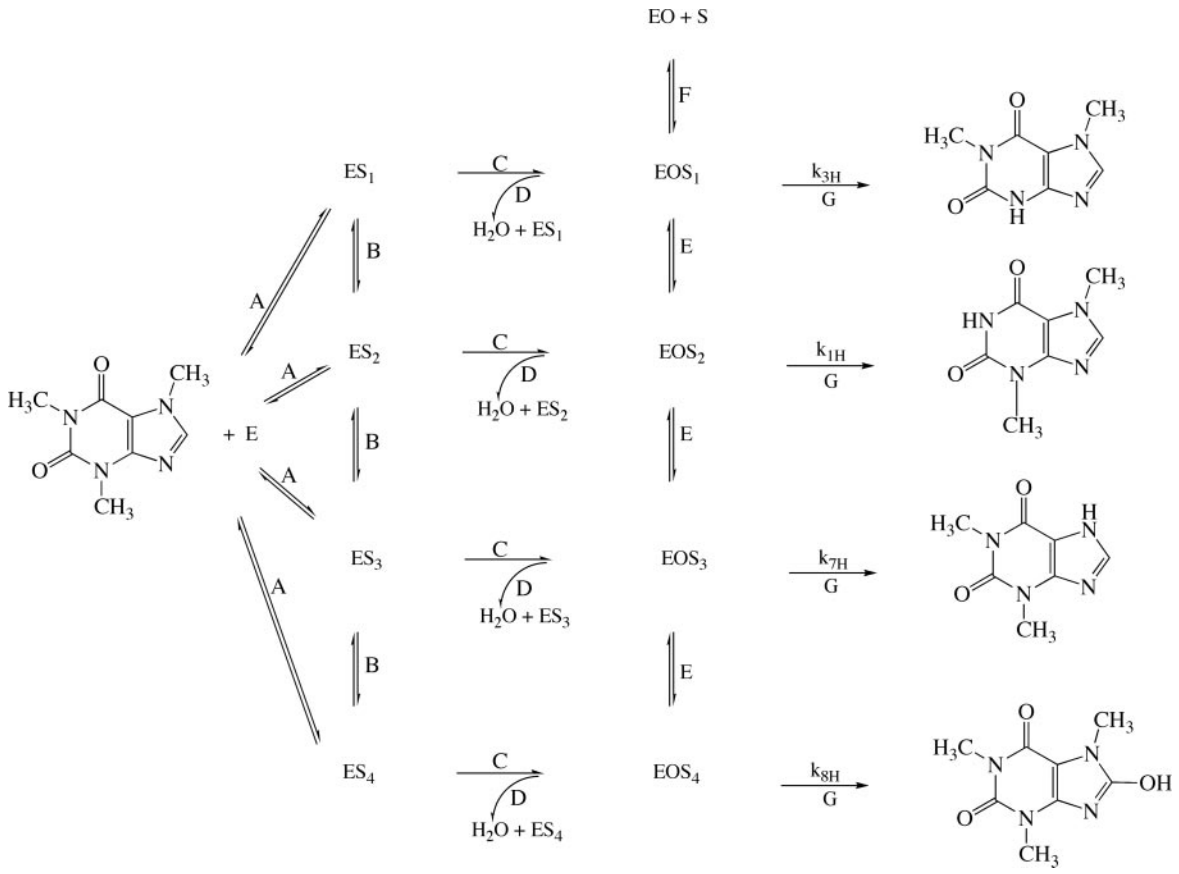


FIG. 2. Potential mechanisms for multiple metabolite formation from caffeine by cytochrome P4501A2. "k_{xh}" represents the rate constant for metabolism at the *x* position of caffeine. Steps are labeled as follows: A, equilibration of the initial binding of substrate to the P450; B, equilibration of substrate orientation within the enzyme active site; C, activation of the P450; D, release of water and regeneration of the enzyme-substrate complex; E, equilibration of substrate orientation within the activated enzyme active site; F, equilibration of substrate on and off the activated P450; and G, oxidation of the substrate and release of product from the P450. The parallel mechanism includes steps A, B, C, and G. The nondissociative mechanism includes all steps except step F. The dissociative mechanism includes all steps, A to G.

TABLE 1

Expected deuterium isotope effects produced by different kinetic mechanisms on nondeuterium abstraction pathways

In this study, nondeuterium abstraction pathways include the formation of TB, TP, and TMU. Values are from Gillette et al. (1994).

Type of Experiment	Magnitude of $^{D}V/K$		
	Nondissociative Mechanism	Dissociative Mechanism	Parallel Mechanism
Competitive	$<1.0^a$	1.0	1.0
Noncompetitive	<1.0	<1 to >1	1.0

[a] An isotope effect of 1.0 indicates no effect. An inverse isotope effect is <1. A normal isotope effect is >1.

