Reductive Dehalogenation by Cytochrome P450$_{CAM}$: Substrate Binding and Catalysis†

Shuying Li‡ and Lawrence P. Wackett*

Department of Biochemistry and Institute for Advanced Studies in Biological Process Technology, Gortner Laboratory, University of Minnesota, St. Paul, Minnesota 55108

Received April 14, 1993; Revised Manuscript Received June 28, 1993®

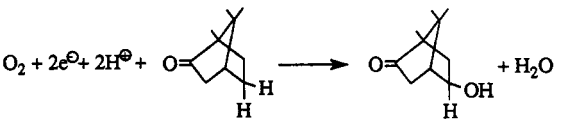
ABSTRACT: Biological reductive dehalogenation reactions are important in environmental detoxification of organohalides. Only scarce information is available on the enzymology underlying these reactions. Cytochrome P450$_{CAM}$ with a known X-ray structure and well-studied oxygenase reaction cycle, has been studied for its ability to reduce carbon–halogen bonds under anaerobic conditions. The reductive reactions functioned with NADH and the physiological electron-transfer proteins or by using artificial electron donors to reduce cytochrome P450$_{CAM}$. Halogenated methane and ethane substrates were transformed by a two-electron reduction and subsequent protonation, β-elimination, or α-elimination to yield alkanes, alkene, or carbene-derived products, respectively. Halogenated substrates bound to the camphor binding site as indicated by saturable changes in the Fe(III)–heme spin state upon substrate addition. Hexachloromethane was bound with a dissociation constant (K_D) of 0.7 μM and caused >95% shift from low- to high-spin iron. Ethanes bearing fewer chlorine substituents were bound with increasing dissociation constants and gave lesser degrees of iron spin-state change. Camphor competitively inhibited hexachloroethane reduction with an inhibitor constant (K_I) similar to the dissociation constant for camphor ($K_I = K_D = 0.9$ μM). Rate determinations with pentachloroethane indicated a 100-fold higher enzyme V/K compared to the second-order rate constant for hematin free in solution. These studies on substrate binding and catalysis will help reveal how biological systems enzymatically reduce carbon–halogen bonds in the environment.

Halogenated organic compounds are the largest single group on the EPA list of priority pollutants (Leisinger, 1983). Many are toxic and persist in the environment. With increasing frequency, bacteria are being identified which are capable of metabolizing haloorganic molecules. Four general biochemical mechanisms of carbon–halogen bond cleavage have been identified, and reductive dehalogenation is most important for the transformation and detoxification of highly chlorinated compounds (Wackett & Schanke, 1992). Only a small number of bacterial pure cultures capable of catalyzing reductive dehalogenation reactions have been isolated (DeWeerd & Suflita, 1990). Little information has been obtained on the biochemical mechanisms operative in reductive dehalogenation.

Metallomacrocycles found in bacteria catalyze reductive dehalogenation reactions *in vitro*. Nickel-containing coenzyme F_{430}, cobalt-containing vitamin B_{12}, and iron–porphyrins (hematin) are redox-active cofactors biosynthesized by diverse bacterial species (Wackett et al., 1989). *In vitro*, the reduced cofactors can catalyze an overall two-electron transfer to carbon–halogen bonds, yielding a free halide anion (Gantzer & Wackett, 1991). *In vivo*, bacterial proteins containing metallocofactors may figure prominently in environmental reductive dehalogenation reactions.

Cytochrome P450$_{CAM}$ monooxygenase is a well-studied heme protein which is known to catalyze nonphysiological reductive dehalogenation reactions (Castro et al., 1985) (Figure 1). The physiological oxidative reaction cycle has been extensively investigated (Yu et al., 1974; Sligar et al., 1991), and an X-ray structure has been solved to a resolution of 1.7 Å (Poulos & Raag, 1992). These extensive data highlight cytochrome P450$_{CAM}$ as an excellent model to better understand bacterial reductive dehalogenation biochemistry. Previously, the binding of halogenated priority pollutants to cytochrome P450$_{CAM}$ had not been investigated. This study filled that gap, and hexachloroethane was found to bind more tightly to Fe(III)–P450$_{CAM}$ than the physiological substrate camphor. Different electron donor systems supported reductive dehalogenation, allowing turnover experiments to be conducted using cytochrome P450$_{CAM}$ alone. A previous study discounted the possibility of carbene intermediates arising during cytochrome P450$_{CAM}$-dependent reduction of halogenated methanes (Castro et al., 1985). In the present study, cytochrome P450$_{CAM}$ catalyzed a single-turnover stoichiometric reduction of $CFCl_3$ to carbon monoxide, indicating a carbene intermediate is on the reaction pathway.

Physiological oxidative reaction



Non-physiological reductive reaction

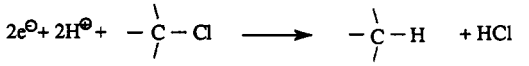


FIGURE 1: Oxidative (top) and reductive (bottom) reactions catalyzed by cytochrome P450$_{CAM}$.

MATERIALS AND METHODS

Bacteria and Enzymes. Pseudomonas putida G786 was grown on camphor. Cytochrome, P450$_{CAM}$, the flavoprotein

† This work was supported by National Institutes of Health Grant GM41235 (to L.P.W.).

* Author to whom correspondence should be addressed. Phone: 612/625-3785. FAX: 612/625-5780.

‡ Present address: Division of Endocrinology and Metabolism, Beth Israel Hospital, Harvard Medical School, Boston, MA 02215.

