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Coenzyme F_{430} as a Possible Catalyst for the Reductive Dehalogenation of Chlorinated C_1 Hydrocarbons in Methanogenic Bacteria[†]

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ABSTRACT: Corrinoids, such as aquocobalamin, methylcobalamin, and (cyanoaquo)cobinamide, catalyze the reductive dehalogenation of CCl_4 with titanium(III) citrate as the electron donor [Krone et al. (1989) *Biochemistry 28*, 4908-4914]. We report here that this reaction is also effectively mediated by the nickel-containing porphinoid, coenzyme F_{430}, found in methanogenic bacteria. Chloroform, methylene chloride, methyl chloride, and methane were detected as intermediates and products. Ethane was formed in trace amounts, and several as yet unidentified nonvolatile compounds were also generated. The rate of dehalogenation decreased in the series of CCl_4, $CHCl_3$, and CH_2Cl_2. With coenzyme F_{430} as the catalyst, the reduction of CH_3Cl to CH_4 proceeded more than 50 times faster than with aquocobalamin. Cell suspensions of *Methanosarcina barkeri* were found to catalyze the reductive dehalogenation of CCl_4 with CO as the electron donor ($E'_0 = -0.524$ V). Methylene chloride was the main end product. The kinetics of $CHCl_3$ and CH_2Cl_2 formation from CCl_4 were similar to those with coenzyme F_{430} or aquocobalamin as catalysts and titanium(III) citrate as the reductant.

Various methanogenic bacteria catalyze the reductive dehalogenation of chlorinated hydrocarbons, such as CCl_4, $CHCl_3$, and perchloroethylene (Egli et al., 1987; Belay & Daniels, 1987; Fathepure & Boyd, 1988a,b). It has been suggested that coenzyme F_{430} is involved in the catalysis of these reactions (Fathepure & Boyd, 1988a,b). Coenzyme F_{430} is a nickel(II) porphinoid (Figure 1) present in all methanogenic bacteria (Diekert et al., 1981). It functions as the prosthetic group of methyl coenzyme M (CoM)[1] reductase, which mediates the final step in methanogenesis (Rouvière & Wolfe, 1988; Ellermann et al., 1988, 1989).

$$CH_3S\text{-}CoM + HS\text{-}HTP \rightarrow CH_4 + CoM\text{-}S\text{-}S\text{-}HTP$$

Electron paramagnetic resonance (EPR) spectroscopic studies indicate that the nickel of enzyme-bound coenzyme F_{430} may undergo redox changes (Albracht et al., 1986, 1988; Jaun & Pfaltz, 1986). In vitro $Ni(I)F_{430}$ [$Ni(II)F_{430}$ reduced with zinc amalgam in dimethylformamide] reacts with methyl iodide to yield methane (Jaun & Pfaltz, 1986, 1988).

$$CH_3I + 2Ni(I)F_{430} + H^+ \rightarrow CH_4 + 2Ni(II)F_{430} + I^-$$

$Ni(I)F_{430}$ is oxidized to $Ni(II)F_{430}$ within the mixing time. In vivo methyl iodide (Laufer et al., 1986) and other halogenated C_1 hydrocarbons (CCl_4, $CHCl_3$, and CH_2Br_2) (Bauchop, 1967; Wolfe, 1971; Belay & Daniels, 1987) are known to specifically inhibit methanogenesis from methyl-CoM, probably by oxidizing the reduced F_{430} bound to methyl-CoM reductase.

We have recently reported that corrinoids catalyze the rapid reductive dehalogenation of CCl_4 to CH_3Cl with titanium(III) citrate in aqueous solution at pH 8.2 (Krone et al., 1989). Evidence is presented here that coenzyme F_{430} is an even more effective dehalogenation catalyst capable of reducing CCl_4 to CH_4.

