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Biodehalogenation: Reactions of Cytochrome P-450 with Polyhalomethanes†

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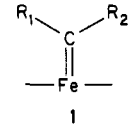
ABSTRACT: The products, stoichiometry, and kinetics of the oxidation of the enzyme cytochrome P-450 cam by five polyhalomethanes and chloronitromethane are described. The reactivity of the enzyme is compared with that of deuteroheme and with the enzyme in its native cell, *Pseudomonas putida* (PpG-786). In all cases, the reaction entails hydrogenolysis of the carbon–halogen bond: $2Fe^{II}P + RCX_n \rightarrow 2Fe^{III}P + RCHX_{n-1}$ (P = porphyrin or P-450 cam in vitro and in vivo). Trichloronitromethane was the fastest reacting substrate, and chloroform was the slowest. The results establish that *P. putida* is a valid whole cell model for the reductase activity of the P-450 complement in these reactions. The reactions of cytochrome P-450 with polyhaloalkanes proceed in a manner quite analogous to other iron(II) proteins in the G conformation. The chemistry observed for the enzyme parallels that of its iron(II) porphyrin active site. Iron-bonded carbenes are not intermediates, and hydrolytically stable iron alkyls are not products of these reactions.

The reactions of cytochrome P-450 with organic halides can be an important means of both activation and detoxification of these xenobiotics in vivo (U.S. Department of Health, Education and Welfare, 1977; Jakoby, 1980; Ullrich, 1977). It is generally held that unsaturated halides are reactivated by conversion to the corresponding epoxides (Henschler, 1977), though the epoxides are not necessary intermediates in the production of halocarbonyl compounds obtained in these processes (Miller & Guengerich, 1982, Liebler & Guengerich, 1983). The nature of the reaction of saturated aliphatic polyhalides with this class of enzyme (Uehleke et al., 1973; Bini et al., 1975; Mansuy et al., 1974; Nastainczyk et al., 1978; Wolf et al., 1977a,b; Nastainczyk et al., 1982; Ahr et al., 1982) is less clear. Of particular interest are the reactions of polyhalomethanes ($R_{4-n}CX_n$, n = 2–4) because the bonding in this class of substrate is typical of that found commonly in anesthetics, solvents, and pesticides. Moreover, visible spectral studies of such reactions (Mansuy et al., 1974; Nastainczyk et al., 1978; Wolf et al., 1977a,b) have suggested the generation of unique "carbene complexes" of the P-450 heme (**1**). Such



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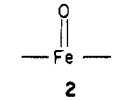

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structures are analogous to the "iron–oxene" formulation (**2**) for the activated oxygen intermediate in P-450-catalyzed oxygen insertion reactions (Ullrich, 1977; Sligar et al., 1977; Griffin et al., 1975; Guengerich & MacDonald, 1984). The unusual bonding in structures like **1** and the "halothane carbene" in particular (**1**, $R_1 = CF_3$ and $R_2 = H$) has received serious attention by theoreticians (Loew & Goldblum, 1980). While the enzyme functions primarily as an oxidase, clearly it has reductase capacity vested in its iron(II) porphyrin form. The reduction of *N*-oxides, epoxides, hydroperoxides, and polyhalomethanes has been noted (Guengerich & Macdonald, 1984). Isolated cases of the reactivity of primarily reduced liver fragments to polyhalomethanes have been reported (Uehleke et al., 1973; Bini et al., 1975; Mansuy, et al., 1974; Nastainczyk et al., 1978, 1983; Wolf et al., 1977a,b; Ahr et al., 1982), but product work is sketchy and contradictory. Thus, the conversion of carbon tetrachloride and bromotri-

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chloromethane to chloroform by reduced liver extracts has been described (Uehleke et al., 1973; Bini et al., 1975) though structures were assigned solely by gas chromatography and yields were not reported. Under similar conditions, the same reactions have been reported to produce carbon monoxide, presumably via a dichlorocarbene intermediate (Wolf et al., 1977a,b). More recently, halothane ($CF_3CHBrCl$) (Ahr et al., 1982) has been found to produce the olefin 1,1-difluoro-2-chloroethene and 2,2,2-trifluoroethyl chloride (the hydrogenolysis product) exclusively. These results are in keeping with the established paths of reactions of alkyl halides with iron(II) porphyrins and and heme proteins (Castro, 1964; Wade & Castro, 1973a,b; Castro & Bartnicki, 1975; Castro et al., 1978; Stotter et al., 1977), but they contradict the original formulation of the "halothane carbene" as an intermediate (Mansuy et al., 1974; Nastainczyk et al., 1978) in these processes.

As part of our study of biodehalogenation processes, we have sought to determine the nature of the reactions of polyhalomethanes with a P-450 enzyme in vivo and in vitro. On the basis of its general oxygen chemistry, P-450 has been assigned a G or globin conformation (Castro, 1980). An important focus of this work was to learn whether, by formation of carbenes from polyhalomethanes, P-450 was uniquely different from other heme proteins of this class.