® Abstract published in *Advance ACS Abstracts*, August 15, 1993.

0006-2960/93/0432-9355$04.00/0

reductase, and putidaredoxin were purified according to published protocols (Gunsalus & Wagner, 1978). The 391-nm:280-nm absorbance ratio of purified cytochrome $P450_{CAM}$ solutions was >1.35, the 325-nm:280-nm absorbance ratio of putidaredoxin was >0.55, and the 280-nm:454-nm absorbance ratio of the flavoprotein was <12. SDS–gel electrophoresis indicated the flavoprotein was ≥95% homogeneous; cytochrome $P450_{CAM}$ and putidaredoxin were ≥90% homogeneous. Camphor-free cytochrome $P450_{CAM}$ was prepared as described by Logan (Logan et al., 1993). The 417-nm/391-nm absorbance ratio of the camphor-free cytochrome $P450_{CAM}$ was ≥1.9. Spinach ferredoxin reductase and ferredoxin were purchased from Sigma Chemical Co. (St. Louis, MO).

Purified cytochrome $P450_{CAM}$ was highly active with camphor under an oxygen-containing atmosphere. Reaction mixtures containing varying amounts, of putidaredoxin yielded data which gave a specific activity of 24 μmol min^{-1} (mg of $P450_{CAM}$)$^{-1}$ by extrapolation to infinite putidaredoxin concentration (Tyson et al., 1972).

Chemicals and Solutions. Halogenated substrate stock solutions were prepared in methanol. The final methanol concentration in all enzyme incubations was ≤2% (v/v). The buffer used in enzyme binding and catalysis studies was 50 mM potassium phosphate, pH 7.4, containing 200 mM KCl. Hematin from bovine blood was from Sigma Chemical Co.

Pentachloroethane Reduction with Various Electron Donors. The assays were carried out at 22 °C in 13-mL vials containing a 1-mL reaction mixture in 50 mM potassium phosphate buffer with 200 mM KCl, pH 7.4. The vials were capped with Teflon-lined rubber septa and made anaerobic by repeatedly cycling between a vacuum pump and an argon gas stream which had passed through an oxygen scrubber as previously described (Wackett et al., 1987). Reactions were initiated by adding the reductant. Pentachloroethane and its reduction product, trichloroethylene (TCE),[1] were analyzed by head space gas chromatography as previously described (Schanke & Wackett, 1992). The reaction mixtures contained 500 μM pentachloroethane and 1.5 μM cytochrome $P450_{CAM}$ with or without 3 μM putidaredoxin. Protein reducing systems used 1.5 μM putidaredoxin reductase and 300 μM NADH, or 1.5 μM spinach ferredoxin reductase, 3 μM spinach ferredoxin, and 600 μM NADPH. The NADH/phenazine methosulfate (PMS) system contained 50 μM PMS and 1.5 mM NADH (Eble & Dawson, 1984). The EDTA/proflavin system contained 500 μM EDTA and 50 μM proflavin, and light was provided as described (Greenbaum et al., 1972). Titanium citrate was prepared following the procedure of Zehnder and Wuhrman (1976), and its concentration in the reaction mixture was 9.0 mM. Sodium dithionite was used at a concentration of 50 mM.

Determination of Reaction Stoichiometry. TCE formation was analyzed by gas chromatography (Schanke & Wackett, 1992). NADH oxidation was analyzed in a parallel sample by withdrawing 20 μL of the reaction mixture, diluting to 400 μL, and measuring the absorption at 340 nm using a Beckman DU-70 spectrophotometer. The rate of NADH oxidation was calculated on the basis of an extinction coefficient of 6.22 mM^{-1} (Gunsalus & Wagner, 1978).

Steady-State Kinetic Determinations. In an anaerobic septum-sealed cuvette, a 700-μL reaction mixture contained 1 μM flavoprotein, 2 μM putidaredoxin, 1 μM cytochrome $P450_{CAM}$, and 1 mM pentachloroethane or 150 μM camphor.

[1] Abbreviations: TCE, trichloroethylene; PMS, phenazine methosulfate; EDTA, ethylenediaminetetraacetic acid; GC/MS, gas chromatography/mass spectrometry.

Table I: Identification of Halogenated Products by Gas Chromatography and Mass Spectrometry

halogenated compound	retention time (min) GC no. 1	GC no. 2	GC/MS	mass spectrometry *m/z*
halomethanes				
$HCCl_2Br$		0.4 (100)[a]	1.8 (60)[c]	164,163,129,127,83
$HCCl_3$		0.3 (100)	1.6 (60)[c]	120,118,85,83
H_2CCl_2		0.1 (100)	1.3 (60)[c]	86,84,57
$HCCl_2F$			1.3 (60)[c]	104,102,85,83
haloethanes				
Cl_2HCCF_3	0.5 (180)		1.1 (50)[b]	153,151,119,117
ClH_2CCF_3		0.4 (140)	0.9 (50)[c]	118,98,83
haloethylenes				
$Cl_2C{=}CCl_2$	2.0 (180)		6.2 (60)[b]	167,166,165
$Cl_2C{=}CF_2$	0.5 (140)		1.0 (50)[c]	134,132
$Cl_2C{=}CHCl$	0.8 (180)		3.2 (60)[b]	131
$Cl_2C{=}CH_2$		1.0 (180)	1.5 (60)[b]	97,96,61

[a] Column temperature (°C) for isothermal chromatography. [b] DB-5 column. [c] DB-1 column.

