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A mechanistic perspective on bacterial metabolism of chlorinated methanes

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Abstract

Chlorinated methanes are important environmental pollutants, which can be metabolized by bacteria. The biotransformation of chlorinated methanes by bacteria has been shown to be due either to gratuitous metabolism (cometabolism) or their use as a source of carbon and energy. The reactions which result in carbon-halogen bond cleavage include substitutive, reductive, oxygenative, and *gem*-elimination mechanisms. Certain methylotrophic bacteria can use dichloromethane as a source of carbon and energy. Dichloromethane dehalogenase catalyzes the first substitutive reaction in this metabolism. The enzyme shows a 10^{10}-fold rate enhancement over the reaction of the bisulfide anion with dichloromethane in water. *Pseudomonas putida* G786 synthesizes cytochrome P-450_{CAM} which catalyzes the gratuitous reduction of chlorinated methanes. These studies with purified enzymes are beginning to reveal more detailed mechanistic features of bacterial chlorinated methane metabolism.

Abbreviations: DNA - deoxyribonucleic acid; k_{cat} - catalytic first order rate constant for an enzyme catalyzed reaction; K_M - Michaelis constant for an enzyme catalyzed reaction; MNDO - modified neglect of diatomic overlap; PIMA - pattern induced multialignment; DCMD - dichloromethane dehalogenase

Introduction

Scope

We have chosen to review the current literature which has mechanistic implications for the metabolism of chlorinated methanes by bacteria. The goal was to correlate the chemical feasibility of certain dehalogenation reactions with the known and proposed biochemical mechanisms. The approach is a selective one; for more broadly based annotations of current literature on the bacterial metabolism of halogenated compounds, excellent recent reviews are available (Hardman 1991; Mohn & Tiedje 1992).

For the purposes of this review, chlorinated methanes will be defined as compounds containing one carbon atom, only hydrogen and halogen substituents, and at least one carbon-chlorine bond (Fig. 1). Even with this restricted definition, 26 chlorinated methanes exist to pose a challenge to microbial metabolic versatility. Most of the discussion in this review will focus on chloromethanes and chlorofluoromethanes.

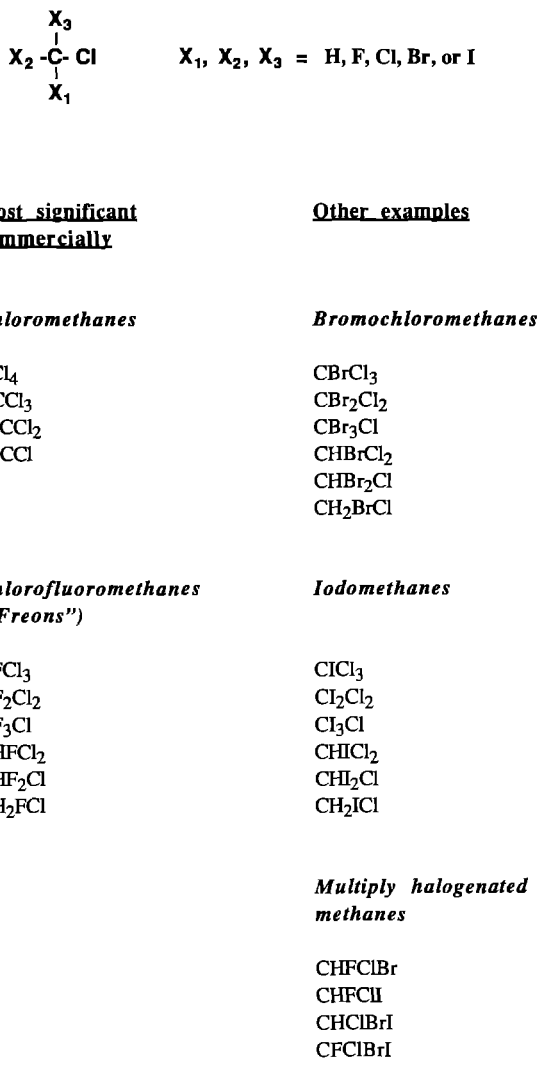


Fig. 1. Chlorinated methanes: substituted methanes containing one or more chlorine substituents and hydrogen and/or halogens.

Significance

The most important compounds in Fig. 1, with respect to their usage and environmental fate, are the chloromethanes and chlorofluoromethanes. As a class, they are stable, low-boiling, non-polar compounds that have usefulness as industrial solvents. This includes usage as a liquid phase for large scale chemical reactions and as degreasing agents for cleaning equipment. They can be easily recovered due to their low boiling points. The chlorofluoromethanes have found widespread usage as refrigerants. Their particularly low boiling points, high stability, and significant heat capacities make them ideal as thermal transfer agents.

All of the compounds on the left side of Fig. 1, with the possible exception of chlorofluoromethane, are in widespread commercial use. Their extensive usage and high volatility have contributed to their entry into the environment. One of them, chloromethane, is a significant natural product (Neidleman & Geigert 1986). Biological synthesis of chloromethane, principally by wood rot fungi, accounts for an environmental efflux of 5×10^9 Kg annually (Rasmussen et al. 1980).

Concerns over the entry of chlorinated methanes into soil, water, and atmosphere reside in the public health consequences from environmental contamination. For instance, chloromethane is directly reactive with biological molecules; reactions with DNA can result in hereditary defects. The more highly chlorinated methanes are generally not reactive with cellular constituents until they have become activated by metabolism (Anders & Pohl 1985). Mammalian liver cytochrome P-450 monooxygenase is principally involved in metabolic activation via hydroxylation which leads to elimination reactions that yield reactive acyl chlorides. This leads to covalent modification of proteins, lipids, and nucleic acids and often underlies the toxic and/or carcinogenic effects of these molecules (Anders & Pohl 1985). Consequently, workplace and environmental exposure to chlorinated methanes is tightly regulated. In contrast, chlorofluoromethanes (Freons) bearing no C-H bonds are not mutagenic (Greim et al. 1977) and are generally non-toxic (Leuschner et al. 1983). This has contributed to their widespread usage. The extreme stability of the Freons, coupled with their low boiling points, causes their long-term survival on and near earth's surface. The reaction of Freons with ozone in the upper atmosphere has contributed to the critical thinning of this protective shield against ultraviolet radiation (Anderson et al. 1991). Thus, the fate of Freons on the earth's surface is currently of great concern.

General Principles

Chemical reactivity of halomethanes

The course of microbial metabolism is determined by: (a) the chemical properties of the substrate and its products, and (b) the available biological macromolecules capable of transforming the substrate. Both of these properties determine the reaction mechanism. General mechanisms of carbon-chlorine bond cleavage are shown in Fig. 2.

Some general chemical properties of the reactions of chlorinated methanes are:

(a) For reactions where the rate-limiting step is the cleavage of the carbon halogen bond, the order of reactivity is: C-I > C-Br > C-Cl > C-F.

(b) The less highly halogenated compounds are more susceptible to hydrolysis.

(c) The more highly halogenated compounds are increasingly inert to hydrolysis and more susceptible to decomposition by reductive and/or radical mechanisms.

(d) For the case of reductive mechanisms of degradation, there can be no β-elimination reactions for C_1 compounds (β-elimination is a common reaction in the case of polychlorinated ethanes-see Schanke and Wackett 1992, and references therein).

