Haloalkene Oxidation by the Soluble Methane Monooxygenase from *Methylosinus trichosporium* OB3b: Mechanistic and Environmental Implications†

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Received December 26, 1989; Revised Manuscript Received March 13, 1990

ABSTRACT: The soluble, three-protein component methane monooxygenase purified from *Methylosinus trichosporium* OB3b is capable of oxidizing chlorinated, fluorinated, and brominated alkenes, including the widely distributed ground-water contaminant trichloroethylene (TCE). The oxidation rates for the chloroalkenes were observed to be comparable to that for methane, the natural substrate, and up to 7000-fold higher than those reported for other well-defined biological systems. The competitive inhibitor tetrachloroethylene was found to be the only chlorinated ethylene not turned over. However, this appears to be due to steric effects rather than electronic effects or the lack of an abstractable proton because chlorotrifluoroethylene was efficiently oxidized. As evidenced by the formation of diagnostic adducts with 4-(*p*-nitrobenzyl)pyridine, the halogenated alkenes were oxidized predominantly by epoxidation. Stable acidic products resulting from subsequent hydrolysis were identified as the major products. However, additional aldehydic products resulting from intramolecular halide or hydride migration were observed in 3–10% yield during the oxidation of TCE, vinylidene chloride, trifluoroethylene, and tribromoethylene. Product analysis of the hydrolysis reaction of authentic TCE epoxide showed little or no 2,2,2-trichloroacetaldehyde (chloral) formation, indicating that atomic migration occurred prior to product dissociation from the enzyme. The occurrence of atomic migration products shows that an intermediate in the substrate to product conversion carries significant cationic character. Such a species could be generated through interaction with a highly electron-deficient activated oxygen in the active site. The oxidation of TCE to TCE epoxide and chloral has also been reported for microsomal cytochrome P-450 [Miller, R. E., & Guengerich, F. P. (1982) *Biochemistry 21*, 1090–1097], suggesting that cytochrome P-450 and methane monooxygenase utilize a similar oxidizing species. A turnover-dependent inactivation of all methane monooxygenase protein components occurred during the oxidation of TCE. Radiolabeling of each of the components during turnover of [1,2-$^{14}C_2$]TCE showed that covalent modification by a diffusible product of the reaction had occurred. Correlation of the rates of inactivation with product formation suggests that the modifying species is a hydrolysis product of TCE epoxide.

The widespread use and indiscriminate disposal of chlorinated ethylenes as polymer substrates, solvents, and degreasers has resulted in a significant adverse impact on the environment (Leisinger & Brunner, 1986). Furthermore, electrophilic, potentially carcinogenic intermediates are formed by cytochrome P-450 mediated metabolism of these compounds after ingestion by mammals (Miller & Miller, 1981). Accordingly, studies of both the anaerobic and aerobic bacterial degradation of chlorinated ethylenes have recently appeared. Under anaerobic conditions, tetrachloroethylene, trichloroethylene (TCE),[1] *cis*- and *trans*-dichloroethylene, and vinylidene chloride are reductively dechlorinated to vinyl chloride (Barrio-Lage, 1986; Vogel & McCarty, 1985). Vinyl chloride, which is both carcinogenic and mutagenic (Maltoni & Lefemine, 1974), was not observed to be dechlorinated. In a recent study using pure cultures, tetrachloroethylene was converted to TCE at a rate of 1.6 pmol min^{-1} (mg of cell protein)$^{-1}$ (Fathepure et al., 1987). Under aerobic conditions, a variety of bacteria have been shown to degrade chlorinated ethylenes (Nelson et al., 1988; Wackett & Gibson, 1988; Arciero et al., 1989; Wackett et al., 1989). Although considerably faster than the anaerobic conversions, the rates of most aerobic oxidations are still rather slow, falling in the range of 0.5–2 nmol min^{-1} (mg of cell protein)$^{-1}$. A common characteristics of the aerobic oxidations appears to be the participation of multicomponent non-heme iron oxygenase enzymes in the degradation (Wackett & Gibson, 1988).

The adventitious oxidation of TCE by aerobic methanotrophic bacteria has been reported (Fogel et al., 1986; Little et al., 1988). Methane monooxygenase was assumed to be responsible for this oxidation, although the reported rates of TCE disappearance were several orders of magnitude slower than rates of methane utilization typical for these bacteria. Methane monooxygenase is unusual in that it is elaborated in both membrane-bound and soluble forms dependent upon growth conditions (Stanley et al., 1983). Recently, much more rapid turnover of TCE (20–150 nmol min^{-1} (mg of biomass)$^{-1}$) has been observed coincident with the elaboration of the soluble form of methane monooxygenase by the methanotroph *Methylosinus trichosporium* OB3b (Oldenhuis et al., 1989; Tsien

† This work was supported by National Institutes of Health Grants GM40466 (J.D.L.) and GM41235 (L.P.W.), a grant from the NSF-sponsored Center for Biological Process Technology of the University of Minnesota (J.D.L.), and a contract from Amoco Corp. (J.D.L.).

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§ Supported in part by a U.S. Public Health Service training grant and a University of Minnesota Graduate School doctoral dissertation fellowship.

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[1] Abbreviations: TCE, trichloroethylene; MOPS, 3-(*N*-morpholino)-propanesulfonic acid.

0006-2960/90/0429-6419$02.50/0

et al., 1989). While these results are strongly suggestive of a role for the soluble form of methane monooxygenase in the oxidation of haloalkenes, the direct demonstration of its involvement requires analogous studies with the purified enzyme. Furthermore, the identification of the intermediate products of haloalkene oxidation can only be determined with certainty through investigation of the reaction catalyzed by the purified soluble enzyme.

We have reported the purification of the soluble methane monooxygenase system (EC 1.14.13.25) from *M. trichosporium* OB3b, consisting of a 40-kDa NADH oxidoreductase, a 16-kDa protein termed component B, and a 245-kDa hydroxylase (Fox & Lipscomb, 1988; Fox et al., 1989). Recent reports from our laboratory (Fox & Lipscomb, 1989) using this enzyme and from Green and Dalton (1989) using the purified methane monooxygenase from *Methylococcus capsulatus* Bath have shown that oxidation of TCE readily occurs. Detailed studies of this reaction are reported here. It is also demonstrated that methane monooxygenase is capable of oxidizing a wide variety of other chloro-, fluoro-, and bromoalkenes at rates comparable to those of other substrates for the enzyme. The rates observed are at least 2 orders of magnitude faster than the rates reported for whole-cell oxidation reactions by nonmethanotrophs and 2–10 times faster than comparable oxidations catalyzed by cytochrome P-450 (Miller & Guengerich, 1982). These findings together with the ubiquitous presence of methanotrophs in soil and water suggest that methane monooxygenase mediated degradation of haloalkenes may be of principal importance on a global scale.

