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Reductive Dehalogenation of Chlorinated C_1-Hydrocarbons Mediated by Corrinoids†

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ABSTRACT: Corrinoids were found to catalyze the reductive dehalogenation of CCl_4 with either titanium(III) citrate or dithiothreitol as electron donors. $CHCl_3$, CH_2Cl_2, CH_3Cl, and CH_4 were detected as intermediates and/or products. In addition, several as yet unidentified nonvolatile compounds were formed. Ethane was a very minor product. The rate of dehalogenation decreased in the series CCl_4, $CHCl_3$, CH_2Cl_2, and CH_3Cl. Organocorrinoids were detected at completion of the reactions, suggesting that the dehalogenation involves the formation and reductive cleavage of alkylcorrinoids as intermediates. However, monoalkylcorrinoids cannot be the only intermediates in this process because the rate of methylcobalamin reduction to methane was much slower than the rate of methane formation from the chlorinated hydrocarbons catalyzed by either aquocobalamin or methylcobalamin. The present findings suggest that the reported slow reductive dehalogenation of CCl_4 and $CHCl_3$ by anaerobic bacteria may be catalyzed by corrinoids present in these microorganisms.

Biological dehalogenation of chlorinated hydrocarbons has been shown to occur via several mechanisms [for reviews, see Müller and Lingens (1986, 1987) and Cook et al. (1988)]. Chlorine can be removed as chloride anion by thiolysis or hydrolysis or by oxidative dehalogenation in the presence of oxygen. More recently, it has been demonstrated that in strictly anaerobic bacteria a reductive dehalogenation also occurs (Bouwer et al., 1981; Bouwer & McCarty, 1983; Vogel & McCarty, 1985; Belay & Daniels, 1987; Dolfing & Tiedje, 1987; Egli et al., 1987, 1989; Fathepure et al., 1987; Fathepure & Boyd, 1988; Bosma et al., 1988). Bouwer et al. (1981) reported that an undefined methanogenic mixed culture de-

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rived from activated sludge was able to degrade $CHCl_3$. Radiolabeled CCl_4 and $CHCl_3$ were converted to CO_2, suggesting a hydrolytic dehalogenation mechanism (Bouwer & McCarty, 1983). Subsequent work with pure cultures showed that many, but not all, anaerobic bacteria catalyze the reduction of CCl_4 to $CHCl_3$ and CH_2Cl_2: *Methanobacterium thermoautotrophicum*, *Desulfobacterium autotrophicum*, *Acetobacterium woodii*, *Clostridium thermoaceticum* (Egli et al., 1987, 1989), and *Methanosarcina barkeri* (Krone, 1988). These bacteria have in common use of the carbon monoxide dehydrogenase/acetyl-CoA pathway for either the degradation or synthesis of acetate (acetyl-CoA) [for literature on the pathway, see Wood et al. (1986), Ljungdahl (1986), Fuchs (1986), and Thauer (1988)]. They also all contain high levels of corrinoids (Pol et al., 1984; Kräutler et al., 1987, 1988; Stupperich & Kräutler, 1988; Stupperich et al., 1988). Reduced corrinoids are known to react with alkyl halides (Wood et al., 1968; Kennedy et al., 1969). It has therefore been proposed that a corrinoid could be involved in the catalysis of the dehalogenation reaction in anaerobic bacteria (Laufer et al., 1987).

Cob(I)alamin reacts with electrophilic agents such as alkyl halides to form alkylcobalamins. This is also the case for multihalogenated C_1-hydrocarbons (Wood et al., 1968; Kennedy et al., 1969). Schrauzer et al. (1972) showed that thiols and dithiols are able to cleave the carbon–cobalt bond of alkylcobalamins and alkylcobaloximes to yield alkanes. Lexa et al. (1979) and Scheffold et al. (1987) suggested that the reduction of alkylcobalamins and alkylcobinamides yields radical anionic intermediates that are unstable and undergo carbon–cobalt cleavage to generate either alkyl radicals or carbanions. Scheffold et al. (1987) pointed out that alkylcorrinoids containing electron-withdrawing groups at the alkyl carbon atom are readily reduced.

