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ANAEROBIC DEHALOGENATION OF HALOTHANE BY RECONSTITUTED LIVER MICROSOMAL CYTOCHROME P-450 ENZYME SYSTEM[1]

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SUMMARY

Cytochrome P-450 from liver microsomes of phenobarbital-treated rabbits catalyzed anaerobic dehalogenation of halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) when combined with NADPH and NADPH-cytochrome P-450 reductase. Cytochromes P-450B$_1$ and P-448 from liver microsomes of untreated rabbits were less active. Triton X-100 accelerated the reaction. Unlike anaerobic dehalogenation of halothane in microsomes, the major product was 2-chloro-1,1,1-trifluoroethane and 2-chloro-1,1-difluoroethylene was negligible. These products were not detected under aerobic conditions, and dehalogenation activity was inhibited by carbon monoxide, phenyl isocyanide and metyrapone.

INTRODUCTION

Halothane is an anesthetic, which is now widely used in clinical anesthesia. This compound is metabolized *in vivo* and excreted in urine as CF_3COOH (1). Volatile metabolites, CF_3CH_2Cl and CF_2CHCl, are also expired from anesthetized rabbits (2). With respect to these metabolites, recent studies have demonstrated that aerobic dehalogenation of halothane in microsomes produces CF_3COOH (3), while anaerobic reaction produces CF_3CH_2Cl and CF_2CHCl (4).

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Moreover, these reactions are inhibited by carbon monoxide and enhanced by pretreatment of animals with phenobarbital (3, 4). These observations indicate that halothane is metabolized by microsomal mixed function oxidase system and metabolites are produced depending upon oxygen tension. In fact, CF_3CH_2Cl and CF_2CHCl are not detected under aerobic conditions (4). Evidence has also been presented on dehalogenation of halothane. Anaerobic addition of halothane to NADPH-reduced microsomes exhibited a difference spectrum, and it is suggested that the spectral species is trifluoroethyl carbene complex of reduced cytochrome P-450 (5). Covalent binding of halothane to liver microsomal proteins (6) and anaerobic release of fluoride from halothane (7) have been reported. The present experiments strongly indicate that anaerobic dehalogenation of halothane is catalyzed by cytochrome P-450 (P-450) and CF_3CH_2Cl is the major product as far as the reconstituted system is concerned.

MATERIALS AND METHODS

NADPH (Oriental Yeast Co.), Triton X-100 (Rohm and Haas Co.), metyrapone (a generous gift from Ciba Geigy, Japan) and halothane (Hohechist, Japan) were used, respectively. Phenyl isocyanide was prepared by the method of Schmidt and Stern (8). All other reagents were of the highest grade commercially available. Rabbit liver microsomes were prepared by the method of Miki et al. (9). Intramuscular injection of phenobarbital was made on rabbits (40 mg per kg) at intervals of 24 hrs for 5 days, and then the rabbits were fasted for 24 hrs. Cytochrome P-450 from phenobarbital-treated rabbit liver microsomes (P-450PB) was purified by the method for purification of cytochromes $P\text{-}450B_1$ and P-448 (9, 10) with a slight modification. The Step 1 preparation in 5 mM potassium phosphate, pH 7.4, containing 0.5% Triton X-100 (w/v), 20% glycerol (v/v) and 1 mM EDTA (5 mM PTGE-buffer) was chromatographed on a CM-Sepharose CL-6B column. The eluate with 20 mM PTGE-buffer was passed through a cytochrome b_5-coupled Sepharose 6B column and the flowthrough fractions were collected. The purified preparation was homogeneous on SDS-gel electrophoresis and the properties were identical to those of P-450 prepared by Imai and Sato (11). Cytochromes $P\text{-}450B_1$ and P-448 from untreated rabbit liver microsomes ($P\text{-}450B_1$ and P-448) were purified by the method of Miki et al. (9, 10). NADPH-P-450 reductase was purified by the method of Yasukochi and Masters (12) with a slight modification. The concentration of P-450 was determined by the method of Omura and Sato (13). NADPH-P-450 reductase activity was assayed by measuring NADPH-cytochrome c reductase activity in 0.3 M potassium phosphate buffer, pH 7.7, containing 0.1 mM EDTA and at 30°C (12). For measurement of anaerobic dehalogenation activity, the reaction mixture contained 0.85-1.1 nmole of P-450, 0.3 unit of NADPH-P-450 reductase, 0.25 μmole of NADPH, 28.5 μmoles of halothane, 100 μmoles of potassium phosphate buffer, pH 7.4, and 0.03% Triton X-100 (w/v) in the total volume of 0.5 ml. A 5 ml test tube containing the reaction mixture was filled with nitrogen gas and sealed by a double cap. The reaction was initiated by injection of NADPH into the test tube, and the reaction was

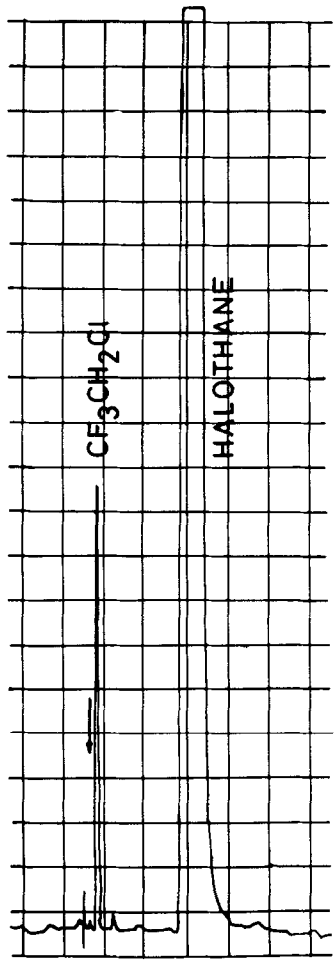

Fig. 1. Gas chromatogram of the metabolite. Anaerobic reaction was made by the reconstituted system containing 2.6 nmoles of P-448. The metabolite was measured at 40°C using DOP column. Other experimental conditions are described in MATERIALS AND METHODS.

carried out at 37°C. After the reaction, an aliquot of the gas phase was taken by a microsyringe and analyzed by a Shimadzu gas chromatograph, model GC-4A. Determination of CF_3CH_2Cl and CF_2CHCl was made using authentic materials.