A variety of spectroscopic studies indicate the hydroxylase component of methane monooxygenase contains an oxo-bridged binuclear iron cluster[2] (Fox et al., 1988, 1989; Ericson et al., 1988). If chemically reduced, the hydroxylase is capable of catalyzing the oxidation of substrates in the absence of the other two protein components, strongly implying that the iron cluster is utilized as the cofactor in the O_2 activation and insertion reaction (Fox et al., 1989). It is shown here that many of the haloalkene oxidation reactions catalyzed by methane monooxygenase result in intramolecular halogen or hydride migration typical of oxygenase reactions catalyzed by cytochrome P-450 (Miller & Guengerich, 1982), in which a highly electron-deficient oxygen atom is the reactive species [see, for example, McMurry and Groves (1986)]. This result suggests that the spin-coupled binuclear iron cluster of the methane monooxygenase hydroxylase component generates an activated oxygen species with reactivity similar in many respects to that of the heme-containing cytochrome P-450 monooxygenases.

Experimental Procedures

Bacterial Strains and Protein Preparations. The growth, purification, and characterization of the soluble methane monooxygenase from *M. trichosporium* OB3b were as previously reported (Fox et al., 1989).

Chemicals. [1,2-$^{14}C_2$]TCE(4.1 mCi/mmol) was obtained from Sigma Radiochemicals, St. Louis, MO. TCE epoxide was synthesized as previously described (Miller & Guengerich, 1982). 2,2-Dichloroacetaldehyde was synthesized by hydrolysis of 2,2-dichloroacetaldehyde diethyl acetal in the presence of 1 M H_2SO_4 at 50 °C. All other chemicals were of the highest purity available and were used without further purification.

Substrate Solubilities. The solubilities of the liquid chlorinated ethylenes were determined at 1 atm by titration into distilled water in sealed vials with no head space. The dissolved chlorinated hydrocarbon concentration was determined by using a Hewlett-Packard 5700A gas chromatograph with a flame ionization detector equipped with a Porapak Q column (6 ft by 0.25 in. i.d. glass column, nitrogen carrier gas flow rate 24 mL/min) at 205 °C. The following solubilities were determined at 23 °C in deionized water: *cis*-1,2-dichloroethylene, 35 mM; *trans*-1,2-dichloroethylene, 31 mM; vinylidene chloride, 25 mM; TCE, 8 mM; tetrachloroethylene, 5 mM. The solubilities of ethylene and vinyl chloride (11 and 13 mM at 23 °C, respectively) were obtained from previously published tables (Braker & Mossman, 1980).

Enzyme Reaction Conditions. Except where noted, the halogenated ethylene (3 μL of the liquid compounds, 3 mL of ethylene or vinyl chloride, 6 mL of trifluoroethylene or chlorotrifluoroethylene gases) was added to a Teflon-sealed 5-mL reaction vial containing hydroxylase (5 nmol, 1.2 mg), component B (10 nmol, 0.16 mg), and reductase (1 nmol, 0.04 mg) in 0.5 mL of 25 mM MOPS, pH 7.5. The reaction was initiated by the addition of 1.5 μmol of NADH, and then the reaction vial was shaken at 23 °C in a constant-temperature bath for 30 min. The reaction products were analyzed as described in the following sections.

Determination of Volatile Products. Head space depletion assays for ethylene and vinyl chloride were performed on a Hewlett-Packard 5890 gas chromatograph with a flame ionization detector equipped with a GS-Q column (Alltech Associates, Deerfield, IL; 30 m by 0.53 μm i.d. capillary column, H_2 carrier gas flow rate 5–15 mL/min, injector and detector temperature 180 °C) at 150 °C. The determination of chloral, fluoral, bromal, and 2,2-dichloroacetaldehyde was performed on a Hewlett-Packard 5890A gas chromatograph with an electron capture detector equipped with a RSL-160 poly(dimethylsiloxane) column (Alltech Associates; 30 m by 0.53 μm i.d. capillary column, H_2 carrier gas flow rate 6.0 mL/min, injector and detector temperature 150 °C) at 30 °C. Chloral was also determined by high-pressure liquid chromatography as described below and by enzymatic assay with alcohol dehydrogenase (equine liver, Sigma) as previously described (Byington & Liebman, 1965). Conversion of chloral to 2,2,2-trichloroethanol in the latter method was confirmed by gas chromatography. CO was quantified by measuring the shift in the Soret maximum upon binding to reduced hemoglobin (Ahr, 1980). In experiments to determine CO, 5.5 mM TCE was added and the reactions were initiated by adding limiting concentrations (80 or 140 nmol) of NADH. These conditions prevented methane monooxygenase dependent oxidation of CO to CO_2.

Determination of Haloalkene Epoxides. The haloalkene epoxides were detected by the method of Miller and Guengerich (1982). The enzyme reaction was quenched at appropriate time points by the addition of 0.5 mL of benzene. The mixture was vortexed and then centrifuged at 10800*g* for 2 min. The benzene layer was transferred to a reaction vial containing 0.25 mL of ethylene glycol/acetone (4:1 v/v) and 100 mM 4-(*p*-nitrobenzyl)pyridine. This mixture was incubated at 80 °C for 60 min. The reaction vial was then cooled to 4 °C, and 0.5 mL of triethylamine/acetone (1:1 v/v) was added to the vial and rapidly mixed. The concentration of TCE epoxide was estimated by using ϵ_{540} = 24 mM^{-1} cm^{-1} (Epstein et al., 1955). TCE, *trans*-dichloroethylene, and tribromoethylene epoxides were also detected directly in the

[2] The chemical state of the bridging oxygen has not been rigorously established. We will refer to the complex as oxo although it may in fact be hydroxo or R-oxo. The protonated form is shown in Figures 3 and 4 for convenience.