(e) Acyl chlorides from chlorinated methane metabolism are highly reactive with cellular nucleophiles or with water. The former reactions may be cytotoxic.

These are general trends, and there can often be exceptions. For instance, with an oxgenative mechanism, based on ease of oxygen insertion into the carbon hydrogen alone, the expected order of reactivity is $HCCl_3 > CH_2Cl_2 > CH_3Cl$. However electronic considerations alone do not dominate in determining relative rates for bacterial oxygenation reactions, since methane monooxygenase can catalyze oxygen insertion into a C-H bond of the physiological substrate methane which at 100 kcal/mol is significantly stronger than those of the chlorinated methanes (March 1985). Eliminative reactions can occur with chloroform following proton abstraction by a strong base (Hine et al. 1956; Fig. 2D) (not shown for biological systems) or with carbon tetrachloride following reductive metal dependent halide displacement (discussed below).

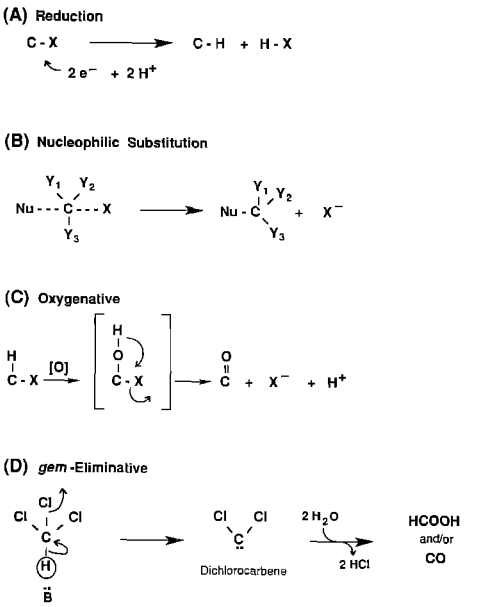


Fig. 2. General mechanisms of carbon halogen bond cleavage. The *gem*-eliminative reaction is illustrated by the well known elimination of chloride from trichloromethyl anion. In the figure, X is Cl, Br, or I; Nu is a nucleophile; B is a strong base.

Cometabolism vs Metabolism

The term cometabolism, as used here, refers to gratuitous metabolism or the transformation of a substrate with no apparent benefit to the organism carrying out the reaction(s). There are numerous examples of cometabolism occurring with chlorinated methanes; for example, as discussed below, all of the wholly chlorinated chloromethanes can be degraded by cometabolic processes in bacteria. In some cases, this gratuitous metabolism can be detrimental. The oxidation of chloroform by the methane monooxygenase of methanotrophs leads to cell toxicity (Alvarez-Cohen & McCarty 1991b), presumably due to hydroxylation and *gem*-elimination of HCl to yield phosgene (Anders & Pohl 1985).

Metabolism, as used here, refers to the transformation of a substrate which results in some benefit to the organism. Typically, the substrate serves

as a carbon source, as a catabolic energy source, or as a final electron acceptor. Chloromethane and dichloromethane are the only chlorinated methanes which have been shown to be metabolized by bacteria, serving as the carbon and energy sources for both aerobic and anaerobic bacteria (see below). The utilization of chlorinated organic compounds as final electron acceptors has been proposed (Tiedje et al. 1987; Freedman & Gossett 1991; Holliger 1992); this ability is most well-documented for the reduction of 3-chlorobenzoate by *Desulfomonile tiedjei* (Mohn & Tiedje 1990). A similar phenomenon is suggested to be occurring in an anaerobic consortium in which a significant fraction of electrons from the oxidation of methanol is being used for reductive dechlorination of tetrachloroethylene (DiStefano et al. 1991). However, there is as yet no clear evidence for the utilization of chlorinated methanes as a final electron acceptor linked to energy metabolism.

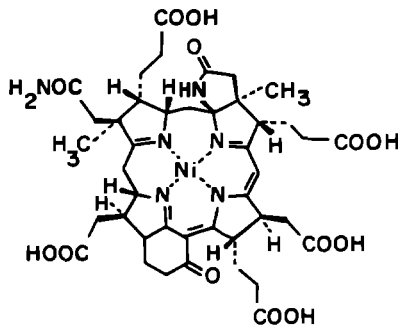
Regardless of the definitions applied to it, metabolism and cometabolism of substrates can be of great importance in biodegradation. For example, trichloroethylene (TCE) is oxidized by methanotrophs at 70% the rate of the physiological substrate methane (Brusseau et al. 1990; Fox et al. 1990) allowing bioremediation schemes of alternating methane and TCE metabolism (Alvarez-Cohen & McCarty 1991a, 1991b). With highly chlorinated recalcitrant compounds, cometabolic degradation processes are potentially extremely valuable.

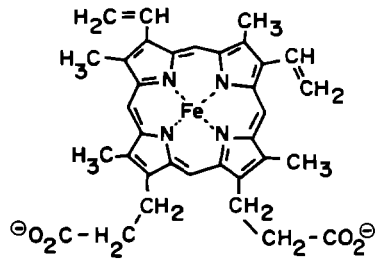
Overview of mechanisms in chlorinated methane metabolism

C_1 compounds span an eight electron redox range from methane to carbon dioxide. For each species more oxidized than methane a physiological equivalent exists to provide entry points for chlorinated methanes into intermediary metabolism (Table 1). In this context, the most obvious bacterial candidates for chlorinated methane metabolism are those capable of metabolizing and cometabolizing other C_1 compounds. Methanotrophic bacteria oxidize methane to carbon dioxide in their principle energy metabolism (Anthony 1982). Methylotrophs lack methane-oxidizing ability but can oxidize methanol and other C_1 compounds such as methylamine. Carboxydobacteria are known to use carbon monoxide as the sole carbon and energy source (Meyer & Schlegel 1983). In anaerobic environments, C_1 reduction reactions occur. Methanogens use carbon dioxide as a final electron acceptor in their energy metabolism. Intermediates are produced which are at the same oxidation level as shown in Table 1 and specific cofactors exist to carry them through the entire eight electron reduction (Wolfe 1985). Acetogens can biosynthesize acetate via condensation of two enzyme-bound C_1-units formally at the carbon monoxide and methanol oxidation levels, respectively (Ragsdale & Wood 1985; Raybuck et al. 1987). Both methanogens and acetogens utilize numerous metalloenzymes in the oxidation-reduction of C_1 intermediates. For example, carbon monoxide dehydrogenase, a nickel iron-sulfur protein, and a corrinoid

Table 1. Oxidation states of C_1 compounds differing by two electrons. Shown are oxygenated and chlorinated C_1 compounds and their physiological equivalents.