Our approach is to compare the chemistry of the enzyme with that displayed by iron porphyrin active sites in homogeneous solution and with that of the enzyme in its native cell. We have employed the bacterium *Pseudomonas putida* (PpG-786, ATCC culture 29608) as a whole cell model for the P-450 complement. The organism was selected by Professor I. C. Gunsalus for its high concentration of the enzyme P-450 cam. Both the organism and the enzyme are well characterized (Griffin et al., 1975; Gunsalus & Wagner, 1978; O'Keefe et al., 1978; Peterson, 1970). In contrast to work with reduced liver microsomes in the presence of excess dithionite, whole cell aerobic metabolism by *P. putida* can in principle allow a determination as to whether or not reductase activity by P-450 cam will be manifest under normal conditions. While the enzyme is known mainly for its capacity to hydroxylate camphor, it exhibits the general properties and structure of an enzyme of the P-450 class (Griffin et al., 1975; Dus, 1982). Homogeneous solutions of iron(II) complexes of deuteroporphyrin IX were studied as active site models for the substrate-affiliated enzyme.

Experimental Procedures

Materials. Chloropicrin, [^{14}C]chloropicrin, dichloronitromethane, chloronitromethane (Castro et al., 1983), and bromotrichloromethane (Castro et al., 1978) were obtained and purified in the manner recently described. Reagent-grade carbon tetrachloride was distilled before use. [^{14}C]Carbon tetrachloride was purchased from New England Nuclear. Deuteroporphyrin was prepared and purified essentially by the method of Caughey (Alben et al., 1968) as described earlier (Castro, 1974).

Pseudomonas putida PpG-786 was grown on camphor according to the procedure outlined by Gunsalus and Peterson (Gunsalus & Wagner, 1978; Peterson, 1970). The enzyme P-450 cam was obtained by the procedure of Gunsalus as were the flavoprotein and putida redoxin (Gunsalus & Wagner, 1978; Peterson, 1970). Final chromatography of P-450 cam was on DEAE-Sephadex A-50, and it showed a typical activity of 14 nmol of NADH s^{-1} (mg of protein)$^{-1}$ in the system NADH, flavoprotein, putida redoxin, camphor, and P-450 cam.

Methods. Typical reactions and workup procedures are given below.

(*A*) *With P. putida.* General reaction conditions and workup procedures with whole cells and the chloronitromethane derivates have been recently described (Castro et al., 1983). The new whole cell product work here with bromotrichloromethane and carbon tetrachloride was conducted in similar fashion except that the chloroform product from both halides was quantitated by direct gas chromatographic analysis of the complete reaction production solution. A 4.5-ft by $^1/_8$-in. Porapak P column was employed with a flame ionization detector.

A cell suspension (0.1 g/mL) in 0.05 M phosphate buffer at pH 7.4 containing initially 2.0×10^{-3} M bromotrichloromethane was allowed to react at room temperature. The reaction was monitored by visible spectroscopy (Figure 1). After 24 h 2-μL aliquots of the entire reaction mixture were analyzed by flame ionization gas chromatography. Bromotrichloromethane had vanished, and a peak coemergent with chloroform appeared at 3.0 min with the Porapak column at 150 °C. Quantitation by comparison with an authentic standard indicated the $CHCl_3$ concentration was $(2.0 \pm 0.1) \times 10^{-3}$ M. Yield was 100%. Qualitative identification was accomplished by passing this VPC emergent directly into a Finnigan quadrapole mass spectrometer. The mass spectrum matched that of chloroform and showed a dominant high mass peak corresponding to the parent $- {}^{35}Cl$ (83). Under the same conditions carbon tetrachloride was also quantitatively converted to chloroform (calculated yield 100 ± 5%).

(*B*) *Reactions with P-450 Cam.* Product work with the enzyme was conducted under turnover conditions, i.e., with an excess of substrate and in the presence of flavoprotein, putida redoxin, and camphor.

(*C*) *Carbon Tetrachloride.* A solution containing 1.5×10^{-4} M P-450 cam, the redoxin, and the reductase was reduced with NADH (initial concentration 2×10^{-3} M). Carbon tetrachloride was added to the reduced iron(II) P-450 solution via hypodermic syringe. The initial concentration of CCl_4 was 2.3×10^{-4} M (3-fold stoichiometric excess). After 24 h the reaction solution was analyzed by gas chromatography in the fashion described above for the whole cell reactions. No carbon tetrachloride remained, and the concentration of chloroform was $(2.4 \pm 0.1) \times 10^{-4}$ M. The protein was in the reduced state at the end of the reaction (excess NADH). Reaction here represented three turnovers of the enzyme.

(*D*) *Bromotrichloromethane.* Under identical conditions, $BrCCl_3$ at 1.1×10^{-4} M (1.5 stoichiometric excess) resulted in $CHCl_3$ at 1.1×10^{-4} M.

(*E*) *Chloropicrin.* As described above, a solution of 7×10^{-5} M P-450 and initially 2.0×10^{-4} M chloropicrin yielded a final product solution that was 1.0×10^{-4} M in nitromethane. The intermediates dichloronitromethane and chloronitromethane were also detected in the manner described previously in the whole studies with chloropicrin and in the studies with iron porphyrin noted below. The yield corresponds to eight turnovers of the enzyme.