Table I: Kinetic Data for Oxidation of Chlorinated Ethylenes by the Soluble Methane Monooxygenase from *M. trichosporium* OB3b[a]

compound	K_m (μM)	V_{max} (milli-units/mg)	% V_{max} of ethylene	turnover no. (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	λ_{max} of pNBP derivative[b,c] (nm)
ethylene	32	858	100	3.5	0.11	560
1-chloroethylene (vinyl chloride)	33	748	87	3.0	0.09	560
cis-dichloroethylene	28	935	108	3.8	0.14	530
trans-dichloroethylene	38	888	103	3.6	0.10	530
1,1-dichloroethylene (vinylidene chloride)	18	648	75	2.7	0.15	none
trichloroethylene	35	682	79	2.8	0.08	520

[a] Assays contained purified hydroxylase (2 nmol), component B (4 nmol), reductase (5 nmol), and NADH (250 nmol) in 25 mM MOPS, pH 7.5, at 23 °C. Activity values are reported relative to the hydroxylase present. [b] The λ_{max} of the product was observed after oxidation of the listed compound with methane monooxygenase followed by derivatization with 4-(*p*-nitrobenzyl)pyridine as described under Experimental Procedures. [c] The 4-(*p*-nitrobenzyl)pyridine derivative of tribromoethylene oxide exhibited λ_{max} = 540 nm.

benzene extraction of the enzymatic reaction mixtures by gas chromatography on RSL-160.

Determination of Acidic Products. Acidic products obtained from the enzymatic oxidation of [1,2-$^{14}C_2$]TCE were separated by high-pressure liquid chromatography on a Bio-Rad Aminex HPX-87H column (solvent 25% acetonitrile in 7.5 mM H_2SO_4, flow rate 0.3 mL/min) and identified by comparison with authentic standards. The retention times observed by monitoring the absorbance at 210 nm were as follows: dichloroacetic acid, 13.3 min; glyoxylic acid, 14.5 min; chloral, 15.8 min; formic acid, 17.8 min. Products were quantified by measuring the radioactivity associated with each observed peak. Acidic products obtained from the enzymatic oxidation of vinylidene chloride, trifluoroethylene, and chlorotrifluoroethylene were separated by high-pressure liquid chromatography on a Polypore H column (solvent 50 mM H_2SO_4, flow rate 0.1–0.4 mL/min) and detected at 220 nm by using an LKB Model 2141 optical detector (Pharmacia/LKB Biotech, Piscataway, NJ). Formate was also determined by observing the production of NADH (ϵ_{340} = 6.2 mM^{-1} cm^{-1}) in the presence of purified *M. trichosporium* OB3b formate dehydrogenase.

Determination of Kinetic Constants. The values of K_m and V_{max} were determined by nonlinear least-squares fitting of hyperbolic plots of initial velocity data obtained from O_2 uptake measurements. Saturating concentrations of the reductase and NADH and optimal concentrations of component B [see Fox et al. (1989)] relative to the hydroxylase were used in all experiments. The chlorinated ethylenes were added to a sealed O_2 electrode chamber filled with air-saturated 25 mM MOPS, pH 7.5, at 23 °C by using a gastight syringe. The inhibition of methane or furan oxidation in the presence of tetrachloroethylene was also determined polarographically. Methane and tetrachloroethylene were prepared as saturated solutions in 25 mM MOPS, pH 7.5. Furan was prepared as a saturated solution (65 mM) in the same buffer. Methane or furan and tetrachloroethylene were added to the sealed O_2 electrode chamber by using gastight syringes. Inactivation during the turnover of TCE was performed in Teflon-sealed 3-mL reaction vials in 0.5 mL of 25 mM MOPS, pH 7.5, at 23 °C with hydroxylase (5 nmol), component B (10 nmol), and reductase (5 nmol). The inactivation reactions were initiated by the addition of 1600 nmol of NADH. Samples were removed at appropriate intervals and assayed for remaining hydroxylation activity. When necessary, the enzymatic reaction was stopped by rapidly drawing a vacuum to remove volatile substrates and products, followed by replacement of O_2-free argon gas. Specific activities of each of the partially inactivated components (1–2 nmol of each component) were determined in the presence of excess concentrations of active preparation of two of the other components [hydroxylase (5 nmol), component B (10 nmol), and reductase (5 nmol)]. The maximum error observed from carry-over of the other protein components during control experiments was 7%. Protection of the enzyme from inactivation during the oxidation of TCE was measured as described above in buffers containing ethylene (3 atm) or cysteine (10 mM).

Covalent Labeling of the Methane Monooxygenase Components. For measurement of covalent incorporation of products resulting from the oxidation of TCE, hydroxylase (5 nmol), component B (10 nmol), reductase (10 nmol), and [1,2-$^{14}C_2$]TCE (15 nmol) were placed into a Teflon-sealed 3.0-mL reaction vial containing 0.5 mL of air-saturated 25 mM MOPS, pH 7.5. The reactions were initiated by the addition of 200 nmol of NADH, and the reaction was allowed to proceed for 15 min at 23 °C with gentle shaking. The reaction mixture was air stripped to remove any residual [1,2-$^{14}C_2$]TCE or other volatile compounds. The ^{14}C-labeled protein components were separated on a 10% SDS–polyacrylamide gel cross-linked with 0.24% *N,N'*-diallyltartardiamide (Young et al., 1980). The gel was sliced and dissolved in periodic–lactic acid, and the amount of ^{14}C incorporated in each peptide was determined by scintillation counting.

Results

Oxidation of Haloalkenes. The soluble methane monooxygenase from *M. trichosporium* OB3b is capable of catalyzing the oxidation of the haloethylenes shown in Tables I and II. The oxidations were determined by head space depletion, polarographic measurements of O_2 uptake, and identification of volatile and acidic products. Tetrachloroethylene was the only haloalkene tested that was not oxidized.