These observations prompted us to investigate the use of corrinoids such as aquocobalamin and (cyanoaquo)cobinamide[1] as catalysts in the reductive dehalogenation of chlorinated hydrocarbons.

Materials and Methods

Cyanocobalamin, hydroxocobalamin hydrochloride, methylcobalamin, and dithiothreitol were obtained from Sigma Chemical Co. Carbon tetrachloride, methylene chloride, and titanium(III) chloride were from Merck, chloroform was from Baker Chemicals, and methyl chloride, methane, and ethane were from Messer Giesheim. ^{13}C-Enriched chloroform was purchased from Icon Services, Inc. All other reagents and chemicals were commercial products of the highest purity and were used as received. (Cyanoaquo)cobinamide was prepared from cyanocobalamin as described by Friedrich and Bernhauer (1956). Titanium(III) citrate solutions were prepared as described by Zehnder and Wuhrmann (1976); the final Ti(III) concentration was 0.09 M.

Standard solutions of CCl_4 (0.062 M), $CHCl_3$ (0.074 M), and CH_2Cl_2 (0.093 M) were prepared by dissolving 0.30 mL of the chlorinated hydrocarbon 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 contain 20 nmol of the gas.

The chlorinated hydrocarbons, CH_4, and C_2H_6 were separated by using a Carlo Erba Strumentazione (GC 6000) gas chromatograph containing a column (6 mm × 1.8 m) of 80/120 carbopack B-DA/4% carbowax (Supelco). The gas chromatograph was equipped with a flame ionization detector. The conditions were as follows: column temperature, 110 °C; injector temperature, 160 °C; detector temperature, 160 °C; N_2 pressure, 1.7×10^5 Pa; H_2 pressure, 0.6×10^5 Pa; air pressure, 0.7×10^5 Pa. Under these conditions, excellent separation of the chlorinated hydrocarbons, methane, and ethane was obtained. Retention times were as follows: CH_4, 31 s; C_2H_6, 37 s; CH_3Cl, 53 s; CH_3OH, 95 s; CH_2Cl_2, 164 s; $CHCl_3$, 406 s; CCl_4, 533 s. Carbon dioxide was determined with the same gas chromatograph using a methanizer at 375 °C and a column (2 mm × 2 m) of 60/80 carbosieve B (Supelco): retention time, 270 s. Hydrogen was measured with a column (2 mm × 1.6 m) of molecular sieve 5Å (Supelco) and a hot wire detector: retention time, 45 s. The conditions for the H_2 measurements were as follows: column temperature, 100 °C; injector temperature, 50 °C; filament temperature, 250 °C; N_2 pressure, 1.3×10^5 Pa.

Acetate was determined by using acetyl CoA synthetase as described by Dorn et al. (1978), and formate was measured as outlined in *Methods of Biochemical Analysis and Food Analysis* (1986).

UV–visible spectra were recorded on a Gilford response recording spectrophotometer. The concentrations of the corrinoid solutions were determined by using the following extinction coefficients: aquocobalamin, $\epsilon_{527} = 8.5 \times 10^3$ M^{-1} cm^{-1}; hydroxocobalamin, $\epsilon_{537} = 9.5 \times 10^3$ M^{-1} cm^{-1}; methylcobalamin, $\epsilon_{528} = 7.9 \times 10^3$ M^{-1} cm^{-1}; (cyanoaquo)cobinamide, $\epsilon_{527} = 8.4 \times 10^3$ M^{-1} cm^{-1} (Friedrich, 1975).

Pulse Fourier-transform ^{13}C (62.9-MHz) NMR spectra were obtained with a Bruker WM-250 spectrometer locked to the resonance of internal ^2H_2O.