Common oxidation states of C_1 compounds	Equivalent chlorinated methane	Physiological C_1 equivalent	Physiological example
CH_4	–	–	end product or oxidizable substrate
CH_3OH	CH_3Cl	CH_3-X where X is O, N, or S	methyl-S-coenzyme M; N^5-methyl tetrahydrofolate
H_2CO	H_2CCl_2	-N-CH_2-N- or -SCH_2OH	N^5, N^{10}-methylene tetrahydrofolate
HCOOH or CO	$HCCl_3$	-N=C-N-	N^5,N^{10}-methenyl tetrahydrofolate
CO_2	CCl_4	C-CO_2^-	ribulose *bis*-phosphate carboxylase product





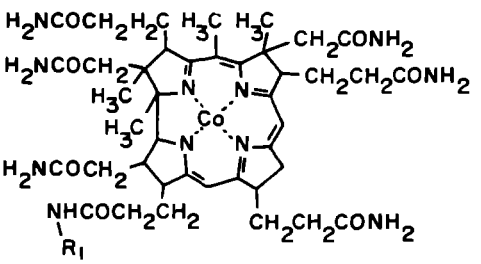


Fig. 3. Transition metal coenzymes: (A) Coenzyme F430, (B) Hematin, and (C) Cobalamin. Complete ligand structures and axial ligands have been omitted for clarity of presentation.

protein are involved in the acetate biosynthetic pathway (Ragsdale & Wood 1985). Methanogens contain high levels of a unique nickel corphinoid cofactor, coenzyme F430, which participates in the ultimate two electron reduction step yielding methane (Pfaltz et al. 1982; Wolfe 1985; Diekert et al. 1980). Methanogens also contain the cobalt-containing corrinoids. Although all the roles of corrinoids are likely not known, methyl group transfer

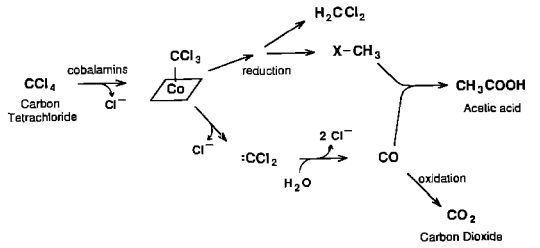


Fig. 4. Proposed mechanism to explain formation of CO_2 and reduced products from carbon tetrachloride (Krone et al. 1989). Bacterial transformations are with acetogens (Egli et al. 1988b), methanogens (Egli et al. 1988a), denitrifiers (Criddle et al. 1990b) and *E. coli* (Criddle et al. 1990a).

to coenzyme M in the methane-yielding pathway has been demonstrated (Wood et al. 1982).

These pathways and their cofactors provide the evolutionary raw material for directed and fortuitous metabolism of chlorinated methanes. In 1987, Vogel et al. described important parameters in the microbial metabolism of halogenated aliphatic compounds. The potential for reactivity between bacterial cofactors and halogenated aliphatic compounds was discussed. Numerous studies had documented the reactions of alkyl halides with hematin, or protoporphyrin IX (Wade & Castro 1973; Klecka & Gonsior 1984), and cobalamins (corrinoids or vitamin B_{12} and its variants) (Schrauzer & Deutsch 1969). Vogel et al. (1987) proposed that coenzyme F430, the nickel-containing corphinoid macrocycle of methanogens, might also be reactive with halogenated organic compounds.

Since 1987, a series of studies have focused on the reactions of chlorinated methanes with bacterial transition-metal coenzymes (Fig. 3). These coenzymes function physiologically in both electron transfer and bond-breaking and forming reactions, consistent with a role in redox mediated carbon-halogen bond cleavage (Gantzer & Wackett 1991). There is evidence in the literature for both reductive carbon-halogen bond cleavage and hydrolytic reactions emanating from reaction of carbene intermediates with bulk solvent water. Several lines of evidence indicate that reactions demonstrated with purified coenzymes *in vitro* are comparable to those observed in *in vivo* microbial transformations. Krone, et al. (1989a,b) showed

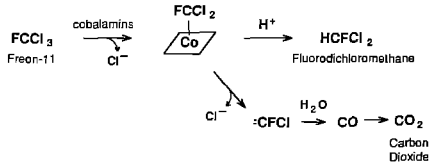


Fig. 5. Proposed reactions of Freon-11 with cobalamins (Krone et al. 1991). Similar reactions have been observed, with different stoichiometeries, with *Methanosarcina barkeri* (Krone & Thauer 1992).

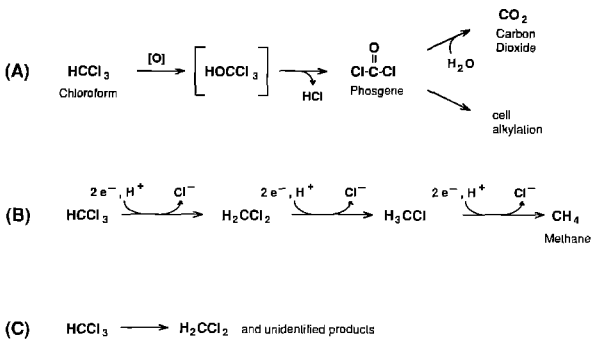


Fig. 6. Chloroform biotransformations by (A) methanotrophs (Oldenhuis et al. 1991) and ammonia oxidizers (Vanelli et al. 1990), (B) anaerobic consortia (Bouwer et al. 1981; Vogel et al. 1987) and methanogens (Krone et al. 1989b), and (C) *Clostridium* sp. (Gälli & McCarty 1989).

that cobalamins (Fig. 3C) and coenzyme F430 (Fig. 3A) catalyzed the reduction of chlorinated methanes (Fig. 4). For example, with carbon tetrachloride (Fig. 4), the rates of the dehalogenation decreased in the series CCl_4, $CHCl_3$ and CH_2Cl_2. Similar reduction reactions and kinetics were observed with *Methanosarcina barkeri* (Krone, 1989b) and, independently, with other anaerobic bacteria (Bouwer et al. 1981; Bouwer & McCarty 1983; Egli et al. 1988b). A further link between the *in vitro* coenzyme reactions and microbial metabolism derived from observations by Egli et al. (1988b). The acetogen *Acetobacterium woodii* catalyzes a relatively rapid conversion of carbon tetrachloride to carbon dioxide coincident with reductive dechlorination to chloroform and dichloromethane. Similar products were observed with cells of *A. woodii* which were autoclaved (Egli et al. 1990). In a study with corrinoids in solution, $< 50\%$ of the starting carbon tetrachloride could be accounted for as volatile hydrocarbon products (Krone et al. 1989b). This discrepancy was resolved in a subsequent study on the reaction of corrinoids with chlorofluoromethanes (Krone et al. 1991). It was demonstrated that halide elimination from a free or cobalt-bound carbanion likely gives rise to carbenes which readily undergo hydrolysis in an aqueous medium (Fig. 5) to yield carbon monoxide; the production of carbenes was suggested as a possible reason for the toxicity of highly halogenated C_1 compounds for various anaerobic bacteria. Carbon monoxide was undetectable by the gas chromatography system used in the previous study (Krone et al. 1989b). Furthermore, these reactions could account for the previous observations that live and autoclaved cells of *A. woodii* gave rise to CO_2 (Fig. 4) (Egli et al. 1988 a,b). Carbon tetrachloride was likely transformed to CO by corrinoids and subsequently oxidized to CO_2 by carbon monoxide dehydrogenase. More recently, fluorotrichloromethane (Freon-11) reduction by *Methanosarcina barkeri* has been demonstrated to yield both fluorodichloromethane, carbon monoxide and fluoride, consistent with a significant *in vivo* carbene pathway (Fig. 5) (Krone et al. 1992).

Chloroform is reactive with reduced coenzymes and is reduced by anaerobic bacteria (Fig. 6B). Additionally, the presence of a C-H bond allows for gratuitous oxidation by non-specific bacterial oxygenases (Fig. 6A). The latter pathway yields phosgene which can react with cellular nucleophiles or undergo hydrolysis to carbon dioxide (Anders & Pohl 1985).