MATERIALS AND METHODS

Coenzyme F_{430} ($\epsilon_{430} = 22\,500$ M^{-1} cm^{-1}) was isolated from H_2/CO_2-grown *Methanobacterium thermoautotrophicum* (strain Marburg) (Diekert et al., 1981). Aquocobalamin ($\epsilon_{527} = 8500$ M^{-1} cm^{-1}) (Friedrich, 1975), dithioerythritol, and dithiothreitol were obtained from Sigma (München, FRG). Carbon tetrachloride, methylene chloride, and titanium(III) chloride were from Merck (Darmstadt, FRG). Chloroform was from Baker Chemicals (Deventer, The Netherlands), and methyl chloride, methane, and ethane were from Messer Griesheim (Düsseldorf, FRG). Titanium(III) citrate solutions, pH 8, were prepared as described by Zehnder and Wuhrmann

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[1] Abbreviations: B_{12a}, cob(III)alamin; B_{12r}, cob(II)alamin; B_{12s}, cob(I)alamin; CoM, coenzyme M; HS-CoM, 2-mercaptoethanesulfonate; CH_3S-CoM, 2-(methylthio)ethanesulfonate; CoM-S-S-HTP, mixed disulfide of HS-CoM and HS-HTP; HS-HTP, *N*-(7-mercaptoheptanoyl)threonine phosphate; Tris, tris(hydroxymethyl)aminomethane.

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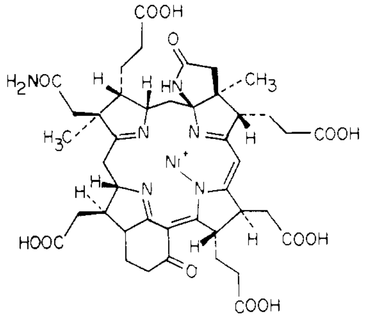

FIGURE 1: Proposed structure of coenzyme F_{430} (Pfaltz et al., 1982, 1985, 1987; Livingstone et al., 1984; Fässler et al., 1985).

(1976); final Ti(III) concentration was 0.09 M.

Methanosarcina barkeri (strain Fusaro) (DSM 804) was from the Deutsche Sammlung von Mikroorganismen (Braunschweig, FRG). Cells were grown on a methanol or an acetate medium and harvested anaerobically. Cell suspensions were prepared in 50 mM imidazole–phosphate buffer, pH 7.4 (Karrasch et al., 1989).

Reactions with coenzyme F_{430} or aquocobalamin as catalyst were carried out in 1-mL anaerobic assay mixtures by using 8-mL serum bottles closed with viton stoppers and wrapped in aluminum foil. Reactions with cell suspensions of *M. barkeri* were performed in 4-mL anaerobic assay mixtures by using 25-mL serum bottles. Except where indicated, the gas phase was N_2 at 1.4×10^5 Pa pressure. Additions and withdrawals were made by syringes. The serum bottles were incubated at 30 °C in a gyrotory water bath shaker at 200 rpm. At time intervals, 0.3-mL gas samples were withdrawn with gastight syringes and analyzed by gas chromatography as described previously (Krone et al., 1989).

Standard solutions of CCl_4 (0.062 M), $CHCl_3$ (0.074 M), and CH_2Cl_2 (0.093 M) were prepared by dissolving 0.3 mL of the chlorinated hydrocarbons in 50 mL of methanol. The gaseous standards (CH_3Cl, CH_4, and C_2H_6) were prepared by injecting 0.25 mL of the gas into a 120-mL serum bottle closed with a viton stopper, containing N_2 at 1.4×10^5 Pa; 0.3 mL of these mixtures contains 20 nmol of the gas.

RESULTS

For the study of coenzyme F_{430} as a dehalogenation catalyst the same assay conditions were chosen as previously described for the reductive dehalogenation of chlorinated C_1 hydrocarbons mediated by corrinoids (Krone et al., 1989). The reactions were performed in Tris-HCl buffer, pH 8.0, at 30 °C. CCl_4, $CHCl_3$, CH_2Cl_2, and CH_3Cl were tested as substrates; titanium(III) citrate and dithiothreitol were tested as reductants. The time course of the formation of intermediates and products was followed by gas chromatography. The kinetics of reductive dehalogenation of chlorinated methanes in *M. barkeri* was also investigated. This methanogenic bacterium is known to contain high levels of coenzyme F_{430} (Diekert et al., 1981; Gorris et al., 1988).

