MOLECULAR PHARMACOLOGY, 23:758–760

Source of the Oxygen Atom in the Product of Cytochrome P-450-Catalyzed *N*-Demethylation Reactions

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Received November 25, 1982; Accepted January 25, 1983

SUMMARY

The source of the oxygen atom in the product of the cytochrome P-450-catalyzed *N*-demethylation of *N*-methylcarbazole was determined by mass spectral analysis of the carbinolamine precursor of formaldehyde formed during incubation in oxygen 18-enriched medium. Initial experiments demonstrated that *N*-(hydroxymethyl)carbazole, the carbinolamine product of the metabolism of *N*-methylcarbazole, did not exchange oxygen with solvent water. When *N*-methylcarbazole was incubated in oxygen 18-enriched medium with purified cytochrome P-450 in the presence of either purified NADPH-cytochrome P-450 reductase and NADPH, cumene hydroperoxide, *t*-butyl hydroperoxide, or peracetic acid, there was no incorporation of oxygen 18 from the medium into *N*-(hydroxymethyl)carbazole. These results clearly demonstrate that the oxygen atom inserted into *N*-methylcarbazole by cytochrome P-450 to yield *N*-(hydroxymethyl)carbazole does not come from the medium and show that the *N*-demethylation reactions catalyzed by cytochrome P-450 proceed in a manner similar to hydroxylation reactions, with the oxygen atom in the product being derived from the oxidant.

INTRODUCTION

Hepatic microsomal cytochrome P-450 can utilize NADPH and molecular oxygen as well as many peroxy compounds to catalyze the hydroxylation or hetero-atom dealkylation of a wide variety of endogenous and exogenous organic compounds (1, 2). Studies utilizing oxygen 18-enriched media have demonstrated that one oxygen atom from molecular oxygen or organic hydroperoxides is incorporated into the product of cytochrome P-450-catalyzed hydroxylation reactions (3–5). The cytochrome P-450-catalyzed *N*-demethylation of tertiary amines is generally accepted to occur by hydroxylation of the *N*-methyl group to form a carbinolamine (*3* in Fig. 1), which is often unstable and spontaneously decomposes to yield formaldehyde and the desmethylamine (6, 7). A carbinolamine intermediate has also been proposed for the cytochrome P-450-dependent oxidative deamination of alicyclic primary amines, where the oxygen atom in the ketone product is derived from molecular oxygen (8). Although oxygen 18 studies of the microsomal *N*-debenzylation of several 4-disubstituted *N*-benzylpiperidines suggested that the oxygen atom in benzaldehyde was derived from molecular oxygen (9, 10), oxygen-labeling studies of cytochrome P-450-catalyzed demethylation reactions have not been possible because of the rapid exchange of the carbonyl oxygen of formaldehyde with solvent water oxygen (11). However, this problem can be circumvented by using the *N*-methylamine substrate *N*-methylcarbazole, which yields a stable isolatable carbinolamine intermediate (12–15).

Several possible mechanisms for the formation of the carbinolamine intermediate (*3*) are shown in Fig. 1. One possible mechanism involves the two-electron-equivalent oxidation of the amine, either directly or via the substrate radical (*1*), to form an iminium cation (*2*), which could then react with water to form *3*. If *2* were the product released from the enzyme, then the oxygen atom in *3* would be derived from the aqueous medium (16–18). Alternatively, *2* could react with the iron-oxo species in the active site of cytochrome P-450 (19) and then the oxygen atom in *3* would be derived from the oxidant. The oxygen atom in *3* would also be derived from the oxidant if the substrate radical (*1*) recombined with the iron-bound oxygen moiety in the active site of cytochrome P-450 (20, 21). In this communication we describe oxygen 18-labeling studies of the cytochrome P-450-catalyzed *N*-demethylation of *N*-methylcarbazole, where the carbinolamine intermediate, NHMC,[4] was analyzed directly. Our experiments were designed using $H_2{}^{18}O$-enriched

This work was supported in part by National Institutes of Health Grant CA-16954 (to P. F. H.).

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[4] The abbreviation used is: NHMC, *N*-(hydroxymethyl)carbazole.

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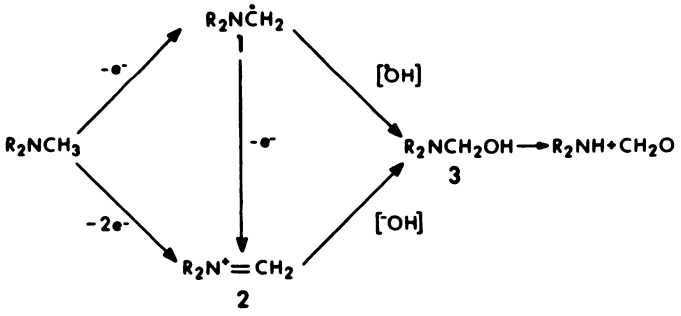

FIG. 1. *Possible mechanisms for the cytochrome P-450-catalyzed N-demethylation of tertiary amines*

buffer as the common medium to allow investigation of the reaction supported by NADPH and molecular oxygen as well as by several other oxidants.