Hyperbolic saturation kinetic behavior was observed for all of the chlorinated ethylenes oxidized by methane monooxygenase. The kinetic constants obtained are listed in Table I. The K_m values observed for ethylene and the haloethylenes are quite similar, which is probably indicative of the structural and chemical similarity of these compounds. Surprisingly, these K_m values are similar to those of methane (25 μM) and furan (20 μM) (Fox et al., 1989), compounds with quite different structures. The nearly constant value of k_{cat}/K_m for all of the chlorinated ethylenes tested (Table I) demonstrates the relative nonselectivity for oxidation of the haloalkenes by methane monooxygenase. Vinylidene chloride and *cis*-dichloroethylene exhibit slightly higher k_{cat}/K_m specificity indexes than the other compounds. However, these two compounds are also distinguished by a high degree of electronic asymmetry present, which may contribute to the increased reactivity. The observation that k_{cat}/K_m for the chlorinated ethylenes is essentially the same as that for ethylene suggests

Table II: Stable Products Observed from the Oxidation of Halogenated Alkenes by Methane Monooxygenase[a]

compound	acidic products	volatile products	oxidation rate relative to that of ethylene (%)
trichloroethylene	glyoxylate (5) dichloroacetate (5) formate (35)	chloral[b] (6) CO (53)	79
vinylidene chloride	glycolate (80)	dichloroacetaldehyde[b] (3)	75
trifluoroethylene	glyoxylate (53) difluoroacetate (43)	fluoral[b] (5)	8
chlorotrifluoroethylene	oxalate (15)		2
tribromoethylene	formate (80)	bromal[b] (5)	1
trichloroethylene epoxide[c]	glyoxylate (5) formate (32)	chloral (<1) CO (55)	

[a] For determinations of acidic products from trichloroethylene, enzymatic reactions contained 1.7 nmol of hydroxylase, 1 nmol of component B, 1 nmol of reductase, 200 nmol of NADH, 1.5 mL of 25 mM MOPS, pH 7.5, and 50 nmol of [1,2-$^{14}C_2$]trichloroethylene. Other enzymatic reactions contained 2 nmol of hydroxylase, 4 nmol of component B, 1 nmol of reductase, 100 nmol of NADH, and 0.2 mL of 25 mM MOPS. The reaction mixtures were analyzed for products after 30 min as reported under Experimental Procedures. The product yields (nmol) are shown in parentheses and are normalized to 100 nmol of trichloroethylene. [b] All volatile products except for CO arise from intramolecular atomic migration that occurs during the enzymatic oxidation reaction. [c] Non-enzyme-catalyzed hydrolysis of authentic trichloroethylene epoxide in 25 mM MOPS, pH 7.5. Dichloroacetate was not determined because the synthetic epoxide contained 30% dichloroacetyl chloride, which was converted to dichloroacetate by hydrolysis. Identical concentrations of products were observed when authentic trichloroethylene epoxide was added to incubations of the methane monooxygenase components in the absence of NADH.

that chlorination per se has little effect on the flux of the reaction.

Since tetrachloroethylene was not oxidized, it was used to investigate the inhibition of methane monooxygenase by a chlorinated ethylene in the absence of complicating turnover reactions. Tetrachloroethylene is an effective inhibitor of both methane and furan oxidation. Figure 1 shows the reciprocal plot obtained for the oxidation of furan in the presence of varied amounts of tetrachloroethylene. At very low tetrachloroethylene concentration (relative to K_i), the inhibition appears to be un- or noncompetitive, suggesting that the inhibitor and substrate bind simultaneously to the enzyme in different sites. This effect may be nonspecific. At slightly higher inhibitor concentrations the inhibition is competitive, suggesting that tetrachloroethylene, and presumably the other halogenated alkenes, bind at the same site as typical methane monooxygenase substrates. The replot of the slope data from Figure 1 (inset) shows that the K_i for tetrachloroethylene is 90 μM. Similar kinetic behavior was observed for the inhibition of methane oxidation by tetrachloroethylene.

Epoxide Formation. Table I shows the absorption maxima observed for the adducts formed between 4-(*p*-nitrobenzyl)-pyridine and the products generated during the oxidation of the chloroethylenes. The observation of these adducts is indicative of the transient formation of product epoxides (Epstein et al., 1955). The appearance of such adducts together with substrate-dependent O_2 uptake (see Table I) was taken as evidence for the oxidation of vinyl chloride and *cis*- and *trans*-dichloroethylene, and no other products were identified. For the other haloethylenes oxidized, all of the major stable products observed were identified and are presented in Table II. Neither semistable epoxides nor formate was detected for vinylidene chloride,[3] trifluoroethylene, and chlorotrifluoroethylene (although it is likely that intermediate epoxides are formed; see below). In contrast, the oxidation of TCE and tribromomethylene yielded semistable epoxides and formate.

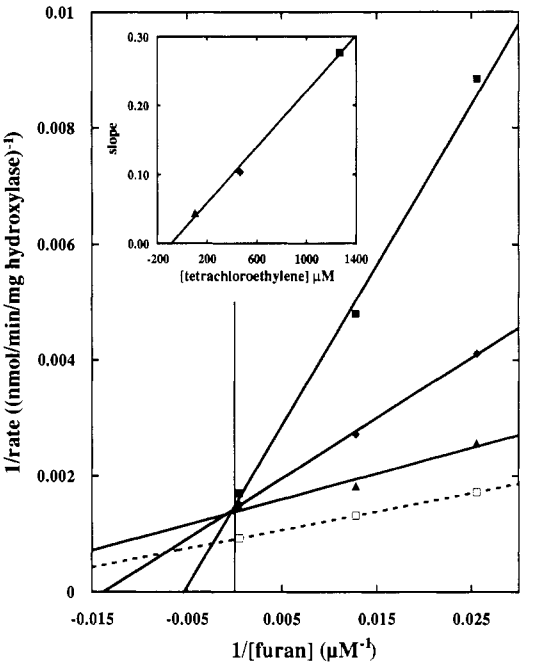

FIGURE 1: Reciprocal plot showing inhibition of the methane monooxygenase catalyzed oxidation of furan in the presence of tetrachloroethylene. The rate of oxidation in the presence of varied concentrations of furan was determined polarographically at fixed tetrachloroethylene concentrations of (□) 0, (▲) 150, (◆) 450, and (■) 1250 μM as described under Experimental Procedures. The rates were determined relative to the amount of hydroxylase component present in the assay. The inset shows the slope replot of the primary data.

Oxidation of Trichloroethylene. During the methane monooxygenase catalyzed oxidation of TCE at pH 7.5 with a stoichiometric concentration of NADH, formate and carbon monoxide account for 88% of the total products. Glyoxylate, dichloroacetate, and chloral were also observed in 5%, 5%, and 6% yield, respectively. A similar product distribution, with the exception of chloral, was observed from the hydrolysis of authentic TCE epoxide in identical buffer solution (Table II). Thus, it is likely that all of the products observed from TCE oxidation except chloral arose from breakdown of TCE epoxide in solution, despite the fact that only about 20% of the TCE oxidized could be trapped as the epoxide by reaction with 4-(*p*-nitrobenzyl)pyridine. Under the conditions used for the experiment, TCE epoxide has a half-life of ~10–20 s (Miller

[3] The instability of the epoxides of vinylidene chloride, trifluoroethylene, and chlorotrifluoroethylene is suggested by the fact that they have not been synthesized and characterized to date. In one attempt to synthesize the epoxide of vinylidene chloride (Bonse et al., 1975), reaction of vinylidene chloride with *m*-chloroperoxybenzoic acid yielded only 2-chloroacetyl chloride. More recently, Liebler and Guengerich (1983) have reported the synthesis and partial characterization of vinylidene chloride epoxide. However, this compound was also observed to freely convert in $CDCl_3$ to 2-chloroacetyl chloride.