It should be emphasized that blank runs like those above but without P-450 did not consume the halides. Independent separate checks with NADH, flavoprotein, or putida redoxin showed each of these components to be inert.

Kinetics. (*A*) *Whole Cell Runs.* Rates were approximated from initial slopes or the initial change in optical density at 540 nm. A buffer suspension of cells like that employed in product work was injected with 1–10 μL of substrate. The spectrum of this solution was run against the same suspension

of cells without substrate (a typical repeat scan is shown in Figure 1). The reaction was independent of the P-450 concentration in the cells but was a linear function of the halide concentration. Approximate first-order "permeation" rates were calculated from initial slopes with the expression $(\Delta OD/\Delta t)/[2\Delta(RX)_0]$.

(*B*) *CO Inhibition.* Cell suspensions gassed with CO were inert even to the fastest reacing substrate, chloropicrin.

(*C*) *The Enzyme.* Purified enzyme with an activity of 14 or more was reduced with dithionite under argon and passed through a short G-10 Sephadex column under argon to remove excess dithionite. The reduced enzyme was collected in argon-purged serum-capped test tubes in an argon glove bag. Aliquots for kinetic analysis were transferred under argon via a 22-gauge hypodermic tubing to argon-purged spectrophotometric cells that were equipped with stopcock and serum cap. Reaction was initiated by injection of the substrate under argon, and initial rates were monitored by following the decrease in iron(II) concentration at 542 nm. A typical repeat scan is shown in Figure 2.

Inhibition by CO was accomplished by bubbling CO through the reduced reaction solution. The substrate was injected under a CO purge.

The Sephadex column was prepared by first purging the dry Sephadex with argon and gentle shaking for 2 days. A thoroughly argon-purged (1 week) buffer solution was transferred under argon to the flask containing the dry-purged Sephadex. The G-10 slowly was transferred to a serum capped column containing 20 mL of purged buffer. The final column was 2.5 × 5 cm. Routinely, 2 mL of the enzyme was collected in a volume of 3 mL and the typical concentration was 1.5×10^{-4} M.

Reactions with Hemes. (*A*) *Chloropicrin.* In a 50-mL three-neck flask equipped as previously described (Wade & Castro, 1973a), 15 μL of [^{14}C]chloropicrin (1.5×10^{-4} mol) was injected under argon into a thoroughly argon-purged homogeneous red solution of 3.0×10^{-4} mol of iron(II) deuteroporphyrin IX in 30 mL of 2:1 *N*-methylpyrrolidone–acetic acid. The heme solution was prepared by the iron powder reduction of 0.18 g (3×10^{-4} mol) of chloroiron(III) deuteroporphyrin IX, and the Fe^0 was removed magnetically before the reaction was begun (Wade & Castro, 1973a). The solution turned brown within a few seconds, and the visible spectrum of the product solution corresponded to iron(III) deuteroporphyrin. The flask was opened to air, and the reaction solution was distilled through a small vigreux column at 65–75 °C/2 mm. The receiver was immersed in a CO_2–isopropyl alcohol bath. The distillation was stopped after collecting approximately 27 mL. An aliquot of the distillate was counted for radioactivity. A complete recovery of products was obtained [$(6.6 \pm 0.1) \times 10^5$ dpm]. Methylene chloride, 15 mL, and H_2O, 75 mL, were added to the distillate. The mixture was shaken and separated. The aqueous phase was extracted three additional times with 5 mL of CH_2Cl_2. The combined CH_2Cl_2 extracts were washed with 5 mL of H_2O and dried over Na_2SO_4. The CH_2Cl_2 product solution was concentrated at 55 °C to a volume of 8 mL. One percent of the counts were lost to the distillate. The radioactive concentrate was subjected to gas chromatography on a $^1/_8$-in. by 2.5-ft Porapak Q column at 145 °C equipped with a thermal conductivity detector. Peaks coemergent with dichloronitromethane (7.5 min) and chloronitromethane (5.5 min) were collected, counted, and subjected to mass spectral, infrared, and NMR analysis. The spectral properties matched exactly those we have reported in the parallel metabolic work (Castro et al., 1983). On the basis of ^{14}C counting, the yields were 71 and 27% for the di- and monochloro derivatives.

The original reaction concentrate, ~3 mL, was placed upon a short Al_2O_3 column. Excess NMP was eluted with methanol; the Fe^{III} porphyrin was brought off the column with acetic acid. This fraction contained no radioactivity. The hemin was demetallated ($Fe^0CH_2Cl_2$/HCl), the dication basified, and the CH_2Cl_2 solution of free porphyrin concentrated and counted for radioactivity. No counts were detectable.

(*B*) *Dichloronitromethane.* In a reaction requiring less than 2 min 0.75×10^{-4} mol of dichloronitromethane and 1.5×10^{-4} mol of deuteroheme in 15 mL of 2:1 NMP–HOAc yielded upon workup like that above $(0.7 \pm 0.05) \times 10^{-4}$ mol of chloronitromethane.

