Biodegradation **4**: 39–50, 1993.

Cosubstrate effects in reductive dehalogenation by *Pseudomonas putida* G786 expressing cytochrome P-450$_{CAM}$

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Received 10 March 1992; accepted in revised form 15 June 1992

Key words: cytochrome P450$_{CAM}$, *Pseudomonas*, chloroethane, reductive dehalogenation

Abstract

Cytochrome P-450$_{CAM}$ was shown to be the primary catalyst mediating reductive dehalogenation of polychlorinated ethanes by *Pseudomonas putida* G786. Under anaerobic conditions, the enzyme catalyzed reductive elimination reactions *in vivo* with the substrates hexachloroethane, pentachloroethane, and 1,1,1,2-tetrachloroethane; the products were tetrachloroethylene, trichloroethylene, and 1,1-dichloroethylene, respectively. *In vivo* reaction rates were determined. No reaction was observed with 1,1,2,2-tetrachloroethane or 1,1,1-trichloroethane. Purified cytochrome P-450$_{CAM}$ was used to measure dissociation constants of polychlorinated ethanes for the enzyme active site. Observed rates and dissociation constants were used to predict the course of a reaction with the three substrates simultaneously. Data obtained from experiments with *P. putida* G786 generally followed the simulated reaction curves. Oxygen suppressed the reductive dechlorination reactions and, in the case of 1,1,1,2-tetrachloroethane, 2,2,2-trichloroacetaldehyde was formed. Significant rates of reductive dechlorination were observed at 5% oxygen suggesting that these reactions could occur under partially aerobic conditions. These studies highlight the potential to use an aerobic bacterium, *P. putida* G786, under a range of oxygen tensions to reductively dehalogenate mixed wastes which are only degraded at very low rates by obligately anaerobic bacteria.

Abbreviations: GC/MS – Gas chromatography/mass spectrometry; P-450$_{CAM}$ – Cytochrome m of the camphor oxidizing system of *P. putida*; pca – Polychlorinated ethane

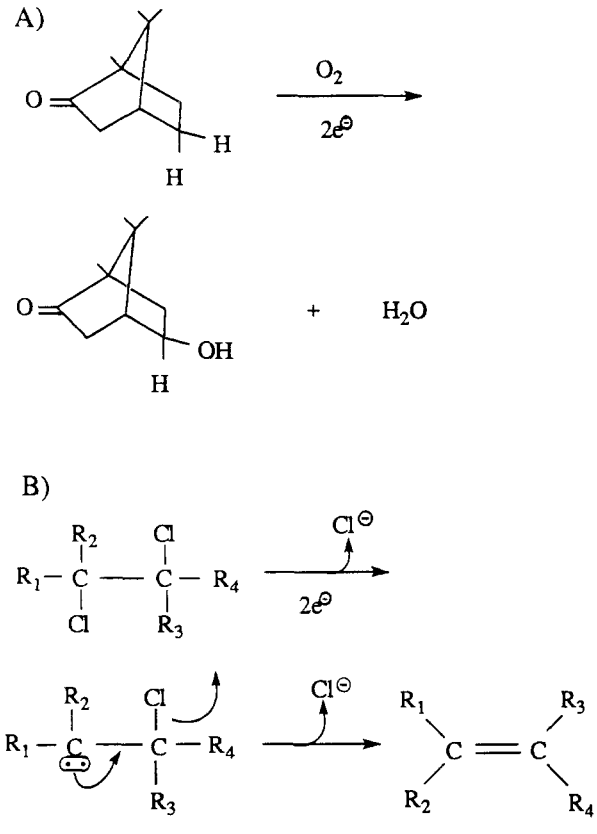
Introduction

There are many differences between biodegradation at the bench scale and bioremediation in practice. Typically in the laboratory, the biodegradation of individual highly-purified compounds is examined as a function of the organism or an enzyme under some defined set of conditions. In most waste treatment situations, the contaminated soils or waters contain more than one toxicant to be degraded. Complex waste mixtures present formidable challenges for the engineering of biological treatment systems. First, toxicity of one or more compounds to the biodegrading organism can prevent degradation of other compounds. Second, different pollutants with similar chemical structures can compete for uptake and enzyme binding sites, and this can profoundly effect biodegradation of individual waste components. The latter problem has been investigated in the present study. The goal was to be-

gin to uncover the most important factors influencing the course of biodegradation when an organism is simultaneously confronted with competing substrates.

The biodegradation reactions chosen for study were the reductive dehalogenation of chlorinated ethanes by *Pseudomonas putida* G786 expressing cytochrome P-450$_{CAM}$. This organism grows on camphor as a sole carbon and energy source under aerobic conditions (Bradshaw et al. 1959). The first reaction in the camphor catabolic pathway, catalyzed by cytochrome P-450$_{CAM}$, is the insertion of one atom from molecular oxygen into the substrate to yield 5-*exo*-hydroxycamphor (Hedegaard & Gunsalus 1965) (Scheme IA). More recently, *P. putida* G786 and purified cytochrome P-450$_{CAM}$ have been demonstrated to catalyze reductive dehalogenation of chlorinated alkanes under anaerobic conditions (Castro et al. 1985). For example, a vicinal dichlorinated ethane typically undergoes a net two electron reduction of one carbon-chlorine bond with subsequent β-elimination to yield an alkene as the first detectable product (Scheme IB).

