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P_{450}/NADPH/O_2- and P_{450}/PhIO-Catalyzed N-Dealkylations Are Mechanistically Distinct

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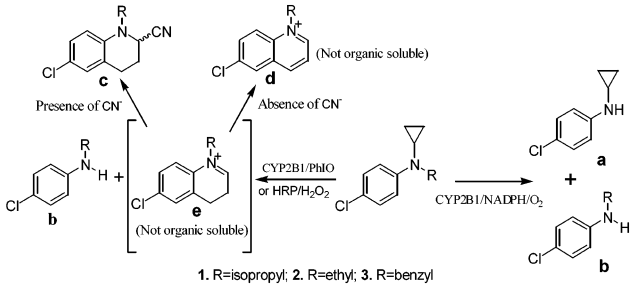
Cytochrome P_{450} (P_{450}) is a family of heme-monooxygenases found in most organisms. Due to its central role, especially with respect to drug metabolism and carcinogenesis, the chemical mechanisms of P_{450} reactions have been extensively studied.[1] Recent cryocrystallographic and ENDOR techniques have unequivocally identified the reaction intermediates Fe(II), Fe(III)O_2, Fe(III)-OOH, and Fe(III)·ROH complexes of P_{450cam}.[2] Although numerous attempts have been made, the proposed final active oxygen species, the ferryl-oxo-π-radical species, has not been observed in the catalytic cycle of any P_{450} so far. Spectroscopic evidence for the formation of the ferryl-oxo species with *m*-chloroperbenzoic acid, in the absence of substrate, has been recently reported with a thermostable P_{450}.[3] On the other hand, catalytically competent ferryl-oxo species have been characterized for chloroperoxidase,[4] horseradish peroxidase (HRP),[5] and oxygen surrogates such as iodosobenzene (PhIO)-activated, model metalloporphyrin systems.[6] The apparent parallels between the reactions catalyzed by P_{450}/NADPH/O_2- and PhIO-supported P_{450} and metalloporphyrin model systems have been taken as strong evidence for the involvement of a similar ferryl-oxo species in P_{450}/NADPH/O_2 reactions.[2] To support this proposal, subtle differences observed in regio- and chemoselectivities,[7] isotope effects,[8] and source of oxygen,[9] etc., between NADPH/O_2- and PhIO-supported P_{450} reactions have been generally attributed to reasons other than the mechanistic differences between the two systems.[6−8] Herein we present the first experimental evidence to demonstrate that the NADPH/O_2- and PhIO-supported P_{450} N-dealkylations are mechanistically distinct and, thus, the P_{450}/PhIO system may not be a good mechanistic model for P_{450}/NADPH/O_2-catalyzed N-dealkylations.

We have previously shown[10] that *N*-cyclopropyl-*N*-alkyl-*p*-chloroaniline derivatives were good N-dealkylating substrates for phenobarbital-treated rat liver microsomal preparations. The present studies show that purified CYP2B1/NADPH/O_2-catalyzed monooxygenation of **1**−**3** produces both N-cyclopropyl and N-alkyl group cleaved products **1a**−**3a** and **1b**−**3b**, respectively, and the partition ratios were dependent on the nature of the N-substituent (Table 1; Scheme 1). The intramolecular isotope effects (k_H/k_D) for the CYP2B1/NADPH/O_2-catalyzed cleavage of N-isopropyl and N-cyclopropyl groups of **1**, which were determined from the partition ratios of appropriately deuterated derivatives, were 2.9 ± 0.1 and 3.0 ± 0.1, respectively. The k_H/k_D's for the cleavages of N-ethyl and N-cyclopropyl groups of **2** were 2.6 ± 0.1 and 2.9 ± 0.1, respectively. Furthermore, no detectable cyclopropyl ring-opened, radical cyclized products, **1c**−**3c**, were observed in the reaction mixtures when the CYP2B1/NADPH/O_2/**1**−**3** reactions were carried out in the presence of CN^- in the incubation medium.[11] (Table 1 and Scheme 1). On the other hand, the HRP/H_2O_2-catalyzed oxidation of **1**−**3** exclusively produces the N-cyclopropyl cleaved products, **1b**−**3b**, and cyclopropyl ring-opened radical cyclized CN^- adducts,[12] **1c**−**3c**, in the presence of CN^- in the incubation medium (Table 1). These results demonstrate that while HRP-catalyzed oxidations of **1**−**3** exclusively proceed through a single electron transfer (SET) from the benzylic nitrogen followed by facile cyclopropyl ring opening and radical cyclization pathway, P_{450}/NADPH/O_2 reactions proceed through a distinct pathway that does not involve the opening of the cyclopropyl ring, but involves the isotope sensitive removal of hydrogen from the C_α of both N-alkyl and N-cyclopropyl substituents. These findings strongly support our previous proposal that P_{450}/NADPH/O_2-mediated N-dealkylations proceed through an initial C_α-HAT rather than a SET pathway.[10]

Scheme 1. Product Profiles of P_{450}- and HRP-Catalyzed N-Dealkylation of **1**−**3** under Various Conditions



