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METABOLISM OF CARBON TETRACHLORIDE TO ELECTROPHILIC CHLORINE BY LIVER MICROSOMES: EXCLUSION OF CYTOCHROME P-450 CATALYZED CHLOROPEROXIDASE REACTION

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SUMMARY: In this report, we have examined the origin of the electrophilic chlorine formed during the microsomal metabolism of carbon tetrachloride and the possibility that liver microsomal proteins catalyze chloroperoxidase or myeloperoxidase halogenation reactions. Studies with stable isotopes of chlorine show that at least 99% of the trapped chlorine originated from carbon tetrachloride. When hydrogen peroxide or cumene hydroperoxide was added to liver microsomes in the presence of chloride ion, no trapped chlorine was observed. Thus, cytochrome P-450 does not catalyze chloroperoxidase type chloride ion oxidation but instead catalyzes a reaction leading to cleavage of a carbon-chlorine bond with concomitant chlorine atom oxidation.

INTRODUCTION

We have recently reported that 2,6-dimethylphenol (DMP) traps an electrophilic form of chlorine as 4-chloro-2,6-dimethylphenol (4-ClDMP) during the microsomal metabolism of carbon tetrachloride (1,Fig.1). This reaction is dependent on both oxygen and NADPH (1) and appears to be catalyzed by cytochromes P-450 (2).

The hemeproteins myeloperoxidase and chloroperoxidase are known to catalyze the oxidation of chloride ions by hydrogen peroxide or organic hydroperoxides to produce electrophilic forms of chlorine (3-6). The reaction catalyzed by myeloperoxidase is mediated by hypochlorous acid whereas chloroperoxidase directly chlorinates substrates susceptible to electrophilic substitution (3-6). Like these enzymes, cytochrome P-450 also uses hydrogen peroxide and

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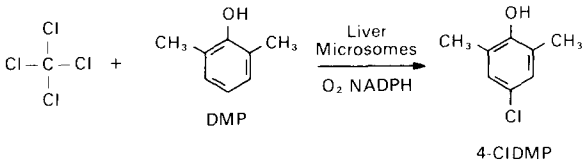

Fig. 1. Trapping of electrophilic chlorine atom by 2,6-dimethylphenol.

organic hydroperoxides to support oxidative drug metabolism (7-9). Moreover, cytochrome P-450 and chloroperoxidase have very similar physical-chemical properties (6,10). For example, both chloroperoxidase and cytochrome P-450 have thiolate-iron ligands (11) as well as ferrous carbon monoxide complex Soret absorbance maxima near 450 nm. In addition, cytochrome P-450 and chloroperoxidase have very similar magnetic circular dichroism and resonance Raman spectra in both their oxidized and reduced states (6,10).

It therefore seemed possible that the chlorine atom trapped during the microsomal metabolism of CCl_4 is the result of oxidation of chloride ion and not CCl_4 metabolism. In the present study this hypothesis was tested and found to be incorrect. Microsomal cytochromes P-450 do not catalyze detectable chloroperoxidase or myeloperoxidase reactions, but instead catalyze an unique reaction which involves the cleavage of a carbon-chlorine bond with concomitant chlorine atom oxidation.

METHODS

NADPH and chloroperoxidase, purified grade, (EC 1.11.1.10) were purchased from Sigma Biochemicals, St. Louis, MO. CCl_4 and DMP were purchased from Aldrich Chemical Co., Milwaukee, WI. Cumene hydroperoxide was obtained from ICN Pharmaceuticals, Plainview, N.Y. and was used without purification. 4-ClDMP and 2-chloro-4,6-dimethylphenol (2-ClDMP) were prepared as previously described (1). CCl_4 was distilled prior to use. [^{35}Cl]CCl_4 (99.7 atom %) was purchased from Isotope Labeling Corp., Whippany, N.J. The isotopic enrichment was verified by mass spectrometry. The purities of CCl_4, unenriched and isotopically enriched, were found to be > 99.9% by GC analysis on Porapak Q (100/120 mesh) and 3% SE-30 on Gas Chrom Q (100/120 mesh) packed columns. [^{37}Cl] NaCl (92 atom %) was obtained from U.S. Services, Inc., Summit, N.J. The isotopic enrichment of [^{37}Cl] NaCl was verified by GCMS after oxidation of chloride with excess H_2O_2 and trapping of HOCl with DMP.