Reductive Dehalogenations with Coenzyme F_{430}. In the absence of a catalyst, CCl_4 was reduced very slowly to $CHCl_3$ by Ti(III). The reaction stopped after 30 min at 10% completion. A reduction to the level of CH_2Cl_2 or CH_3Cl was not observed. When coenzyme F_{430} was added to the system, the rate of CCl_4 conversion to $CHCl_3$ increased by a factor of approximately 10. CH_2Cl_2, CH_3Cl, and methane were also formed as products (Figure 2A). The concentration of $CHCl_3$ increased while CCl_4 was detectable in the gas phase, but decreased rapidly after the CCl_4 was depleted. Such a time course would be expected if $CHCl_3$ were an intermediate. The recovery of the volatile products was about 60%.

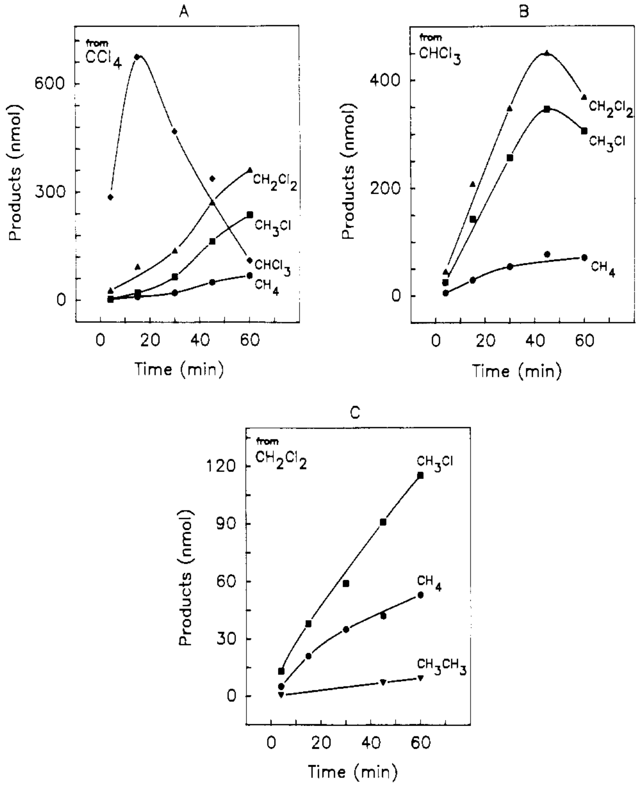

FIGURE 2: Coenzyme F_{430} catalyzed reductive dehalogenation of CCl_4 (A), $CHCl_3$ (B), and CH_2Cl_2 (C) with titanium(III) citrate as electron donor. The assays were performed in the dark at 30 °C in 8-mL serum bottles with N_2 as gas phase. The 1-mL reaction mixtures contained 2.2 μmol of the chlorinated hydrocarbons (added as methanolic solutions), 27 μmol of titanium(III) citrate, and 46 nmol of coenzyme F_{430} in 0.66 M Tris-HCl buffer, pH 8.0.

The kinetics of dehalogenation with $CHCl_3$ and with CH_2Cl_2 as substrate are presented in Figure 2B,C. The rates of dehalogenation decreased in the series CCl_4, $CHCl_3$, and CH_2Cl_2. Methyl chloride conversion to CH_4 (Figure 3A) and CH_2Cl_2 reduction to CH_3Cl (Figure 2C) proceeded at similar rates.

The reduction of CH_3Cl to CH_4 catalyzed by coenzyme F_{430} was studied in more detail. The effects of coenzyme F_{430} and CH_3Cl concentrations on the rate of CH_4 formation are given in Figure 3B,C. For comparative purposes, the rates of reduction with aquocobalamin (B_{12a}) as catalyst are also given. The results show that coenzyme F_{430} is at least 50 times more efficient than aquocobalamin in mediating CH_3Cl reduction to CH_4.

Small amounts of ethane were also generated from CCl_4, $CHCl_3$, and CH_2Cl_2 but not from CH_3Cl. The highest rates were observed with CH_2Cl_2 as substrate. A time course of ethane formation from CH_2Cl_2 is shown in Figure 2C.

Coenzyme F_{430} also catalyzed the reductive dehalogenation of CCl_4 when dithiothreitol was used as reductant. However, the rates were only a fraction of those observed with Ti(III), and the reaction essentially stopped at the level of $CHCl_3$.