(*C*) *Chloronitromethane.* A reaction mixture of chloronitromethane and deuteroheme, exactly like that above, was directly analyzed by gas chromatography after 24 h A 3-ft Porapak P column at 85 °C was employed. Quantitation by comparison with an authentic standard of nitromethane indicated a yield at 90 ± 10%. The trapped substance had all of the properties of the authentic material (Castro et al., 1983).

(*D*) *Carbon Tetrachloride.* Reaction was conducted and worked up in the fashion described above for chloropicrin except that *n*-heptane rather than CH_2Cl_2 was employed to extract the product distillate. A reaction of 12 mL of 2×10^{-3} M $Fe^{II}D$ (2.4×10^{-5} mol) with 1.0×10^{-2} M carbon tetrachloride indicated 50% of the iron(II) was oxidized in 1 day. Gas chromatographic analysis of the heptane concentrate as described for whole cells (vide infra) indicated a yield of $(0.6 \pm 0.06) \times 10^{-5}$ mol (100%) of chloroform. The mass spectrum of the trapped peak was that indicated above. Identical reactions with $^{14}CCl_4$ (2.85×10^5 dpm/μL) confirmed these observations. Moreover, in these runs following the separation of the porphyrin by chromatography, no counts were found associated with the hemin or the porphyrin derived from it after demetallation.

(*E*) *Bromotrichloromethane.* As described above for CCl_4, 2.4×10^{-5} mol of $Fe^{II}D$ and 1.2×10^{-5} mol of the bromide yielded 1.2×10^{-5} mol (100%) of chloroform.

(*F*) ^{14}*C Counting.* Radioactivity was assayed by liquid scintillation counting with a Packard Tricarb Model 3255 liquid scintillation counter. All counts were quench corrected by the channels ratio method employing an automatic external standard. The counting cocktail employed was Packard Instagel. In all cases, enough counts were taken to ensure a maximum error of 1% at the 95% confidence level.

(*G*) *Kinetics.* Rates were monitored by visible spectroscopy at 627 nm (appearance of Fe^{III}) in the manner described earlier (Wade & Castro, 1973). A second-order plot for carbon tetrachloride at stoichiometric ratios of reactants is shown in Figure 3. In contrast, the sequential reduction of chloropicrin and dichloronitromethane to nitromethane exhibits a multiphasicity in the optical density vs. time plots. Minimal estimates of these rates were taken from initial slopes at lower concentrations of substrates.

Results

Stoichiometry. The organic products from the oxidation of all of the iron(II) complexes studied in this work are listed in the right-hand column of Table I. In all cases, with the hemes in solution, the enzyme, or intact cells, the yields were quantitative within experimental error. In all cases the iron(II) complex was converted to the corresponding iron(III) derivative. The general stoichiometry observed is given in eq 1,

$$2P\ Fe^{II} + R_2CX_2 + H^+ \rightarrow 2P\ Fe^{III} + R_2CHX + X^- \quad (1)$$

Table I: Rates and Products of Oxidation of Iron(II) Porphyrin in Solution, P-450 cam, and *P. putida*

substrate	*P. putida* (s^{-1})[a]	P-450 cam (L mol^{-1} s^{-1})	$Fe^{II}D$ (L mol^{-1} s^{-1})	product
Cl_3CNO_2	5.4	160	$\geq 8 \times 10^4$	Cl_2CHNO_2
Cl_2CHNO_2	0.5	15	1.1×10^4	$ClCH_2NO_2$
$BrCCl_3$	0.3	5.4	3.1×10^2	$HCCl_3$
CCl_4		2.3	34	$HCCl_3$
$ClCH_2NO_2$	0.15	1.7	2.4	CH_3NO_2

[a] Calculated assuming an even distribution of Fe^{II} porphyrin.

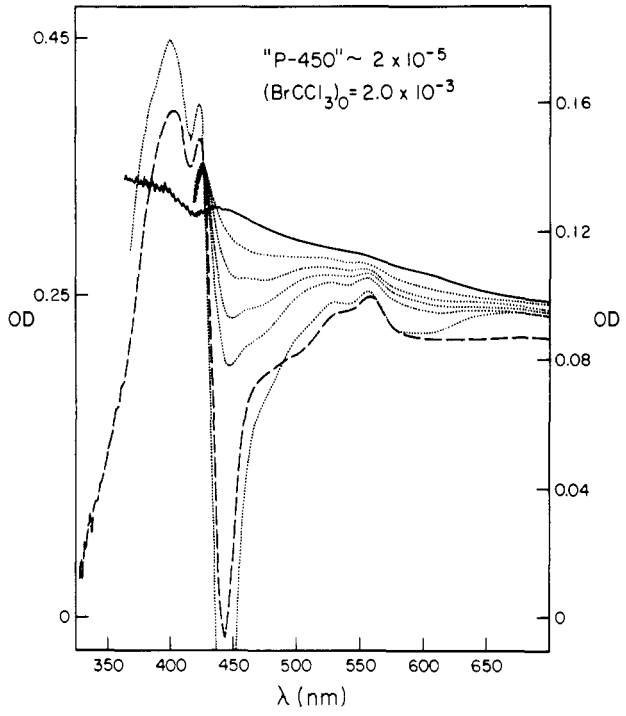

FIGURE 1: Difference spectra of a cell suspension of *P. putida* vs. cells + $BrCCl_3$: (solid line) time 0; (dotted lines) repeat scans at 10, 20, 30, 40, and 230 min (right ordinate); (dashed line) after 1 day (left ordinate).