Synthesis of the trideuteromethylated internal standards used after noncompetitive incubations with commercial caffeine (d_0) and CYP1A2 has been described previously (Regal et al., 1998). When 3-CD_3-caffeine was the substrate, commercially available metabolites (TB, TP, TMU) were used as the internal standards. Quantification of PX in the noncompetitive experiments always utilized the corresponding d_3 analog since metabolism of both labeled (d_3) and unlabeled caffeine results in d_0-PX formation. The d_0 internal standards were purified as described for their d_3 analogs. Table 2 summarizes the substrates used, the expected metabolites, and the corresponding internal standards, when appropriate.

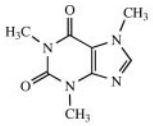
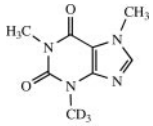
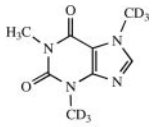
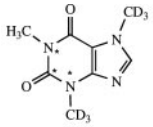
CYP1A2 Expression and Membrane Fractions. Recombinant virus containing the human *CYP1A2* gene was used to infect *Taenia nigricans* HSB 1–4 insect cells on 150 × 25 mm plates, grown in 25 ml of HyQCCM-3 medium, supplemented with 7% fetal bovine serum. Cells were infected at a multiplicity of infection of at least 10. After 24 h, 25 μl of sterile filtered 3 mg/ml hemin chloride in 0.1 M ammonium hydroxide was added. Cells were pelleted at 72 h after infection, resuspended in 50 mM potassium phosphate, pH 7.4, 20% glycerol, 1 mM EDTA, and 1 mM dithiothreitol, and stored at 70°C until further use. Membrane fractions were prepared according to described methods (Haining et al., 1997).

Addition of Reductase and Cytochrome b_5 to CYP1A2 Preparations. The cytochrome b_5 and reductase were expressed and purified by standard procedures (Miyata et al., 1989; Shen et al., 1989). The initial concentration of the expressed CYP1A2 membrane preparation was at least 4 μM, to avoid the addition of excessive volumes to the incubations. For every equivalent of expressed CYP1A2 (membrane preparations) to be used, three equivalents of rat reductase were added and the mixture incubated in a 27°C water bath for 10 min. This was followed by the addition of 10 mM phosphate buffer (pH 7.4) and 0.3% sodium cholate. Cholate is believed to facilitate incorporation of the reductase into the membrane since incubations without it resulted in low levels of caffeine turnover. After another incubation period of 10 min at 27°C, one equivalent of cytochrome b_5 was added, followed by another 10 min at 27°C. The final concentration of cholate within the actual incubations was less than 0.06%. Supplementation of the commercial CYP1A2 microsomes involved the addition of supplemental reductase and cytochrome b_5, in a comparable fashion, minus the addition of the phosphate buffer and the cholate. Both sources of CYP1A2 required supplemental reductase to see the expected turnover numbers (1 min^{-1}; Gu et al., 1992). For each experiment using expressed CYP1A2, one batch of enzyme was prepared, followed by distribution to the individual incubations, allowing the assumption that the enzyme is identical throughout each experiment.

Incubations. Human liver microsomes rich in CYP1A2 content (HL103) were prepared as previously described (Raucy and Lasker, 1991). Cytochrome P450 content was measured by the method of Omura and Sato (1964), in the

TABLE 2

Composite of substrates, expected metabolites, and internal standards

Substrate	Type of Experiment	Metabolites	Internal Standards (Noncompetitive Experiment Only)
 Caffeine	Both competitive and noncompetitive	All d_0	All d_3
 3-CD_3-Caffeine (d_3)	Both competitive and noncompetitive	d_0 PX d_3 TB d_3 TP d_3 TMU	d_3 PX d_0 TB d_0 TP d_0 TMU
 3,7-CD_3-Caffeine (d_6)	Competitive only	d_3 PX d_6 TB d_3 TP d_6 TMU	N/A
 2-^{13}C-1,3-$^{15}N_2$-Caffeine	Competitive only	All 2-^{13}C-1,3-$^{15}N_2$- labeled	N/A

presence of α-naphthoflavone, and protein content was determined by the bicinchoninic acid method (Smith et al., 1985). Expressed, microsomal CYP1A2 was purchased from commercial sources until an expression system in insect cells was established. Membrane fractions were prepared and supplemented in one tube per experiment, followed by the enzyme being aliquoted to the individual incubations. This ensured that the enzyme was identical in each incubation.

The general method for the incubations with caffeine as well as the separation and quantification of the metabolites has been previously published (Regal et al., 1998). Metabolite formation was previously shown to be linear with respect to time and protein content. In the presence of expressed CYP1A2 membrane preparations, it was necessary to add superoxide dismutase and catalase, to obtain linearity over enough time to form sufficient amounts of metabolites. Competitive isotope effects ($^{D}V/K$) for the formation of TB, TP, and TMU were measured in incubations with a 1:1 ratio of d_0:d_3 caffeine. A 1:1 ratio of either d_3:^{13}C, $^{15}N_2$ or d_0:d_6 caffeine was used for the measurement of competitive isotope effects on PX formation. Membrane preparations of CYP1A2 were the enzyme source in the competitive experiments (100 pmol of enzyme per incubation; final [P450] = 0.2 μM) and control incubations with only one substrate (d_0) were performed at the same time. The ratio of CYP1A2 to rat reductase to human cytochrome b_5 was 1:3:1. The residual amounts of glycerol and cholate did not affect the CYP1A2-catalyzed metabolism of caffeine or its analogs. Noncompetitive incubations (^{D}V and $^{D}V/K$) included a single substrate (d_0 or d_3) and commercially expressed CYP1A2 microsomes (d_0, 100 pmol of enzyme; d_3, 200 pmol of enzyme; final [P450] = 0.2 or 0.4 μM, respectively). The substrates, expected metabolites, and internal standards, when applicable, are summarized in Table 2. Metabolites were separated by reverse phase HPLC before derivatization and quantification by GC-MS (Regal et al., 1998).