Dichloromethane is metabolized as a sole source of carbon and energy or it may be oxidized gratuitously by the soluble methane monooxygenase of methanotrophic bacteria (Colby et al. 1977; Patel et al. 1982) (Fig. 7). The latter pathway generates reactive formyl chloride and, by subsequent hydrolysis, formate (Fig. 7B). Both aerobic and anaerobic bacteria utilize dichloromethane as a carbon and energy source. The anaerobic systems are consortia and the mechanism(s) of dehalogenation have yet to be defined (Freedman & Gossett 1991; Stromeyer et al. 1991) (Fig. 7C). Aerobic dichloromethane metabolism by methylotrophs has been

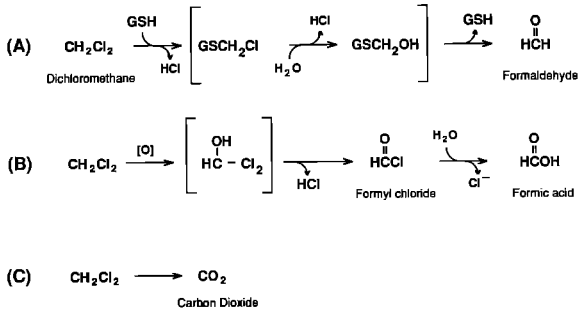


Fig. 7. Dichloromethane metabolism. (A) Proposed mechanism for methylotroph dichloromethane dehalogenase (Kohler-Staub & Leisinger 1985), (B) Methanotrophic oxidation (Patel et al. 1982) following pathway proposed for mammalian cytochrome P-450 (Anders & Pohl 1985), and (C) unknown mechanism for anaerobic consortia (Freedman & Gosset 1991; Stromeyer et al. 1991).

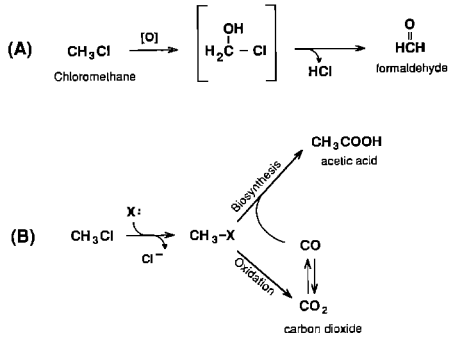


Fig. 8. Chloromethane metabolism by (A) aerobic methylotrophs (Hartmans et al. 1986) and methanotrophs (Patel et al. 1982), and (B) an anaerobic acetogen (Traunecker et al. 1991).

studied extensively. Two distinct enzymes that transform dichloromethane to formaldehyde have been purified to homogeneity (Kohler-Staub & Leisinger 1985; Scholtz et al. 1988) and they are discussed in greater detail below (Fig. 7A).

Chloromethane is a prevalent natural product (Neidleman & Geigert 1986; Wuosmaa & Hager 1990) and contains significant chemical energy for release by oxidative metabolism. It is not surprising that bacteria capable of utilizing chloromethane as a sole source of carbon and energy have been identified (Hartmans et al. 1986; Traunecker et al. 1991). An anaerobic homoacetogen is known to grow on chloromethane (Fig. 8B). A pathway has been proposed that feeds into acetogenesis although the identity of the dehalogenation catalyst remains undefined. Methanotrophs are also known to gratuitously oxidize chloromethane (Fig. 8A) similarly to other chlorinated methanes which contain a C-H bond.

Currently, it is difficult to assess the quantitative contribution of cobalamins, coenzyme F430, hematin, and other potential biocatalysts in microbial transformations of chlorinated methanes on a global scale. These biological cofactors have demonstrated reactivity when free in solution (Wade & Castro 1973; Krone et al. 1989a; Krone et al. 1989b; Gantzer & Wackett 1991) and in enzyme bound form (Castro et al. 1985; Wood et al. 1968; Holliger 1992). Further studies are required to determine the ecological significance and biotechnological potential of these biocatalysts.

Specific examples

Dichloromethane dehalogenase

Dichloromethane, or methylene chloride, is a widespread industrial solvent. It is significantly soluble in water, ~150 mM at saturation (Dean 1985). Mammalian exposure to chronic low concentrations or high concentrations may lead to cancer or immediate death, respectively (Anders & Pohl 1985). These toxicities are due to hydroxylation of dichloromethane by liver cytochrome P-450 monooxygenase. The hydroxylation and subsequent spontaneous elimination reactions lead to formyl chloride formation (Fig. 9). Formyl chloride may undergo a second *gem*-elimination reaction yielding carbon monoxide which binds tightly to the oxygen carrying protein hemoglobin. Animal exposure to 400 ppm dichloromethane in air can cause death due to asphyxiation. Intracellular formyl chloride also undergoes direct reactions with cellular nucleophiles, including DNA. Misrepair of DNA can lead to genetic changes with one manifestation being carcinogenesis.

Overlaid on these public health consequences is the relative abiotic stability of dichloromethane in the environment. In water at neutral pH and 25 °C, dichloromethane undergoes hydrolysis with a half-

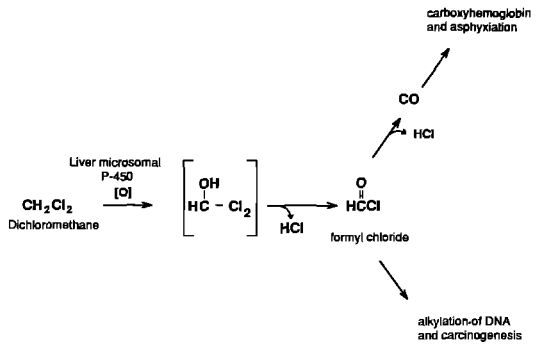


Fig. 9. Metabolic activation of dichloromethane in mammalian liver.

life of 704 years (Mabey & Mill 1978; Roberts 1991). The combined properties of toxicity and environmental persistance have caused the inclusion of dichloromethane on the Environmental Protection Agency's Priority Pollutant list.

It is of great interest that both aerobic and anaerobic bacteria have been identified which are capable of growth on dichloromethane. The aerobic bacteria have been studied extensively. Most pure culture isolates have been obtained by Leisinger and coworkers in Switzerland (Stucki et al. 1981a; Gälli 1986; Scholtz et al. 1988) but similar organisms have been isolated in the United States (D. Paone, Celgene Corporation, personal communication). In all cases, the dichloromethane degrading strains are methylotrophic bacteria. The first demonstrated product of dichloromethane metabolism by these organisms is formaldehyde (Stucki et al. 1981b), which can undergo further oxidation for energy or be assimilated for biosynthesis. The reaction is formally hydrolytic (Fig. 7A) although it is postulated not to occur via direct hydroxide attack on dichloromethane (Kohler-Staub & Leisinger 1985). In general, hydrolysis or other nucleophilic displacement reactions of dihalomethanes are very sluggish compared to their methyl halide counterparts (Moelwyn-Hughes 1949; Roberts & Gschwend 1992). For example, methyl bromide reacts with hydroxide ion 230 fold faster than does dibromomethane. This heightens interest in determining the biological mechanism(s) of dichloromethane hydrolysis.

