Vol. 201, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1994 Pages 373-378

Oxidation of Low Molecular Weight Chloroalkanes by Cytochrome $P450_{CAM}$

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Received April 4, 1994

SUMMARY: Cytochrome $P450_{CAM}$ from *Pseudomonas putida* G786 oxidized chlorinated ethanes and 1,2-dichloropropane. The rate of NADH oxidation decreased with decreasing chlorine substitution. The single detectable oxidation products of 1,1,1-trichloroethane and 1,2-dichloropropane were 1,1,1-trichloroethanol and chloroacetone, respectively. Organic product formation was largely uncoupled from NADH oxidation.

Chloroalkanes have been used extensively as solvents and synthetic intermediates (1). Some of these compounds are acutely or chronically toxic or are suspected carcinogens and teratogens (2). Bacterial enzymes capable of metabolizing these halogenated compounds are being identified with increasing frequency. Dehalogenases may confer on a bacterium the ability to grow on halogenated alkanes. For example, haloalkane dehalogenase and 2-chloroacetate halidohydrolase function in certain facultative methylotrophic bacteria during growth on 1,2-dichloroethane (3). In contrast, methanotrophic bacteria expressing soluble methane monooxygenase oxidize numerous chloroalkanes and alkenes (4,5) but the bacteria do not grow on those compounds. Similarly, *Pseudomonas putida* G786 expressing cytochrome $P450_{CAM}$ monooxygenase (EC 1.14.15.1) metabolizes chloroalkanes (6,7) but the metabolism does not provide carbon and energy for the organism.

Cytochrome P450 monooxygenases are found in bacteria, plants and mammals (8). The membrane-bound mammalian enzymes biotransform structurally diverse xenobiotic compounds, including chloroalkanes (9,10). The soluble bacterial enzyme from *P. putida* G786 oxidizes camphor, a carbon source for the bacteria. Mammalian cytochrome P450s (11) and cytochrome $P450_{CAM}$ (12) each catalyze reductive and oxidative dechlorination and the respective reactions are modulated by oxygen tension (13). The reductive reactions with chloroalkanes have been most extensively studied.

Cytochrome $P450_{CAM}$ has served as the paradigm for understanding structure and mechanism for the broad class of cytochrome P450 monooxygenases. A high resolution X-ray structure is available (14) and intermediates in the monooxygenation reaction pathway have been

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0006-291X/94 $5.00

delineated (15). Protein engineering of cytochrome $P450_{CAM}$ has been reported (16,17). It is proposed that cytochrome $P450_{CAM}$ may be redesigned by directed mutagenesis for enhanced activity with chlorinated substrates of environmental concern (18). To do this, it is essential to further characterize the reactions of the wild-type enzyme. Reductive dechlorination reactions have been studied (19,20). Oxidative reactions have only been reported for the halogenated substrates 1,1,2-trichloroethane and 1,2-dibromo-3-chloropropane (7,12). In this study, seven chlorinated substrates were examined. Unlike previous studies, measurements were made here of substrate-dependent NADH oxidation rates, substrate binding-induced heme-iron spin state changes, and degree of coupling of NADH oxidation to organic product and H_2O_2 formation. Rates of NADH oxidation decreased with decreasing chlorine substitution and organic product formation was largely uncoupled from NADH oxidation.

MATERIALS AND METHODS

Reagents: All chemicals used were reagent grade or better. Pentachloroethane and 2,3-dichloro-1-propanol were purchased from TCI America Co., Portland, OR and Kodak Co., Rochester, NY, respectively. All other chlorinated substrates and standards were obtained from Aldrich Co., Milwaukee, WI. Biochemical reagents were purchased from Sigma Chemical Co., St. Louis, MO.