Results

Supplementation of Expressed CYP1A2 with Reductase and Cytochrome b_5. Due to low turnover numbers in the presence of the commercially expressed CYP1A2 microsomes, the effects of supplemental reductase and cytochrome b_5 were examined. Turnover was enhanced by as much as 10-fold (Table 3). Hence, the remaining incubations with expressed CYP1A2 were supplemented with rat reductase and human cytochrome b_5, in a ratio of 1:3:1 P450/reductase/b_5.

TABLE 3

Effect of reductase and cytochrome b_5 on CYP1A2-catalyzed caffeine turnover

CYP1A2 microsomes (BD Gentest) were preincubated with varying amounts of reductase and b_5, in the presence of 5 mM d_0-caffeine, in duplicate (described under *Materials and Methods*). PX formation was quantitated by HPLC.

Enzyme Components	PX Peak Area
	AU × *s*
No additions	N.D.[a]
1:1 Reductase:P450	0.56
1:1:1 Reductase:b_5:P450	0.73
5:1 Reductase:P450	1.02[b]

AU, absorbance units; N.D., not detected by HPLC.
[a] When quantified by GC-MS, the turnover number was 0.2 min^{-1} (n = 3).
[b] When quantified by GC-MS, the turnover number was 2.0 min^{-1} (n = 3).

The commercially expressed CYP1A2 was found to be more stable than the expressed CYP1A2 membrane preparations, based on the linearity profiles of metabolite formed versus time (data not shown). Thus, shorter incubation times were used with the latter system as well as incorporation of superoxide dismutase and catalase into the incubations, which provided further stabilization. Presumably, the membrane composition of the two sources of expressed 1A2 was different, due to differences in the expression systems (human lymphoblastoid cells versus insect cells). The supplemental reductase and cytochrome b_5 were readily incorporated into the commercial, lymphoblastoid microsomes. However, membrane preparations of the CYP1A2 expressed in insect cells required the addition of sodium cholate to see comparable turnover numbers.

Competitive Experiments. Competitive isotope effect experiments were performed to determine the potential involvement of the nondissociative mechanism in the CYP1A2-catalyzed metabolism of

TABLE 4

Competitive experiments: isotope effect on metabolite ratio

Membrane preparations of expressed CYP1A2 were preincubated with reductase and b_5, followed by incubations with a 1:1 ratio of either d_0:d_6 caffeine (PX formation) or d_0:d_3 caffeine (TB and TP formation). The total concentration of caffeine was 5 mM. Conditions are described under *Materials and Methods*. Estimations of isotope effects were determined from the ratio of each d_0 metabolite to the corresponding d_3 metabolite ± S.D. ($n = 3$).

Site of Metabolism	$^{D}V/K$[a]
N-3 (PX)	7.0 ± 0.10
N-1 (TB)	0.58 ± 0.07[b]
N-7 (TP)	0.65 ± 0.02[b]

[a] Effects are significantly different from 1.0 ($P < 0.01$).
[b] Differences are statistically insignificant ($0.05 < P < 0.10$).

caffeine. For the determination of the isotope effects associated with PX formation, incubations with a 1:1 mixture of d_0- and d_6-caffeine resulted in isotope effects which were large and normal ($^{D}V/K = 7.0$; Table 4). A similar magnitude was also seen in the presence of human liver microsomes (HL103; data not shown). Since there was concern that the second CD_3 functionality might be affecting this magnitude, the experiment was repeated with a 1:1 mixture of d_3- and ^{13}C, $^{15}N_2$-caffeine and the effect on PX formation was the same (data not shown). For the metabolites that did not arise from pathways involving deuterium abstraction (TB, TP), incubations with 1:1 mixtures of d_0- and d_3-caffeine resulted in inverse isotope effects ($^{D}V/K \approx 0.6$; Table 4). Comparison of these latter effects with Table 1 indicated that the nondissociative mechanism was responsible for the formation of PX, TB, and TP from caffeine. Thus, the caffeine molecule is able to perform orientational changes within the substrate binding pocket of the activated perferryl species of CYP1A2, resulting in kinetically distinguishable EOS complexes. The rate constants for the orientational changes of caffeine within the active site of CYP1A2 must be comparable in magnitude (if not larger) to the rate constant for hydrogen abstraction, i.e., these EOS species must be relatively stable.