A major step in developing mechanistic insight came with the purification of dichloromethane dehalogenase from *Hyphomicrobium* DM2 (Kohler-Staub & Leisinger 1985). The purified protein transformed dichloromethane, dibromomethane, diiodomethane, and bromochloromethane to formaldehyde, but not a series of chlorinated ethanes, propanes, and olefins. This same enzyme was shown to be present in other methylotrophic bacteria via immunological methods (Kohler-Staub et al. 1986). This class of dichloromethane dehalogenases is known as the group A enzymes. More recently, a distinct dichloromethane dehalogenase was found in strain DM11 and it was denoted as a type B dehalogenase (Scholtz et al. 1988). Both groups of dehalogenase enzymes required glutathione for enzyme activity and this proved to be an important mechanistic clue. Leisinger and coworkers proposed that the glutathione thiolate may be the initial nucleophile functioning in chloride displacement with the resultant facile hydrolysis of an intermediate chloromethylthioether (Fig. 7A). This proposition was consistent with the observations of Ahmed and Anders (1976,1978) that rat liver protein extracts catalyze the glutathione-dependent hydrolysis of dichloromethane. More recently, cloning and sequencing of the bacterial group A dichloromethane dehalogenase gene (group A) revealed primary sequence homology with glutathione S-transferases which are abundant in mammalian liver (LaRoche & Leisinger 1990). Glutathione S-transferases are reported to enhance the nucleophilicity of the glutathione thiolate at neutral pH for attack on various electrophilic cosubstrates (Keen et al. 1976; Graminsky et al. 1989), consistent with the Leisinger model.

It is instructive to compare these enzyme-catalyzed rates with chemical rates for thiolytic attack on dichloromethane and other dihalomethanes. The thiolate anion is a stronger nucleophile than the hydroxide anion (March 1985), yet its uncatalyzed reaction with dichloromethane is slow; for example, a second order rate constant of 6.02×10^{-6} s^{-1} M^{-1} (Table 2) is reported for the reaction of dichloromethane with bisulfide anion (HS^-) (Roberts & Gschwend 1992). The comparable second order rate constant for an enzyme catalyzed reaction is the parameter k_{cat}/K_M. Recent work in

our laboratory has identified the liver glutathione S-transferase isozymes which are active in dichloromethane metabolism. This allows us to use the data of Ahmed and Anders (1976,1978) to determine an aggregate k_{cat}/K_M value for the liver transferases with dichloromethane. The value of 3.4 s^{-1} M^{-1} is an average for all the transferases, taking into account their estimated amounts in liver extracts. These mammalian enzymes show a rate enhancement of ~ 10^5 over the uncatalyzed reaction. The group B dehalogenase from bacterial strain DM11 shows a k_{cat}/K_M of 6.1 × 10^4 s^{-1} M^{-1}, fully 10^{10} greater than the uncatalyzed reaction of thiolate anion with dichloromethane. The slower dichloromethane dehalogenase from bacterial strain DM4 (group A) still shows rate enhancements of ~ 10^9 over the reaction of bisulfide anion with dichloromethane in water.

Further efforts are underway to better understand the enzyme reaction mechanism. Both DM2 (group A) and DM11 (group B) dehalogenase types have been shown to transform CD_2Cl_2 and CD_2Br_2, respectively, to *dideutero*-formaldehyde, consistent with nucleophilic displacement of halide substituents (Gälli et al. 1982; Bao-li & Wackett, unpublished data). Although chloromethane is generally more reactive with nucleophiles, it is not a substrate turned over by the enzyme; neither methanol nor S-methylglutathione were observed in incubations of enzyme, chloromethane, and glutathione (Bao-li & Wackett, unpublished data). The enzyme must exercise control over the reaction course and this is likely central to the observed large rate acceleration. Kohler-Staub & Leisinger (1985) observed that 1,1-dichloroethane did not undergo dehalogenation, although it was a competitive inhibitor, which further demonstrates the specificity of the catalyzed reaction.

It is instructive to compare a series of dihalomethane substrates for reactivity with enzyme and thiolate model systems. In Table 2, the reactivity of CH_2Cl_2, CH_2Br_2, CH_2BrCl with HS^- increases in the ratio 1.0, 28.9, 40.2, respectively. The comparative DM11 enzyme reaction parameter k_{cat}/K_M increases in the ratio 1.0, 4.4, 5.7 for the same series. The trend is the same but the lower magnitude of change suggests that the enzyme is doing more than directing the glutathione thiolate in nucleophilic attack on dihalomethanes. A prominent feature of enzyme catalysis is proximity effects or increasing the effective molarity of cosubstrates in the active site to bring them together for reaction (Walsh 1979; Jencks 1975). The glutathione S-transferases are thought to catalyze reactions by proximity effects and by deprotonating the glutathione thiol group at neutral pH thereby increasing its nucleophilicity (Jakoby & Keen 1977; Douglas 1987; Liu et al. 1992; Armstrong, 1991; Graminski et al. 1989). The bacterial enzymes show a k_{cat}/K_M of ~ 10^4 greater than most of the glutathione S-transferases. Approximately 10^3 of this second order rate enhancement is due to the K_M term (Bao-li & Wackett, unpublished data and Ahmed & Anders, 1976). Further studies are required to better define the mechanisms underlying the relatively rapid second order rate constants observed for the bacterial enzymes. Experiments using affinity labels and a putative mechanism-based inactivator have been

Table 2. Kinetic parameters for the reaction of dihalomethanes with bisulfide anion, rat liver glutathione S-transferase, and bacterial strain DM 11 dichloromethane dehalogenase (type B).

Substrate	Bisulfide anion (HS^-)		Glutathione transferase		Dichloromethane dehalogenase		
	k_{obs} (s^{-1} M^{-1})[1]	Relative k	k_{cat}/K_m (s^{-1} M^{-1})[2]	Relative k	k_{cat}/K_m (s^{-1} M^{-1})[3]	Relative k	k_{enzyme}/k_{HS^-}
CH_2Cl_2	6.02 × 10^{-6}	1.0	3.4	1.0	6.1 × 10^4	1.0	1.0 × 10^{10}
CH_2Br_2	1.74 × 10^{-4}	28.9	18.6	5.5	2.7 × 10^5	4.4	1.6 × 10^9
CH_2BrCl	2.42 × 10^{-4}	40.2	19.3	5.7	3.5 × 10^5	5.7	1.4 × 10^9

[1] Data from Roberts and Gschwend (1992).
[2] Data from Ahmed & Anders(1976); Blocki & Wackett (unpublished).
[3] Bao-Li & Wackett (unpublished).

initiated. These studies should uncover active site residues participating in catalysis and help delineate the reaction cycle.

A comparative enzymology approach is also fruitful, building on LaRoche and Leisinger's (1990) observation that the bacterial dehalogenase primary structure places it in the glutathione S-transferase supergene family. Classes within the family have been designated alpha, mu, and pi. Meyer et al. (1991) have expanded the supergene family to include a fourth class, theta. The theta class includes a minor isozyme from rat liver (5–5) which fails to bind to glutathione affinity columns and shows slightly greater activity with dichloromethane than does the bacterial enzyme. Other glutathione S-transferase isozymes have been purified from rat liver and assayed for activity with dichloromethane (Blocki & Wackett, unpublished data). While the alpha class isozymes had no detectable activity, the mu class isozymes 3–3, 3–4, and 4–4 had specific activities in the range of 5–40 nmol/min per mg protein or one hundred to one thousand fold lower than the bacterial dehalogenase and liver isozyme 5–5.