wherein P may be porphyrin, P-450 cam, or the enzyme in its native cell. These processes with chloropicrin are somewhat more complex than normal because both the first and second reduced products are themselves reactive substrates. The general sequence we have established with *P. putida* (Castro et al., 1983) also obtains with P-450 and with the hemes in homogeneous solution (eq 2). Each step proceeds according

$$Cl_3CNO_2 \rightarrow Cl_2CHNO_2 \rightarrow ClCH_2NO_2 \rightarrow CH_3NO_2 \quad (2)$$

to the stoichiometry given in eq 1 and requires two hemes.[1] Chloroform is slow to react or inert on the time scale of our experiments. Thus, bromotrichloromethane and carbon tetrachloride react to produce chloroform quantitatively (eq 3)

$$XCCl_3 \xrightarrow{P\ Fe^{II}} HCCl_3 \quad (3)$$

in all systems. The symbolism and stoichiometry is that noted

[1] (a) With the cells (but not the enzyme or heme), a small portion of chloropicrin is biologically hydrolyzed to carbon dioxide (4%) (Castro et al., 1983). Also, there is some consumption of this substrate by dead cells. (b) We have previously demonstrated that nitromethane will oxidize high-spin hemes. The overall product in this case is methylamine, but the process is slow and can be held at the nitromethane state (Ong & Castro, 1977).

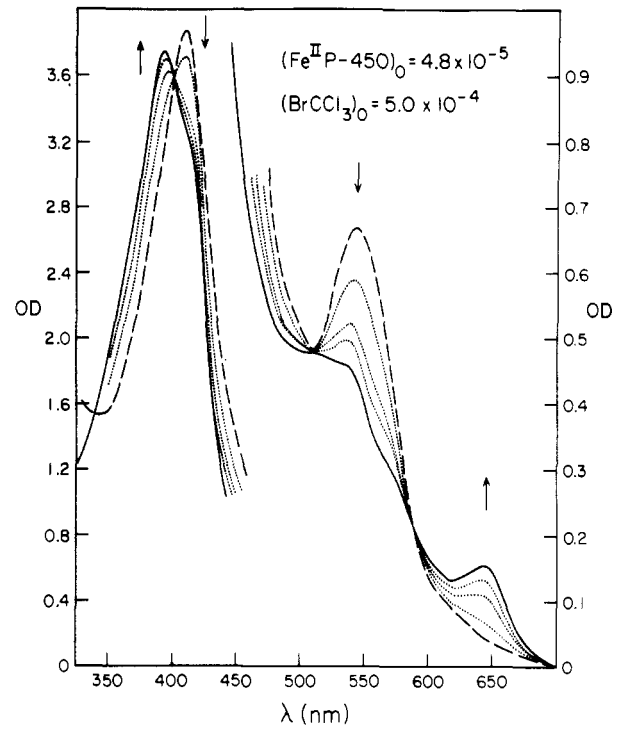

FIGURE 2: Repeat spectra of the oxidation of P-450 cam by $BrCCl_3$: (dashed line) time 0; (dotted lines) repeat scans at 1, 5, and 10 min; (solid line) at 1 h.

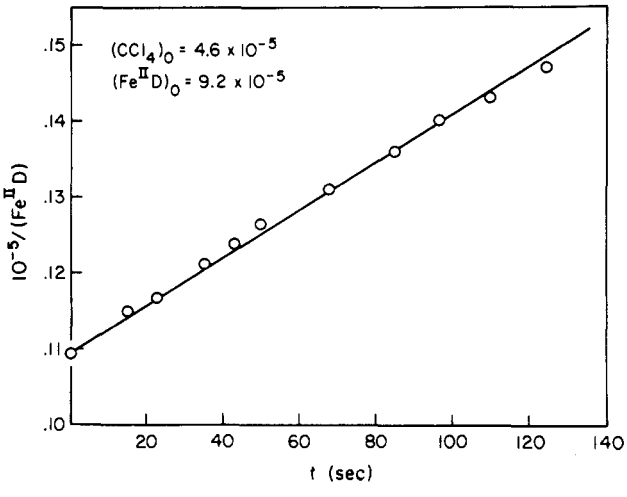

FIGURE 3: Oxidation of deuteroheme by CCl_4 at stoichiometric ratios.

above, and X = Cl or Br. The capacity of this enzyme to rapidly and catalytically dehalogenate such powerful alkylating agents as the polyhalonitroalkanes is astonishing.