EXPERIMENTAL PROCEDURES

Materials. *N*-Methylcarbazole and NHMC were synthesized as previously described (14). Both compounds were >99% pure as judged by high-pressure liquid chromatography. Cumene hydroperoxide was obtained from Matheson, Coleman, and Bell (Cincinnati, Ohio); *t*-butyl hydroperoxide from ICN Pharmaceuticals, Inc. (Irvine, Calif.); peracetic acid from Pfaltz and Bauer, Inc. (Stamford, Conn.); and NADPH from Sigma Chemical Company (St. Louis, Mo.). Dilaurylphosphatidylcholine was obtained from Serdary Research Laboratories (London, Ont., Canada) as a chloroform solution. Before use, the chloroform was removed under a stream of nitrogen, and a 1 mg/ml aqueous suspension was made by sonication. Oxygen 18 water was obtained in 97% enrichment from KOR, Inc. (Cambridge, Mass). All other materials were reagent-grade and were obtained from commercial sources.

Enzyme preparations. Cytochrome P-450$_{LM_2}$ was purified from liver microsomes of phenobarbital-pretreated rabbits by the procedure of Coon *et al.* (22). The preparation had a specific content of 15.7 nmoles of cytochrome P-450 per milligram of protein. NADPH-cytochrome P-450 reductase was purified from liver microsomes of phenobarbital-pretreated rabbits (23, 24) and had a specific content of 10.5 nmoles of flavin per milligram of protein. Both preparations gave only one band upon sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

Assay procedure. The mixtures contained potassium phosphate buffer (0.1 M), pH 7.7, or phosphate buffer containing 19.4% $H_2{}^{18}O$, cytochrome P-450$_{LM_2}$ (0.99 nmole), dilaurylphosphatidylcholine (30 μg), *N*-methylcarbazole (0.5 mM), and either NADPH-cytochrome P-450 reductase (0.99 nmole) and NADPH (0.5 mM), cumene hydroperoxide (3 mM), *t*-butyl hydroperoxide (3 mM), or peracetic acid (1.5 mM) in a final volume of 1.0 ml. The incubation mixtures were preincubated at 30° for 3 min. The reaction mixtures containing NADPH-cytochrome P-450 reductase were initiated by the addition of NADPH and incubated for 15 min. The reactions supported by the peroxy compounds were initiated by the addition of the oxidant and incubated for 5 min. All incubations were carried out in duplicate. The incubation mixtures were extracted twice with 3 ml of ethyl acetate containing 1% diethylamine, and the extracts were prepared for chromatographic analysis as previously described (14). The dried extracts were allowed to react with bis(trimethylsilyl)trifluoroacetamide (Supelco, Inc., Bellefonte, Pa.), evaporated to dryness under a stream of nitrogen, and redissolved in ethyl acetate; aliquots (1.0 μl) were injected onto a Finnigan 3200 gas chromatograph/mass spectrometer equipped with a INCOS 2300 data system and a 30-m fused silica capillary column (0.25 mm inner diameter) coated with SE-54 (J and W Scientific, Rancho Cordoba, Calif.). Duplicate splitless injections were made from each sample with the column held at 60°. After 1 min, the column was heated to 250° at 64°/min. The carrier gas was helium at a flow rate of 3 ml/min. The trimethylsilyl ether of NHMC had a retention time of 10.7 min. Ionization was by electron impact with the filament operated at 70 eV.

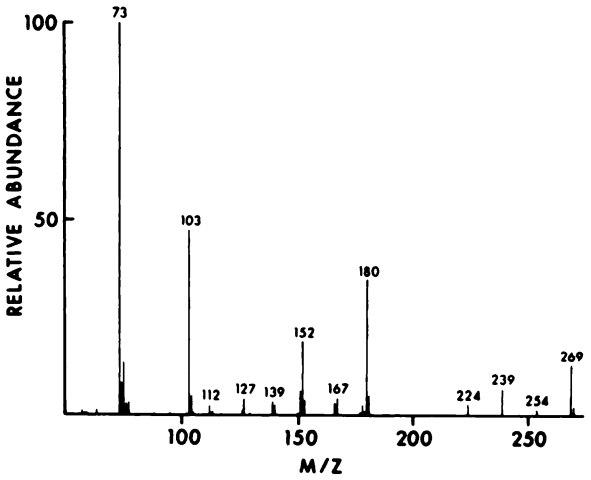

FIG. 2. *Mass spectrum of the trimethylsilyl ether of NHMC*
Ions were observed at m/z 269 $(M)^+$, m/z 254 $(M\text{-}CH_3)^+$, m/z 239 $(M\text{-}[CH_3]_2)^+$, m/z 224 $(M\text{-}[CH_3]_3)^+$, and m/z 180 $(M\text{-}OSi[CH_3]_3)^+$.