RESULTS

Identification of metabolites: Anaerobic dehalogenation of halothane was performed by the reconstituted system, and metabolites were assayed by gas chromatography. Under the chromatographic conditions employed, only two peaks were observed (Fig. 1), one of which was identified as CF_3CH_2Cl and the other

Table I. Obligatory components for the anaerobic dehalogenation activity (A) and the specificity of the activity for P-450 isozymes (B).

(A) Obligatory components		(B) Specificity for P-450 isozymes	
Components	Activity[a)]	P-450	Activity[a)]
Complete system[b)]	5.0 (100%)	P-450PB	5.0 (100%)
- P-450PB	0.4 (8%)	P-450B_1	4.0 (80%)
- NADPH-P-450 reductase	undetectable	P-448	2.8 (56%)
- NADPH	undetectable		
- Triton X-100	2.4 (48%)		

a) The activity is expressed as nmoles of CF_3CH_2Cl formed / nmole of P-450 per 30 min, and the concentration of each P-450 isozyme was 2.2 nmoles per ml. The reconstituted system as described in MATERIALS AND METHODS was used.
b) P-450PB was used in this reconstituted system.

as unreacted halothane. The peak of CF_2CHCl was negligible (arrow in Fig. 1). Even when P-448 was replaced by either P-450PB or P-450B_1, the peaks of CF_3CH_2Cl and halothane were observed, but the peak of CF_2CHCl could not be detected.

Obligatory components for anaerobic dehalogenation of halothane: As shown in Table I, NADPH-P-450 reductase and P-450 were essential for the reaction and NADPH was required as electron donor. It was also shown that the maximum activity was attained in the presence of 0.03% Triton X-100. The activity was dependent upon the concentration of P-450PB and increased in a linear fashion with increasing concentration of P-450PB at least up to 4 nmoles per ml. The activity was also dependent upon the concentration of NADPH-P-450 reductase and saturated at around 0.3 unit per nmole of P-450PB. When P-450PB was replaced by either P-448 or P-450B_1, the activity with P-448 and P-450B_1 was 56% and 80% of that with P-450PB, respectively.

Effect of inhibitors on the reaction: As shown in Table II, phenyl isocyanide and metyrapone strongly inhibited the reaction. When nitrogen gas phase in the test tube was replaced by carbon monoxide, the activity decreased to 24% that of the control activity. The extent of inhibition by carbon monoxide was less than that observed with anaerobic reaction in microsomes (4). The reaction under air did not produce any CF_3CH_2Cl or CF_2CHCl.

Table II. Effect of inhibitors on the anaerobic dehalogenation activity.

Conditions	Residual activity[a]	Per cent residual activity (%)
Complete system[b]	3.8	100
+ Ethanol (0.02%)	4.1	108
+ Phenyl isocyanide (20 μM)[c]	0.3	8
+ Metyrapone (10 mM)[c]	0.3	8
under carbon monoxide[d]	0.9	24
under air[d]	undetectable	0

a) The activity is expressed as nmoles of CF_3CH_2Cl formed / nmole of P-450 per 30 min.
b) The concentration of each component is described in MATERIALS AND METHODS, and P-450PB (1.7 nmole/ml) was used in this reconstituted system.
c) Phenyl isocyanide and metyrapone were dissolved in ethanol, and the concentration of ethanol in the reaction mixture was 0.02%.
d) Nitrogen gas in the sealed test tube was replaced by either carbon monoxide or air.
Values in parentheses represent the final concentration of the inhibitors and ethanol.

DISCUSSION

The present investigation has strongly indicated that anaerobic dehalogenation of halothane is catalyzed by P-450-dependent mixed function oxidase system. In fact, the maximum activity was attained when the reconstituted system containing P-450PB was used. This result is consistent with the observation that anaerobic dehalogenation of halothane in microsomes is enhanced by pretreatment of rabbits with phenobarbital (4). However, the reconstituted system containing either P-448 or P-450B_1 also showed the activity, indicating that the reaction is not necessarily specific for P-450PB, but halothane is catalyzed by these P-450 isozymes. As to the reaction mechanism, several problems remain to be solved. The reason why CF_2CHCl is not produced by the reconstituted system is unknown. Some other microsomal component which is associated with the formation of CF_2CHCl might exist, or it might be due to loss of the mode of binding of P-450 to microsomal membrane. The formation of trifluoroethyl carbene complex of reduced P-450 does not seem to reconcile with the present results, since elimination of chloride and bromide from the halothane molecule along with the complex formation has been suggested (5).

In connection with this problem, 5 nmoles of CF_3CH_2Cl per nmole of P-450 were produced after the reaction for 30 min, and the reaction continued further. Therefore, the complex, even if it exists, might be a chloride containing intermediate. Two possible reaction mechanisms have been proposed for anaerobic dehalogenation of halothane, in which a radical species was assumed as an intermediate (14). However, it is unknown whether CF_3CH_2Cl is formed by either a two-electron or one-electron metabolic reduction of halothane. Moreover, the radical species has not been identified yet. The elucidation of these problems must await further investigations.

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