In contrast to the product profiles of P_{450}/NADPH/O_2/**1**−**3** reactions, the P_{450}/PhIO/**1**−**3** reactions exclusively produce the N-cyclopropyl cleaved products with little or no N-alkyl cleaved products. In addition, P_{450}/PhIO/**1**−**3** reactions produce the N-cyclopropyl group cleaved (**1b**−**3b**) and cyclopropyl ring-opened radical cyclized products (**1c**−**3c**) in the presence of CN^- in the incubation medium, similar to the HRP-catalyzed reactions (Scheme 1 and Table 1). The control experiment revealed that the formation of the products was strictly dependent on the presence of the active enzyme and PhIO in all cases. The above results show that P_{450}/PhIO and HRP/H_2O_2 systems favor the initial SET from the benzylic nitrogen, whereas, P_{450}/NADPH/O_2 favors the HAT from the C_α of the substrate, under competitive conditions. These results are also consistent with the previous observations that the chemistry of P_{450}/PhIO-catalyzed N-dealkylations is similar to that of HRP rather than P_{450}/NADPH/O_2. For example, both HRP- and P_{450}/PhIO-catalyzed N-dealkylations display high intramolecular isotope effects in comparison to low isotope effects of similar P_{450}/NADPH/O_2-supported reactions.[8] In addition, the formation of nitrogen cation radical intermediates has been observed for both P_{450}/PhIO and HRP systems,[8] but similar species have not been detected in P_{450}/NADPH/O_2-mediated reactions.

Although the observed differences in product profiles of P_{450}/PhIO reactions could simply be due to nonaccessibility of the C_α-H's of the substrates to the active oxygen species due to the steric

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Table 1. Product Distributions of CYP2B1/NADPH/O_2-, CYP2B1/PhIO-, and HRP/H_2O_2-Catalyzed N-Dealkylation of **1**−**3**

	% product distribution[a]								
reaction	**1a**	**1b**	**1c**	**2a**	**2b**	**2c**	**3a**[c]	**3b**[c]	**3c**[c]
CYP2B1/NADPH/O_2[b]	30(1)	70(1)		47(1)	53(1)		20(1)	80(1)	
CYP2B1/NADPH/O_2/CN^-[d]	27(2)	73(2)	ND	48(1)	52(1)	ND	22(1)	78(1)	ND
CYP2B1/PhIO[b]	ND	>98		ND	>98		ND	>98	
CYP2B1/PhIO/CN^-[d]	ND	50(2)	50(2)	ND	15(2)	85(2)	ND	40(2)	60(2)
HRP/H_2O_2[b]	ND	>98		ND	>98		ND	>98	
HRP/H_2O_2/CN^-[d]	ND	32(1)	68(1)	ND	8 (2)	92(2)	ND	25(2)	75(2)

[a] The product ratios were averages of at least three independent determinations. The standard deviations for the last significant figures are given in parentheses. **a**, 4-chloro-*N*-cyclopropylaniline; **b**, 4-chloro-*N*-alkylaniline; **c**, CN^-, adduct. [b] The intermediates **d** and **e** (Scheme 1) are not organic soluble and could not be detected under the experimental conditions, and % products were calculated based on the detectable products, **a** and **b**. [c] In CYP2B1/NADPH/O2/**3** reactions a significant amount of an unidentified aromatic ring hydroxylated product was also detected; % products were calculated based on total N-dealkylated products. [d] The % product ratios were calculated assuming that trapping of **e** by CN^- was 100%. ND, not detected.

effects of the remnant PhI, the previous literature evidence[13] and the following experimental observations argue against such a possibility: (a) The product distribution patterns of *N*-cyclopropylaniline derivatives were not altered when the steric bulk at the benzylic nitrogen of the substrate was significantly altered by substituting N-Me, N-Et, N-*i*Pr, and N-benzyl for both systems; (b) structure optimization of these substrates shows that the nitrogen atoms are sterically relatively more shielded than the C_α-H's; (c) the product profiles of all the above substrates were not altered when the P_{450}/NADPH/O_2 or P_{450}/PhIO reactions were carried out in the presence of excess PhI (up to 2 mM) in the reaction mixtures; (d) product profiles were not changed when the wild-type CYP2B1 is replaced with the active site double mutant I114A/F297G, which has an enlarged active site and has been shown to produce multiple hydroxylation products from progesterone including 16α, 15α, 21, and two other unknown hydroxylated products[14] (data not shown); (e) no ring hydroxylated or any other products of PhI or PhIO were detected in any of the reaction mixtures. Therefore, the differences of the product distribution patterns of the PhIO- and NADPH-supported P_{450} reactions of the above substrates are most likely associated with the differences of the chemistries of the two systems.[15]

As mentioned above, PhIO-supported model metalloporphyrin-catalyzed hydroxylations and epoxidations have been commonly used as models for P_{450}-catalyzed reactions, and the active oxygen species of these reactions is widely believed to be the ferryl-oxo species.[2] However, numerous recent model studies suggest that the catalytically relevant active oxygen species in these reactions may not be the ferryl-oxo species, but could be a complex between the iron(III)-porphyrin and the oxidant.[16−18] Therefore, a parallel attractive possibility for the above observations is that the catalytically relevant oxygen species of P_{450}/PhIO reactions could also be a similar complex between the PhIO and the heme center of the enzyme, which is distinct from active oxygen species of the P_{450}/NADPH/O_2 and favors the SET from the benzylic nitrogen rather than the HAT from the C_α of the substrate. However, additional experimental evidence is necessary to clearly define the contrasting activities of P_{450}/NADPH/O_2 and P_{450}/PhIO with respect to the above substrates.

The unexpected mechanistic differences observed in the P_{450}/NADPH/O_2 and P_{450}/PhIO systems with respect to the above substrates provide a unique opportunity to further exploit the relative chemistries of these systems, which would be valuable in understanding the nature of their active oxygen species. In addition, our results demonstrate that the P_{450}/PhIO system may not be a good mechanistic model for P_{450}/NADPH/O_2-catalyzed N-dealkylations.

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Supporting Information Available: Experimental methods and GC and GS-MS traces of the reaction products (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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