Using EPR spectroscopy, we tried to determine whether Ni(II)F_{430} was reduced to Ni(I)F_{430} (Jaun & Pfaltz, 1986) by Ti(III) under the assay conditions. However, this was not feasible because the signal of Ti(III) at $g = 2$ was too broad and too intense. Attempts to observe possible changes in the UV–vis spectrum of coenzyme F_{430} also failed because the intense black color of Ti(III) masked the coenzyme F_{430} spectrum. After oxidation with air to Ti(IV), which is colorless, the typical UV–vis spectrum of Ni(II)F_{430} was seen,

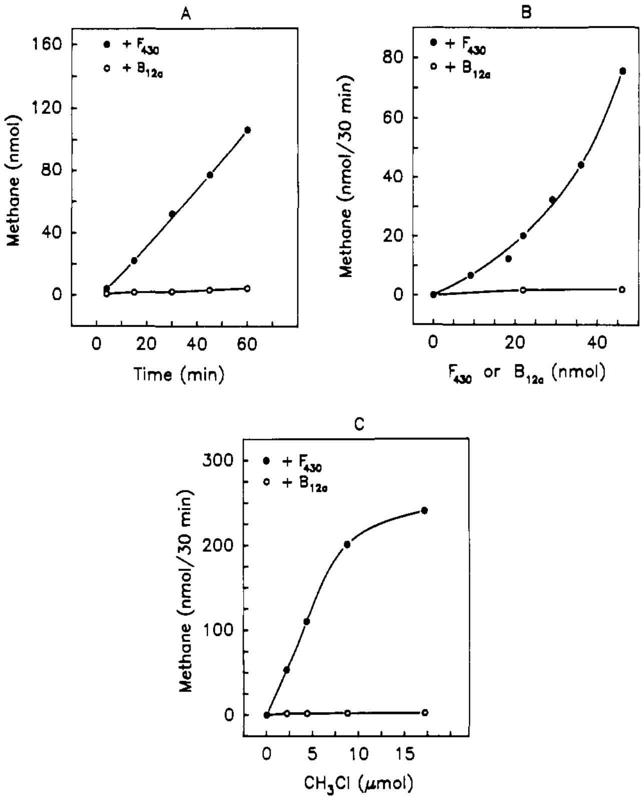

FIGURE 3: Coenzyme F_{430} catalyzed or aquocobalamin-catalyzed reduction of CH_3Cl to CH_4 with titanium(III) citrate as electron donor. (A) Time dependence; (B) dependence on catalyst concentration; (C) dependence on CH_3Cl concentration. The assays were performed in the dark at 30 °C in 8-mL serum bottles with N_2 as gas phase. The 1-mL reaction mixtures contained 2.2 µmol of CH_3Cl (0.05 mL of gaseous CH_3Cl) or, as indicated, 27 µmol of titanium(III) citrate, 46 nmol of coenzyme F_{430}, 46 nmol of aquocobalamin, or concentrations indicated in 0.66 M Tris-HCl buffer, pH 8.0.

indicating that coenzyme F_{430} was not irreversibly changed during the reductive dehalogenation.

Reduction of Protons and Disulfides by Ti(III). In the dehalogenation assays, some H_2 was always formed. However, under our assay conditions, H_2 was formed, even in the absence of coenzyme F_{430}, and addition of the nickel porphinoid did not stimulate the reaction.

Corrinoids were recently shown to catalyze the reduction of disulfides with titanium(III) citrate (Hedderich & Thauer, 1989). In contrast, coenzyme F_{430} mediated this reaction relatively poorly (Figure 4).

Reductive Dehalogenations with M. barkeri. The experiments with *M. barkeri* were performed with methanol- and acetate-grown cells suspended in 50 mM imidazole–phosphate buffer, pH 7.4, containing 5 mM dithioerythritol and incubated at 30 °C under an atmosphere of 6% carbon monoxide. This methanogenic bacterium mediates, via an active carbon monoxide dehydrogenase, the conversion of CO plus H_2O into CO_2, two e^-, and two H^+, supplying the cells with reducing equivalents (Bott & Thauer, 1989). The redox potential of the CO_2/CO couple ($E'_0 = -0.524$ V) (Thauer et al., 1977) is of the same order of magnitude as that of the Ti(IV)/Ti(III) couple ($E'_0 = -0.480$ V) [Krone et al., 1989; see also Jones and Pickard (1980)].