Oxygen 18 incorporation into NHMC was quantitated by taking the ratio of the integrated ion current at m/z 271 $(M[^{18}O])^+$ to that at m/z 269 $(M[^{16}O])^+$ under the gas chromatographic peak due to NHMC (see Fig. 2 for the complete mass spectrum of the trimethylsilyl ether of NHMC).

RESULTS AND DISCUSSION

Initial experiments were carried out to determine whether the carbinolamine of *N*-methylcarbazole, NHMC, exchanged oxygen with the aqueous medium. Synthetic NHMC was incubated in potassium phosphate buffer (0.1 M), pH 7.7, and in phosphate buffer containing 19.4% $H_2{}^{18}O$ for 15 min at 30° in duplicate. The incubation mixtures were extracted twice with ethyl acetate containing 1% diethylamine, and the extracts were processed and analyzed as described under Experimental Procedures. The ratio of m/z 271:269 was 5.25 ± 0.07% for NHMC incubated in phosphate buffer and 5.70 ± 0.28% for NHMC incubated in $H_2{}^{18}O$-enriched phosphate buffer, indicating that NHMC does not significantly exchange oxygen with solvent water.

TABLE 1

Oxygen isotope content of the NHMC formed by cytochrome P-450$_{LM2}$

N-Methylcarbazole was incubated with cytochrome P-450$_{LM2}$ and the indicated oxidants in phosphate buffer and in phosphate buffer containing 19.4% $H_2{}^{18}O$. The oxygen isotope content of the NHMC formed was determined by monitoring the ions in the mass spectrum at m/z 271 and m/z 269. The incubation conditions and analytical procedure are described under Experimental Procedures. The data presented are average values of duplicate determinations, with the individual values varying by less than 10%.

Oxidant	m/z 271:269	
	$H_2{}^{16}O$	$H_2{}^{18}O$
	%	%
Molecular oxygen (NADPH-cytochrome P-450 reductase + NADPH)	5.90	5.80
Cumene hydroperoxide	6.00	5.75
t-Butyl hydroperoxide	6.05	5.80
Peracetic acid	5.65	5.90

Experiments were carried out to determine the source of the oxygen atom in the NHMC formed during the cytochrome P-450$_{LM_2}$-catalyzed *N*-demethylation of *N*-methylcarbazole supported by several different oxidants. The results are shown in Table 1. In all four oxidant systems, NHMC was the major metabolite of *N*-methylcarbazole. Small amounts of the three ring-hydroxylated metabolites (13–15) were observed but were not analyzed for oxygen isotope content because the oxidant has previously been established to be the source of the oxygen atom in the product of cytochrome P-450-catalyzed aromatic hydroxylation reactions (5). The average ratio of m/z 271:269 for the NHMC formed in $H_2{}^{16}O$ phosphate buffer was 5.90 ± 0.30% in all four oxidant systems, with the individual determinations ranging from 5.2 to 6.1%. In $H_2{}^{18}O$-enriched phosphate buffer, the average ratio of m/z 271:269 was 5.81 ± 0.18%, with the individual determinations ranging from 5.6 to 6.2%.

These results indicate that ^{16}O from the oxidant rather than ^{18}O from the medium is incorporated into the product of the cytochrome P-450-catalyzed *N*-demethylation of *N*-methylcarbazole when the reaction is supported by NADPH and molecular oxygen, cumene hydroperoxide, *t*-butyl hydroperoxide, or peracetic acid. Our results with purified cytochrome P-450$_{LM_2}$ are consistent with the results of a recent oxygen 18 study of the NADPH-dependent oxidation of *N*-methylcarbazole by rat liver microsomes (25). The data are inconsistent with the mechanism of cytochrome P-450-catalyzed demethylation reactions proposed by Griffin and co-workers (16–18), where the imine (*2* in Fig. 1) dissociates from the enzyme and reacts with water to form the carbinolamine (and ultimately formaldehyde and the desmethylamine). Although this mechanism may be operative in the demethylation of aminopyrine (16–18), it is clearly not a general mechanism for the *N*-demethylation reactions catalyzed by cytochrome P-450. If *2* is actually an intermediate in the demethylation of *N*-methylcarbazole, then it must react with an oxygen species derived from the oxidant in the active site of cytochrome P-450. Alternatively, *2* may not be involved as an intermediate in demethylation reactions, and the substrate radical (*1* in Fig. 1) may react with the iron-bound oxygen species in the active site of cytochrome P-450 to form the carbinolamine (*3* in Fig. 1) directly, as has been proposed by White and Coon (21). While the results presented here cannot distinguish between these two mechanisms, they do establish that, as in hydroxylation reactions (3–5), the oxygen atom in the product of *N*-demethylation reactions catalyzed by cytochrome P-450 is derived from the oxidant and not the aqueous medium.

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