Liver microsomes from phenobarbital treated (100 mg/kg for 3 days) male Sprague Dawley rats (180-250 g) were prepared as previously described (1). Unless otherwise indicated, incubation mixtures contained 4 mg of microsomal protein, 1.0 mM NADPH, 20 mM HEPES buffer (pH 7.5), 5 mM substrate and 1 mM DMP in a total volume of 2 ml. The incubations were conducted in sealed vials (rubber septum) at 37°C under an atmosphere of air. After 30 min, an internal standard, 2-chloro-4,6-dimethylphenol (400 ng in 50 µl methanol), was added, and the incubation mixtures were extracted immediately with diethyl ether (6 ml). The ether layer was evaporated to dryness in a water bath at room temper-

ature under a very gentle stream of nitrogen, and the residue was dissolved in 50 µl of methanol. Samples were analyzed for the internal standard (retention time 1.1 min) and 4-ClDMP (retention time 1.9 min) by GC/EIMS in the selective ion mode as previously described (1).

Incubations with chloroperoxidase (20 µg protein) were conducted at 37°C in 100 mM potassium phosphate buffer (pH 4.8) with DMP (7 mM) and potassium chloride (20 mM) in a total volume of 2 ml. The reaction was initiated by addition of H_2O_2 (2 mM). After 10 min, the incubation mixtures were analyzed for 4-ClDMP as described for the microsomal incubations.

RESULTS

To determine the origin of the trapped chlorine atom, liver microsomes, DMP and NADPH were incubated with CCl_4, CCl_4 plus [^{37}Cl] NaCl, [^{35}Cl] CCl_4, or [^{35}Cl]CCl_4 plus [^{37}Cl] NaCl. The reaction mixture was analyzed by gas chromatography mass spectrometry for the molecular ions of 4-ClDMP containing ^{35}Cl and ^{37}Cl at m/z 156 and 158, respectively. Analysis of microsomal incubation mixtures containing CCl_4 (unenriched) revealed a metabolite peak (retention time 1.9 min, Fig.2A) which had been previously identified as 4-ClDMP (1). The ratio of ion current at m/z 156 to 158 of 3:1 corresponds to the expected natural abundance ratio of chlorine isotopes. The ratio of ion currents for internal standard 2-chloro-4,6-dimethylphenol (retention time 1.1 min) was also 3:1. As shown in Fig.2B, when 100 mM [^{37}Cl] NaCl (92 atom %) was incubated with CCl_4, the ratio of ion currents at m/z 156 to 158 remained 3:1, indicating the chloride ion was not incorporated into 4-ClDMP. In contrast, analysis of microsomal incubations with [^{35}Cl] CCl_4 (99.7 atom %) revealed a metabolite peak at m/z 156 (Fig.2C) but none at m/z 158, demonstrating that the trapped chlorine atom came from CCl_4. To confirm these results, incubations were performed with both [^{35}Cl] CCl_4 and [^{37}Cl] NaCl. As shown in Fig.2D, ion current was observed only at m/z 156 while the ratio of m/z 156 to 158 in the internal standard peak remained approximately 3:1. These studies demonstrate that the origin of the electrophilic chlorine atom formed during metabolism of CCl_4 with rat liver microsomes, oxygen and NADPH is CCl_4 and not chloride ion.