Table III: Inactivation of Methane Monooxygenase Components during Oxidation of Trichloroethylene[a]

component	% activity remaining		
	no additions	plus ethylene	plus cysteine
hydroxylase	6	28	34
component B	42	56	62
reductase	53	75	86

[a] Samples of inactivation reactions performed as described in Figure 2 were withdrawn at 10 min and assayed polarographically for the ability of the reported component to enhance the hydroxylation activity of saturating or optimal concentrations of the other two components. Ethylene (3 atm) or cysteine (10 mM) was added to the reaction mixtures prior to the addition of NADH.

& Guengerich, 1982), allowing a significant fraction to be hydrolyzed before it can be trapped.

As shown in Table II, the amount of chloral that arises from spontaneous breakdown of synthetic TCE epoxide alone or from incubation in the presence of the methane monooxygenase components in the absence of NADH is below the detection limit. Thus, it is likely that chloral was generated in the active site of the enzyme during catalysis. The yield of chloral from the enzymatic reaction was not significantly changed by pH in the range of 6.5–8.7 or by temperature in the range of 15–45 °C. Chloral was not further oxidized on the time scale of these experiments, as no trichloroacetic acid was detected in reaction mixtures. As shown in Table II, haloacetaldehydes consistent with hydride, fluoride, or bromide migration were also observed during the oxidations of other halogenated ethylenes.

Turnover-Dependent Enzyme Inactivation. Incubation of the methane monooxygenase components with TCE in the absence of NADH caused no change in specific activity. However, as shown in Figure 2, the enzyme-catalyzed oxidation of TCE resulted in time-dependent enzyme inactivation. Complete inactivation required that approximately 200 molecules of TCE be turned over per molecule of hydroxylase component. The rate of inactivation was slowed by the addition of the substrate ethylene (see Table III), suggesting that oxidation of TCE occurred at the active site located on the hydroxylase component. These results do not allow discrimination between inactivation caused by a reactive product that is not released from the enzyme active site and one that is diffusible. However, inactivation was also found to be inhibited by the addition of the nucleophile cysteine to the reaction buffers (Table III), suggesting that the inactivating reagent was diffusible. Therefore, the utilization of TCE and the formation of the diffusible products TCE epoxide and chloral were monitored throughout the inactivation reaction (Figure 2, inset). During the oxidation, the concentration of epoxide rapidly attained a steady-state level, reflecting production by the enzyme-catalyzed reaction and subsequent degradation by nonenzymatic hydrolysis in solution and/or protein inactivation. Upon complete utilization of TCE or cessation of the enzymatic reaction, the epoxide rapidly disappeared. Chloral was formed with no observable lag time, consistent with the proposal that it is formed directly from TCE and not TCE epoxide. Furthermore, chloral was observed to be a stable product even when insufficient TCE was provided to completely inactivate the enzyme.

For inactivation reactions as shown in Figure 2, the presence of 10 mM cysteine increased the level of epoxide present at 1 and 3 min by 10% and 40%, respectively, presumably due to the increased enzyme activity afforded by the protective effects of the exogenous nucleophile. Since this increase in epoxide concentration was associated with a decrease in the

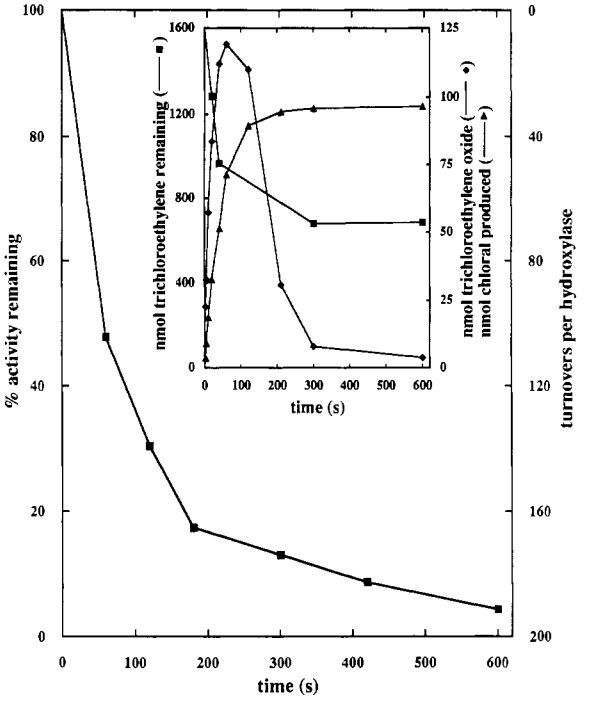

FIGURE 2: Time course of inactivation of methane monooxygenase during the oxidation of trichloroethylene. Inactivation experiments containing hydroxylase (5 nmol), component B (10 nmol), reductase (5 nmol), NADH (1600 nmol) and trichloroethylene (1600 nmol) in 0.5 mL of 25 mM MOPS, pH 7.5, were performed as described under Experimental Procedures. The left ordinate axis shows the percent activity remaining while the right ordinate axis shows the number of turnovers of trichloroethylene per molecule of hydroxylase. The inset shows the time course of utilization of trichloroethylene (■) and the formation of trichloroethylene epoxide (◆) and chloral (▲) during the inactivation reaction. Levels of these compounds were determined by gas chromatography as described under Experimental Procedures.

inactivation rate, it is unlikely that the epoxide itself was the reagent responsible for the inactivation. Thus, either a hydrolysis product of the epoxide or chloral is a more likely modifying reagent. However, chloral is unlikely to be the inactivating reagent because incubation of the methane monooxygenase components with chloral did not result in inactivation.