All reactions were carried out in 8-mL serum bottles closed with viton stoppers and wrapped in aluminum foil. The anaerobic reaction components were injected into the serum bottles which were first rigorously deaerated with N_2 at 1.4×10^5 Pa pressure. The reaction mixtures (1 mL) were incubated at the desired temperatures in a shaking water bath (New Brunswick Scientific). At time intervals 0.3-mL gas samples were withdrawn with a gastight syringe and analyzed by gas chromatography.

Results

Reductive Dehalogenation of Chlorinated Hydrocarbons. Aquocobalamin catalyzed the reductive dehalogenation of CCl_4, $CHCl_3$, CH_2Cl_2, and CH_3Cl by Ti(III) citrate. The results of typical experiments are presented in Figure 1. The progress curves demonstrate that the chlorinated hydrocarbons were successively dehalogenated to their lower homologues and finally to methane. Traces of ethane (<1 nmol) and of two unidentified volatile products were also formed from CCl_4, $CHCl_3$, and CH_2Cl_2. Neither methanol nor formic acid was produced in these reactions, indicating that a hydrolytic dehalogenation did not occur. Schrauzer et al. (1972) reported that the methyl carbanions formed in the reductive cleavage of methylcobinamide can react with CO_2 to form acetate. Our reaction mixtures contained sodium bicarbonate, which was used to neutralize the Ti(III) citrate solution (Zehnder & Wuhrmann, 1976). However, no acetate could be detected in our reaction mixtures. It should be noted that the recovery of the identified gaseous products is less than 50%. This low recovery is not due to loss of the products. The use of viton stoppers prevented the loss of the chlorinated hydrocarbons by interaction with the stoppers. Furthermore, in control experiments, in which zinc in 10% aqueous ammonium chloride

[1] (Cyanoaquo)cobinamide denotes a mixture of the two isomers (*Coα*-cyano)(*Coβ*-aquo)cobinamide and (*Coα*-aquo)(*Coβ*-cyano)cobinamide.

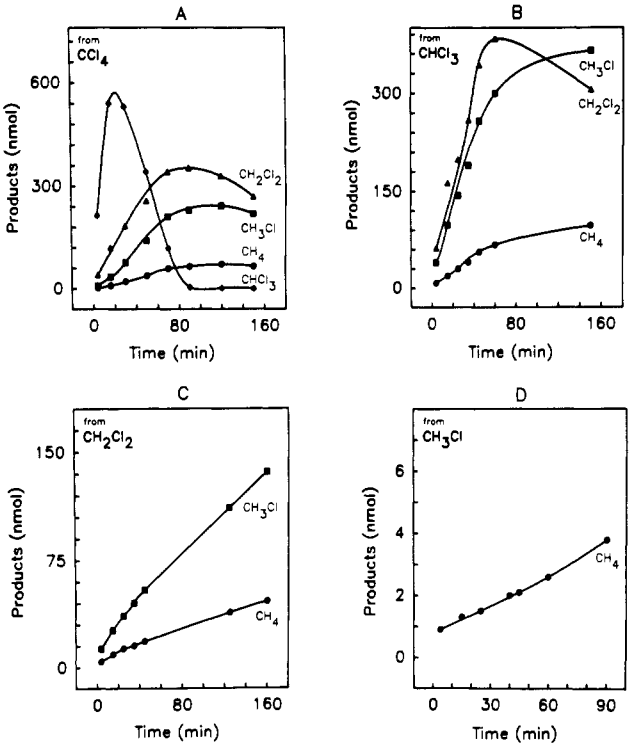


FIGURE 1: Corrinoid-catalyzed reductive dehalogenation of CCl_4 (A), $CHCl_3$ (B), CH_2Cl_2 (C), and CH_3Cl (D) with Ti(III) as electron donor. The assays were performed in the dark at 30 °C in 8-mL serum bottles with N_2 as the gas phase. The 1-mL reaction mixtures contained 2.2 μmol of chlorinated hydrocarbon, 27 μmol of Ti(III) citrate, and 46 nmol of aquocobalamin in 0.66 M Tris buffer, pH 8.2. At the indicated time intervals, 0.3-mL samples of the head space were analyzed by gas chromatography.

was used as the reducing agent, the recovery of methane derived from CCl_4 and $CHCl_3$ was quantitative. These results suggest that, in addition to the gaseous products, one or more nonvolatile compounds are formed.