The reaction was initiated by adding NADH. NADH oxidation was followed continuously by spectrophotometry. To determine the enzyme k_{cat} with pentachloroethane, the substrate was varied at three fixed putidaredoxin concentrations. The k_{cat} was obtained from a replot of the apparent k_{cat} against putidaredoxin concentration and extrapolation to infinite concentration. The pentachloroethane concentration in the liquid phase was calculated using its Henry's Law constant (see below) (Gossett, 1987). Steady-state kinetic parameters were determined with hexachloroethane using camphor as an inhibitor. Varying amounts of hexachloroethane in 14 μL of methanol were added, and initial rates of NADH oxidation were analyzed at camphor concentrations of 0, 1.0, and 3.2 μM, respectively. The hexachloroethane concentration in the liquid phase was calculated using its Henry's Law constant (Gossett, 1987).

Determination of the Rate Constant for Heme Free in Solution. Hematin solutions were made anaerobically in 10% NaOH and diluted in water as needed. The concentration of the heme solution was determined by the absorbance at 580 nm (Lemberg & Legge, 1949). The assay of pentachloroethane reduction and TCE formation was performed by gas chromatography (Schanke & Wackett, 1992). A 1-mL reaction mixture contained 2 μM heme, 9.0 mM titanium(III) citrate, and varied concentrations of pentachloroethane. Then at a fixed pentachloroethane concentration, heme was varied from 0.5 to 2 μM to determine the dependence of reaction rate on heme concentration. Finally, the heme concentration was fixed, and Ti(III) was varied to ensure the reaction was zero order with respect to reductant.

Identification of Reaction Products. Cytochrome $P450_{CAM}$ (10 μM) was incubated with various halogenated substrates (1.0 mM) under an argon atmosphere with 3 mM titanium(III) citrate as the electron donor. Control reactions with only titanium(III) citrate failed to yield detectable products. Halogenated products were analyzed using a Varian or Hach-Carle AGC-100 gas chromatograph, each equipped with a flame ionization detector and an AT-1000 column (Alltech Associates, Deerfield, IL). The nitrogen carrier gas flow rate was 30 mL/min. The products were also analyzed by gas chromatography/mass spectrometry using an electron-capture detector and a DB-1 or DB-5 column (J & W Scientific) coupled to a Kratos MS-25 mass spectrometer. Products were identified by gas chromatography using two independent conditions and by mass spectrometry (Table I). Carbon monoxide was determined as previously described (Fox et al., 1990).

Table II: Pentachloroethane Reduction by Cytochrome P450$_{CAM}$ with Various Electron Donors

electron donor (all except controls contain P450$_{CAM}$)	rate of TCE formation (nmol/min)		
	+putidaredoxin	−putidaredoxin	−P450$_{CAM}$ (control)
NADH + flavoprotein + putidaredoxin	10.1 ± 0.7	<0.2	<0.2
NADPH + spinach ferredoxin reductase + spinach ferredoxin	8.8 ± 1.0	1.8 ± 0.3	<0.2
NADH + phenazine methosulfonate	10.0 ± 0.7	0.4 ± 0.1	<0.2
sodium dithionite	10.6 ± 0.7	5.2 ± 0.3	2.0 ± 0.3
light + EDTA/proflavin	10.3 ± 0.4	11.7 ± 0.7	0.3 ± 0.1
titanium(III) citrate	10.2 ± 1.0	12.4 ± 1.4	0.7 ± 0.3

Determination of K_D and Spin-State Changes. K_D and spin-state changes were measured in 50 mM potassium phosphate buffer, pH 7.4, containing 200 mM KCl using a Beckman DU-7400 diode array spectrophotometer as described (Logan et al., 1993; Fisher & Sligar, 1985).

Hexachloroethane Inhibition of Camphor Hydroxylation. In a 1.5-mL septum-sealed air-saturated cuvette, 1-mL reaction mixtures contained 1 μM cytochrome P450$_{CAM}$, 2 μM putidaredoxin, 1 μM flavoprotein, 200 μM camphor, and 300 μM NADH with or without 100 μM hexachloroethane. NADH oxidation was followed, and the reaction was stopped at 4 min by extraction into 1 mL of chloroform. The sample was worked up as described (Gelb et al., 1982). The extracts were analyzed for camphor and 5-hydroxycamphor by GC/MS using a DB-wax column (30 m × 0.5 μM) and a Kratos MS-25 mass spectrometer.

Measurement of Henry's Law Constants. The Henry's Law constants for pentachloroethane and hexachloroethane were measured according to the methods outlined by Gossett (1987).

RESULTS

Purified Cytochrome P450$_{CAM}$ Catalyzes Reductive Dehalogenation with Various Electron Donors. In a previous study, intact *P. putida* G786 cells induced with camphor catalyzed the reduction of pentachloroethane to trichloroethylene (TCE). In this study, purified cytochrome P450$_{CAM}$ was shown to catalyze the same reaction. With NADH and the physiological electron-transfer proteins, TCE was formed at a rate of 10.1 nmol/min in a reaction mixture containing 1.5 nmol/mL cytochrome P450$_{CAM}$ (Table II). Note that the enzyme components were not optimized in these experiments, and so the specific activity of the cytochrome P450$_{CAM}$ component with this substrate is underestimated (see below). However, the data indicated that alternative protein electron donor systems may be used in reductive dehalogenation experiments. Spinach ferredoxin, its flavoprotein reductase, and NADPH were capable of supporting the reaction, albeit at a lower rate. The presence of putidaredoxin increased the rate substantially.