Enzyme purification and assays: Cytochrome $P450_{CAM}$, putidaredoxin, and putidaredoxin reductase were obtained from *Pseudomonas putida* PpG786 grown on camphor as previously described (21). The 390 nm:280 nm absorbance ratio of purified $P450_{CAM}$ solutions was >1.3, the 280 nm:454 nm absorbance ratio of putidaredoxin reductase was <12, and the 325 nm:280 nm absorbance ratio of putidaredoxin was >0.5. Camphor-free cytochrome $P450_{CAM}$ was obtained as previously described by (21). Enzyme and reagent concentrations in assays were 0.5 μM $P450_{CAM}$, 10 μM putidaredoxin, 0.5 μM putidaredoxin reductase, 50 mM potassium phosphate, pH 7.4, and 200 mM KCl.

Calculation of percent spin state change: Chlorinated substrates were incubated with cytochrome $P450_{CAM}$ at 22°C in a spectrophotometric cell as previously described (20). Substrate-induced changes in the spin state of the heme iron atom were measured as described (22). The data were expressed as a percent change from low to high spin iron.

NADH oxidation rates and hydrogen peroxide determination: NADH oxidation was measured at 22°C as the decrease in absorbance at 340 nm using a Beckman DU-7400 spectrophotometer and an extinction coefficient of 6.22 mM^{-1} cm^{-1}. Chlorinated substrates were added from stock solutions in methanol. To insure enzyme saturation with substrate, incubations were performed with 100 μM, 1 mM, and 10 mM substrate. Solutions with 1 mM concentrations of substrate were saturating in all cases and data are presented for this concentration. Control incubations with saturating camphor yielded a turnover number of 13.6 s^{-1}. Hydrogen peroxide was quantitated using iron thiocyanate (23). Reactions were initiated by adding NADH to 200 μM. The reactions were sampled at 2-3 minute intervals until NADH was depleted. Hydrogen peroxide formation was uniformly proportional to the amount of NADH oxidized over the entire time course. Hydrogen peroxide formation was negligible in the absence of chlorinated substrates.

Identification of organic reaction products and stoichiometries: Enzyme solutions and reagents were mixed as described above in 10 ml vials with teflon-lined septa. Incubations were conducted overnight with gentle shaking at 22°C. Both heat killed controls and vials repeatedly flushed with CO failed to yield detectable products. Chlorinated products were analyzed by extracting the reaction mixtures with an equal volume of ethyl acetate and injecting the organic phase on a Hewlett Packard 5890 gas chromatograph equipped with an electron capture detector and a HP 3396A integrator. A DB-wax column (J & W Scientific, Holsom, CA) was eluted with a thermal gradient of 60-200°C over 30 minutes. Product stoichiometries were determined by including incubations with varying concentrations of NADH. Products were also analyzed by gas spectrometry/mass spectrometry (GC/MS) using a DB-wax column coupled to a Kratos MS25 double focus magnetic sector GC/MS.

GC analysis of synthetic chloroacetone gave a single peak with a retention time of 4.2 minutes. The single observed oxidation product of 1,2-dichloropropane had an identical retention time. GC/MS analysis of the standard and the enzyme product gave major mass fragments with m/z 94, 92, 77. GC analysis of synthetic 2,2,2-trichloroethanol produced a single peak eluting at 3.7 minutes. The single observed oxidation product of 1,1,1-trichloroethanol had an identical retention time. GC/MS analysis of both peaks gave a set of major mass fragments with m/z 133, 119, 117, 115, 113.

RESULTS

NADH oxidation: The rate of NADH oxidation by reconstituted $P450_{CAM}$ systems was examined for a series of chlorinated ethanes and 1,2-dichloropropane. NADH was oxidized more rapidly with the more highly chlorinated C_2 substrates. The reactions with 1,2 dichloropropane and 1,1,1-trichloroethane gave the slowest initial rate of NADH oxidation (Table 1).