The results, presented in Figure 1, show that the rate of reductive dehalogenation decreased in the series $CCl_4 > CHCl_3 > CH_2Cl_2 > CH_3Cl$. Indeed, the dehalogenation of CH_3Cl was extremely slow; only 2–3 nmol of CH_4 was formed from 2.2 μmol of CH_3Cl in 1 h. The low rate of methane formation from CH_3Cl was partially due to the low solubility of this gaseous hydrocarbon in the reaction mixture, as the rate of methane formation from CH_3I under identical reaction conditions was approximately 20 nmol/h.

As expected, the rates of dehalogenation were increased by raising the incubation temperature, by increasing the concentration of the corrinoid catalyst, and by increasing the concentration of the chlorinated hydrocarbon. The rates of CCl_4 dehalogenation increased approximately 2-fold by raising the temperature from 30 to 60 °C, by increasing the corrinoid concentration from 46 to 92 μM, and by increasing the CCl_4 concentration from 2.2 to 4.4 mM.

Of the reducing agents tested, only Ti(III) citrate and DTT[2] functioned in the reductive dehalogenation. Sodium borohydride, stannous chloride, sodium dithionite, and carbon monoxide did not serve as reducing agents. In contrast, zinc in 10% aqueous ammonium chloride was a very effective reducing system even in the absence of aquocobalamin.

The reductive dehalogenation of CCl_4 with DTT as the electron donor and aquocobalamin as the catalyst is shown in Figure 2. The progress curves demonstrate that, in the presence of 100 μmol of DTT, the dehalogenations of CCl_4 and $CHCl_3$ were fast, but the rate of the reaction slowed down considerably at the CH_2Cl_2 stage. Indeed, methyl chloride and methane production were extremely slow; only traces (3–4 nmol) were formed from 2.2 μmol of CCl_4 in a 2-h incubation.

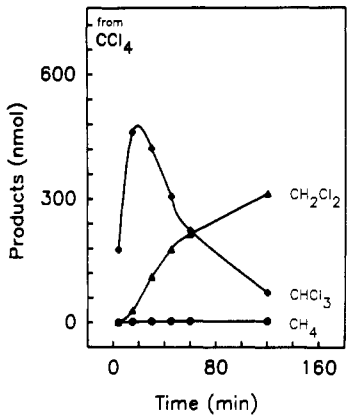


FIGURE 2: Corrinoid-catalyzed dehalogenation of CCl_4 with DTT as electron donor. The reaction conditions were as in Figure 1; 100 μmol DTT was the reducing agent. The rate of CH_4 formation was 5 nmol/120 min, and that of CH_3Cl formation only 1 nmol/120 min.

Table I: Reductive Dehalogenation of $CHCl_3$ with Ti(III) as Electron Donor Catalyzed by Different Corrinoids[a]

corrinoid present	formation (nmol/4 min) of CH_4	CH_3Cl	CH_2Cl_2
none	1	4	6
aquocobalamin	44	276	252
(cyanoaquo)cobinamide	92	448	360
methylcobalamin	44	56	68

[a] The assays were performed in the dark at 60 °C in 8-mL serum bottles with N_2 as the gas phase. The 1-mL reaction mixtures contained 2.2 μmol of $CHCl_3$, 27 μmol of Ti(III) citrate, and 110 nmol of the indicated corrinoid in 0.66 M Tris buffer, pH 7.