The generation of a diffusible modifying reagent would reasonably be expected to affect the activity of all three of the monooxygenase components. As shown in Table III, the specific activity of the reductase and component B are also substantially decreased as the hydroxylase is inactivated in the absence of cysteine. Since the observed rate of turnover is sensitive to both the relative and absolute concentrations of all three components (Fox et al., 1989), it is not unexpected that the inactivation time course shown in Figure 2 does not follow any integer order or hyperbolic saturation kinetics. As also shown in Table III, the addition of cysteine to the inactivation buffers results in protection of the activity of all three components in roughly the same amount, further supporting the proposal that a diffusible modifying reagent is responsible for the inactivation.

Covalent Incorporation of a Reaction Product into the Enzyme. If, as suggested above, the product responsible for inactivation of the enzyme is free in solution, modification of the enzyme would be expected globally on the enzyme surface rather than specifically in the active site. To test this proposal and to show that TCE was the source of the modifying reagent, the reaction was carried out with [1,2-$^{14}C_2$]TCE. As shown in Table IV, the inactivation is accompanied by the covalent

Table IV: Incorporation of ^{14}C into Components of Methane Monooxygenase during Oxidation of [1,2-$^{14}C_2$]Trichloroethylene[a]

component	control (−NADH) dpm	control (−NADH) pmol of ^{14}C	complete system (+NADH) dpm	complete system (+NADH) pmol of ^{14}C	% of total ^{14}C incorpd
hydroxylase					
α subunit	32	<8	483	54	10
β subunit	51	<8	1153	128	24
γ subunit	37	<8	227	25	5
reductase	40	<8	1931	214	39
component B	65	<8	1099	122	22

[a] Hydroxylase (5 nmol), component B (10 nmol), reductase (10 nmol), and [1,2-$^{14}C_2$]trichloroethylene (15 nmol) were placed into a Teflon-sealed 2-mL reaction vial containing 500 μL of air-saturated buffer. The reactions were initiated by the addition of 200 nmol of NADH and allowed to go to completion (~15 min). The ^{14}C-labeled protein components were separated, and incorporated radioactivity was determined as described under Experimental Procedures.

modification of each of the methane monooxygenase protein components and each subunit of the hydroxylase. The distribution of radioactivity is roughly in accord with the estimated exposed surface area of each component,[4] further supporting the proposal of a diffusible modifying reagent. The stoichiometry of incorporation indicates that ^{14}C from only about 1 out of 30 molecules of [1,2-^{14}C]TCE oxidized is incorporated into the protein components. Thus, a substantial portion of the modifying reagent is rendered unreactive by competing reactions such as hydrolysis. It is notable that under the conditions described in Table IV only about 3 turnovers can occur per hydroxylase molecule. On the basis of the results shown in Figure 2, little enzyme inactivation would have occurred. Thus, any potentially reactive active-site groups should be largely unmodified. Nevertheless, even at this early stage in the reaction, all of the protein components are labeled.

Discussion

It has been shown here that the soluble methane monooxygenase purified from the Type II methanotroph *M. trichosporium* OB3b can catalyze the oxidation of a variety of halogenated ethylenes at high rates relative to other well characterized biological systems. These results provide the first detailed evidence that soluble methane monooxygenase is responsible for the previously observed ability of methanotrophs to degrade halogenated alkenes. Epoxides, the major products of the oxidation reactions, are shown to undergo isomerization or hydrolysis in aqueous media to primarily volatile or readily biodegradable materials, lending environmental significance to this activity of methane monooxygenase. Haloaldehydes, secondary products in some cases, are shown to derive from halide or hydride migration occurring in a catalyzed reaction while the substrate is bound to the enzyme. This reaction may be indicative of the general mechanism of catalysis utilized by the enzyme as discussed below.

Substituent Effects. The rate of oxidation among the chlorinated ethylenes is remarkably insensitive to position and degree of substitution. Of the substituted ethylenes tested, only tetrachloroethylene was not turned over, and in view of the reactivity of chlorotrifluoroethylene, this is probably related to steric rather than electronic effects. The oxidation of chlorotrifluoroethylene, which contains no abstractable hydrogen atom, suggests that methane monooxygenase initiates oxidation of haloalkenes by direct attack on the substrate π bond. The small rate differences noted among the chlorinated ethylenes that are turned over also appear to be related primarily to steric effects. For example, on the basis of the selectivity index shown in Table I, vinylidene chloride should be oxidized the most rapidly of the three dichloroethylenes. Yet *cis*-dichloroethylene, in which one side of the π bond is always exposed, exhibited 30% faster turnover. Accordingly, the similarity of the turnover numbers for vinylidene chloride and TCE suggests that an advantageous orientation within the active site is restricted by the presence of *gem*-chloro substitution. The oxidation rates of ethylenes substituted by halogens other than chlorine show a much larger range. For the trisubstituted ethylenes, the rate decreases in the order TCE > trifluoroethylene > tribromoethylene. While electronic effects clearly play a role in the decreased reactivity observed, the higher rate observed for the fluoro- over the bromo-substituted analogue suggests that steric effects are significant in these cases as well.

Epoxide and Haloaldehyde Formation. In contrast to the turnover rates, the products detected from the oxidation of the haloalkenes are highly dependent on the position, type, and degree of halogen substitution in the haloalkene. However, this is likely to reflect the isomerization and hydrolysis reactions of intermediate epoxides in solution rather than a substrate-dependent variation in the enzyme mechanism. For all of the haloalkenes investigated here, either epoxides were directly detected or the products isolated from the enzymatic reaction mixtures were consistent with hydrolysis of an intermediate epoxide.[3] Thus, it is likely that epoxide formation is a common feature in the mechanism of haloalkene oxidation by methane monooxygenase. Haloaldehydes account for a small percentage of the products from enzyme-catalyzed oxidation of several haloethylenes. These products must arise from halide or hydride migration. Since little or no halide migration occurs when synthetic TCE epoxide is added to the buffering solution used for the enzyme reaction, it is likely that the haloaldehyde-forming reactions occur in parallel with the epoxide-forming reactions in the active site of the enzyme.