The recent availability of x-ray crystallographic structures for pig lung pi glutathione S-transferase isozyme (Reinemer 1991) and rat liver mu isozyme 3–3, (Liu et al. 1992) focus attention on potential bacterial-mammalian enzyme sequence homology to provide information on the secondary and tertiary elements of these enzyme structures. Application of the Chou-Fasman (1978) secondary structure algorithm to the N-terminal pi glutathione S-transferase primary sequence yielded predicted structures in agreement with elements seen in the crystal structure (Table 3). Similarly, Table 3 shows that the predicted N-terminal secondary elements of DM4 dichloromethane dehalogenase are congruent with other glutathione S-transferases.

The N-terminus of the pig lung pi glutathione S-transferase contributes significantly to the glutathione binding site: three out of the ten total enzyme glutathione contact points lie within the first 13 amino acids. Tyrosine-7, at the end of β-sheet 1, is within hydrogen bonding distance of the cysteinyl thiolate of enzyme bound glutathione and is highly conserved amongst all the transferases. Arginine-13 at the N-terminus of the first α-helix affords the primary binding site for the α-carboxy group of the γ-glutamyl group of glutathione. A helix dipole (α-helix residues 15–25 of the pig lung pi transferase and 14–24 of isozyme 3–3) has been recognized as contributing constructively to the electrostatic field near the sulfhydryl group of glutathione (Reinemer 1991; Liu et al. 1992).

A search of the Swissprot, EMBL, GenBank, and PIR-30 databanks provided 72 glutathione S-transferase amino acid sequences from human, rat, mouse, Helminth, pig, chicken, maize, fruit fly, house fly, yeast, wheat, fluke, *Caenorhabditis, Serratia marcescens,* and *Platessa.* Given that a major portion of the N-terminus contributes to the glutathione binding site, and that only 30 residues were available for rat 5–5 and *S. marcescens* trans-

Table 3. Comparison of secondary structural elements for bacterial dichloromethane dehalogenase from strain DM4 and mammalian glutathione S-transferases.

Glutathione S-transferase	Evidence for structure	Amino acid sequence of defined secondary structure[1]			Reference
		β-sheet	β-turn	α-helix	
pig pi	X-ray crystal structure	3–7	10–13	15–25	Reinemer et al. 1991
pig pi	Chou-Fasman rules	2–8	10–13	13–27	this study
rat liver 3–3	X-ray crystal structure	–	–	14–24	Liu et al. 1992
rat liver 3–3	Chou-Fasman rules	2–7	8–11	15–34	this study
Bacterial DM4[2]	Chou-Fasman rules	0–6	9–12	14–33	this study

[1] The amino acid numbering starts with 1 at the N-terminus unless otherwise indicated.

[2] Aligned such that tyrosine-18 of DM4 is in register with tyrosine-5 of GST 5 according to Fig. 6 of Meyer et al. (1991).

ferases, the 30 N-terminal residues from each sequence were aligned (Fig. 10) using the PIMA (pattern induced multi-alignment) algorithm of Smith and Smith (1990). Similar results were obtained when the complete sequences were run (Blocki & Wackett, unpublished data). A higher similarity score indicates stronger sequence homology. As predicted by Meyer et al. (1991), both fruit fly glutathione S-transferase and the dichloromethane dehalogenase (DCMD) appear to align in association with rat GST 5–5 in the theta family (Fig. 10).

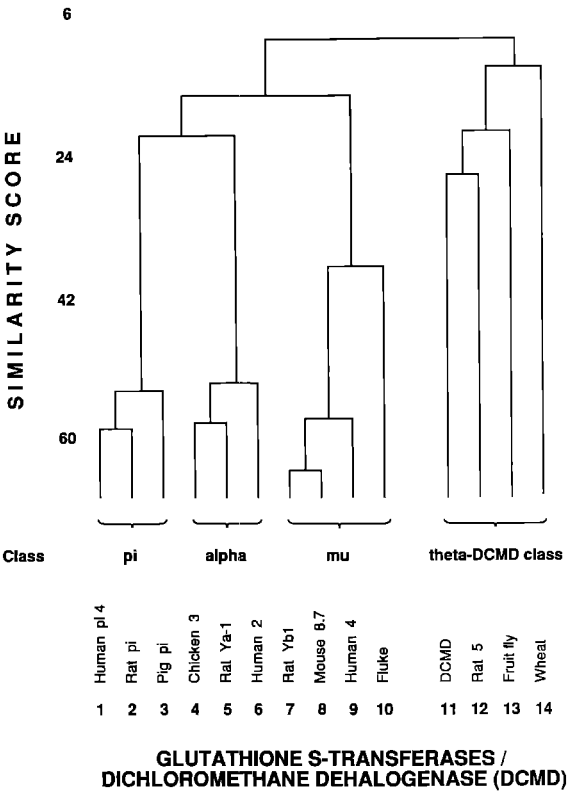


Fig. 10. PIMA generated cladogram showing primary sequence homology for the N-terminal regions of dichoromethane dehalogenase (DCMD) and selected glutathione S-transferases. Data bank loci of references listed below correspond to the entries on the x-axis from left to right: (1) HUMGSTP14; (2) XURTG; (3) S13780; (4) CHKCL3; (5) XURTG; (6) A25909; (7) A25510; (8) A28946; (9) 50719; (10) A26484; (11) DCMA, (dichoromethane dehalogenase, DCMD, LaRoche & Leisinger 1990); (12) S1435; (13) XUFF11; (14) TAGSTAGST. Dichloromethane dehalogenase was compared with residues 14–43 in line with transferase sequences 1–30. As indicated by Meyer et al. (1991), this places DCMD Tyr-18 in register with Tyr-5 of rat subunit 5.

Reductive dehalogenation by cytochrome P-450$_{CAM}$

Environmental reductive dehalogenation reactions often occur at very low rates and this may reflect the unsuitability for assimilation of highly chlorinated compounds into intermediary metabolism. For example, the carbon atom in carbon tetrachloride is in the same oxidation state as is CO_2. Carbon tetrachloride cannot serve as an energy source for a microorganism and it can provide carbon for assimilation only after the cleavage of four carbon-to-chlorine bonds. Carbon tetrachloride can theoretically serve as a final electron acceptor; its redox potential for reduction to chloroform in aqueous solution at pH 7.0 is 516 mV (Krone et al. 1989a), making it accessible to biological electron donors (Vogel et al. 1987). In the environment, carbon tetrachloride would compete as an electron acceptor with carbon dioxide, sulfate, nitrate and other globally significant electron sinks. Thus, it is not surprising that biological carbon tetrachloride reduction proceeds slowly in anaerobic ecosystems and that a significant amount of that metabolism may be fundamentally cometabolic. Carbon tetrachloride coreduction is catalyzed by pure cultures of an acetogenic bacterium (Egli et al. 1988b), methanogens (Egli et al. 1988a), a denitrifying *Pseudomonas* sp. (Criddle et al. 1990b), *Escherichia coli* (Criddle et al. 1990a) and a *Clostridium* sp. (Gälli & McCarty 1989). These organisms have not been shown to benefit from the transfer of electrons to carbon tetrachloride. The specific intracellular catalyst(s) responsible for carbon tetrachloride reductions were not elucidated in the examples above. Cobalamin participation is suggested by the rapid formation of CO_2 with *Acetobacterium woodii* which might be derived from an intermediate dichlorocarbene (Egli et al. 1988b; Krone et al. 1991). Furthermore, it is unknown whether transition metal coenzymes are participants in coreductive reactions largely as freely diffusible intracellular molecules or while coordinated in enzyme active sites.