There is currently avid interest in developing a greater understanding of the mechanisms of bacterial reductive dehalogenation reactions. Progress, however, has been slowed by difficulties in obtaining pure cultures from anaerobic consortia showing reductive dehalogenation activity (Tiedje et al. 1987). A rare pure culture is *Desulfomonile tiedjei*, which utilizes 3-chlorobenzoate as a final electron acceptor in an energy linked reduction to benzoate (Dolfing 1990; Mohn & Tiedje 1990). This system is beginning to yield to biochemical inquiry. Recently, crude cell extracts were shown to be active in dehalogenation under anaerobic conditions (DeWeerd & Suflita 1990). Further progress in this field requires a greater knowledge of the enzyme structures and mechanisms capable of mediating reductive dehalogenation. The cytochrome P-450$_{CAM}$ system offers an excellent opportunity for making inroads into reductive dehalogenation, building on the detailed knowledge of the oxygenase reaction cycle (Gunsalus et al. 1974) and the x-ray crystal structure (Poulos et al. 1985).



Scheme I

In this study, organic cosubstrate and oxygen effects on reductive dehalogenation by cytochrome P-450$_{CAM}$ were investigated *in vivo* and *in vitro*. The model substrates were polychlorinated ethanes, an important class of industrial solvents and ubiquitous groundwater pollutants (Westerick et al. 1984). Kinetic and substrate binding parameters were investigated for each substrate individually. These were, in turn, used to predict substrate preferences for mixed substrate reactions. Predicted relative rates were compared to experimentally determined mixed substrate reactions. Inhibition of reductive dechlorination by the potential cosubstrate oxygen was also investigated. These parameters are of fundamental importance for developing more effective bacterial systems to degrade highly chlorinated compounds in mixed waste situations and under varying oxygen tensions.

Materials and methods

Growth and harvesting of P. putida G786

Defined minimal salts buffer (MSB media), at pH 7.0 (Stanier et al. 1966) and containing 0.2% (w/v) L-arginine hydrochloride was used for cell growth and resuspension during *in vivo* experiments. Cultures were incubated at 30 °C with shaking at 200 rpm, and the liquid volume did not exceed 25% of the total flask volume. To induce the production of cytochrome P-450$_{CAM}$, D-(+)- camphor was added in N,N-dimethylformamide to a final concentration of 1 mM. The cultures were grown until the absorbance at 600 nm attained 1.5 using a Beckman DU 70 spectrophotometer. The cells were harvested by centrifugation, washed once in fresh MSB media, and resuspended in MSB media containing 0.2% (w/v) L-arginine hydrochloride to an absorbance at 600 nm of 10–12. The cells were then used immediately for *in vivo* assays. For enzyme purification experiments, *P. putida* G786 was grown in a fermenter with a 13 l working volume using previously described methods (Gunsalus & Wagner 1978).

In vivo *assays*

Whole cell assays were carried out at 22 °C in 10 ml anaerobic septum vials containing one ml of cell suspension. The vials were capped with Teflon-lined rubber septa and made anaerobic with argon. Substrates were prepared as stock solutions in methanol, and 100 nmol was added to the reaction vials by a gas tight syringe (Hamilton, Reno, NV). Less than 80 µmol of methanol was added to prevent depressing the activity of the cells. In multi-substrate assays, the substrates were added simultaneously to the reaction vials. Substrate disappearance and product formation were monitored as described in 'Analytical methods'.

For assays containing oxygen, the reaction vials were prepared similarly. Prior to the addition of substrate to the vials, an appropriate volume of argon was withdrawn from the vials using a gas-tight syringe and replaced with oxygen to give a final headspace concentration of 0–30% (v/v). The vials were shaken vigorously, the respective substrate was added, and the vials incubated as above. After 5 minutes, the vials were sacrificed by addition of 1 ml diethyl ether, vortexed for 10 seconds, and centrifuged at 5000× g for 10 min at 4 °C in a Beckman J2–21 centrifuge. The ether extract was then analyzed by gas chromatography.

Enzyme purification

Cytochrome P-450$_{CAM}$ was purified using protocols developed by Gunsalus and coworkers (Gunsalus & Wagner 1978). The 417:280 nm ratio of the final cytochrome P-450$_{CAM}$ preparation was 1:1. The protein was stored frozen in buffer containing 200 µM camphor. Before use, the enzyme was incubated with 50 mM dithiothreitol and passed over a PD-10 gel filtration column (Pharmacia, Piscataway, New Jersey) to remove camphor and dithiothreitol. This yielded native enzyme free of substrate as judged by the optical spectra of the ferric, the camphor-bound ferric, and the ferrous CO-bound forms of the enzyme.

Measurement of dissociation constants

Substrate binding in the active site of cytochrome P-450$_{CAM}$ induces a change in the heme iron spin state from low to high spin; this can be observed as a shift in the Soret peak from 417 to 390 nm (Gunsalus et al. 1974). This large change in the optical spectrum was used to determine the dissociation constants and the maximal spin state conversion caused by polychlorinated ethane binding to cytochrome P-450$_{CAM}$.