Due to the selection of d_3 analogs to be used as internal standards for the d_0 metabolites and vice versa, competitive experiments that involved coincubation with d_0 and d_3 or d_6 substrates only allowed the determination of metabolite ratios. In addition, the mixture of d_0 and d_3 substrates was only useful for the measurement of metabolite ratios for TB, TP, and TMU formation. Mixtures of d_0 and d_6 or d_3 and ^{13}C, $^{15}N_2$ substrates were necessary to determine the metabolite ratios for PX. For these reasons, competitive isotope effects on total metabolism were unattainable.

Noncompetitive Experiments. The isotope effects for CYP1A2-catalyzed N-3 demethylation of caffeine was assessed in the presence of HL103 at saturating substrate concentrations (5 mM). The isotope effect on PX formation was large and normal ($^{D}V = 8.5$; data not shown). In the presence of commercially expressed CYP1A2, the noncompetitive isotope effects for PX formation were again large and normal ($^{D}V = 10.8$; Table 5). The large magnitude of these isotope effects indicates that the intrinsic isotope effect (^{D}k) is also substantial. In addition, there was a small amount of switching to TB formation ($^{D}V = 0.7$) but not enough to cover the loss in total metabolism ($^{D}V_{total} = 3.4$).

Noncompetitive experiments at sub-K_m concentrations of caffeine (100 μM) revealed normal isotope effects for PX formation ($^{D}V/K = 7.7$; Table 5), further supporting the fact that the intrinsic isotope effect is large and normal. Inverse isotope effects for TB formation were observed ($^{D}V/K \approx 0.65$). The results for TP formation were complicated in that there was an inseparable, nonxanthine, derivatization side-product. This contaminant contributed significantly to the peak area of d_0-TP at low formation rates, i.e., in the noncompetitive experiments, leading to false, "normal" isotope effects (Table 5). Peak areas for the d_0-TP were significantly larger in the competitive experiments and, hence, the interference due to this contaminant was minimal. As demonstrated in the competitive experiment, the TP isotope effect should have been an inverse effect ($^{D}V/K \approx 0.6$; Table 4). Despite this, the results for TB further support the conclusion that the nondissociative mechanism is involved in caffeine metabolism. Furthermore, this system did not exhibit classical metabolic switching in that there was a significant isotope effect on total metabolism ($^{D}V/K_{total} = 2.9$; Table 5), which is indicative of water formation as a result of reduction of the perferryl species (Gillette et al., 1994).

Discussion

Competitive isotope effect experiments readily provide evidence for or against the involvement of the nondissociative mechanism (Gillette et al., 1994; Table 1). Such experiments are independent of substrate concentration and only $^{D}V/K$ values can be evaluated (Nelson and Trager, 2003). In addition, masking factors that result from

TABLE 5

Noncompetitive isotope effects on ratios of formation rates (effects significantly different from 1.0 ($P < 0.01$)

Commercial CYP1A2 microsomes supplemented with reductase and b_5 were the enzyme source and incubations were performed as described under *Materials and Methods*. Turnover numbers are presented as nmol min^{-1} nmol $P450^{-1}$. Estimations of isotope effects were calculated from the mean ± S.D. for each d_0 metabolite ($n = 3$) divided by the mean ± S.D. for each d_3 metabolite ($n = 3$).

Site of Metabolism	d_0-Caffeine	d_3-Caffeine	d_0-Caffeine/d_3-Caffeine
^{D}V ([caffeine] = 5 mM)			
N-3 (PX)	1.01 ± 0.08	0.093 ± 0.007	10.8 ± 1.14[a]
N-1 (TB)	0.133 ± 0.006	0.186 ± 0.001	0.72 ± 0.03[a]
N-7 (TP)	0.081 ± 0.004	0.083 ± 0.006	0.98 ± 0.01[b]
C-8 (TMU)	N.D.	N.D.	
Total metabolism	1.22 ± 0.08	0.362 ± 0.009	3.37 ± 0.23[a]
Ratio (PX/TB/TP)	12.5: 1.6: 1.0	1.1: 2.2: 1.0	
$^{D}V/K$ ([caffeine] = 100 μM)			
N-3 (PX)	0.107 ± 0.002	0.014 ± 0.002	7.69 ± 0.97[a]
N-1 (TB)	0.016 ± 0.001	0.024 ± 0.001	0.67 ± 0.07[a]
N-7 (TP)	0.008 ± 0.000	0.007 ± 0.001	1.16 ± 0.08[c]
C-8 (TMU)	N.D.	N.D.	
Total metabolism	0.131 ± 0.002	0.045 ± 0.002	2.93 ± 0.06[b]
Ratio (PX/TB/TP)	13.4: 2.0: 1.0	2.0: 3.4: 1.0	

N.D., not detected.
[a] Ratios are significantly different from 1.0 ($P < 0.005$).
[b] $0.1 < P < 0.25$.
[c] $0.025 < P < 0.05$.

the rate constants prior to the isotopically sensitive step are cancelled by the presence of both substrates. Thus, there are fewer rate constants that affect the observable isotope effects. As mentioned above, the competitive isotope effects for TB and TP (Table 4) led to the conclusion that the nondissociative mechanism accounts for metabolite formation from caffeine.