Cell suspensions of *M. barkeri* rapidly converted CCl_4 to $CHCl_3$ and CH_2Cl_2 (Figure 5A). CCl_4 was dehalogenated within the first 25 min. During this time, the concentration of $CHCl_3$ rapidly increased. After 25 min, the $CHCl_3$ concentration decreased, and CH_2Cl_2 and minor amounts of CH_3Cl were formed concomitantly. The small amount of methane detected may have been generated from endogenous sources or from reduction of CCl_4.

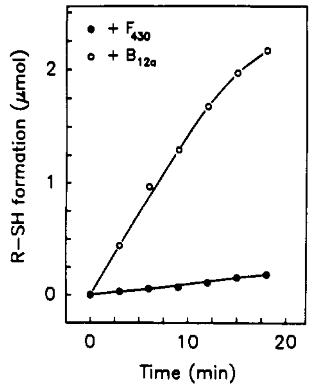

FIGURE 4: Coenzyme F_{430} catalyzed or aquocobalamin-catalyzed reduction of CoM-S–S-HTP to HS-CoM and HS-HTP (R-SH) with titanium(III) citrate as electron donor. The assays were performed at 60 °C in 8-mL serum bottles with N_2 as the gas phase. The 0.5-mL reaction mixtures contained 1 µmol of CoM-S–S-HTP, 5 µmol of titanium(III) citrate, and 0.25 nmol of aquocobalamin or coenzyme F_{430} in 50 mM Tris-HCl buffer, pH 7.6. The 25-µL samples were analyzed for thiols with Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)] as described in Hedderich and Thauer (1988).

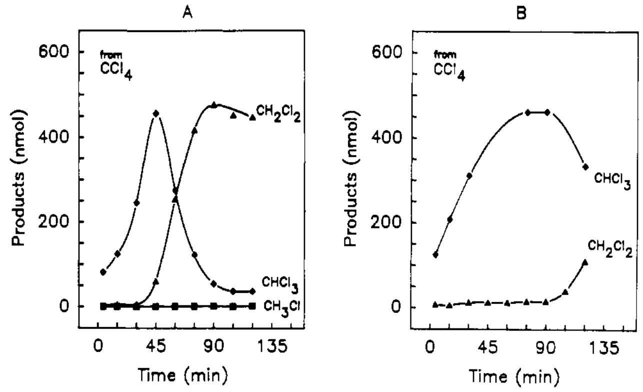

FIGURE 5: Reductive dehalogenation of CCl_4 to $CHCl_3$, CH_2Cl_2, and CH_3Cl by *M. barkeri*. (A) Reactions catalyzed by cell suspensions of methanol-grown *M. barkeri* with carbon monoxide as electron donor; (B) reaction catalyzed by heat-denatured cell suspensions of *M. barkeri* with titanium(III) citrate as electron donor. The assays were performed in the dark at 30 °C in 25-mL serum bottles with 6% CO and 94% N_2 at 1.5 × 10^5 Pa as gas phase. The 4-mL reaction mixtures contained (A) 2.2 µmol of CCl_4 (35 µL of methanolic solution), 20 µmol of dithioerythritol, and 30 mg of *M. barkeri* (15 mg of protein) in 50 mM imidazole–phosphate buffer, pH 7.4, (B) 2.2 µmol of CCl_4 (35 µL of methanolic solution), 20 µmol of dithioerythritol, 27 µmol of titanium(III) citrate, and 30 mg of *M. barkeri* (15 mg of protein), heated for 10 min in a boiling water bath in 50 mM imidazole–phosphate buffer, pH 7.4.

The dehalogenation kinetics were also determined with $CHCl_3$, CH_2Cl_2, and CH_3Cl as substrates. At saturating concentrations, the rates of CCl_4 and $CHCl_3$ dehalogenation were almost identical [20 nmol h^{-1} (mg of protein)$^{-1}$], while the conversion of CH_2Cl_2 to CH_3Cl was significantly lower [maximally 5 nmol h^{-1} (mg of protein)$^{-1}$]. No reduction of CH_3Cl to methane was observed. The background formation of methane [<1 nmol min^{-1} (mg of protein)$^{-1}$] was not enhanced upon addition of CH_3Cl.