A Beckman DU 7400 diode array spectrophotometer was used. In a typical experiment, from 0.4 to 2 µM P-450$_{CAM}$ was present in either a 1.4 or 3.5 ml cuvette (1 cm pathlength) containing 50 mM potassium phosphate buffer at pH 7.5 and 200 mM KCl. The chlorinated compound tested was added from a stock solution in methanol (the methanol alone had no effect on the optical spectrum of P-450$_{CAM}$ at the levels used). The cuvette was sealed either before the addition of chlorinated compound by a rub-

ber serum stopper, and the compound injected through the stopper, or the compound was gently injected at the bottom of the cuvette, and a teflon stopper immediately placed on the cuvette. The headspace was typically 10 to 50 µl, and no correction was made for the amount of headspace. The sample was scanned, and then an excess of camphor was added, and the sample scanned again. The raw spectral data were analyzed by the treatment shown in Scheme II.

$$\Delta A = \frac{(A_s^{390} - A_s^{417}) - (A_o^{390} - A_o^{417})}{(A_c^{390} - A_c^{417}) - (A_o^{390} - A_o^{417})}$$

Scheme II

In Scheme II, ΔA is the net change in absorbance for a particular trial with a polychlorinated ethane; A_s^{390} and A_s^{417} are the absorbances at 390 and 417 nm of the P-450$_{CAM}$ when incubated with a particular concentration of polychlorinated ethane; A_o^{390} and A_o^{417} are the absorbances at 390 and 417 nm of the P-450$_{CAM}$ in the ferric state with no test compound or camphor present and A_c^{390} and A_c^{417} are the absorbances at 390 and 417 nm of the P-450$_{CAM}$ when camphor is present.

For all the polychlorinated ethanes tested, except for hexachloroethane, the value of the dissociation constant was sufficiently large relative to the P-450$_{CAM}$ concentration that it was appropriate to analyze the data using a simple hyperbolic binding equation (Scheme III).

$$\Delta A = \frac{\Delta A_{max}\,[pca]}{[pca] + K_d}$$

Which may be linearized:

$$\frac{1}{\Delta A} = \frac{1}{\Delta A_{max}} + \frac{K_d}{[pca]\,\Delta A_{max}}$$

Scheme III

In Scheme III, [pca] is the concentration of the polychlorinated ethane being determined, K_d is the dissociation constant, ΔA is defined as above, and ΔA_{max} is the maximum change in absorbance seen when the concentration of pca is extrapolated to infinity.

The determination of the dissociation constant for hexachloroethane required the use of a Scatchard plot, since the magnitude of the dissociation constant was comparable to the amount of enzyme required for reliable optical data. The data were analyzed according to Scheme IV.

$$\frac{[Hex_b]}{[Hex_f]\,[P\text{-}450_{cam}]} = \frac{-1}{K_d}\,\frac{[Hex_b]}{[P\text{-}450_{cam}]} + \frac{1}{K_d}$$

Where $[Hex_b]$ is the concentration of hexachloroethane bound to P-450$_{CAM}$ and is calculated:

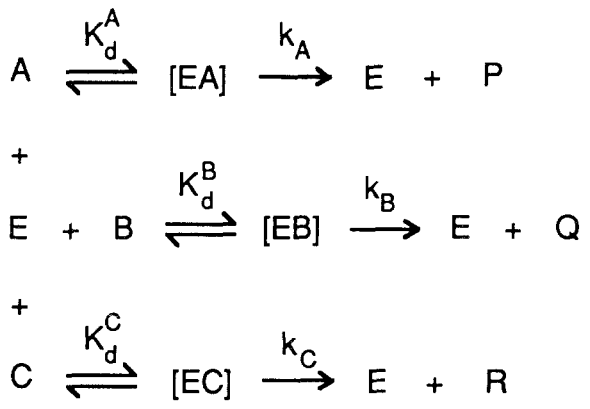
$$[Hex_b] = \frac{A_s^{390} - A_s^{417}}{\Delta A_{max}} \times [P\text{-}450_{cam}]$$

Scheme IV

ΔA_{max} is the maximum change in absorbance seen when the concentration of hexachloroethane is extrapolated to infinity, which was obtained by using a double reciprocal plot of the points obtained at hexachloroethane concentrations at least 10 fold greater than the P-450$_{CAM}$ concentration. $[Hex_f]$ is the free concentration of hexachloroethane and is the difference between the total and bound concentrations of hexachloroethane. Other symbols have their previously defined meanings.

Simulation of reaction time courses

Theoretical simulations of the mixed substrate reaction containing hexachloroethane, pentachloroethane, and 1,1,1,2-tetrachloroethane were performed assuming that the reaction could be described by a rapid equilibrium model typical of enzyme catalyzed reactions. Scheme V depicts the equilibria resulting when an enzyme is incubated with three mutually competitive substrates.



Scheme V

In Scheme V, E is free enzyme, cytochrome P-450$_{cam}$; A, B, and C are hexachloroethane, pentachloroethane, and 1,1,1,2-tetrachloroethane, respectively; K_d^A, K_d^B, and K_d^C, are the dissociation constants; EA, EB, and EC are the P-450$_{cam}$ -hexachloroethane, P-450$_{cam}$-pentachlorothane, and P-450$_{cam}$-1,1,1,2-tetrachloroethane complexes, respectively; k_A, k_B, and k_C are the rate constants for the reaction of the various enzyme-substrate complexes to form their respective products; and P is tetrachloroethylene, Q is trichloroethylene, and R is 1,1-dichloroethylene.