The visible spectrum of the final product solution corresponded to the iron(III) complex in all cases. Some specific examples are illustrated in Figures 1 and 2 of the following section.

Kinetics. Apparently contrasting behavior is typified by the spectra shown in Figures 1 and 2 for the response of whole cell suspensions and the isolated enzyme to bromotrichloromethane. While the overall process is the same, it will be noted that the iron(II) enzyme in solution is consumed in logarithmic fashion but the iron(II) "concentration" in the cells diminishes at a constant rate. This was a general phenomenon for all substrates. Thus, the rate of oxidation of the enzyme in solution is dependent on the concentration of the iron(II) protein, but this is not true for the enzyme in the cell suspensions. We

Table II: Initial Rates of Oxidation of 5.8×10^{-5} M P-450 cam in the Presence of Camphor or Carbon Monoxide

substrate (mol/L)	camphor (mol/L)	CO (mol/L)	k (L mol^{-1} s^{-1})
CCl_4			
1.0×10^{-3}	1.0×10^{-4}		2.2
5.0×10^{-3}	1.0×10^{-5}		2.4
5.0×10^{-3}	1.0×10^{-5}	$\sim 10^{-3}$[a]	0
$BrCCl_3$			
1.0×10^{-3}	1.0×10^{-5}		5.2
1.0×10^{-3}	1.0×10^{-4}		5.4
1.0×10^{-3}	1.5×10^{-5}	$\sim 10^{-3}$	0
5.0×10^{-3}	1.5×10^{-5}		5.1
5.0×10^{-3}	3.0×10^{-5}		5.9
5.0×10^{-3}	4.5×10^{-5}		5.9
5.0×10^{-3}	6.0×10^{-5}		5.6
Cl_3CNO_2			
0.4×10^{-3}	1.0×10^{-5}		160
1.0×10^{-3}	1.0×10^{-5}	$\sim 10^{-3}$	0

[a] Saturated; cf. Experimental Procedures.

have established the following general rate laws for iron(II) porphyrins and P-450 cam in solution (Figures 2 and 3)

$$\frac{-d[Fe^{II}]}{dt} = \frac{d[Fe^{III}]}{dt} = k[Fe^{II}P][RX]$$

for cell suspensions (Figure 1)

$$\frac{-d[Fe^{II}]}{dt} = \frac{-2d[RX]}{dt} = k[RX]$$

The lack of iron dependence in the rate law for whole cells indicates that permeation of the cell by the organic halide rather than reactivity at iron is rate limiting.

It will be noted that the more reactive halides Cl_3CNO_2 and Cl_2CHNO_2 oxidize hemes in solution 5–700 times faster than they oxidize the enzyme. On the other hand, the slowest reacting substrate, $ClCH_2NO_2$, oxidizes both hemes and P-450 at rates that are similar.

The first-order "rates" approximated for whole cells (assuming the irons were homogeneously distributed) cannot directly be compared with those of the enzyme or heme. The relatively small spread in these numbers is consistent with a rate-limiting permeation in these cases (Figure 3).

Influence of Ligands. (*A*) *Ligation of Enzyme.* Camphor is well-known to ligate this enzyme with an accompanying change of low- to high-spin iron(II). Presumably, an axial ligand is removed from iron by a conformation change near the active site attendant upon substrate binding. It was of interest then to learn whether or not the binding of camphor influenced the rates of reaction with the halides. Experiments to probe this question were performed, and the results are presented in Table II. Camphor does not influence the rates of reaction at these concentrations, even though it is bound to the enzyme. The product of the oxidation in the presence of adequate camphor is the iron(III)–P-450–camphor adduct, λ_{max} 392, 646.

(*B*) *Ligation at Iron.* Ligation at iron, however, seriously inhibited these reactions. Carbon monoxide at saturating concentrations in all cases generated the iron carbonyl, and this species was inert. With whole cells or the isolated enzyme, even the fastest reacting substrate, chloropicrin, was inert.

Discussion

The major finding of our work is that the general character and nature of the oxidation of hemes by polyhalmomethane is the same whether the heme is in homogeneous solution, ensconced in the active site of the enzyme P-450 cam, or in its natural biological framework in the bacteirum *P. putida*. At all levels of bioorganization represented here, the transformation observed is a reductive hydrogenolysis of the carbon halogen bond (eq 1). In all cases, within experimental error this transformation is quantitative. Moreover, the kinetic studies of these reactions accord with the mechanisms generally established for the oxidation of hemes and a wide range of heme proteins by organic halides (Castro, 1964; Wade & Castro, 1973a,b; Castro & Bartnicki, 1975; Castro et al., 1978; Stotter et al., 1977).