Nonprotein redox systems were also effective in supporting cytochrome P450$_{CAM}$-dependent pentachloroethane reduction (Table II). All supported substantial dechlorination rates in the presence of putidaredoxin. However, only titanium(III) citrate and light/EDTA/proflavin were active in the absence of putidaredoxin to catalyze pentachloroethane reduction at rates comparable to the physiological three-component enzyme system at NADH. These results and the controls lacking cytochrome P450$_{CAM}$ are consistent with the reductive dechlorination reaction being carried out exclusively by the cytochrome P450 component.

Reaction Stoichiometry and Initial Rates of NADH Oxidation. Reaction stoichiometries were obtained by spectrophotometric and gas chromatographic determinations of reactant and product concentrations. In a previous study, a 1:0.9 ratio of pentachloroethane consumed to trichloroethylene formed was observed when oxygen was excluded from the reaction mixture (Logan et al., 1993). In the present study, the ratio of NADH oxidized to pentachloroethane consumed was 1.0 ± 0.1, indicating complete coupling between NADH oxidation and pentachloroethane reduction.

In a reaction mixture containing 350 μM NADH, 1 μM flavoprotein, 2 μM putidaredoxin, and 1 μM cytochrome P405$_{CAM}$ in 50 mM phosphate buffer, pH 7.5 with 200 mM KCl, the NADH oxidation rate with camphor under air was 1.4 ± 0.1 μmol min^{-1} (mg P450)$^{-1}$. Under the same conditions, the initial rate of NADH oxidation with pentachloroethane under argon was 1.0 ± 0.1 μmol min^{-1} (mg of P450)$^{-1}$.

Enzyme V/K Compared to the Second-Order Rate Constant with Free Heme. The second-order rate constant for the reaction of heme and pentachloroethane in the presence of titanium(III) citrate was 2 × 10^3 L mol^{-1} s^{-1}. The reaction was first-order each with respect to heme and pentachloroethane. The reaction was essentially zero-order with respect to Ti(III) at the concentrations used in these experiments. The reaction was negligible in the absence of heme or titanium. To determine the maximal velocity as a function of cytochrome P450$_{CAM}$, NADH and putidaredoxin reductase were added to reaction mixtures at saturating concentrations, and putidaredoxin was added at varied concentrations. At each putidaredoxin concentration, initial rates of NADH oxidation were determined for a range of initial pentachloroethane concentrations. This yielded a series of double-reciprocal plots at each putidaredoxin concentration (Tyson et al., 1972). From these, the k_{cat} was determined to be 1.5 s^{-1} by extrapolation of a straight line fit of the points to infinite putidaredoxin concentration. The apparent K_m, however, did not vary linearly with pentachloroethane concentration, precluding its accurate determination. We used the K_D value of 7 μM, determined by equilibrium binding studies described below, to obtain k_{cat}/K_D. The validity of using K_D as a substitute for K_m in V/K determinations has been discussed previously (Atkins & Sligar, 1988). The k_{cat}/K_D was 2.1 × 10^5 L m^{-1} s^{-1} or 2 orders of magnitude greater than the reaction of free heme and pentachloroethane in solution.

Reaction Products with Chloromethanes and Chloroethanes. Purified cytochrome P450$_{CAM}$ was incubated under an argon atmosphere with a series of halogenated methanes and ethanes. Either NADH and the physiological electron-transfer proteins or titanium(III) citrate was used as the reductant. Reaction products were determined by gas chromatograhpy and mass spectrometry as described under Materials and Methods. Control incubations omitting cytochrome P450$_{CAM}$, NADH, or titanium(III) citrate did not yield detectable products.

As had been reported previously (Castro et al., 1985), CCl_4 and $BrCCl_3$ were reduced by two electrons to yield $CHCl_3$ (Table III). The latter compound was not further reduced nor was it transformed when added as the initial substrate.

Table III: Binding and Anaerobic Turnover of Halogenated Substrates by Fe(III)–Cytochrome P450$_{CAM}$

substrate	product(s)	substrate binding to Fe(III)–P450$_{CAM}$ K_D	% spin-state conversion
halomethanes			
Br_2CCl_2	$HBrCCl_2$	>2 mM	47
$BrCCl_3$	$HCCl_3$	>2 mM	41
CCl_4	$HCCl_3$	>2 mM	36
$FCCl_3$	CO	>2 mM	31
$HBrCCl_2$	CH_2Cl_2	>2 mM	22
$HCCl_3$	ND[a]	>2 mM	14
haloethanes			
Cl_3CCCl_3	$Cl_2C{=}CCl_2$	0.7 μM ± 0.1	≥95
Cl_3CCF_3	HCl_2CCF and $CCl_2{=}CF_2$	105 μM ± 5	73
Cl_2HCCCl_3	$ClHC{=}CCl_2$	7 μM ± 1	70
ClH_2CCCl_3	$H_2C{=}CCl_2$	150 μM ± 10	50
$BrClHCCF_3$	ClH_2CCF_3	1.2 mM ± 0.1	45
$Cl_2HCCHCl_2$	ND	100 μM ● 20	30
Cl_3CCH_3	ND	400 μM ± 50	35
BrH_2CCF_3	ND	1.9 mM ± 0.1	32

[a] ND = none detected.