Using the rapid equilibrium assumption (*eg.* see Segel, 1975), the differential rate equations were derived :

$$\frac{v_A}{V_{max_A}} = \frac{\frac{[A]}{K_d^A}}{1 + \frac{[A]}{K_d^A} + \frac{[B]}{K_d^B} + \frac{[C]}{K_d^C}}$$

$$\frac{v_B}{V_{max_B}} = \frac{\frac{[B]}{K_d^B}}{1 + \frac{[A]}{K_d^A} + \frac{[B]}{K_d^B} + \frac{[C]}{K_d^C}}$$

$$\frac{v_C}{V_{max_C}} = \frac{\frac{[C]}{K_d^C}}{1 + \frac{[A]}{K_d^A} + \frac{[B]}{K_d^B} + \frac{[C]}{K_d^C}}$$

Scheme VI

In Scheme VI, v_A, v_B, v_C, are the observed rates of the reaction for substrates A, B, and C at some time t; $V_{max_A} = k_A [E]$, $V_{max_B} = k_B [E]$, $V_{max_C} = k_C [E]$. The theoretical time traces of reduction were calculated using the values for the dissociation constants in Table 2, values of V_{max} derived from Table 1, and by numerically integrating the equations simultaneously, using the computer program Mathematica (Wolfram Research, Champaign, Illinois) on a Macintosh SE/30 computer.

Analytical methods

Single substrate reactions were analyzed by headspace gas chromatography using a Hach-Carle gas chromatograph equipped with a flame ionization detector. An AT-1000 packed column (Alltech Associates, Deerfield, IL) was used as previously described (Schanke & Wackett, 1992). Reaction mixtures containing oxygen or multiple substrates were analyzed using a Hewlett Packard gas chromatograph equipped with an electron capture detector. An RSL-160 capillary column (Alltech Associates, Deerfield, IL) was used to detect products. Carrier gas flow equaled 8 ml/min. The temperature program used was 35 °C for 8 minutes, followed by a linear gradient to 100 °C at 15 °C/min. Compounds and retention times are as indicated: 1,1-dichloroethylene (1.2 min), trichloroethylene (4.7 min), 2,2,2-trichloroacetaldehyde (5.2 min), tetrachloroethylene (10.3 min). Stoichiometries were determined by comparing peak areas from reaction mixture extracts with peak areas determined from standard curves using authentic compounds. Gas chromatography/ mass spectrometry was performed using a Kratos MS-25 instrument with an ionization energy of 70 eV; a DB-5 capillary column (1 mm thick, 30 m long) at 60 °C was used.

Materials

D-(+) camphor and chloral hydrate were purchased from Sigma Chemical Co. (St. Louis, MO); L-arginine hydrochloride, pentachloroethane, hexachloroethane, 1,1,1,2-tetrachloroethane, trichloroethy-

lene, tetrachloroethylene, and 1,1-dichloroethylene, were purchased from Aldrich Chemical Co. (Milwaukee, WI). Diethyl ether was obtained from Mallinckrodt (Paris, KY). Bathke Co. (Minneapolis, MN) supplied 100% argon and 100% oxygen. HPLC grade methanol was purchased from EM Science (Gibbstown, NJ). N,N-dimethylformamide was obtained from Fisher Scientific (Fair Lawn, NJ). Other reagents used were of the highest purity commercially available.

Results and discussion

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

In initial experiments, *P. putida* G786 was grown on minimal media plus arginine and camphor, and then resuspended in fresh minimal media containing 0.2% (w/v) arginine and 100 μM pentachloroethane under an argon atmosphere. Pentachloroethane was observed to decrease with the concomitant stoichiometric formation of trichloroethylene. The identity of the product, indicated by GC retention time, was confirmed by mass spectrometry. Cells killed by boiling for 5 minutes showed no significant product formation. The addition of 0.5 mM camphor completely inhibited the transformation of pentachloroethane to trichloroethylene. In parallel experiments, *P. putida* G786 was grown on arginine in the absence of camphor and then assayed for reductive dehalogenation activity. The observed activity was 15% that of camphor grown cells. Carbon monoxide difference spectra revealed that cytochrome P-450 was present in cell extracts prepared from arginine-grown cells, but at a lower level than that found in camphor grown *P. putida* G786. These observations are consistent with previous reports which showed that cytochrome P-450$_{CAM}$ monooxygenase components are found at low, but significant levels in uninduced cells (Koga et al. 1986).

To confirm the *in vivo* data, cytochrome P-450$_{CAM}$ was purified. Cytochrome P-450$_{CAM}$ catalyzed the reduction of pentachloroethane to trichloroethylene when excess titanium (III) citrate was used to provide electrons to reduce the heme iron center. Previous studies have shown that titanium (III) citrate reduces Fe (III)-porphyrins to Fe (II)-porphyrins (Schanke & Wackett 1992). These *in vivo* and *in vitro* experiments indicated that reductive dehalogenation of polychlorinated ethanes by *P. putida* G786 is largely, if not wholly, due to the presence of cytochrome P-450$_{CAM}$. This conclusion is consistent with previous studies on reductive dehalogenation of chlorinated aliphatic compounds catalyzed by *P. putida* G786 and purified cytochrome P-450$_{CAM}$ (Castro et al. 1985; Castro & Belser 1990).