There are several ways to approach the calculation of the intrinsic isotope effect (^{D}k). Whereas neither intramolecular isotope effects (Iyer et al., 1997; Nelson and Trager, 2003) nor a comparison between deuterium and tritium isotope effects (Northrop, 1977) were applicable, the true isotope effects can be directly obtained from the product ratios for the protio and deuterio substrates, as shown below (Atkinson et al., 1994; Nelson and Trager, 2003).

$$[(P1_H)/(P2_H)]/[(P1_D)/(P2_D)] = [(P1_H)/(P1_D)]/[(P2_H)/(P2_D)] = {}^{D}k \quad (1)$$

P1 and P2 represent two metabolites formed from the same substrate. The resultant numbers for PX relative to TB and TP indicate that the true competitive isotope effects fall between 10.8 and 12.1, respectively. Since the labeled substrate has three carbon-deuterium bonds at the main site of oxidation, and the mechanism of *N*-dealkylation involves the abstraction of a single hydrogen (Miwa et al., 1983), these isotope effects are the product of one primary and two secondary isotope effects (PS^2; Atkinson et al., 1994). If we assume that the secondary isotope effect is approximately 1.2, this would indicate that the primary isotope effect falls between 7.5 and 8.4.

The theoretical maximum for an intrinsic or primary isotope effect is approximately 9 (Bell, 1974; Shea et al., 1983). Combined primary and secondary isotope effects of 11.8 to 13.2 are considered to be the maximal effects that could be observed for P450-catalyzed oxidative cleavage of any carbon-hydrogen bond (Jones and Trager, 1987; Jones et al., 1990; Atkinson et al., 1994). The similarity between the published and the estimated isotope effects for caffeine N-3 demethylation implies that there is a highly symmetrical transition state, one that is neither reactant- nor product-like, and that there is extensive C–H bond stretching within this transition state. It also implies that the bond being broken and the bond being formed have similar energies (Karki et al., 1995).

To provide additional verification of the mechanism as well as further characterization of the caffeine-CYP1A2 interaction immediately before oxidation, noncompetitive experiments were performed. Use of the isotope effects obtained for TB and TP under V_{max} conditions (Table 5) leads to a value between 11 and 15 for the true isotope effects on PX formation (PS^2; Atkinson et al., 1994). Similar results were obtained from the noncompetitive experiments at sub-K_m concentrations of caffeine ($PS^2 = 11.5$; $^{D}k \approx 8.0$; Table 5). The contribution of the N-7 demethylated metabolite (TP) was inaccurate and ignored, due to the inseparable contaminant mentioned earlier. Thus, there is general agreement in the magnitude of the combined as well as the intrinsic isotope effects between the competitive and noncompetitive experiments.

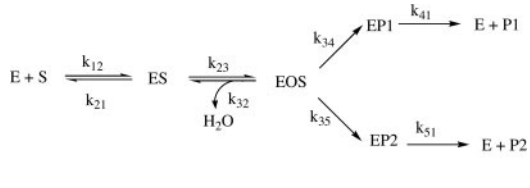
According to Northrop (Northrop, 1977), and as shown for the following simple kinetic scheme, expression of the intrinsic isotope effect ($k_{3H}/k_{3D} = {}^{D}k$) within the noncompetitive isotope effects on V_{max} (^{D}V) depends on the ratio of catalysis to product release (k_{3H}/k_{3D}).

$$E \underset{k_2}{\overset{k_1 S}{\rightleftharpoons}} ES \xrightarrow{k_3} EP \xrightarrow{k_5} E + P$$
$$^{D}V = \frac{V_H}{V_D} = \frac{k_{3H}/k_{3D} + k_{3H}/k_5}{1 + k_{3H}/k_5} \quad (2)$$

This "ratio of catalysis" is a masking factor in that its magnitude determines the ability to either partially or completely observe the intrinsic isotope effect. Arguments in the literature indicate that it is possible to ignore the potential masking by equilibrium constants associated with the catalytic cycle of the P450s (Iyer et al., 1997). Due to the magnitude of the true noncompetitive isotope effects on PX formation ($PS^2 \geq 11$), the conclusion was drawn that product release is not rate-limiting for the CYP1A2-catalyzed metabolism of caffeine.

Interestingly, the noncompetitive sub-K_m isotope effects on total turnover were greater than one ($^{D}V/K_{total} = 2.9$; Table 5). In other words, the decrease in the rate of PX formation was not offset by a commensurate increase in the rates of formation of the other metabolites. In the absence of new metabolite(s), the lack of a compensatory switch indicates significant water formation (Gillette et al., 1994), whereas the overall formation of water would not be expected to change significantly (Atkins and Sligar, 1986; Atkins and Sligar, 1988). No new metabolites were detected. Yun et al. (2001) have shown significant water formation by human CYP1A2; thus, this particular attribute does not appear to be caffeine-specific.