There is currently evidence for the functioning of the heme protein cytochrome P-450_{CAM} in reductive dehalogenation reactions catalyzed by *Pseudomonas putida* G786 (Castro et al. 1985; Lam & Vilker 1987). For example, both whole cells and purified enzyme reduce carbon tetrachloride and bromotrichloromethane to yield chloroform. In more recent studies, whole cell reactions were shown to be strongly inhibited by (+)-camphor (Logan et al. 1992), consistent with the idea that cytochrome P-450_{CAM} is the major or only catalyst responsible for reductive dehalogenation in *P. putida* G786.

P. putida G786 is a derivative of *P. putida* G1 which was isolated by I. Gunsalus and coworkers using (+)-camphor as an enrichment substrate (Bradshaw et al. 1959). The first step in camphor metabolism is a hydroxylation reaction yielding *5-exo*-hydroxycamphor (Fig. 11A). This regio- and enantiospecific reaction is catalyzed by cytochrome P-450_{CAM} monooxygenase (Gunsalus et al. 1974). Detailed study of this enzyme, principally by the laboratories of I.C. Gunsalus, S.G. Sligar, and T. Poulos, has contributed greatly to current knowledge on oxygenase structure and function. A high resolution x-ray structure of cytochrome P-450_{CAM} is now available (Poulos et al. 1985) and key steps along the oxygenative reaction cycle have been delineated (Sligar et al. 1991; Poulos & Raag 1992). Cytochrome P-450_{CAM} accepts two electrons in the course of activating molecular oxygen for reaction with camphor. In *P. putida* G786, cytochrome P-450_{CAM} reduction is accomplished by electron transfer from NADH via two accessory redox active proteins, putidaredoxin and putidaredoxin reductase (Fig. 12). Electron transfer to the cytochrome P-450 heme iron can occur in the absence of oxygen allowing alternative heme chemistry including reductive dehalogenation (Fig. 11B, Fig. 12). For this to occur, the polypeptide surrounding the heme must provide a suitable environment for substrate binding to occur. As an example, our laboratory has determined that hexachloroethane binds to the active site of cytochrome P-450_{CAM} with a dissociation constant (K_D) of 0.6 μM (Fig. 13), an affinity comparable to the binding of the physiological substrate camphor (Fisher & Sligar 1985). Previous studies have shown that tight substrate binding expels water molecules from the active site modulating the heme iron from low to high spin with a concomitant raising of the midpoint potential by 137 mV (Sligar & Gunsalus 1976; Poulos & Raag 1992). In the physiological reaction cycle, these changes facilitate cytochrome P-450_{CAM} reduction by putidaredoxin allowing oxygen binding in the next step. It is currently unclear how the events associated with substrate binding effect reductive dehalogenation reactions. Previous work in our laboratory has shown that the substrate K_D increases and the percent low to high spin iron conversion decreases as a function of decreasing chlorination of ethane (Logan et al. 1992). These substrate binding parameters should also be determined for chlorinated methanes given the known reactivity of carbon tetrachloride and bromotrichloromethane with cytochrome P-450_{CAM}. It is of interest that the rates of reduction of halogenated ethanes decreased with decreasing dechlorination (Logan et al. 1992). Further work is required to assess the relative importance of substrate binding and/or substrate reactivity parameters on the rate of the reaction.

Fukui et al. (1963) have shown that the redox midpoint potential decreased for halogenated methanes of decreasing halogenation and across the series C-I > C-Br > C-Cl. Polarographically determined redox potentials were shown to correlate with the energy of the lowest unoccupied molecular orbital (LUMO). By this analysis, the ease of reduction corresponds to the energy required to put one electron into a carbon halogen σ^* or antibonding orbital (Hanzlick 1981). One electron input with bond cleavage yields a halogen anion and a carbon centered radical. Radical intermediates have been observed, for example in the reduction of carbon tetrachloride by mammalian cytochrome P-450 isozymes (Anders & Pohl 1985). Such intermediates remain to be demonstrated with bacterial cytochrome P-450_{CAM} where the wealth of structure-function detail could be used to derive maximal mechanistic insight.

Theoretical calculations using the MNDO method provide comparative data on the reduction potentials of halogenated methanes which are useful

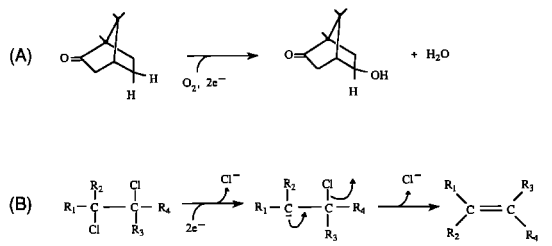


Fig. 11. Reactions catalyzed by cytochrome P-450$_{CAM}$. (A) Physiological hydroxylation of camphor, (B) reductive elimination of a polychlorinated ethane.

to predict reactivity with the heme center of cytochrome P-450 (Luke & Loew 1986). This analysis is based on the assumption that the first one electron transfer from the heme iron to a chlorinated methane is the rate determining step in reductive dehalogenation. It is instructive to compare the theoretical reactivities for a series of halogenated methanes with experimental data obtained using *P. putida* G786 containing cytochrome P-450$_{CAM}$. As indicated in Table 4, bromotrichloromethane, bromodichloromethane, carbon tetrachloride, fluorochloromethane and iodochloromethane are predicted to readily accept one electron from reduced heme in an exothermic reaction. Of these, the first three have been shown to be reactive with liver microsomal P-450; iodochloromethane had not been examined experimentally (Luke & Loew 1986). Data for theoretical calculations were not available for dibromodichloromethane but general reactivity trends indicate it would be reactive with reduced hematin. Chloroform was in the questionable category, based on MNDO calculations, but experimentally, it undergoes slow reaction with reduced hematin (Klecka & Gonsior 1984) and with mammalian microsomal cytochrome P-450 (Wolf et al. 1977).

Previous studies showed that cytochrome P-450$_{CAM}$ reductively dehalogenated bromotrichloromethane and carbon tetrachloride (Castro et al. 1985) and this was confirmed in the present study using camphor-induced *P. putida* G786. As anticipated, dibromodichloromethane also underwent reduction by cells expressing P-450$_{CAM}$(Table 4). In contrast, the incubation of fluorotrichloromethane with *P. putida* G786 did not yield detectable products. Previous studies showed fluorotrichloromethane underwent reduction in rat liver preparations, presumably by microsomal cytochrome P-450 (Wolf et al. 1978). MNDO calculations indicated that the reduction potentials of fluorotrichloromethane and carbon tetrachloride are nearly identical (Luke & Loew 1986). These data suggest that parameters other than the reduction potential should be investigated to develop a more complete understanding of reductive dehalogenation by cytochrome P-450$_{CAM}$. For example, the substrate-cytochrome P-450$_{CAM}$ binding equilibrium, and its