Turnover-Dependent Inactivation. The results presented here strongly imply that neither TCE nor the immediate enzyme-catalyzed oxidation products, TCE epoxide and chloral, are responsible for the inactivation reaction. However, it is also clear that TCE oxidation is required and that the putative modifying intermediate is labile in solution, which causes the inactivation reaction to stop when TCE turnover is completed. Thus, it is likely that the modifying reagent is a diffusible intermediate derived from the non-enzyme-catalyzed hydrolysis or isomerization of TCE epoxide, such as glyoxyl chloride, formyl chloride, dichloroacetyl chloride, or dichlorocarbene (Miller & Guengerich, 1982). Among these intermediates, the acyl chlorides are highly reactive and are known from previous studies (Henschler et al., 1979; Miller & Guengerich, 1982) as well as those presented here to be hydrolyzed to nonreactive acidic products in solution. Acyl chlorides also react rapidly with nucleophiles (March, 1985), which would appear to account for the protective effect described here for cysteine. In contrast, TCE epoxide is relatively stable in the presence of nucleophiles such as methanol (Kline & Van Duuren, 1977); thus it is reasonable that the steady-state epoxide concentration would rise during the TCE turnover reaction conducted in the presence of cysteine.

Comparison with Haloalkene Oxidation by Methane Monooxygenase from M. capsulatus. Green and Dalton (1989)

[4] A diffusible modifying reagent should be expected to react primarily with the surface of each component. By making the nominal assumption that the components are globular, the observed incorporation can be scaled for the relative surface areas. The scaled incorporations are 43%, 43%, and 14% for the reductase, component B, and hydroxylase components, respectively.

have reported that methane monooxygenase from *M. capsulatus* Bath will catalyze the oxidaton of TCE and vinylidene chloride. Vinylidene chloride was reported to be oxidized at about 5% of the rate of TCE, while in the current study the two haloalkenes were found to be oxidized at esssentially the same rate. No intermediate epoxide was reported for either substrate, but the final products observed were similar to those reported here and are consistent with such an intermediate. Interestingly, no formation of chloral was observed from the oxidation of TCE. It is not known whether this represents a difference in the methods used to detect the relatively small amount of chloral formed or whether it is reflective of a true difference in the reactions catalyzed by the two enzymes. As reported here for the *M. trichosporium* enzyme, oxidation of TCE and vinylidene chloride by the *M. capsultaus* enzyme caused inactivation. On the basis of the higher rate of inactivation during vinylidene chloride oxidation, it was postulated that the most likely reactive species was an enzyme-bound intermediate that preceded the formation of monochloroacetic acid. In contrast, we have presented evidence here that the reactive species is diffusible.

Comparison with Oxidation of Trichloroethylene by Other Oxygenases. TCE and other haloalkenes have also been shown to be oxidized by cell-free extracts of *Pseudomonas putida* F1 containing toluene dioxygenase (S. R. Householder and L. P. Wackett, unpublished observation) and by mammalian microsomal cytochrome P-450 (Guengerich et al., 1979; Ortiz de Montellano et al., 1982; Miller & Guengerich, 1982; Liebler & Guengerich, 1983). It has been shown that toluene dioxygenase initially catalyzes addition of activated O_2 across a π bond of the toluene aromatic ring to form a *cis*-dihydrodiol (Gibson et al., 1968). In the case of TCE, such an intermediate could lead to dehalogenation, but it is unlikely to evoke epoxide formation or chloride migration. In contrast, the oxidation of TCE by cytochrome P-450 (Miller & Guengerich, 1982) yields both TCE epoxide and chloral, as observed for methane monooxygenase. The reaction mechanism of cytochrome P-450 has been extensively investigated [see, for example, McMurry and Groves (1986)]. It is quite likely that the reactive species is a heme-bound oxene stabilized by two electrons drawn from the heme and the iron, respectively, to yield an oxo-Fe(IV) π cation radical (Hamilton, 1974; Hrycay et al., 1976). A single, highly electron-deficient oxygen species could evoke both epoxide formation and chloride migration by partitioning from a common intermediate subsequent to electron abstraction and radical recombination. Thus, assuming a similar reaction strategy is followed by methane monooxygenase, environmental factors might be expected to have a profound influence on the partitioning ratio. Indeed, despite the ability of both methane monooxygenase and cytochrome P-450 to catalyze TCE epoxide and chloral formation, chloral is formed in only small amounts from the methane monooxygenase reaction, while it is the major product from the cytochrome P-450 reaction. Moreover, methane monooxygenase exhibits much lower K_m values and higher V_{max} values than cytochrome P-450 for all of the haloalkenes that have been studied for both enzymes.

As observed for methane monooxygenase, cytochrome P-450 monooxygenase is inactivated during the oxidation of haloalkenes (Guengerich et al., 1979; Ortiz de Montellano et al., 1982; Liebler & Guengerich, 1983). This inactivation has been demonstrated to involve both heme alkylation and protein modification (Ortiz de Montellano et al., 1981; Guengerich, 1986). The former may reflect the proximity of a reactive site on the heme (Kunze et al., 1983) or the relatively long residence time of the product in the active site of cytochrome P-450 (Ortiz de Montellano, 1982; Liebler & Guengerich, 1983), while the latter appears to be similar to that described here for methane monooxygenase.

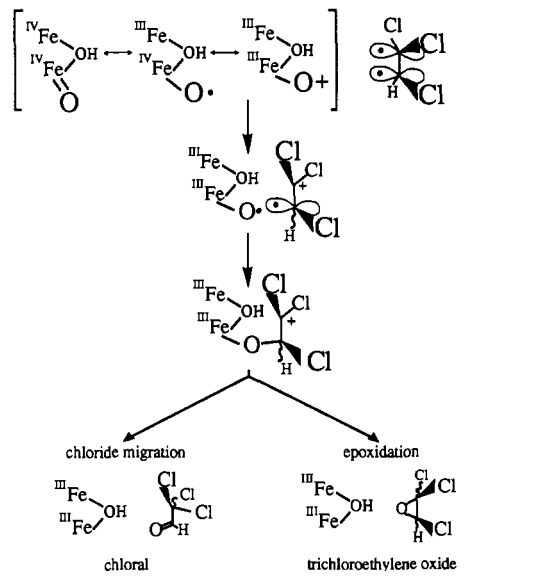

FIGURE 3: Proposed mechanism for the oxidation of trichloroethylene by methane monooxygenase.