Formation of multiple metabolites, as a result of rapid equilibration of the multiple EOS complexes, allows the observation of isotope effects within $^{D}V/K$ by providing a siphon for the excess EOS_D (Jones et al., 1986; Korzekwa et al., 1989; Higgins et al., 1998; Nelson and Trager, 2003). Such a branch point leads to the "unmasking" of the isotope effect. Partial reduction of the activated perferryl species to water can have the same effect (Gillette et al., 1994; Higgins et al., 1998). Thus, water formation as well as minor switching to TB and TP facilitated the observation of isotope effects on ^{D}V and $^{D}V/K$. A representative branched mechanism is shown, as well as the equation explaining the relationship between the rate constants and the observable isotope effects on V/K (Gillette et al., 1994; Nelson and Trager, 2003; eq. 3). This mechanism presents water formation as a branch-point, i.e., the associated rate constant (k_{32}) appears in the denominator of the masking factor, increasing the magnitude of the observable isotope effects.



$$^{D}V/K_{P1} = \frac{k_{34H/3D} + \dfrac{k_{34H}(1/k_{23} + 1/k_{21})}{k_{35}(1/k_{23} + 1/k_{21}) + k_{32}/k_{23}}}{\dfrac{k_{34H}(1/k_{23} + 1/k_{21})}{k_{35}(1/k_{23} + 1/k_{21}) + k_{32}/k_{23}}} \quad (3)$$

Rapid equilibration among the kinetically distinguishable EOS complexes (presented as one entity above) results in an obvious preference for oxidation at the N-3 position of caffeine. There could be an innate chemical reactivity within caffeine, facilitating the preference of oxidation at the N-3 methyl group. However, preliminary estimations of the change in the heats of formation associated with hydrogen abstraction, resulting in a carbon-centered radical at each of the three methyl groups on caffeine, did not indicate a thermodynamic preference for the N-3 position on caffeine ($\Delta H_{N1} = -26.8$ kcal/mol; $\Delta H_{N3} = -25.9$ kcal/mol; $\Delta H_{N7} = -24.6$ kcal/mol). It is also possible that the preference for N-3 demethylation could be the result of a preferred conformation of caffeine in response to the active site architecture. In fact, it has previously been assumed that caffeine sits perpendicular to the

CYP1A2 active site with the N-3 position closest to the heme (Lewis, 1995; Lozano et al., 1997). Recent NMR studies have provided evidence for caffeine sitting parallel to the heme during the initial interaction with the P450 (Regal and Nelson, 2000). Similar studies with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Modi et al., 1997) and CYP2D6 or lauric acid and CYP102 (Modi et al., 1996) have indicated that altered binding modes which are more consistent with the known sites of oxidation exist after reduction of the enzyme. Thus, it is apparent that substrate movement later in the P450 catalytic cycle plays a role in determining the site(s) of oxidation.

It has been shown that the magnitude of the isotope effects for *N*-dealkylation cannot be used to distinguish between the electron or hydrogen abstraction pathways (Karki et al., 1995). However, amides are believed to go through a hydrogen abstraction mechanism based on the observation that chemical methods for electron abstraction show significantly smaller isotope effects than those seen for amide *N*-dealkylation (Hall et al., 1989). In the few instances examined, relatively large "intrinsic" isotope effects have been observed for the *N*-dealkylation of amides ($^{D}V/K \geq 6$; Hall and Hanzlik, 1990; Constantino et al., 1992). The large isotope effects for the N-3 demethylation of caffeine ($^{D}V/K = 11.5–11.7$) are in agreement with the summation of Hall et al. (1989), that P450-catalyzed *N*-demethylation of amides is associated with a large intrinsic kinetic deuterium isotope effect.

In conclusion, large intermolecular isotope effects for the CYP1A2-catalyzed *N*-3 demethylation of caffeine have been observed. The observed competitive isotope effects for the nondeuterium abstraction pathways (TB, TP) indicate that there is equilibration of the kinetically distinguishable EOS complexes via the nondissociative mechanism, leading to the formation of at least three primary metabolites. This equilibration occurs at comparable (if not faster) rates relative to the rate of hydrogen abstraction and at rates competitive with reduction of the perferryl species to water formation. The true isotope effects for PX formation indicate that hydrogen abstraction proceeds through a symmetrical transition state and that product release is not rate-limiting. Water formation is believed to have facilitated the observation for the isotope effects, in combination with a small amount of switching to TB and TP, by providing a siphon for the excess EOS_D. Because it was a significant branchpoint in the presence of the labeled caffeine, water formation presumably occurs in the presence of unlabeled caffeine.

Acknowledgments. Special thanks to Dr. Charles Kasper (University of Wisconsin-Madison) for the rat reductase expression system, to Dr. R. Kato (Keio University, Tokyo, Japan) for the human cytochrome b_5 cDNA, and to Dr. Frank Gonzalez (National Institutes of Health, Bethesda, MD) for the human CYP1A2 cDNA. The recombinant wild-type CYP1A2 baculovirus was kindly supplied by Drs. R. Haining and A. Rettie (University of Washington, Seattle, WA). The contributions of Dr. Keith Laidig (University of Washington) as a consultant on the computer-generated changes in heats of formation were greatly appreciated.

