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Microsomal P_{450}-Catalyzed N-Dealkylation of *N*,*N*-Dialkylanilines: Evidence for a C_α−H Abstraction Mechanism

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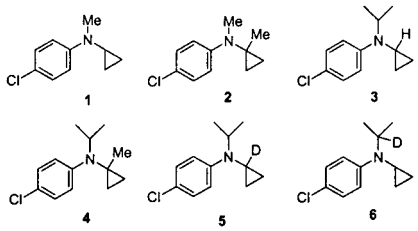
Cytochrome P_{450} (P_{450}) enzymes are a family of nonspecific heme monooxygenases that are responsible for the metabolism and detoxification of xenobiotics. These enzymes are shown to catalyze a variety of monooxygenations, including aliphatic and aromatic hydroxylations, oxygenation of N, S, and Se compounds, epoxidation of olefins, and N- and O-dealkylation of substituted amines and ethers.[1] Due to its central role in xenobiotic metabolism, the mechanism of P_{450} has been extensively studied.[1,2] Despite these efforts, some aspects of the mechanism still remain controversial. For example, the widely accepted early proposal that P_{450}-catalyzed N-dealkylation of *N*,*N*-dialkylamines proceeds through a single-electron-transfer (SET) mechanism[3] was later challenged in favor of the C_α−H abstraction mechanism.[4] However, the importance of the SET mechanism in P_{450}-catalyzed N-dealkylations has been re-emphasized, based on the isotope effects, redox potential correlations, and other studies.[5] In the present study, we have used a series of *N*-alkyl-*N*-cyclopropyl-*p*-chloroaniline probes[6] (**1**−**6**) to examine whether the P_{450}-catalyzed N-dealkylations proceed through a C_α−H abstraction and/or a SET mechanism, using phenobarbital-induced rat liver microsomal P_{450} enzymes (P_{450}) as a model system.[7]



HPLC and GC−MS analysis of the aromatic products from the P_{450} incubations of **1**[8] revealed the presence of one major (Table 1) and a second minor product.[9] The retention time comparisons and the GC−MS analysis confirmed that these were *N*-(*p*-chlorophenyl)cyclopropylamine and *N*-(*p*-chlorophenyl)-*N*-methylaniline, respectively (Table 1). These results parallel the product distribution of the isosteric acyclic analogue, *N*-methyl-*N*-isopropyl-*p*-chloroaniline, that also produces *N*-isopropyl-*p*-chloroaniline and a small amount of *N*-methyl-*p*-chloroaniline under similar incubation conditions (data not shown). The *N*-(1-methylcyclopropyl) derivative **2** was also a good substrate for the enzyme, with activity comparable to that of **1**, but produced only the N-demethylated product with no detectable (a trace by GC−MS) N-decyclopropylated products. On the other hand, the *N*-isopropyl derivative **3**, which was also a good substrate for the enzyme, partitioned between N-decyclopropylation (65.4%) and N-deisopropylation (34.6%) under identical incubation conditions (Table 1). However, parallel to the product distribution of **2**, the *N*-(1-methylcyclopropyl)-*N*-isopropyl derivative **4** produced the N-deisopropylated product with only a trace of N-decylopropylated product (Table 1).

The parallel product distributions of **2** and **4** suggest that the C_α−H of the cyclopropyl ring could play a role in the P_{450}-catalyzed N-decyclopropylation of these derivatives. To test this possibility, we have examined *N*-(1-deuteriocyclopropyl)-*N*-isopropyl derivative **5** (D content >98% by ^{1}H NMR and MS) with the enzyme. As shown in Table 1, the cyclopropyl C_α−D substitution resulted in the inversion of the product distribution, yielding a 36.3% ± 0.2 N-decyclopropylation and a 63.7% ± 0.2 N-deisopropylation (Table 1). The intrinsic primary deuterium isotope effect (k_H/k_D) calculated from the partition ratios of **3** and **5** was 3.2 ± 0.1 for the N-decyclopropylation. We have also synthesized and examined the corresponding *N*-(2-deuterioisopropyl) derivative **6** (D content >98%) with the enzyme. The k_H/k_D calculated from the partition ratios of **3** and **6** was 3.1 ± 0.1 for the N-deisopropylation under identical experimental conditions. These results clearly demonstrate that the P_{450}-catalyzed N-deisopropylation and N-decyclopropylation reactions proceed through similar mechanisms and that the C_α−H's of cyclopropyl and isopropyl substituents are removed at similar isotopically sensitive steps along the catalytic pathways. In addition, the similar magnitudes of k_H/k_D for the two pathways and the insignificant N-decyclopropylation of the cyclopropyl C_α−Me derivatives **2** and **4** clearly suggest that P_{450}-catalyzed N-decyclopropylation could not be accounted for by the normal isotope-insensitive cyclopropyl ring-opening pathway.