Mechanistic Implications. An atomic migration reaction such as that resulting in chloral formation implies the intermediacy of a cationic substrate species and hence the generation of a powerful electrophile during the oxidation reaction (Daly et al., 1972). Thus, the observation of products resulting from atomic migration argues against the generation of a nucleophilic oxygen intermediate such as an enzyme-bound peroxide-level oxygen for use in catalysis (Entsch et al., 1976). The formation of a powerful electrophile similar to the oxene species of cytochrome P-450 (Hamilton, 1974) would require cleavage of the O–O bond prior to substrate oxidation during catalysis by methane monooxygenase. Such a species would presumably have to be stabilized in some manner in order to first promote O–O bond cleavage and then to preserve the highly reactive oxene long enough to allow a specific reaction with substrate to occur. The Fe(IV) π cation radical of cytochrome P-450 is proposed to effect this stabilization by providing two reducing equivalents to complement the six valence electrons of oxene (McMurry & Groves, 1986). We have recently shown that the hydroxylase component of methane monooxygenase alone is competent for O_2 activation and substrate hydroxylation upon chemical reduction (Fox et al., 1989). Since the hydroxylase contains only μ-oxo-bridged binuclear iron clusters (Fox et al., 1988), it must be assumed that this cofactor effects the required stabilization of the activated oxygen species in some manner. One mechanism that would allow the binuclear iron cluster to provide the two reducing equivalents required to stabilize the oxene resulting from O–O bond cleavage is shown in Figure 3. The reactivity with haloalkenes described here is consistent with oxidation mediated by such an oxenoid species. In addition, a common intermediate from which the observed 15-fold partitioning between formation of TCE epoxide and chloral could occur is shown in Figure 3. While the ability of the spin-coupled binuclear iron cluster to delocalize electron density would facilitate stabilization of the proposed oxene intermediate, no evidence for the formation of an Fe(IV)-containing cluster has been adduced either from our work or from the results of numerous model studies (Armstrong et al., 1984; Murch et al., 1986; Vincent et al., 1988).

Table V: Relative Rates of Oxidation of Trichloroethylene by Different Biological Oxidation Systems[a]

	rate of oxidation (nmol min^{-1} mg^{-1})	reference
enzyme systems		
methane monooxygenase		
M. trichosporium OB3b	682	this work
M. capsulatus Bath	<80	Green & Dalton, 1989
cytochrome P-450		
rat liver microsomes	20–500	Miller & Guengerich, 1982
live organisms		
M. trichosporium OB3b (methane monooxygenase)	20–150	Oldenhuis et al., 1989; Tsien et al., 1989
strain G-4 (toluene 2-monooxygenase)	*b*	Shields et al., 1989
Pseudomonas mendocina (toluene 4-monooxygenase)	2	Winter et al., 1989
P. putida F1 (toluene dioxygenase)	2	Wackett & Gibson, 1988
Nitrosomonas europaea (ammonia monooxygenase)	~1	Arciero et al., 1989
Mycobacterium sp. (propane monooxygenase)	~0.5	Wackett et al., 1989
methanotroph strain 46-1 (methane monooxygenase)	<0.1	Little et al., 1988
methanotrophic consortium	~0.03	Fogel et al., 1986

[a] For enzymatic oxidations, rates of oxidation are listed relative to the oxygenase component; for oxidations by live organisms, rates are listed relative to cell mass present for *M. trichosporium* and with respect to mg of whole-cell protein for the others. [b] Initial rates not specified.

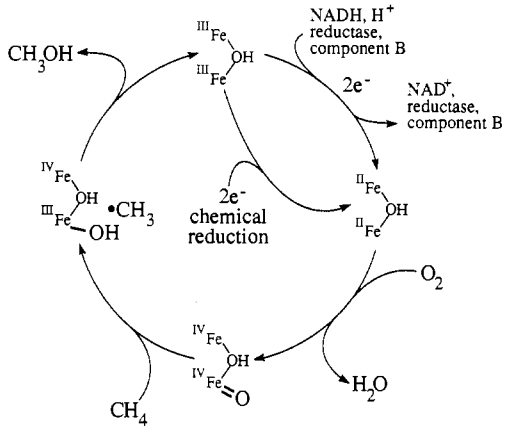

FIGURE 4: A catalytic cycle for methane monooxygenase.

On the basis of the studies reported here and the results of our previous studies (Fox et al., 1989), a proposal for the catalytic cycle of methane turnover by methane monooxygenase has been formulated as shown in Figure 4. It is proposed that the oxo-bridged iron cluster of the hydroxylase component first accepts two electrons either from its biological reducing system consisting of NADH, the reductase, and component B or from a chemical reducing system such as methyl viologen and dithionite. Dioxygen is proposed to add to this state and undergo heterolytic cleavage to form water and the reactive oxene, which abstracts a hydrogen atom from methane. Reabstraction of hydroxyl by the methane radical would yield the product and return the enzyme to its original state. In contrast, Green and Dalton (1989) have proposed that the electrons are donated one at a time with O_2 binding to the mixed valent state of the cluster. However, our studies suggest that the mixed valent state is unreactive with O_2 (Fox et al., 1989). Moreover, the redox characteristics of the successive steps in the reduction of the hydroxylase strongly favor the introduction of two reducing equivalents (Fox et al., 1988, 1989). Consequently, our proposed cycle invokes the fully reduced state of the enzyme as the catalytically relevant state.

Environmental Implications. Recently, considerable interest has been generated in the possible use of methanotrophs for the controlled biodegradation of TCE in contaminated ground water and soils [see, for example, Tsien et al. (1989)]. We have shown here that soluble methane monooxygenase can oxidize four of the five chlorinated ethylenes classified by the U.S. Environmental Protection Agency as priority toxic pollutants, as well as other halogenated alkenes. As considerable concern has been raised over the biological production of vinyl chloride as a product of the anaerobic dehalogenation of chlorinated ethylenes (Vogel & McCarty, 1985), it is noteworthy that vinyl chloride is readily oxidized by soluble methane monooxygenase from *M. trichosporium* OB3b. The rates of reactivity reported here for the purified soluble enzyme are much higher than the values previously observed during studies with other purified enzyme systems or other bacterial cells (Table V). More recent whole-cell studies of *M. trichosporium* OB3b containing soluble methane monooxygenase now indicate that the haloalkenes are oxidized at rates consistent with the specific activities reported here for the purified enzyme (Oldenhuis et al., 1989; Tsien et al., 1989). At present, however, no information is available concerning the presence of the soluble methane monooxygenase in environmental samples.

ACKNOWLEDGMENTS

We thank Dr. R. S. Hanson for many valuable discussions, Jay E. Dege and Wayne A. Froland for help with initial experiments, David R. Jollie for the purified formate dehydrogenase used in these studies, and Gregory A. Brusseau for assistance and advice with gas chromatography.

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