Other bromochloromethanes underwent two-electron reduction. In all cases, bromine was selectively displaced, consistent with the reported ease of carbon–bromine bond reduction in comparison to carbon–chlorine bond reduction (Hanzlick, 1981). Initially, halogenated products were not observed in enzyme incubation mixtures with $CFCl_3$. This was curious since theoretical calculations indicate $CFCl_3$ to be as reactive as CCl_4 with reduced heme (Luke & Loew, 1986). In subsequent UV–visible spectroscopic experiments, the major absorption band at 408 nm of substrate-free reduced cytochrome P450$_{CAM}$ shifted to 448 nm upon the addition of $CFCl_3$. The extinction coefficient of the intermediate was similar to that described for Fe(II)–CO P450$_{CAM}$. EPR studies revealed no P450 iron signal, consistent with the assignment of this species as Fe(II)–CO P450$_{CAM}$. Addition of reduced hemoglobin to the $CFCl_3$–P450$_{CAM}$ reaction mixture caused a decrease in the 448-nm peak with the formation of a peak at 419 nm. The new peak at 419 nm can be attributed to the formation of Fe(II)–CO–hemoglobin by competitive removal of CO from Fe(II)–CO–P450$_{CAM}$. The transfer of a carbene to hemoglobin would be highly unlikely. Fe(II)–CO P450$_{CAM}$ is stable in the absence of hemoglobin. Thus, we would not expect to see multiple turnovers of cytochrome P450$_{CAM}$ with $CFCl_3$ (Figure 3). This explains the failure to detect significant disappearance of substrate or halogenated product formation by gas chromatography.

In contrast to the halomethanes, vicinally substituted polychlorinated ethanes largely reacted with cytochrome P450$_{CAM}$ to undergo concomitant reduction and elimination (Table III). Reductive elimination with vicinal dihalide substrates yielding alkenes has been observed in numerous studies with iron–porphyrins (Wade & Castro, 1973; Schanke & Wackett, 1992) and in electrochemical studies (Connors et al., 1988). With cytochrome P450$_{CAM}$, hexachloroethane yielded tetrachloroethylene, pentachloroethane yielded trichloroethylene, and 1,1,1,2-tetrachloroethane yielded 1,1-dichloroethylene. 1,1,2,2-Tetrachloroethane and 1,1,1-trichloroethane failed to yield detectable products. When the carbon β to the carbon undergoing reduction contained three fluorine substituents, direct reductive dechlorination was observed. 1,1,1-Trichlorotrifluoroethane yielded a nearly equal mixture of 1,1-dichlorodifluoroethylene and 1,1-dichloro-2,2,2-trifluoroethane. Haloethane ($BrClHCCF_3$) underwent a very slow reaction to yield 1-chloro-2,2,2-trifluoroethane as the major product.

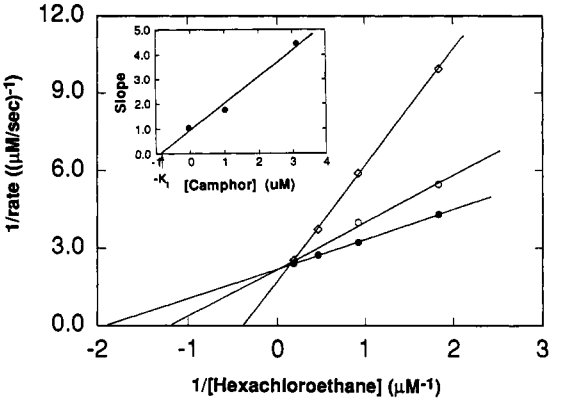


FIGURE 2: Reciprocal plot showing inhibition of anaerobic cytochrome P450$_{CAM}$ catalyzed reduction of hexachloroethane in the presence of camphor. The rate of reduction was determined spectrophotometrically by following NADH oxidation at 340 nm. Measurements at varied concentrations of hexachloroethane were made at fixed camphor concentrations of (●) 0, (○) 1, and (□) 3.2 μM. Each reaction mixture contained 1 μM cytochrome P450$_{CAM}$, 2 μM putidaredoxin, and 1 μM flavoprotein. The inset shows the slope replot of the primary data.

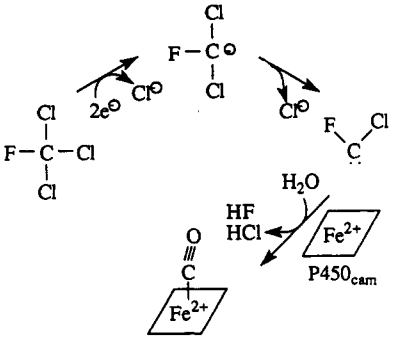


FIGURE 3: Proposed pathway of CO formation from $CFCl_3$.

Substrate Binding. Previous studies of substrate binding have largely focused on camphor-like compounds. Our cytochrome P450$_{CAM}$ preparations showed binding properties with camphor and tetramethylcyclohexane that were consistent with published data. In Table III, we report K_D and spin-state conversion data observed with halogenated substrates. The halomethanes uniformly showed K_D values >2 mM. Low water solubilities precluded a more definitive determination of these parameters. The precent spin-state conversion varied from 14 to 47% concomitant with the increase in size of the halomethane. The haloethanes are bound relatively tightly. In fact, hexachloroethane is bound (K_D = 0.7 μM) and shows spin-state conversion data comparable to the physiological substrate camphor (K_D = 0.84 μM; Fisher & Sligar, 1985). The tight binding and the observed changes in the spin state of the active-site heme iron suggest chlorinated substrates bind in the same enzyme cavity as camphor.