The overall two electron reduction of RX to RH proceeds in three discreet steps:

(i) ligation at iron

$$P\ Fe^{II} + RX \rightarrow P\ Fe^{II}(XR)$$

(ii) carbon–halogen bond cleavage

$$P\ Fe^{II}(XR) \rightarrow [P\ Fe\cdots X\cdots R]^{*} \rightarrow P\ Fe^{III}X + R\cdot$$

(iii) rapid scavenging of the radical by heme

$$P\ Fe^{II} + R\cdot \xrightarrow{\text{fast}} (P\ FeR) \xrightarrow[\text{or Solv-H}]{H^{+}} P\ Fe^{III} + RH$$

With hemes in homogeneous solution, step ii is rate limiting as it is here with the polyhalomethanes. It will be noted from Table I that the rates for heme oxidation parallel what might be expected for the bond dissociation energies of the carbon–halogen bond and or the stabilities of the generated radicals. With heme proteins, however, because of the steric constraints associated with a given conformation, actually getting to iron, i.e., ligation (step i), can be rate limiting. This has been demonstrated most thoroughly with cytochrome *c* (Castro & Bartnicki, 1975), but hemoglobin and myoglobin both show substantial steric effects in these processes (Castro et al., 1978). The relative rates $k_{P\text{-}450}/k_{heme}$ in Table I can be taken as a rough measure of steric constraint imposed by the P-450 cam on these processes. For a rapid reacting substrate, e.g., dichloronitromethane, this ratio is 1/700. On the other hand, with those substrates for which the inherent rate of carbon–halogen scission by heme is slow the above rate ratio increases significantly and approaches 1 ($ClCH_2NO_2$). The size of the substrate can be all determining. Thus, hemes are readily oxidized by the insecticide DDT (Castro, 1964; Wade & Castro, 1973). While not in Table I, we find P-450 cam, in solution or in *P. putida*, is inert to this large substrate.

The Protein Conformation. Rates for the oxidation of P-450 cam by $BrCCl_3$ would place it somewhere between myoglobin (2.5 L mol^{-1} s^{-1}) and hemoglobin (8.0 L mol^{-1} s^{-1}) in steric accessibility to iron. On the other hand, P-450 cam is oxidized more rapidly by CCl_4 than the globins. Since camphor does not influence these rates (Table II), the gross conformation of the protein and its effective redox geometry are not affected by this substrate even though its binding induces some change within the protein matrix. The reactivity demonstrated here is consistent with a heme protein in the G conformation.

Whole Cells. The spectral changes observed for P-450 in the cells show clearly that the enzyme is oxidized by the halides. Moreover, carbon monoxide inhibits these reactions. Unfortunately though, the visible spectra do not allow a direct measure of the rate of oxidation of the enzyme in the cells. We presume these iron-independent rates are most reasonably permeation rates, though they could result from an interplay of other reductive apparati in the cell with P-450. This latter possibility seems unlikely since it would imply a steady-state concentration of iron(II) P-450, and this is clearly not observed. While it cannot be established by the rate data alone, the

inhibition by carbon monoxide is compelling evidence that the mechanism operating with the enzyme and hemes in solution is also operating within the cell.

Hemes. The hemes in homogeneous solution react as would be predicted from earlier work; step ii above is rate limiting. An important point is that none of these reactions afford carbene products. The results with ^{14}C-labeled CCl_4 or Cl_3CNO_2 allow a particularly sensitive assessment of the fate of the carbon moiety. All of the counts were found in the reduction products, and none were associated with the hemin or the porphyrin after demetallation. However, the reactions of hemes with substrates of this class, in heterogeneous millieu, in the presence of a massive excess of reductant may take an additional course (Mansuy, 1980). The general character of these reactions do not however parallel that exhibited by the enzyme.

Clearly carbenic intermediates or derived products are *not* generated in *any* of these reactions. We have no evidence for this occurrence with any heme protein or heme in homogeneous solution. P-450 cam is no exception to these general observations. Given the fact that the microsomal enzyme, where it has been studied (vide infra), yields products of a similar nature, it is unlikely that carbenes are generated with any P-450 enzyme at all. Most notable in this regard is the recent study with halothane reported by Ullrich. Whereas earlier the reaction had been hypothesized to produce the first example of an iron carbene (λ_{max} 470), the new work (Nastainczyk et al., 1982; Ahr et al., 1982) proposes an iron alkyl to account for the spectral observations. The products of the reaction (CF_3CH_2Cl and $CF_2{=}CHCl$) are consistent with the chemistry observed herein and that generally established earlier.

It would seem then that there is no compelling experimental evidence in the literature in support of the generation of carbenes by the P-450 enzymes. An exception is the report that carbene adducts and carbon monoxide are produced from tetrahalomethanes and reduced rat liver microsomes under basic conditions. The method of carbon monoxide analysis relied upon difference spectra obtained after addition of hemoglobin to the liver microsomal suspension. This method is not without ambiguity. However, the more revealing observation was that CO was also generated from the haloforms $CHBr_3$, CHI_3, and, to a lesser extent, $CHCl_3$. The carbenes generated by reductive processes from these latter species would be monohalocarbenes of the form H–C–X, and they could hydrolyze to formaldehyde but not carbon monoxide. On the other hand, a base-catalyzed generation of CX_2 from the haloforms is well established. We note that acid-washed microsomes produced no CO upon exposure to substrate. Assuming carbon monoxide was actually obtained, these results could be chemically accommodated by the sequence

$$CX_4 \xrightarrow{\text{P-450}} CHX_3 \xrightarrow{\text{base}} {}^{-}CX_3 \rightarrow CX_2 \rightarrow CO$$

The first conversion would be in keeping with the present findings and those described by others. It is likely the only chemical act performed by P-450 in these studies.