References

Atkins WM and Sligar SG (1986) Metabolic switching in cytochrome P-450cam: deuterium isotope effects on regiospecificity and the monooxygenase/oxidase ratio. *J Am Chem Soc* **109:**3754–3760.

Atkins WM and Sligar SG (1988) Deuterium isotope effects in norcamphor metabolism by cytochrome P-450cam: kinetic evidence for the two-electron reduction of a high-valent iron-oxo intermediate. *Biochemistry* **27:**1610–1616.

Atkinson JK, Hollenberg PF, Ingold KU, Johnson CC, Le Tadic MH, Newcomb M, and Putt DA (1994) Cytochrome P450-catalyzed hydroxylation of hydrocarbons: kinetic deuterium isotope effects for the hydroxylation of an ultrafast radical clock. *Biochemistry* **33:**10630–10637.

Bell RP (1974) Liversidge Lecture. Recent advances in the study of kinetic hydrogen isotope effects. *Chem Soc Rev* **3:**513–544.

Berlioz C, Falconnet J, Désage M, and Brazier J (1987) Synthesis of 1-C^2H_3 theophylline and 3-C^2H_3 theophylline. *J Labelled Compd Radiopharm* **24:**275–289.

Constantino L, Rosa E, and Iley J (1992) The microsomal demethylation of N,N-dimethylbenzamides. Substituent and kinetic deuterium isotope effects. *Biochem Pharmacol* **44:**651–658.

Darbyshire JF, Gillette JR, Nagata K, and Sugiyama K (1994) Deuterium isotope effects on A-ring and D-ring metabolism of testosterone by CYP2C11: evidence for dissociation of activated enzyme-substrate complexes. *Biochemistry* **33:**2938–2944.

Ebner T, Meese CO, and Eichelbaum M (1995) Mechanism of cytochrome P450 2D6-catalyzed sparteine metabolism in humans. *Mol Pharmacol* **48:**1078–1086.

Gillette JR, Darbyshire JF, and Sugiyama K (1994) Theory for the observed isotope effects on the formation of multiple products by different kinetic mechanisms of cytochrome P450 enzymes. *Biochemistry* **33:**2927–2937.

Gu L, Gonzalez FJ, Kalow W, and Tang BK (1992) Biotransformation of caffeine, paraxanthine, theobromine and theophylline by cDNA-expressed human CYP1A2 and CYP2E1. *Pharmacogenetics* **2:**73–77.

Haining RL, Hunter AP, Sadeque AJ, Philpot RM, and Rettie AE (1997) Baculovirus-mediated expression and purification of human FMO3: catalytic, immunochemical and structural characterization. *Drug Metab Dispos* **25:**790–797.

Hall LR and Hanzlik RP (1990) Kinetic deuterium isotope effects on the N-demethylation of tertiary amides by cytochrome P-450. *J Biol Chem* **265:**12349–12355.

Hall LR, Iwamoto RT, and Hanzlik RP (1989) Electrochemical models for cytochrome P-450. N-demethylation of tertiary amides by anodic oxidation. *J Org Chem* **54:**2446–2451.

Harada N, Miwa GT, Walsh JS, and Lu AY (1984) Kinetic isotope effects on cytochrome P-450-catalyzed oxidation reactions. Evidence for the irreversible formation of an activated oxygen intermediate of cytochrome P-448. *J Biol Chem* **259:**3005–3010.

Higgins L, Bennett GA, Shimoji M, and Jones JP (1998) Evaluation of cytochrome P450 mechanism and kinetics using kinetic deuterium isotope effects. *Biochemistry* **37:**7039–7046.

Horning MG, Haegele KD, Sommer KR, Nowlin J, Stafford M, and Thenot JP (1976) Metabolic switching of drug pathways as a consequence of deuterium substitution, in *Proceedings of the Second International Conference on Stable Isotopes* (Klein ER and Klein PD eds), pp 41–54, CONF-751027, NTIS, Springfield, VA.

Iyer KR, Jones JP, Darbyshire JF, and Trager WF (1997) Intramolecular isotope effects for benzylic hydroxylation of isomeric xylenes and 4,4′-dimethylbiphenyl by cytochrome P450: relationship between distance of methyl groups and masking of the intrinsic isotope effect. *Biochemistry* **36:**7136–7143.

Jones JP, Korzekwa KR, Rettie AE, and Trager WF (1986) Isotopically sensitive branching and its effect on the observed intramolecular isotope effects in cytochrome P-450 catalyzed reactions: a new method for the estimation of intrinsic isotope effects. *J Am Chem Soc* **108:**7074–7078 [Correction (1988) *J Am Chem Soc* **7110:**2018].

Jones JP, Rettie AE, and Trager WF (1990) Intrinsic isotope effects suggest that the reaction coordinate symmetry for the cytochrome P-450 catalyzed hydroxylation of octane is isozyme independent. *J Med Chem* **33:**1242–1246.