Hydrogen peroxide production: The reconstituted enzyme system used here produced undetectable amounts of H_2O_2 during camphor oxidation, consistent with previous studies (24). Chlorinated substrates stimulated NADH oxidation and H_2O_2 formation (Table 1). During the oxidation of 200 μM NADH, H_2O_2 was formed as a uniform percentage of the amount of NADH consumed. Depending on the substrate, hydrogen peroxide formation accounted for 33-86% of the NADH oxidized.

Organic reaction products and stoichiometries: No products were detected from aerobic incubations of cytochrome $P450_{CAM}$ with hexachloroethane. Previously, pentachloroethane and 1,1,1,2-tetrachloroethane were shown to be oxidized to trichloroacetic acid (25) and 2,2,2-trichloroacetaldehyde (22), respectively. In this study, the oxidation of 1,1,2,2-tetrachloroethane produced dichloroacetic acid as demonstrated with an HPLC organic acid column (5). In a

Table 1. Cytochrome $P450_{CAM}$-catalyzed oxidation of low molecular weight chloroalkanes. NADH oxidation rates are reported for saturating substrate concentrations. The observed rate of NADH oxidation with camphor, under the conditions used, was 13.6 s^{-1}. Other parameters were measured as described in Materials and Methods.

Substrate	NADH Oxidation Rate (s^{-1})	% of Rate with Camphor	H_2O_2 Formation (s^{-1})	% Spin State Change (Reference)	Organic Products (Reference)
Hexachloroethane	3.0 ± .5	22	1.0 ± 0.1	>95 (20)	None[1]
Pentachloroethane	2.4 ± .3	18	0.8 ± 0.1	70 (20)	Trichloroacetic Acid (25)
1,1,1,2-Tetrachloroethane	1.9 ± .2	14	1.0 ± 0.1	50 (20)	2,2,2-Trichloro-acetaldehyde (22)
1,1,2,2-Tetrachloroethane	1.5 ± .3	11	0.5 ± 0.1	30 (20)	Dichloroacetic Acid[1]
1,1,2-Trichloroethane	1.0 ± .1	7	0.7 ± 0.1	12[1]	Chloroacetic Acid (7)
1,1,1-Trichloroethane	0.7 ± .1	5	0.6 ± 0.1	35 (20)	2,2,2-Trichloroethanol[1]
1,2-Dichloropropane	0.7 ± .1	5	0.3 ± 0.1	19[1]	Chloroacetone[1]

[1]This study.

previous study using intact *P. putida* G786, 1,1,2-trichloroethane was oxidized to chloroacetic acid and glyoxylic acid (7). Glyoxylic acid may not have been a primary oxidation product.

Of the compounds studied here, 1,1,1-trichloroethane and 1,2-dichloropropane are the most prevalent environmentally. Chloroacetone was the single identified product from 1,2-dichloropropane oxidation. 1,2-Dichloropropanol and 2-chloropropionaldehyde were looked for but not detected. Chloroacetone was quantified at several different limiting NADH concentrations yielding a stoichiometry of 1 mol chloroacetone per 100 mol NADH oxidized ($r^2 = 0.997$). Trichloroethanol was the sole product of 1,1,1-trichloroethane oxiation. This reaction was even more uncoupled; a ratio of 1 mol product to 10,000 mol NADH oxidized was observed over a range of NADH concentrations ($r^2 = 0.992$). The product was derived from a cytochrome $P450_{CAM}$-dependent reaction as indicated by the absence of product in CO-inhibited enzyme controls.

DISCUSSION

This study extends the characterization of chloroalkane oxidation by cytochrome $P450_{CAM}$. In one of the previous two studies, the reaction of 1,1,2-trichloroethane was examined with intact *P. putida* G786 (7), precluding detailed studies of relevant enzyme parameters. Recently, the oxidation of 1,2-dibromo-3-chloropropane was studied using purified enzyme components (12). NADH oxidation and H_2O_2 formation was not examined in that study, but the rate of 1,2-dibromo-3-chloropropane oxidation was 0.57 s^{-1} which is significantly greater than our observed rate with the sterically less bulky substrate 1,2-dichloropropane (0.7 s^{-1} NADH oxidation x 0.01 organic product formed/NADH oxidized = 0.007 s^{-1} for organic product formation). In both studies, C_2 of the substituted propane was oxidized to yield the respective haloacetone product.