When the experiments were performed under N_2 rather than CO as the gas phase, both the rate and extent of dehalogenation were more than 50% lower. The residual dehalogenation activity can be explained by the finding that the harvested cells still contained endogenous reducing power as evidenced by their ability to reduce CO_2 to CH_4 in the absence of exogenous electron donors.

The ability of *M. barkeri* to mediate the reduction of chlorinated methanes with CO was almost completely lost when the cells were incubated for 10 min at 100 °C. This heat treatment caused the permeabilization of the cells as well as the inactivation of carbon monoxide dehydrogenase and other enzymes. Suspensions of these heat-treated cells catalyzed, however, the formation of $CHCl_3$ and CH_2Cl_2 from CCl_4 upon addition of titanium(III) citrate (Figure 5B). The rates were similar to those observed with CO as electron donor before the inactivation (Figure 5A). These findings suggest that the reductive dehalogenation by *M. barkeri* involves heat-stable cell components capable of using Ti(III) as electron donor.

DISCUSSION

Coenzyme F_{430} was found to catalyze the reductive dehalogenation of chlorinated C_1 hydrocarbons with titanium(III) citrate ($E'_0 = -0.480$ V) as the reducing agent. Neither dithiothreitol ($E'_0 = -0.330$ V) nor cysteine ($E'_0 = -0.210$ V) (Cleland, 1964) served as reductant. The redox potentials for the chlorinated methane couples calculated from the free energies of formation (CCl_4, $CHCl_3$, and CH_2Cl_2 in the liquid state, CH_3Cl and CH_4 in the gaseous state, 1 M Cl^- in aqueous solution, pH 7) (Wagman et al., 1968) are as follows: $CCl_4/CHCl_3$, $E'_0 = +0.516$ V; $CHCl_3/CH_2Cl_2$, $E'_0 = +0.439$ V; CH_2Cl_2/CH_3Cl, $E'_0 = +0.421$ V; and CH_3Cl/CH_4, $E'_0 = +0.438$ V. These redox potentials show that the reduction of the chlorinated methanes by dithiothreitol or cysteine is feasible thermodynamically. Jaun and Pfaltz (1986) measured the redox potential of the pentamethyl ester of coenzyme F_{430} ($F_{430}M$) in dimethylformamide. Cyclic voltammograms of $F_{430}M$ showed a single one-electron wave at −1.32 V vs the ferricenium/ferrocene couple (0.815 V), at −0.84 V vs the 0.1 M calomel electrode (0.335 V), and thus at −0.504 V vs the hydrogen electrode (0.0 V) (Clark, 1972). Assuming that this potential is also valid for coenzyme F_{430} in aqueous solution, a redox potential of −0.504 V indicates that coenzyme F_{430} can be partially reduced by titanium(III) citrate, but not by dithiothreitol or cysteine. The requirement for the reduction of coenzyme F_{430} is, thus, most likely the reason why only Ti(III) functioned as the electron donor in the nickel porphinoid mediated dehalogenation reactions. We propose that $Ni(II)F_{430}$ is reduced by Ti(III) to $Ni(I)F_{430}$, which then reacts with the chlorinated methanes. Organometallic intermediates are probably formed in the sequence of reactions leading to the final products (Jaun & Pfaltz, 1988). The formation of alkylnickel intermediates in the reduction of alkyl halides by macrocyclic Ni(I) complexes has been described by Bakac and Espenson (1986a). The same authors (Bakac & Espenson, 1986b) showed that the reaction of these alkylnickel complexes with alkyl halides yielded alkanes, alkenes, and coupled products. In accord with their observations, the coenzyme F_{430} catalyzed dehalogenation of CCl_4, $CHCl_3$, and CH_2Cl_2 by Ti(III) produced small amounts of ethane and significant quantities of nonvolatile, as yet, unidentified products. The formation of ethane from these halogenated hydrocarbons indicates the intermediacy of C_1 radicals. Dolbier and Burkholder (1988) have provided evidence that polyhalogenated methanes yield carbenes upon reductive dehalogenation. It is of interest to note that the reductive dehalogenation of CH_3Cl to CH_4 by coenzyme F_{430} does not generate ethane as a side product.