Aquocobalamin and also methylcobalamin and (cyanoaquo)cobinamide function as catalysts in the reductive dehalogenation (Table I). (Cyanoaquo)cobinamide was clearly the most effective catalyst, not only in the successive dehalogenation of $CHCl_3$ but also in the final reduction to CH_4.

Identification of Alkylcorrinoids as Intermediates in the Dehalogenation. In order to identify corrinoid intermediates in the reductive dehalogenation, a reaction mixture containing $CHCl_3$, (cyanoaquo)cobinamide, and a limiting amount of Ti(III) citrate in Tris buffer, pH 7.0, was incubated in an anaerobic cuvette in the dark at 60 °C for 1 h. After that time, the dark brown Ti(III) complex was completely oxidized to the colorless Ti(IV) form. The visible absorption spectrum of this reaction mixture (Figure 3A) is very similar to that of (methylaquo)cobinamide (Friedrich, 1975). Indeed, exposure of the solution to visible light generated a spectrum characteristic of diaquocobinamide, confirming the presence of a photolabile carbon–cobalt bond. An absorption spectrum of a similar reaction mixture containing an excess of DTT rather than Ti(III) citrate is shown in Figure 3B. The absorption maximum at 466.5 nm is at somewhat higher wavelength than that of (methylaquo)cobinamide (462 nm), suggesting the presence of (chloromethyl)- or (dichloromethyl)cobinamides. Exposure of this reaction mixture to light generates a spectrum characteristic of cob(II)inamide, the expected corrinoid formed in the presence of excess DTT. Direct evidence for (chloromethyl)- and (dichloromethyl)cobinamides as intermediates in the reductive dehalogenation

[2] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol.