The uniqueness of P-450 is manifest in its oxygen chemistry. In most tissue and in *P. putida* the reactive form of the enzyme is the "iron oxene". At reasonable concentrations, however, polyhalomethanes can compete with oxygen for binding at the iron(II) center (step i above). Thus, if the bond cleavage step (step ii above) proceeds rapidly enough, reduction of the halide can ensue. In this sense, the reactivity of P-450 cam to this class of substrate is consistent with that of other heme proteins in the G conformation (Castro et al., Belser, 1978). The reductase capacity of the enzyme resembles that of myoglobin and oxymyoglobin, though P-450 appears to be less inhibited by oxygen. Thus, the P-450 enzymes can display a remarkable chemical flexibility toward substrate.

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Registry No. P-450, 9035-51-2; $ClCH_2NO_2$, 1794-84-9; Cl_3CNO_2, 76-06-2; Cl_2CHNO_2, 7119-89-3; Cl_3CBr, 75-62-7; CCl_4, 56-23-5; CH_3NO_2, 75-52-5; Cl_3CH, 67-66-3.

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Reaction of Hemerythrin with Disulfides†

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ABSTRACT: The reactions of hemerythrin from *Phascolopsis gouldii* with the specific sulfhydryl reagents 5,5′-dithiobis(2-nitrobenzoate), 2,2′-dithiodipyridine, and 4,4′-dithiodipyridine were studied at 25 °C. Spectrophotometric measurements showed that 1 mol of disulfide reacted per protein subunit consistent with a single cysteine at residue 50. Reaction leads to dissociation of the octameric structure of the native protein to monomers. The first-order rate constants at 25 °C and pH 9.0 for reactions of methemerythrin [$(1.5 \pm 0.3) \times 10^{-3}$ s^{-1}] and metazidohemerythrin [$(4.0 \pm 0.3) \times 10^{-3}$ s^{-1}] are independent of both the concentration and the nature of the disulfide. The reactions of methemerythrin are strongly inhibited by ClO_4^- ion, which however has no effect on the rates of those of metazidohemerythrin. The first-order kinetic behavior is ascribed to a conformational change involving the protein controlling the reaction, and this slow change appears to dominate a number of the reactions of hemerythrin.

Hemerythrin is an iron-containing respiratory protein in certain marine organisms whose structure and properties have been well characterized (Henderickson, 1978; Loehr & Loehr, 1979; Stenkamp & Jensen, 1979; Wilkins & Harrington, 1983; Klotz & Kurtz, 1984). Interest in the protein has been revitalized by the apparent similarity of its active site to those of several iron proteins from disparate sources. These include ribonucleotide reductase (Sjoberg & Gräslund, 1983; Sjoberg et al., 1983), purple acid phosphatases from beef spleen (Davis & Averill, 1982) and from pig allantoic fluid (also called uteroferrin; Antanaitis & Aisen, 1983; Debrunner et al., 1983), and component A of methane monooxygenase from *Methylococcus capsulatus* (Bath) (Woodland & Dalton, 1984; M. P. Woodland and R. Cammack, private communication).

The native hemerythrin from the coelomic fluid of the sipunculid *Phascolopsis gouldii* is octameric (Klotz & Keresztes-Nagy, 1963; Wilkins & Harrington, 1983). It has a single cysteine at residue 50, and although this is some distance [approximately 15 Å (Stenkamp et al., 1978a)] from the binuclear iron site, a small sequence of residues around 50 plays a critical role in some important reactions of the protein. Treatment with a number of sulfhydryl reagents including salyrganic acid, PCMB,[1] PMB, and NEM promotes dissociation of the octamer to the monomer (Keresztes-Nagy & Klotz, 1963; Garbett et al., 1971; Rao & Keresztes-Nagy, 1973; Clarke et al., 1979). Octameric hemerythrin from *Themiste dyscritum* has cysteine residues at 9 and 50. In many other respects, the proteins from the two sources are very similar (Dunn et al., 1977). The interaction of a number of mercurials with *T. dyscritum* hemerythrin has been recently examined (Clarke et al., 1979). Here again, binding of the bulky PHMB or NEM to the sulfur of cysteine disrupts

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoate); 2-PDS, 2,2′-dithiodipyridine; 4-PDS, 4,4′-dithiodipyridine; PHMB, *p*-(hydroxymercuri)benzoate; PCMB, *p*-(chloromercuri)benzoate; PMB, *p*-mercuribenzoate; CMNP, 2-(chloromercuri)-4-nitrophenol; NEM, *N*-ethylmaleimide; Mes, 4-morpholineethanesulfonic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; SDS, sodium dodecyl sulfate.

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