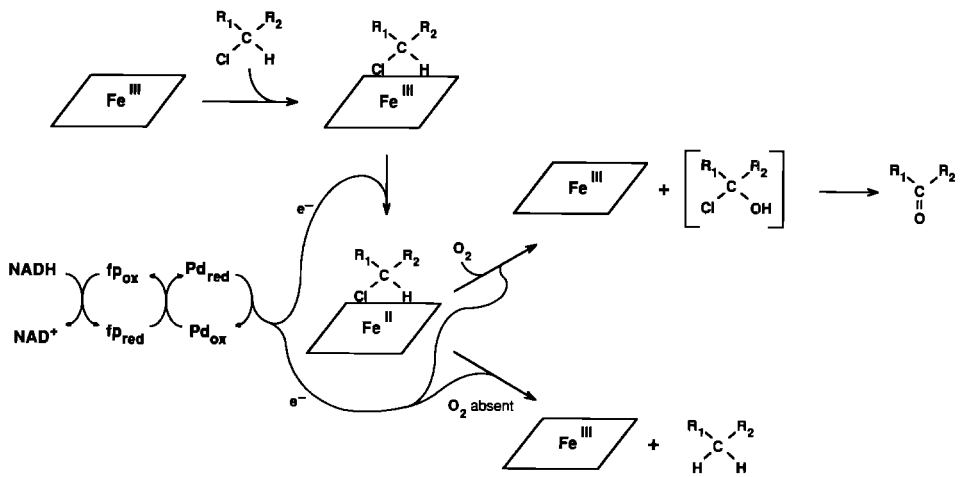


Fig. 12. Electron transfer in the cytochrome P-450$_{CAM}$ reaction cycle for reductive and oxidative reactions. The heme in cytochrome P-450$_{CAM}$ is depicted as a square; Fp denotes the putidaredoxin reductase; Pd denotes putidaredoxin; ox denotes the oxidised and red the reduced states of the putidaredoxin reductase and putidaredoxin.

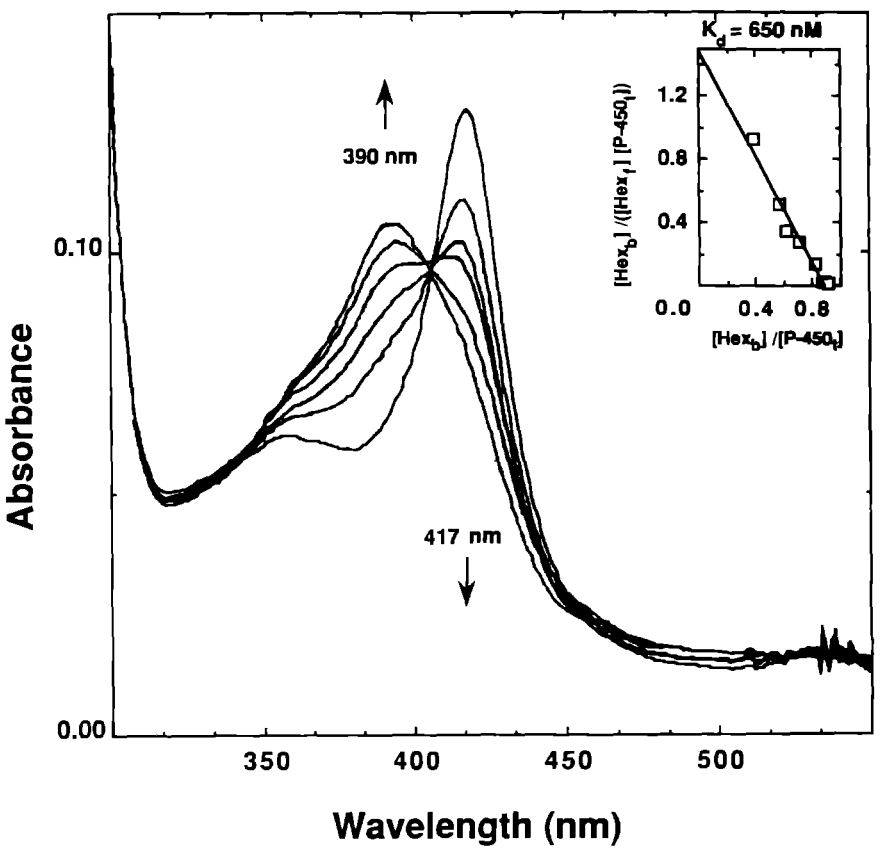


Fig. 13. Electronic spectra of cytochrome P-450$_{CAM}$ upon consecutive additions of hexachloroethane showing low to high spin state conversion. Inset is a Scatchard plot treatment of the data to derive the dissociation constant.

ancillary effect on heme iron redox properties and active site water population, could effect carbon-halogen bond reductions. In Table 4, chloroform is shown to effect a 14% low to high spin iron conversion at saturation. Based on the correlations between spin state equilibria and heme iron redox potential previously described by Fisher and Sligar (1985), the cytochrome P-450$_{CAM}$-chloroform complex is predicted to have a heme center reduction potential of −243 mV. Given that putidredoxin transfers electrons to P-450$_{CAM}$ with a reduction potential of −196 mV, interprotein electron transfer (Fig. 12) or other active site parameters could play a role in the failure of chloroform to undergo reduction by *P. putida* G786. It should be noted that hexachloroethane, which readily undergoes reduction by cytochrome P-450$_{CAM}$, causes > 95% spin conversion upon binding and should raise the heme reduction potential to −170 mV, comparable to that caused by camphor binding. Further studies with purified enzyme components will help resolve these questions.

Future directions

Detailed studies on the mechanisms of enzymatic dehalogenation require pure bacterial cultures to obtain purified enzymes. These prerequisites have been met infrequently to date for enzymes metabolizing chlorinated methanes. The known examples include dichloromethane dehalogenase which is required for certain methylotrophs to grow on dihalomethanes. Methane monooxygenase and cytochrome P-450$_{CAM}$ are well-studied enzymes which are responsible for cometabolic transformation of

Table 4. Reductive dehalogenation of chlorinated methanes by *P. putida* G786 (experimental) and hematin (theoretical) and cytochrome P-450$_{CAM}$ heme-iron spin state conversion.

Compound	Dehalogenated?		% Spin state conversion[3]
	Theoretical[1]	Experimental[2]	
Br_2CCl_2	Yes	Yes	47
$BrCCl_3$	Yes	Yes[4]	41
$ClCCl_3$	Yes	Yes[4]	36
$FCCl_3$	Yes	No	31
$HCBrCl_2$	Yes	Yes	22
H_2ICCl	Yes	No	ND[5]
$HCCl_3$	marginal	No	14
H_3CI	marginal	No	ND
$HCFCl_2$	No	No	ND

[1] Based on MNDO calculations for reaction of hematin with halogenated compounds (Luke & Loew 1986).
[2] Determined in this study using camphor induced *P. putida* G786 and gas chromatographic determination of products.
[3] Cytochrome P-450$_{CAM}$ conversion to high spin iron with substrate at saturation determined by previously described methods (Fisher & Sligar 1985; Logan et al 1992).
[4] Previously demonstrated (Castro et al 1985) and confirmed in this present study.
[5] Not determined

chlorinated methanes *in vitro* and *in vivo*. Further studies on these enzymes will give rise to deeper insights into substitutive, oxygenative, and reductive mechanisms of dehalogenation, respectively.

As microorganisms are increasingly exposed to chlorinated solvents, new organisms, and perhaps new catabolic pathways, will be selected for over time. Some of these, with the ability to feed chlorinated methanes into trunk metabolic pathways, will provide new resources for the rapid degradation of chlorinated methanes.

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