Steady-state kinetic experiments were conducted to further explore halogenated substrate binding. It was not possible to monitor camphor hydroxylation aerobically in the presence of hexachloroethane by following NADH oxidation due to uncoupling with the latter substrate. It was possible to monitor hexachloroethane reduction anaerobically using camphor as an inhibitor. Camphor was a competitive inhibitor of hexachloroethane reduction (Figure 2). The K_I for camphor was 0.9 μM, in good agreement with the reported K_D of 0.84 μM for camphor.

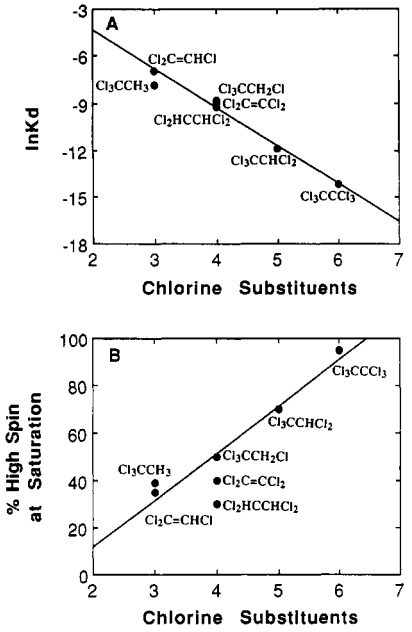


FIGURE 4: ln binding constants (A) and percent spin-state conversion (B) as a function of degree of chlorination for C_2 halogenated compounds.

In separate experiments, gas chromatography/mass spectrometry was used to determine if hexachloroethane inhibited camphor oxidation to 5-*exo*-hydroxycamphor in air-saturated buffer. In fixed time point assays, hexachloroethane significantly decreased the yield of 5-*exo*-hydroxycamphor. At the end of the experiment, the enzyme was reduced, exposed to CO, and shown to retain the 450-nm peak indicative of native enzyme. This result mitigates against hexachloroethane inhibiting the reaction due to protein denaturation.

DISCUSSION

In this study, reductive dehalogenation by purified cytochrome P450$_{CAM}$ was investigated. Electrons for reduction could be provided by putidaredoxin, putidaredoxin reductase, and NADH or by alternative donor systems. These results provide the first data about halogenated substrate binding. The data indicate that halogenated substrates bind to the same site as the physiological substrate camphor and some of them form tight ES complexes with cytochrome P450$_{CAM}$. The rate of reductive dehalogenation, up to 2 μmol min^{-1} (mg of cytochrome P450$_{CAM}$)$^{-1}$ with pentachloroethane, has potential environmental significance. A comparison of second-order rate constants for enzyme and free heme provides a backdrop for beginning to understand how enzymes may facilitate reductive dehalogenation reactions in nature.

Halogenated Substrate Binding. Binding was monitored spectroscopically as the conversion of low- to high-spin-state iron accompanying substrate addition to Fe(III)–P450$_{CAM}$. For the methane series (K_D > 2 mM), a K_D could not be more precisely determined due to substrate solubility and mass-transfer limitations. The chlorinated ethanes bound much more tightly. The C_2 chlorocarbons showd an inverse linear correlation between their ln K_D and the number of chlorine substituents (Cl_3 to Cl_6) (Figure 4A). Indeed, hexachloroethane bound to cytochrome P450$_{CAM}$ as tightly as the physiological substrate camphor. This is particularly striking given that 2 kcal/mol of binding energy is imparted to camphor by a hydrogen bond between the substrate carbonyl oxygen and tyrosine-96 (Atkins & Sligar, 1988). Comparable hydrogen bond energies would not likely contribute to hexachloroethane binding. This highlights the potential strength of hydrophobic interactions in protein active-site interactions with complementary-sized halogenated compounds.

This study also reported the percent enzyme converted to high-spin iron when saturated with halogenated substrates. The percent spin-state change has been correlated with the observed midpoint potential for the heme iron (Fisher & Silgar, 1985). Data from X-ray crystallography support the idea that hydrophobic substrates exclude water molecules from the heme environment, facilitating the changes in spin state and redox potential. A comparable correlation has not been found for substrate K_D values. For example, 3-bromocamphor and tetramethylcyclohexanone bind to cytochrome P450$_{CAM}$ with similar K_D values (Fisher & Sligar, 1985). However, 3-bromocamphor is much more effective in binding to the high-spin enzyme form, thus effecting a higher percent spin-state change. In the present study with halogenated alkanes, substrate K_D and induced percent spin-state change were also observed to vary independently. Brominated halomethanes caused moderate spin-state conversion but bound relatively weakly (K_D > 2 mM). Haloethanes bound more tightly and showed a wide variability in changing the heme iron spin state (from 30 to >95%). Within the haloethane series, spin-state change varied linearly with the number of chlorine substituents (Figure 4B).

The data in the present study indicate that halogenated substrates and camphor bind to the same site on the enzyme. This is suggested first by the tight fit of hexachloroethane and pentachloroethane. These substrates also cause substantial conversion from low- to high-spin iron which has previously been correlated with expulsion of water molecules from the camphor binding site (Poulos & Raag, 1992). It was observed that camphor competitively inhibited hexachloroethane reduction with a K_I near the known K_D for camphor. Conversely, hexachloroethane inhibited camphor hydroxylation in an air-saturated reaction mixture.