The reductive dehalogenations catalyzed by coenzyme F_{430} and aquocobalamin differ in two respects: (i) Coenzyme F_{430} is only able to mediate reductive dehalogenations with Ti(III) as the reductant. In contrast, the corrinoids are active catalysts with both Ti(III) and dithiothreitol as reducing agents. (ii) Coenzyme F_{430} catalyzes the reduction of CH_3Cl to CH_4 almost 50 times faster than aquocobalamin. The first difference is most likely due to the more positive redox potential of the B_{12a}/B_{12s} (Co[III]/Co[I]) couple at −0.400 V (Savéant et al., 1979). Second, the more efficient dehalogenation of CH_3Cl by coenzyme F_{430} may be the consequence of differences in the stabilities of the organometallic bonds of the nickel and cobalt complexes. Since we have been unable to detect changes in the absorption spectrum of coenzyme F_{430}, we assume that the carbon–nickel bond of the alkyl coenzyme F_{430} intermediate is more labile than the corresponding carbon–cobalt bond of the alkyl corrinoids.

It is also of interest to consider the different physiological functions of coenzyme F_{430} and the corrinoid coenzymes in the methanogens and acetogens. The former is involved in the reduction of the methyl group of methyl coenzyme M to methane, while the latter participate in methyl transfer reactions (Kräutler, 1987). A second intriguing difference between these coenzymes is that aquocobalamin, and presumably other corrinoids, are able to catalyze the reduction of disulfides with Ti(III) as the electron donor (Hedderich & Thauer, 1988), while the nickel porphinoid mediates this reaction only poorly (Figure 4).

M. barkeri contains, besides high concentrations of coenzyme F_{430} and corrinoids (Gorris & Van der Drift, 1986), also the *b* and *c* type cytochromes (Kühn & Gottschalk, 1983). Klecka and Gonsior (1984) have reported that iron porphyrins were able to catalyze the reduction of CCl_4 to $CHCl_3$ with cysteine as the electron donor; only minute amounts of CH_2Cl_2 were formed. Thus, the iron porphyrins, cobalt corrinoids, and nickel porphinoids have to be considered to be the heat-stable cell components capable of catalyzing the reductive dehalogenation of C_1 hydrocarbons in *M. barkeri.*

Carbon monoxide was found to be the most effective electron donor for the reduction of chlorinated methanes in *M. barkeri.* It has been demonstrated that the corrinoids and cytochromes in this bacterium can be reduced by CO via the carbon monoxide dehydrogenase system (Terlesky et al., 1986; Kemner et al., 1987; Terlesky & Ferry, 1988). Evidence has also been presented that in *M. barkeri* CO can be used as the electron donor for the coenzyme F_{430} dependent reduction of methyl coenzyme M to methane. These findings indicate that all three transition metals containing macrocycles could be catalysts in the in vivo reductive dehalogenation of CCl_4.

Methanogenic bacteria are the only organisms known to contain coenzyme F_{430}. They are, however, not the only anaerobes that are able to mediate reductive dehalogenation of halogenated hydrocarbons [Egli et al., 1988; Gälli & McCarthy, 1989a,b; see Krone et al. (1989) for additional references]. However, this observation does not exclude coenzyme F_{430} as a catalyst in the reductive dehalogenation in *M. barkeri* because more than one coenzyme could be involved in these reactions. Indeed, the rates of dehalogenation by this organism were found to be relatively high.

All anaerobic bacteria, including the methanogens, that are able to dehalogenate chlorinated hydrocarbons contain high levels of corrinoids. Some of these, such as *M. thermoautotrophicum* and *Acetobacterium woodii*, do not contain cytochromes (Dangel et al., 1987). In contrast, *Desulfobacter hydrogenophilus*, which does contain cytochromes (Schauder et al., 1987; Möller-Zinkhan & Thauer, 1988) but lacks corrinoids (Kräutler et al., 1988), is unable to dehalogenate chlorinated hydrocarbons.

ACKNOWLEDGMENTS

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Registry No. $CHCl_3$, 67-66-3; CH_2Cl_2, 75-09-2; CH_3Cl, 74-87-3; CH_4, 74-82-8; CCl_4, 56-23-5; coenzyme F_{430}, 73145-13-8.

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