Chloroalkane-dependent NADH oxidation was less than the camphor-dependent rates (5-22%) but still significant (0.7-3.0 s^{-1}) compared to rates reported for mammalian microsomes containing cytochrome P450. By comparison, 1,1,1-trichloroethane and 1,1,2,2-tetrachloroethane stimulated rat liver microsomal CO-inhibitable NADPH oxidation by 0.02 s^{-1} (9). The liver microsomal reactions produced little H_2O_2 and NADPH oxidation was largely coupled to product formation.

The degree of chlorination significantly effects binding of C_2 alkanes to cytochrome $P450_{CAM}$. For example, hexachloroethane binds with a dissociation constant (K_D) of 0.7 μM (20) and causes a >95% spin state change of the heme iron. By comparison, C_2 alkanes with 3 chlorine substituents induce more modest (12-35%) spin state changes and bind more weakly (K_D = 150 μM). 1,1,1-Trichloroethane weakly stimulates NADH oxidation, yields a relatively high percentage of H_2O_2 per NADH consumed, and is poorly oxidized to 1,1,1-trichloroethanol. Preliminary experiments indicate that pentachloroethane oxidation is more tightly coupled to NADH oxidation (25) and this substrate binds more tightly to cytochrome $P450_{CAM}$ (K_D = 7 μM and % spin state change = 70%) than the trichloroethanes.

Recent molecular dynamics simulations compared hexachloroethane and 1,1,1-trichloroethane, each bound in the active site of cytochrome $P450_{CAM}$ over a 150 psec trajectory

(26). The most striking observation was a large difference in substrate mobilities. Hexachloroethane showed no rotations along an axis perpendicular to the C-C bond and a fairly small B-factor of 34 $Å^2$. Trichloroethane shows substantially greater mobility in the active site. It undergoes rotation and has a calculated B-factor of 89 $Å^2$. In other studies, dynamical simulations and experimental oxidation parameters have been compared for cytochrome $P450_{CAM}$ with styrenes (23), nicotine (27), ethylbenzene (16) thiocamphor and norcamphor (17). The present study, coupled to complimentary computational methods (26), contributes further knowledge about cytochrome $P450_{CAM}$ with organohalide substrates.

The major finding of this study is that low molecular weight chlorinated alkanes are poorly oxidized by cytochrome $P450_{CAM}$. As observed here, substrates not resembling camphor may show low coupling between NADH oxidation and substrate oxygenation. In another example, the reaction with styrenes is only 2% coupled (23). In this context, wild-type cytochrome $P450_{CAM}$ may not be the best choice for enzymatic oxidation of chlorinated pollutants. Altered forms of cytochrome $P450_{CAM}$, derived from site-directed or random mutagenesis protocols, should be examined for improved oxidation rates and coupling efficiencies.

ACKNOWLEDGMENTS

We acknowledge the expert assistance of Tom Krick and Rick Riemer for gas chromatography/mass spectrometry. We appreciate the help of Earl Ringquist in growth of *P. putida* G786 using the Biological Process Technology Institute pilot plant. This research was supported by EPA cooperative agreement no. EPA/CR820771-01-0.