Previously, camphor was reported not to inhibit reductive dechlorination of CCl_4, $BrCCl_3$, and Cl_3CNO_2 (Castro et al., 1985). Most of the incubations in this previous study contained camphor at substoichiometric concentrations with respect to cytochrome P450$_{CAM}$. However, even at 1.7 equiv of camphor with respect to enzyme, inhibition was not observed. In that study, reactions were monitored spectrophotometrically by following the one-electron oxidation of Fe(II)–cytochrome P450 to Fe(III). It was assumed that this measurement was reflective of the two-electron reduction of a carbon–halogen bond. We conducted similar spectrophotometric experiments and found that camphor, present in excess of cytochrome P450$_{CAM}$, significantly inhibited the anaerobic $BrCCl_3$-dependent oxidation of Fe(II)–cytochrome P450. The reason for the apparent discrepancy between our data and those reported previously (Castro et al., 1985) is not apparent. However, our observations of halogenated substrate-dependent iron spin-state changes, low dissociation constants, and the kinetic inhibition data are all consistent with halogenated substrates and camphor binding to the same site on cytochrome P450$_{CAM}$.

Reductive Dehalogenation Rates. In the present study, reactions were followed by the chromatographic determination of two-electron-reduced halocarbon products. NADH oxidation was also determined spectrophotometrically and shown to be stoichiometric with pentachloroethane reduction. The rate of pentachloroethane reduction, at saturating putidaredoxin concentrations, approached 2 μmol min^{-1} (mg of protein)$^{-1}$ or a turnover number of 1.5 s^{-1}. We used k_{cat}/K_D

to estimate the enzyme second-order rate constant with pentachloroethane. This value was 100 times greater than the collisional reaction between free heme and pentachloroethane in solution under the conditions used. The modest rate acceleration affected by the enzyme is likely due to proximity effects (Walsh, 1979) given the relatively tight binding of pentachloroethane determined in this study (K_D = 7 μM).

It is also of interest to compare *in vivo* and *in vitro* rates of pentachloroethane reductive dehalogenation. In a previous study, *P. putida* G786 transformed pentachloroethane to trichloroethylene at a rate of 0.9 nmol min^{-1} (mg of whole cell protein)$^{-1}$. Assuming that cytochrome P450$_{CAM}$ comprised 1.4% of the cell protein (Gunsalus & Wagner, 1978), the *in vitro* rate is 66 nmol min^{-1} (mg of cytochrome P450$_{CAM}$)$^{-1}$. This is considerably lower than the *in vitro* V_{max} of 2 μmol min^{-1} (mg of cytochrome P450$_{CAM}$)$^{-1}$ obtained at saturating concentrations of the accessory electron-transfer proteins and NADH. These observations indicate the need for further biochemical investigations to determine rate-determining steps in biodegradation as a prelude to any use of *P. putida* G786 or similar bacteria as biodehalogenation catalysts.

Reaction Products. As has been previously observed, halomethanes largely underwent a direct two-electron reduction, and vicinally substituted haloethanes underwent reduction and elimination to yield alkenes. Similar reactions have been reported with iron–porphyrins free in solution. With haloethanes containing a $-CF_3$ group, direct two-electron reduction occurred. With CCl_3CF_3, both β-elimination and direct two-electron reduction were observed. With halothane, only the latter reaction was observed. The β-fluorine substituents show a lesser propensity for elimination, likely due to the strength of the C–F bond. However, the factors controlling enzymatic reduction and protonation vs reduction and elimination pathways are not currently well-defined. Mammalian liver microsomal cytochrome P450 transforms halothane to a mixture of chlorodifluoroethylene and chlorotrifluoroethane (Ahr et al., 1982). Subtle active-site differences between bacterial and mammalian cytochrome P450 enzymes may underlie the different product partitioning.

It has been proposed that reported reduction of halomethanes to carbene intermediates in cytochrome P450 reaction mixtures is artifactual: that carbenes actually arise from enzymatic two-electron reduction and nonenzymatic base-catalyzed deprotonation and elimination (Castro et al., 1985). In this study, cytochrome P450$_{CAM}$ reacted with $CFCl_3$ to yield CO as the major product. Only a trace amount of $CHFCl_2$ was detected. Furthermore, no free CO was found in reaction mixtures. These data are consistent with the major pathway of $CFCl_3$ turnover as shown in Figure 3. Fluorine substituents are known to stabilize carbenes (Dolbier & Burkholder, 1990). A similar carbene pathway to CO has been proposed for reduction of $CFCl_3$ by cobalamins and by a methanogenic bacterium (Krone et al., 1991; Krone & Thauer, 1992). The current data reported here do not discriminate between potential free or iron-bound carbene intermediates. Rapid spectroscopic methods may prove useful in observing these or other intermediate species.

Environmental Implications. The reactions studied here are nonphysiological. It is likely that evolutionary forces have not been operative in optimizing cytochrome P450$_{CAM}$ to bind and reduce halogenated substrates. Despite this, our observed pentachloroethane reduction rates are much faster than reported environmental reductive dehalogenation rates for this and comparable compounds (Egli et al., 1988). It is suspected that a significant incidence of biological reductive dehalogenation reactions occurring in soils and sediments may be nonphysiological or gratuitous reactions with hydrophobic contaminants that diffuse nonspecifically through cell membranes (Egli et al., 1990). Some of these environmental reductive reactions may take place with enzymes resembling cytochrome P450$_{CAM}$. Further studies with cytochrome P450$_{CAM}$ may reveal mechanistic details to better understand reductive dehalogenation in the environment. Additionally, laboratory-directed evolution to improve biodehalogenation catalysts will require further insights into halogenated substrate binding and reduction by well-studied enzymes like cytochrome P450$_{CAM}$.