To determine if in the absence of CCl_4 liver microsomal proteins would catalyze a myeloperoxidase or chloroperoxidase reaction with peroxidase cofactors, H_2O_2 (20 mM) or cumene hydroperoxide (2 mM) was incubated with liver

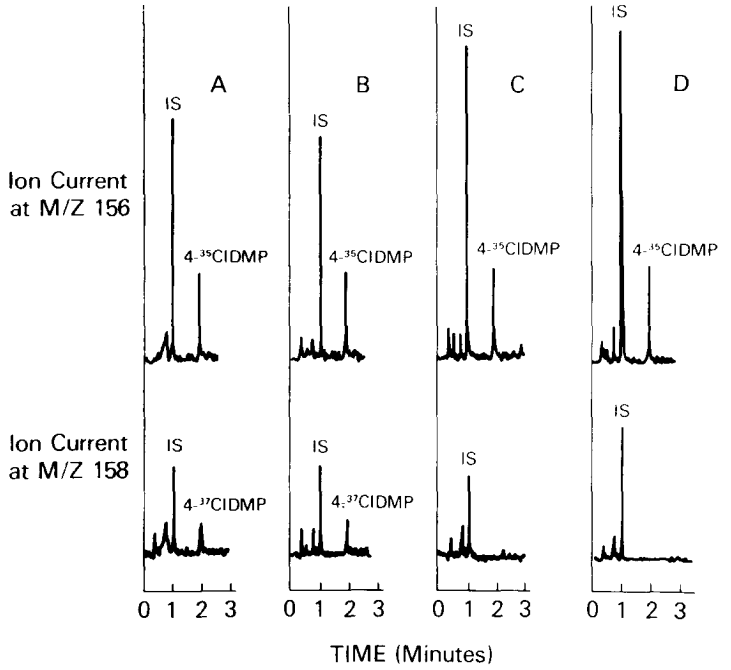

Fig. 2. Gas chromatography mass spectrometry selective ion monitoring of 4-ClDMP (retention time 1.9 min). Analysis of incubation mixtures containing: A) CCl_4 (unenriched), B) CCl_4 (unenriched) plus [^{37}Cl] NaCl, C) [^{35}Cl] CCl_4, and D) [^{35}Cl] CCl_4 plus [^{37}Cl] NaCl. Ion current at m/z 156 represents 4-ClDMP containing a ^{35}Cl atom. Ion current at m/z 158 represents 4-ClDMP containing a ^{37}Cl atom. Internal standard (IS) 2-ClDMP (natural abundance of chlorine isotopes, retention time 1.1 min), an isomer of 4-ClDMP, was added for quantitation and to insure proper operation of the mass spectrometer.

microsomes, KCl (100 mM) and DMP. Under these conditions, no 4-ClDMP (<25 p-moles) was detected. However, during incubation of mixtures of DMP (7 mM), KCl (20 mM), and H_2O_2 (2 mM) with chloroperoxidase (20 μg), more than 200 nmoles of 4-ClDMP were formed per minute. Moreover, when hypochlorous acid (20 nmoles) was added to liver microsomes and DMP, approximately 100 pmoles of 4-ClDMP was obtained. These studies demonstrate that the failure to observe a chloroperoxidase or myeloperoxidase reaction in liver microsomes is not due to ineffective trapping of halogenating species by DMP.

DISCUSSION

The results of these studies establish that the chlorine atom trapped during microsomal metabolism of CCl_4 is exclusively derived from CCl_4 and not chloride ion. Therefore, cytochrome P-450 catalyzes an unprecedented reaction leading to carbon-chlorine bond cleavage with concurrent oxidation

of chlorine atom. A chloroperoxidase-type reaction as the mechanism of electrophilic chlorine formation is specifically excluded. Although these data do not establish the pathway of elecrophilic chlorine formation during the metabolism of CCl_4, they do place important constraints on the possible mechanisms and are consistent with both oxidative and reductive pathways previously postulated for this reaction (1). The studies also establish that cytochrome P-450 or other microsomal proteins, under the conditions of this study, do not catalyze detectable chloroperoxidase or myeloperoxidase reactions at the concentration of hydroperoxides which support oxidative drug metabolism (7-9).

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