Products, yields, and rates of anaerobic polychlorinated ethane transformation in vivo

Camphor-induced *P. putida* G786 was incubated anaerobically in separate parallel experiments with

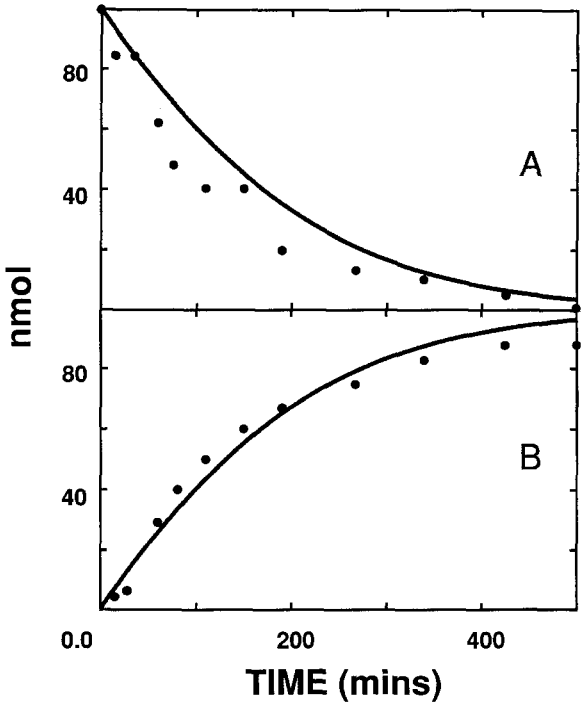


Fig. 1. Anaerobic incubation of P. putida G786 showing (A) disappearance of 1,1,1,2 tetrachloroethane and (B) appearance of 1,1 dichloroethylene. Experimental points (•) are shown for the times sampled. The curves are theoretical traces derived using the values of V_{max} and K_d in Tables 1 and 2, a simple Michaelis Menten mechanism where $K_m = K_d$, and by numerical integration.

hexachloroethane, pentachloroethane, 1,1,1,2-tetrachloroethane, 1,1,2,2-tetrachloroethane, and 1,1,1-trichloroethane. Substrate depletion and product appearance were monitored by GC, as described in the Methods section. A representative set of experimental points are shown in Fig. 1 with 1,1,1,2-tetrachloroethane added at an initial amount of 100 nmol per vial. A non-linear decay in the substrate concentration was matched by a reciprocal appearance of the product, 1,1-dichloroethylene. The identity of the product was confirmed in separate experiments using GC/MS. The product yield was not quite stoichiometric, accounting for 85% of the substrate consumed.

In a similar fashion, data were obtained for all of the substrates indicated above (Table 1). The only detectable products of these reductive elimination reactions were alkenes bearing two fewer chlorine substituents than the starting material (Scheme I). These were also the major products previously observed in non-biological model reactions with hematin and vitamin B_{12} as the catalysts (Schanke & Wackett, 1992). The product yields with *P. putida* G786 ranged from 85–92%. With 1,1,2,2-tetrachloroethane and 1,1,1-trichloroethane, no substrate depletion or product formation was observed in incubations conducted for up to 10 h. The rates of the reactions were determined for the active substrates and normalized on the basis of total cell protein (Table 1). The *in vivo* specific activities ranged from 138 pmol min^{-1} per mg protein for 1,1,1,2-tetrachloroethane to 1322 pmol min^{-1} per mg protein for hexachloroethane as the substrate. These rates are slow in comparison to the estimated *in vivo* specific activity of 600 nmol min^{-1} per mg protein for the hydroxylation of the physiological substrate camphor (assuming cytochrome P-450_{CAM} to comprise 2.5% of the total cell protein). However, the rates shown in Table 1 are significant in comparison to reductive dehalogenation reactions reported for anaerobic bacterial cultures (Mikesell & Boyd 1990; Egli et al. 1987; Egli et al. 1988).

In vitro *cytochrome P-450_{CAM} K_D and spin-state parameters*

A key early step in the cytochrome P-450_{CAM} hydroxylation reaction cycle is organic substrate binding with consequent conversion of the heme iron from low to high spin (Sligar & Gunsalus 1976; Fisher & Sligar 1985). This transition raises the midpoint potential of the iron center, facilitating electron transfer from putidaredoxin to the heme. The reaction cycle for reductive dehalogenation likely follows these same initial steps. To begin to investigate this, substrate binding (K_d) and spin-state conversion parameters were determined with chlorinated ethanes and ethylenes.

Substrate binding studies were carried out with purified cytochrome P-450_{CAM}. Dissociation constants (K_d) were determined spectrophotometrically. Precautions were taken to maintain known concentrations of volatile chlorinated compounds in cuvettes, and these are described in the Methods section. A representative set of spectral changes observed with 1,1,1,2-tetrachloroethane is shown in Fig. 2. The addition of substrate to the oxidized enzyme caused a sequential decrease in the percentage of the heme low spin form, as evidenced by the decrease in the band at 417 nm. An increase in absorbance at 390 nm indicated the formation of a

Table 1. Identification and initial rate of formation of products formed during anaerobic incubation of polychlorinated ethanes with *Pseudomonas putida* G786

Substrate	Product	Yield (%)	Rate (pmol/min/mg protein)
Hexachloroethane	Tetrachloroethylene	92	1322
Pentachloroethane	Trichloroethylene	90	890
1,1,1,2 Tetrachloroethane	1,1 Dichloroethylene	85	138
1,1,2,2 Tetrachloroethane	None detected	–	–
1,1,1 Trichloroethane	None detected	–	–