Table 1. Product Distribution of Rat Liver Microsomal P_{450}-Catalyzed N-Dealkylation of *N*-Cyclopropyl-*N*-alkyl-*p*-chloroanilines (A), N-decylopropylation (B), and N-Demethylation or N-Deisopropylation[a]

	microsomal P450		Fe(III)/pyridine	
	%A	%B	%A	%B
1	6.7 ± 1.2	93.3± 1.2	90.1 ± 3.1	9.9 ± 3.4
2	trace	>98	86.2 ± 2.0	13.8 ± 2.0
3	64.6 ± 0.7	35.4 ± 0.7	100	nd[b]
4	trace	>98	100	nd
5	36.3± 0.2	63.7 ± 0.2	100	nd
6	84.9 ± 0.1	15.1 ± 0.1	100	nd

[a] Percent conversions under standard enzymatic reaction conditions[8] are (**1**) 11.5 ± 0.1, (**2**) 8.7 ± 1.6, (**3**) 9.8 ± 0.3, (**4**) 7.3 ± 0.3, (**5**) 10.6 ± 0.1, and (**6**) 9.6 ± 0.3 (averages of three or more experiments). [b] nd, not detected.

We have also examined the chemistry of SET intermediates of **1**−**6** that were generated from the well-characterized single-electron oxidant, $Fe^{III}(phen)_3^{3+}(PF_6)_3^{3-}$,[10] in CH_3CN in the presence of the mild base, pyridine. Under these SET conditions,[4a] both **1** and **2** preferentially produced the N-decyclopropylated products relative to N-demethylated products (Table). In addition, all *N*-isopropyl derivatives (**3**−**6**) exclusively and readily produced the corresponding N-decyclopropylated product without a trace of N-deisopropylated products. Furthermore, the product distributions of **3**−**6** were not altered when pyridine was replaced with the stronger base triethylamine[12] (data not shown). These results, especially the exclusive formation of the N-decyclopropylated product from **4**, which has no C_α−H on the cyclopropyl ring, and similar partition of **1** and its C_α−Me derivative **2** between N-demethylation and N-decyclopropylation, clearly demonstrate that the cyclopropyl C_α−H does *not* play a role in the N-decyclopropylation of these

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derivatives under chemical conditions. Therefore, the N-decyclopropylation of these derivatives under chemical conditions must be exclusively due to the opening of the cyclopropyl ring of the SET intermediate, the nitrogen cation radical. These results further suggest that the rates of cyclopropyl ring opening are greater than the rates of deprotonation of the $C_\alpha-$Me protons, which are much greater than the deprotonations of either the *N*-isopropyl or the cyclopropyl $C_\alpha-$H's of the nitrogen cation radical intermediate under the experimental conditions. These findings are in excellent agreement with the abundant literature evidence that the opening of the *N*-cyclopropyl ring of the nitrogen cation radical is very fast.[11]

The above results clearly demonstrate that, while the chemical SET-initiated N-decyclopropylation exclusively proceeds through the opening of the cyclopropyl ring of the cation radical intermediate, the P_{450}-catalyzed N-decyclopropylation primarily proceeds through the removal of the cyclopropyl $C_\alpha-$H without (or prior to) the opening of the cyclopropyl ring. Therefore, if the SET mechanism is operative in P_{450}-catalyzed N-dealkylations of these derivatives, then the rate of isotope-sensitive deprotonation of the cyclopropyl and/or isopropyl $C_\alpha-$H's must be much faster than the rate of cyclopropyl ring opening of the cation radical intermediate requiring a much stronger base than pyridine or triethylamine[12] in the active site of the enzyme.