Jones JP and Trager WF (1987) The separation of the intramolecular isotope effect for the cytochrome P-450 catalyzed hydroxylation of n-octane into its primary and secondary components. *J Am Chem Soc* **109:**2171–2173 [Correction (1988) *J Am Chem Soc* **2110:**2018].

Karki SB, Dinnocenzo JP, Jones JP, and Korzekwa KR (1995) Mechanism of oxidative amine dealkylation of substituted N,N-dimethylanilines by cytochrome P-450: application of isotope effect profiles. *J Am Chem Soc* **117:**3657–3664.

Korzekwa KR, Trager WF, and Gillette JR (1989) Theory for the observed isotope effects from enzymatic systems that form multiple products via branched reaction pathways: cytochrome P-450. *Biochemistry* **28:**9012–9018.

Lewis DF (1995) Three-dimensional models of human and other mammalian microsomal P450s constructed from an alignment with P450102 (P450bm3). *Xenobiotica* **25:**333–366.

Lozano JJ, Lopez-de-Brinas E, Centeno NB, Guigo R, and Sanz F (1997) Three-dimensional modelling of human cytochrome P450 1A2 and its interaction with caffeine and MeIQ. *J Comput-Aided Mol Des* **11:**395–408.

Miwa GT, Walsh JS, Kedderis GL, and Hollenberg PF (1983) The use of intramolecular isotope effects to distinguish between deprotonation and hydrogen atom abstraction mechanisms in cytochrome P-450- and peroxidase-catalyzed N-demethylation reactions. *J Biol Chem* **258:**14445–14449.

Miyata M, Nagata K, Yamazoe Y, and Kato R (1989) Isolation and characterization of human liver cytochrome b5 cDNA. *Pharmacol Res* **21:**513–520.

Modi S, Gilham DE, Sutcliffe MJ, Lian LY, Primrose WU, Wolf CR, and Roberts GC (1997) 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine as a substrate of cytochrome P450 2D6: allosteric effects of NADPH-cytochrome P450 reductase. *Biochemistry* **36:**4461–4470.

Modi S, Sutcliffe MJ, Primrose WU, Lian LY, and Roberts GC (1996) The catalytic mechanism of cytochrome P450 BM3 involves a 6 A movement of the bound substrate on reduction. *Nat Struct Biol* **3:**414–417.

Nelson SD and Trager WF (2003) The use of deuterium isotope effects to probe the active site properties, mechanism of cytochrome P450-catalyzed reactions and mechanisms of metabolically dependent toxicity. *Drug Metab Dispos* **31:**1481–1498.

Northrop D (1977) Determining the absolute magnitude of hydrogen isotope effects, in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland WW, O'Leary MH, and Northrop DB eds), pp 122–152, University Park Press, Baltimore.

Omura T and Sato R (1964) The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification and properties. *J Biol Chem* **239:**2379–2385.

Raucy JL and Lasker JM (1991) Isolation of P450 enzymes from human liver. *Methods Enzymol* **206:**577–587.

Regal KA, Howald WN, Peter RM, Gartner CA, Kunze KL, and Nelson SD (1998) Subnanomolar quantification of caffeine's in vitro metabolites by stable isotope dilution gas chromatography-mass spectrometry. *J Chromatogr B Biomed Sci Appl* **708:**75–85.

Regal KA and Nelson SD (2000) Orientation of caffeine within the active site of human cytochrome P450 1A2 based on NMR longitudinal (T1) relaxation measurements. *Arch Biochem Biophys* **384:**47–58.

Shea JP, Nelson SD, and Ford GP (1983) MNDO calculation of kinetic isotope effects in model cytochrome P-450 oxidations. *J Am Chem Soc* **105:**5451–5454.

Shen AL, Porter TD, Wilson TE, and Kasper CB (1989) Structural analysis of the FMN binding

domain of NADPH-cytochrome P-450 oxidoreductase by site-directed mutagenesis. *J Biol Chem* **264:**7584–7589.
Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, and Klenk DC (1985) Measurement of protein using bicinchoninic acid. *Anal Biochem* **150:**76–85.
Tassaneeyakul W, Birkett DJ, McManus ME, Veronese ME, Andersson T, Tukey RH, and Miners JO (1994) Caffeine metabolism by human hepatic cytochromes P450: contributions of 1A2, 2E1 and 3A isoforms. *Biochem Pharmacol* **47:**1767–1776.
Yun CH, Miller GP, and Guengerich FP (2000) Rate-determining steps in phenacetin oxidations by human cytochrome P450 1A2 and selected mutants. *Biochemistry* **39:**11319–11329.
Yun CH, Miller GP, and Guengerich FP (2001) Oxidations of p-alkoxyacylanilides catalyzed by human cytochrome P450 1A2: structure-activity relationships and simulation of rate constants of individual steps in catalysis. *Biochemistry* **40:**4521–4530.

Address correspondence to: Dr. Sidney Nelson, Dept. of Medicinal Chemistry, University of Washington School of Pharmacy, Box 357610, Seattle, WA 98195-7631. E-mail: sidnels@u.washington.edu