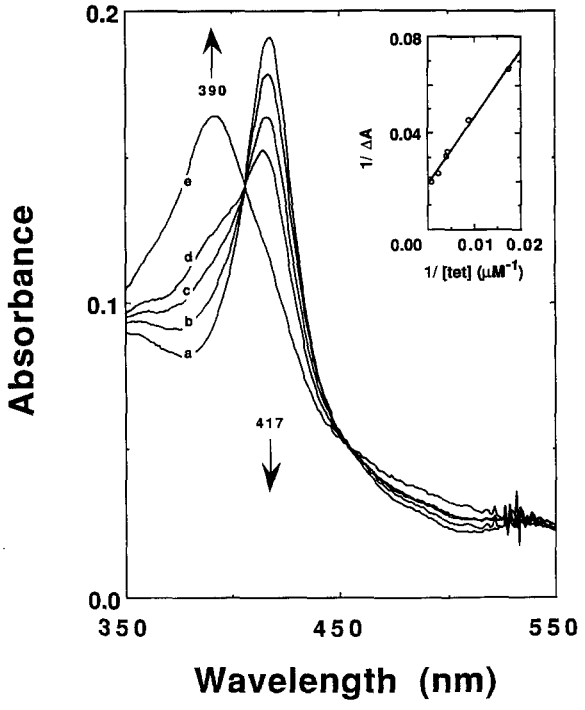


Fig. 2. Determination of the dissociation constant (K_d) for the binding of 1,1,1,2-tetrachloroethane to cytochrome P-450$_{CAM}$. Shown are representative scans observed for P-450 incubated with various concentrations of 1,1,1,2-tetrachloroethane: (a) none, (b) 57 μM, (c) 250 μM, (d) 1.2 mM, (e) a control, with 100 μM camphor present. Other conditions were as described in Materials and Methods. Inset: Plot of the data used to determine the value of the dissociation (K_d) constant for 1,1,1,2-tetrachloroethane.

high spin iron center concomitant with substrate binding. At saturation, the limit of high spin iron reached 50% (Table 2). This is similar to that observed for the hydroxylatable substrates *d*-fenchone or thiocamphor (Fisher & Sligar 1985; Raag & Poulos 1991). In a control experiment, tetramethylcyclohexanone at saturation yielded 27% high spin protein, similar to the value of 22% previously reported by Fisher & Sligar (1985). Hexachloroethane, which undergoes dehalogenation more rapidly under the conditions examined, showed a saturating spin state conversion of ≥ 95% (Table 2). The physiological substrate, for comparison, also afforded a ≥ 95% spin state conversion. An increase in % spin state conversion has been linked to increased water expulsion from the active site, facilitated electron transfer into the heme, and a lowering of the activation energy for ferric to ferrous iron reduction (Fisher & Sligar 1985; Raag & Poulos 1989). These parameters may be involved in the observed reductive dehalogenation rate differential. The bulkier, more highly chlorinated compounds could cause greater water expulsion from the active site, facilitating greater spin state conversion and ultimately faster substrate reduction. It should also be noted that the more highly chlorinated compounds have more positive redox potentials, making them thermodynamically more susceptible to reduction (Vogel et al 1987). This latter physical property may also account for the higher dechlorination rates observed with increasing chlorine substitution of the substrate (Luke & Loew 1986).

The spectral titration also allowed a determination of the dissociation constant (K_d) for substrate binding in the cytochrome P-450$_{CAM}$ active site. The inset to Fig. 2 illustrates the double reciprocal plot for 1,1,1,2-tetrachloroethane showing a linear correlation coefficient of 0.99 and a derived K_d of 150 μM. Other K_d values were derived in a similar fashion. Again, hexamethylcyclohexanone was used as a positive control. It was determined to have a K_d of 32 μM, which agrees well with the published value of 34 μM (Fisher & Sligar 1985). In general, the greater the chlorine substitution of the C_2 compound, the lower the K_d. Indeed, hexachloroethane was bound tightly enough to necessitate the use of a Scatchard plot for analyzing the data. The K_d was 0.7 μM, indicating hexachloroethane is bound with similar affinity as the physiological substrate camphor at 0.84 μM (Fisher & Sligar 1985).

Table 2. Dissociation constants and maximal percent spin state conversion of various polychlorinated ethanes for cytochrome P-450$_{CAM}$*.

Compound	K_d (μM)	% High spin
hexachloroethane	0.7 ± 0.1	≥ 95
pentachloroethane	7 ± 1	70
1,1,1,2-tetrachloroethane	150 ± 10	50
1,1,2,2-tetrachloroethane	100 ± 20	30
1,1,1-trichloroethane	400 ± 50	35
tetrachloroethylene	130 ± 10	40

* Dissociation constants and maximal spin state conversion were determined as described in Materials and Methods.