The current SET mechanism for P_{450}-catalyzed N-dealkylations suggests that the active site $[P-FeO]^{2+}$ species rather than a protein-derived base is responsible for the C_α deprotonation of the nitrogen cation radical intermediate.[5] Studies with lignin peroxidase and other model systems have recently shown that the $[P-FeO]^{2+}$ species is less basic than pyridine (ca. $pK_a = 7$),[13] which is in agreement with the electron-deficient nature of this species. Therefore, even if the electronic and steric constraints of the P_{450} active site significantly increase the basicity of $[P-FeO]^{2+}$, it is highly unlikely that $[P-FeO]^{2+}$-mediated deprotonation of the cyclopropyl and/or isopropyl $C_\alpha-$H's could completely obscure the highly favorable cyclopropyl ring-opening pathway.[14] Therefore, the above results could *not* simply be explained by the proposed SET mechanism for P_{450}-catalyzed N-dealkylations. On the other hand, all the above observations, including (a) lack of significant N-decyclopropylated products from $C_\alpha-$Me-cyclopropyl derivatives (**2** and **4**); (b) similar k_H/k_D's with magnitudes consistent with a $C_\alpha-$H abstraction[4a,15] for P_{450}-catalyzed N-decyclopropylation and N-deisopropylation; (c) lack of evidence for significant ring opening in P_{450}-catalyzed N-decyclopropylation, although the chemical SET-initiated N-decyclopropylation exclusively proceeds through a cyclopropyl ring-opening mechanism; and (d) similarity of the relative product distributions of the P_{450}-catalyzed N-dealkylations of *N*-cyclopropyl derivative **1** and the isosteric acyclic analogue *N*-isopropyl-*N*-methylaniline, are highly consistent with a $C_\alpha-$H abstraction mechanism.[16] The preference for N-demethylation over N-decylopropylation of **1** and **2** must be due to the preferential H abstraction from Me over C_α-cyclopropyl, probably due to steric reasons. However, further experimental evidence may be necessary to completely rule out the SET mechanism of P_{450}-catalyzed N-dealkylations.

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(6) Compounds **1**−**6** were synthesized according to the procedure of Chaplinski and de Meijere using the corresponding *N*-isopropyl or *N*-methyl formanilides or acetanilides in good yields (Chaplinski, V.; de Meijere, A. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 413). All structures were confirmed by standard spectroscopic techniques; the purities were determined by HPLC and GC−MS analyses and were found to be better than 98%. To prevent the ring *p*-hydroxylation, the *p*-chloroderivatives were used.

(7) Phenobarbital-induced rat liver microsomal preparations primarily contain CYP2B1 and minor amounts of related CYP2B2. The isotopic studies of the N-dealkylation of substituted *N*,*N*-dimethylanilines show that the kinetic and mechanistic behavior of phenobarbital-treated rat liver microsomal P450 preparation is identical to the purified CYP2B1 (Karki, S. B.; Dinnocenzo, J. P.; Jones, J. P.; Korzekwa, K. R. *J. Am. Chem. Soc.* **1995**, *117*, 3657). Therefore, the conclusions drawn from the partition ratios should not be significantly affected by the use of rat liver microsomes rather than the purified CYP2B1.

(8) Reactions were carried out in a solution containing 30 μL of rat liver microsomes (protein, 42 mg/mL; P_{450}, 98 nM/mL), 50 mM KPi buffer, pH 7.4, 1 mM EDTA, and 75 mM KCl and 4.0 mM substrate in a total volume of 1.0 mL. The reactions were initiated by adding NADPH (final concentration 1.2 mM) after preincubation at 37 °C for 5 min. The reactions were incubated for 1 h at room temperature, quenched with saturated $NaHCO_3$, and extracted with EtOAc. The organic layer was dried over a stream of N_2, redissolved in MeOH, and analyzed by HPLC−UV at 254 nm and by GC−MS. HPLC separations were carried out on a C_{18} column (250 × 4.6 mm, 5 μm) using 50 mM NaOAc, pH 4.5, CH_3CN, and MeOH (30:60:10) as the mobile phase. All the products were analyzed by HPLC and GC−MS and quantified by HPLC based on the standard curves of authentic material.

(9) In addition to these two products, small (<2%) and variable amounts of ring hydroxylated products were also detected in most incubation mixtures.

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