REFERENCES

1. Leisinger, T. (1983) Experientia **39,** 1183-1191.
2. Parker, J.C., Casey, G.E., and Bahlman, L.J. (1979) Am. Ind. Hyg. Assoc, J. **40,** 46-60.
3. van den Wijngaard, A.J., van der Kamp, K.W.H.J., van der Ploeg, J., Pries, F., Kazemier, B. and Janssen, D.B. (1992) Appl. Environ. Microbiol. **58,** 976-983.
4. Oldenhuis, R., Vink, R.L.J.M., Janssen, D.B. and Witholt, B. (1989) Appl. Environ. Microbiol. **55,** 2819-2826.
5. Fox, B.G., Borneman, J.G., Wackett, L.P. and Lipscomb, J.D. (1990) Biochemistry **29,** 6419-6427.
6. Lam, T. and Vilker, V.L. (1987) Biotechnol. Bioeng. **29,** 151-159.
7. Castro, C.E. and Belser, N.O. (1990) Environmental Toxicology and Chemistry **9,** 707-714.
8. Nelson, D.R., Kamataki, T., Waxman, D.J., Guengerich, F.P., Estabrook, R.W., Feyereisen, R., Gonzalez, F.J., Coon, M.J., Gunsalus, I.C., Gotoh, O., Okuda, K. and Nebert, D.W. (1993) DNA Cell Biol. **12,** 1-51.
9. Ivanetich, K.M. and van den Honert, L.H. (1981) Carcinogenesis **2,** 697-702.
10. Salmon, A.G., Jones, R.B. and Mackrodt, W.C. (1981) Xenobiotica **11,** 723-734.
11. Ahr, H.J., King, L.J., Nastainczyk, W., and Ullrich, V. (1980) Biochemical Pharmacology **29,** 2855-2861.
12. Koe, G.S. and Vilker, V.L. (1993) Biotechnol. Prog. (1993) **9,** 608-614.
13. Anders, M.W. and Pohl, L.R. (1985) In Bioactivation of Foreign Compounds (M.W. Anders, Ed.), p. 283-315, Academic Press, New York.
14. Poulos, T.L., Finzel, B.C., Gunsalus, I.C., Wagner, G.C., and Kraut, J. (1985) J. Biol. Chem. **260,** 16122-16130.
15. Gunsalus, I.C., Wagner, G.C., and Debrunner, P.G. (1980) In Microsomes, Drug Oxidations and Chemical Carcinogenesis (M.J. Coon, A. Conney, R. Estabrook, H. Gelboin, J. Gillette, and R. O'Brien, Eds.), pp. 233-242, Academic Press, New York.

16. Loida, J.P. and Sligar, S.G. (1993) Biochemistry **32,** 11530-11538.
17. Paulsen, M.D., Filipovic, D., Sligar, S.G., and Ornstein, R.L. (1993) Protein Sci. **2,** 357-365.
18. Ornstein, R.L., Bass, M.B., Paulsen, M.D., and Arnold, G.E. (1991) in Proceedings of Hazmacon 91 (Bursztynsky, T., ed.), J.T. Litho, Concord, CA, p. 470-480.
19. Castro, C.E., Wade, R.S. and Belser, N.O. (1985) Biochemistry **24,** 204-210.
20. Li, S. and Wackett, L.P. (1993) Biochemistry **32,** 9355-9361.
21. Gunsalus, I.C., Wagner, G.C. (1978) Methods in Enzymology **52,** 166-188.
22. Logan, M.S., Newman, L.M., Schanke, C.A. and Wackett, L.P. (1993) Biodegradation **4,** 39-50.
23. Fruetel, J.A., Collins, J.R., Camper, D.L., Loew, G.H. and Ortiz de Montellano, P.R. (1992) J. Am. Chem. Soc. **114,** 6987-6993.
24. Atkins, M.W. and Sligar, S.G. (1987) J. Am. Chem. Soc. **109,** 3754-3760.
25. Li, S. (1993) Ph.D. Dissertation, University of Minnesota.
26. Paulsen, M.D. and Ornstein, R.L. (1994) J. Comp. Aided Mol. Des. (in press).
27. Jones, J.P., Trager, W.F. and Carlson, T.J. (1993) J. Am. Chem. Soc. **115,** 381-387.