Prediction of biodegradation rates for single and mixed substrate reactions

In addition to mechanistic insights, the K_d values are potentially useful for predicting *in vivo* biodegradation rates for single and mixed substrate situations. In the experiments below, theoretical and experimental reactions were investigated at initial substrate concentrations of 100 nmol per vial. This concentration gave significant rates for all substrates and was significantly below concentrations showing toxic effects on *P. putida* G786. Interpretation and simulation of experimental results were performed using a rapid equilibrium Michaelis-Menten model. The values for V_{max} for each substrate were determined by using the initial observed rates, the values for the dissociation constants in Table 2, and by using the Michaelis Menten equation. The V_{max} values were calculated to be, in pmol min^{-1} per mg protein: hexachloroethane (1330), pentachloroethane (952), and 1,1,1,2-tetrachloroethane (331). These V_{max} parameters and K_d values were used to numerically integrate the Henri Michaelis Menten equation to calculate a theoretical reaction time trace; the simulated reaction curves for 1,1,1,2-tetrachloroethane disappearance and 1,1-dichloroethylene formation are shown in Fig. 1. The simulated curves reasonably agreed with the actual disappearance and formation of substrate and product, respectively. Similar analyses were performed with pentachloroethane and hexachloroethane (data not shown). The initial rate of the reactions were correctly predicted by the simulated time traces; however, the experimentally-determined reaction rates declined more rapidly than did the simulated time traces. Preliminary simulations (not shown) suggest that the partitioning of the substrates into the gaseous phase is of significance; a more complete simulation would probably require a determination of the Henry's law constants for the substrates under the experimental conditions used here.

Biodegradation of a mixture of substrates *in vivo* is a situation more closely resembling real field conditions than single substrate studies. Therefore, we considered a model to predict the time traces of reduction of hexachloroethane, pentachloroethane, and 1,1,1,2-tetrachloroethane when all three substrates were present simultaneously. The reaction was modeled assuming that all three substrates are competitive inhibitors of each other (see scheme V). A simultaneous numerical integration of the equations in scheme VI was performed and is shown in Fig. 3A. The predicted utilization of the substrates was sequential, in the order: hexachloroethane, pentachloroethane, and 1,1,1,2-tetrachloroethane.

The potential usefulness of the simulation was then tested by performing the equivalent biological experiment. *P. putida* G786 was incubated under an argon atmosphere with 100 nmol each of hexachloroethane, pentachloroethane, and 1,1,1,2-tetrachloroethane. Products were determined for each substrate at identical timepoints, as shown in Fig. 3B. The same general order of substrate preference was observed in the whole cell incubations as in the model, suggesting that the mutual competition between the three substrates, based upon their affinities for $P\text{-}450_{CAM}$, is an important factor in the reaction. However, the predicted rates of substrate utilization were faster than those actually observed, and the simulation does not predict the observed simultaneous utilization of hexachloroethane and pentachloroethane in the initial phase of the incubation. As was the case for the predicted and experimental time traces for the reduction of single substrates, preliminary work indicates that the partitioning of the substrates into the gaseous phase must be included into a model which would be a more complete and accurate description of the experimental data.

Oxygen cosubstrate effects on reductive dechlorination

The physiological hydroxylation of camphor by cytochrome $P\text{-}450_{CAM}$ requires the binding of both camphor and molecular oxygen. As elucidated by Gunsalus and coworkers, the organic substrate is bound first, followed by electron input to the ferric heme iron. Molecular oxygen is then coordinated by the ferrous iron center. Transfer of a second electron into cytochrome P-450 leads to oxygen – ox-

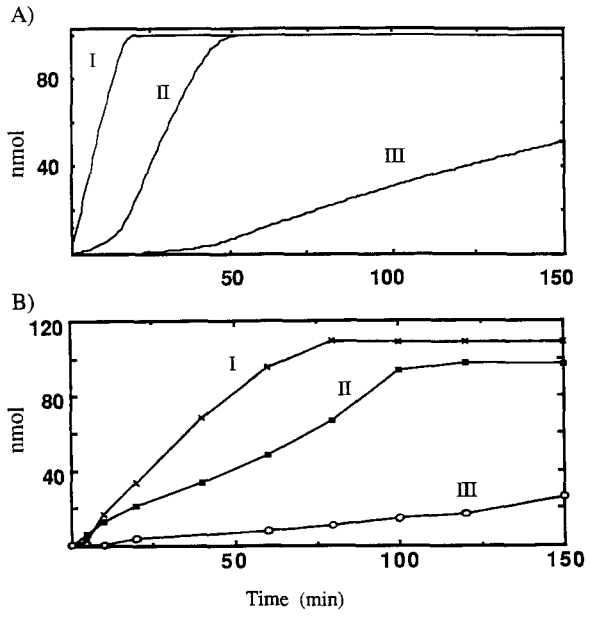


Fig. 3. Formation of tetrachloroethylene(I), trichloroethylene (II), and 1,1-dichloroethylene(III) from hexachloroethane, pentachloroethane, and 1,1,1,2-tetrachloroethane, respectively, when all three substrates are present simultaneously. (A) Theoretical simulation based upon the experimentally derived values in Tables 1 and 2 and Scheme VI. (B) Experimentally observed formation of product from cells incubated as described in Materials and Methods.

ygen bond scission and subsequent substrate hydroxylation. In reductive dechlorination reactions under an argon atmosphere, the two one electron inputs can effect an overall two electron reduction of the carbon-chlorine bond. Oxygen would be anticipated to intervene in the reductive cycle and thus inhibit the formation of products derived from the reductive pathway.

In *in vivo* experiments, the disappearance of the three chlorinated substrates was examined separately in vials containing an air atmosphere (21% oxygen). The initial depletion of hexachloroethane was very slow. In contrast, pentachloroethane and 1,1,1,2-tetrachloroethane were consumed more quickly in the initial phase of the incubations. Product elucidation studies proved to be informative. With pentachloroethane, trichloroethylene formation accounted for only ~10% of the substrate consumed, suggesting that significant oxygenation of the C-H bond was occurring. This possibility was investigated in greater detail using 1,1,1,2-tetrachloroethane. Aerobic reaction mixtures were extracted with diethyl ether and analyzed by gas chromatography. Two chlorinated compounds were detected. The first was dichloroethylene and the second comigrated with authentic 2,2,2-trichloroacetaldehyde, or chloral. These data indicated that polychlorinated ethanes containing C-H bonds react with cytochrome P-450$_{CAM}$ in air via a mixture of oxidative and reductive pathways (Fig. 4). Note that similar observations have been reported with 1,1,2-trichloroethane as the substrate (Castro & Belser 1990).