ACKNOWLEDGMENT

We thank Dr. Michael Logan for performing some of the binding experiments and for helpful discussion, Dr. Kristoffer Andersson for help with the EPR experiment, and Dr. John Lipscomb and Dr. Harry Hogenkamp for helpful discussions. We acknowledge the assistance of Dr. Thomas Krick in GC/MS analysis and Ms. Bonnie Allen for help in the preparation of the manuscript.

REFERENCES

Ahr, H. J., King, C. J., Nastainczyk, W., & Ullrich, V. (1982) *Biochem. Pharmacol. 31*, 383–390.
Atkins, W. M., & Sligar, S. G. (1988) *J. Biol. Chem. 263*, 18842–18849.
Castro, C. E., Wade, R. S., & Belser, N. O. (1985) *Biochemistry 24*, 204–210.
Connors, T. F., Arena, J. V., & Rusling, J. F. (1988) *J. Phys. Chem. 92*, 2810–2816.
DeWeerd, K. A., & Suflita, J. M. (1990) *Appl. Environ. Microbiol. 56*, 2999–3005.
Dolbier, W. R., Jr., & Burkholder, C. R. (1990) *J. Org. Chem. 55*, 589–594.
Eble, K. S., & Dawson, J. H. (1984) *Biochemistry 23*, 2068–2073.
Egli, C., Scholtz, T., Cook, A. M., & Leisinger, T. (1988) *FEMS Microbiol. Lett. 43*, 257–261.
Egli, C., Stromeyer, S., Cook, A. M., & Leisinger, T. (1990) *FEMS Microbiol. Lett. 68*, 207–212.
Fisher, M. T., & Sligar, S. G. (1985) *J. Am. Chem. Soc. 107*, 5018–5019.
Fox, B. G., Borneman, J. G., Wackett, L. P., & Lipscomb, J. D. (1990) *Biochemistry 29*, 6419–6427.
Gantzer, C. J., & Wackett, L. P. (1991) *Environ. Sci. Technol. 25*, 715–721.
Gelb, M. H., Heimbrook, D. C., Mälkönen, P., & Sligar, S. G. (1982) *Biochemistry 21*, 370–377.
Gossett, J. M. (1987) *Environ. Sci. Technol. 21*, 202–208.
Greenbaum, E., Austin, R. H., & Gunsalus, I. C. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1273–1276.
Gunsalus, I. C., & Wagner, G. C. (1978) *Methods Enzymol. 52C*, 166–188.
Hanzlick, R. P. (1981) *Biochem. Pharmacol. 30*, 3027–3030.
Krone, V. E., & Thauer, R. K. (1992) *FEMS Microbiol. Lett. 90*, 201–204.
Krone, V. E., Thauer, R. K., Hogenkamp, H. P. C., & Steinbach, K. (1991) *Biochemistry 30*, 2713–2719.
Leisinger, T. (1983) *Experientia 39*, 1183–1191.
Lemberg, R., & Legge, J. W. (1949) in *Hematin Compounds and Bile Pigments*, pp 169–174, Interscience, New York.
Logan, M. S., Newman, L. M., Schanke, C. A., & Wackett, L. P. (1993) *Biodegradation 4*, 39–50.
Luke, B. T., & Loew, G. H. (1986) *Int. J. Quantum Chem. Quantum Biol. Symp. No. 12*, 99–112.
Poulos, T. L., & Raag, R. (1992) *FASEB J. 6*, 674–679.

Schanke, C. A., & Wackett, L. P. (1992) *Environ. Sci. Technol. 26*, 830–833.

Sligar, S. G., Filipovic, D., & Stayton, P. S. (1991) *Methods Enzymol. 206*, 31–49.

Tyson, C. A., Lipscomb, J. D., & Gunsalus, I. C. (1972) *J. Biol. Chem. 247*, 5777–5784.

Wackett, L. P., & Schanke, C. A. (1992) in *Metal Ions in Biological System* (Sigel, H., & Sigel, A., Eds.) pp 329–356, Marcel Dekker, Inc., New York, Basel, and Hong Kong.

Wackett, L. P., Honek, J. F., Begley, T. P., Wallace, V., Orme-Johnson, W. H., & Walsh, C. T. (1987) *Biochemistry 26*, 6012–6018.

Wackett, L. P., Orme-Johnson, W. H., & Walsh, C. T. (1989) in *Metal Ions and Bacteria* (Beveridge, T. J., & Doyle, R. J., Eds.) pp 165–206, Academic Press, New York.

Wade, R. S., & Castro, C. E. (1973) *J. Am. Chem. Soc. 95*, 226–230.

Walsh, C. T. (1979) in *Enzymatic Reaction Mechanisms*, W. H. Freeman, San Francisco.

Yu, C.-A., Gunsalus, I. C., Katagiri, M., Suhara, K., & Takemori, S. (1974) *J. Biol. Chem. 249*, 94–101.

Zehnder, A. J. B., & Wuhrmann, K. (1976) *Science 194*, 1165–1166.