Further experiments were conducted to quantitatively determine the inhibitory effects of oxygen, at different concentrations, on the reductive limb of these reactions for each substrate individually. This analysis necessitated examining initial reaction rates at short intervals to preclude significant ox-

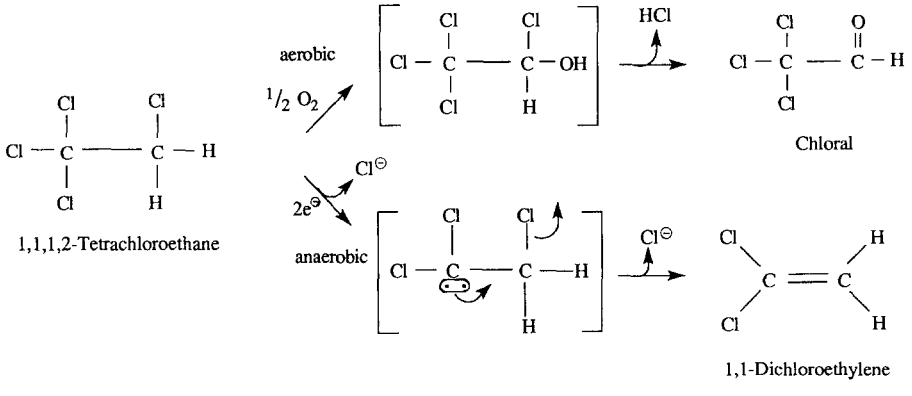


Fig. 4. Partitioning of the reaction of 1,1,1,2-tetrachloroethane with cytochrome P-450$_{CAM}$ under aerobic conditions.

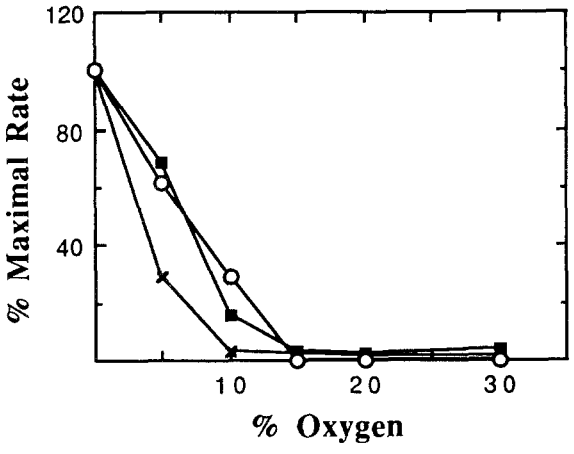


Fig. 5. Oxygen inhibition of product formation from polychlorinated ethanes by *P. putida* G786. The 100% rate is defined as the rate under anaerobic conditions. The percent oxygen refers to its fractional volume in the reaction headspace. The product curves are for the following starting materials: hexachloroethane(X); pentachloroethane(□)1,1,1,2-tetrachloroethane (○).

ygen depletion due to cellular metabolism during the course of the experiment. The reductive products were quantitatively determined as before and plotted as a function of oxygen concentration. As seen in Fig. 5,the reduction of hexachloroethane was most sensitive to oxygen inhibition. In all cases, however, significant reductive dechlorination still occurred with 5% (v/v) oxygen in the culture headspace. The degree of inhibition was less than previously observed for mammalian microsomes containing cytochrome P-450 (Ahr et al. 1982). Rigorous comparisons are rendered difficult due to potential differences in oxygen transfer from gas to liquid phases in these different experiments. However, the conditions of lowered oxygen tension used here are not unlike those found in waters and soils. The use of an aerotolerant organism for catalyzing reductive dehalogenation may circumvent the necessity for engineering complex anaerobic/aerobic two-staged reactor systems for degrading polychlorinated hydrocarbons (Fathepure & Vogel 1991). This points out the potential for practical usage of *P. putida* G786 or similar organisms for catalyzing reductive dehalogenation under conditions suitable for *in situ* or bioreactor-mediated biodegradation.

Conclusions

These studies begin to define the important parameters for predicting cosubstrate effects on reductive dehalogenation by *P. putida* G786. Cytochrome $P\text{-}450_{CAM}$ is very well understood structurally and functionally, allowing detailed molecular studies on how cosubstrate effects become manifest. Knowledge gained from a study of cytochrome $P\text{-}450_{CAM}$ may help unravel other more ill-defined biodehalogenation systems. The findings presented here serve as a prelude to further studies on developing high-velocity reductive biodehalogenation systems which would, most usefully, tolerate significant oxygen tensions.

Acknowledgements

We thank Dr. Stephen Sligar, University of Illinois, for providing us with *P. putida* G786. We thank Tom Krick for performing GC/MS. The assistance of Ms. Mary Jo Keefe in the preparation of the manuscript is gratefully acknowledged. The research was supported by National Institutes of Health Grant GM41235 (to LPW).

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