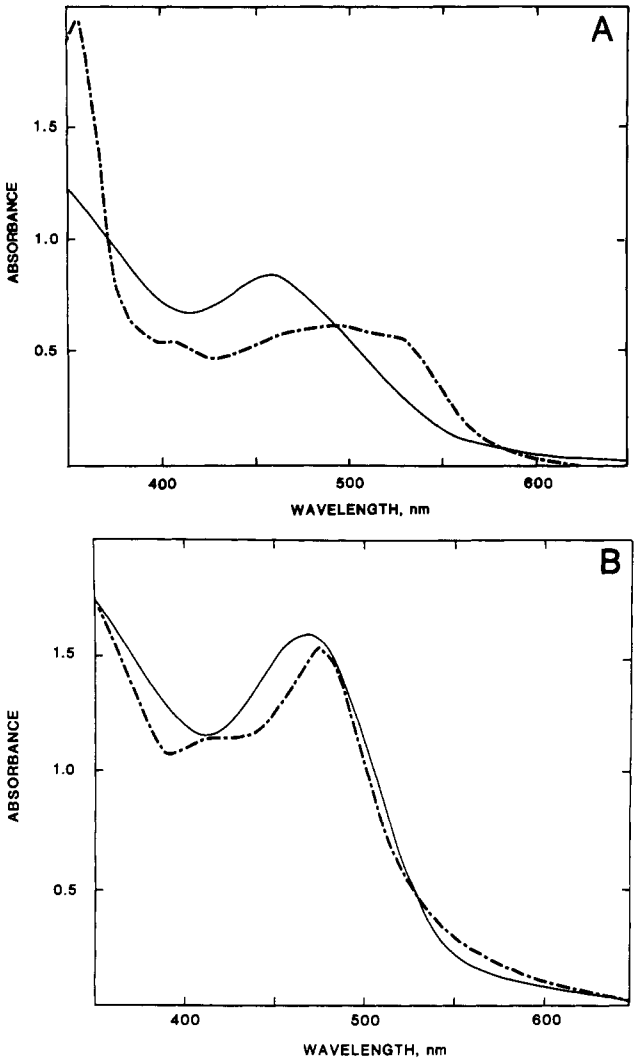


FIGURE 3: Visible absorption spectra of the alkylcorrinoid formed during the dehalogenation of $CHCl_3$ with Ti(III) citrate (A) or DTT (B) as reducing agents. The reactions were performed in the dark at 60 °C in a cuvette with N_2 as the gas phase. The 1-mL reaction mixtures contained 2.2 μmol of $CHCl_3$, 9 μmol of Ti(III) citrate or 100 μmol of DTT, and 110 nmol of (cyanoaquo)cobinamide in 0.85 M Tris buffer, pH 7.0. After an 120-min incubation, the spectra (—) were recorded; the cuvettes were then exposed to visible light to generate the second spectrum (- - -).

was obtained from experiments using ^{13}C-enriched chloroform. A reaction mixture containing 83 μmol of [^{13}C]$CHCl_3$, 540 μmol of Ti(III) citrate, and 2.4 μmol of (cyanoaquo)cobinamide was incubated under anaerobic conditions in the dark at 50 °C for 2 h. The corrinoids were then isolated by the usual phenol extraction procedure. A proton-decoupled ^{13}C NMR spectrum of the corrinoids dissolved in ^2H_2O showed three resonances at −0.05, 20.4, and 38.1 ppm. The resonance at −0.05 ppm corresponds to the Co–methyl moiety of (methylaquo)cobinamide; the other two resonances can be assigned to the (chloromethyl)- and (dichloromethyl)cobinamides, respectively.

Effect of pH on the Reductive Dehalogenation by Ti(III) Citrate. The reductive dehalogenation of CCl_4 to CH_4 by Ti(III) citrate in the presence of (cyanoaquo)cobinamide showed a strong pH dependence (Figure 4). The rate of methane formation from CCl_4 was slow at acidic pH; however, at alkaline pH the rate of methane formation increased with increasing pH. The rates of the successive dehalogenation of CCl_4 had a similar pH profile. At pH 10 the dehalogenation of CCl_4 to $CHCl_3$ occurred even in the absence of the corrinoid

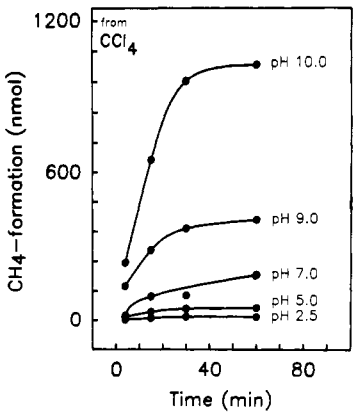


FIGURE 4: Effect of pH on the corrinoid-catalyzed reductive dehalogenation of CCl_4 to CH_4 with Ti(III) as electron donor. The assays were performed in the dark at 60 °C in 8-mL serum bottles with N_2 as the gas phase. The 1-mL reaction mixtures contained 2.2 μmol of CCl_4, 27 μmol of Ti(III) citrate, and 110 nmol of (cyanoaquo)-cobinamide in 0.66 M buffer. The buffers used were glycine buffer for pH 2.5 and 9.0, acetate buffer for pH 5.0, Tris buffer for pH 7.0, and ethylamine buffer for pH 10.0. At the indicated time intervals, 0.3-mL samples of the head space were analyzed by gas chromatography.

Table II: Methylcobalamin-Catalyzed Reductive Dehalogenation of CCl_4, $CHCl_3$, CH_2Cl_2, and CH_3Cl to Methane with Ti(III) as Electron Donor[a]

assays	formation of CH_4 (nmol/15 min)
methylcobalamin alone	2
+CH_3Cl	2
+CH_2Cl_2	7
+$CHCl_3$	16
+CCl_4	36

[a] The assays were performed in the dark at 60 °C in 8-mL serum bottles with N_2 as the gas phase. The 1-mL reaction mixtures contained 2 μmol of chlorinated hydrocarbon, 27 μmol of Ti(III) citrate, and 110 nmol of methylcobalamin in 0.66 M Tris buffer, pH 7.

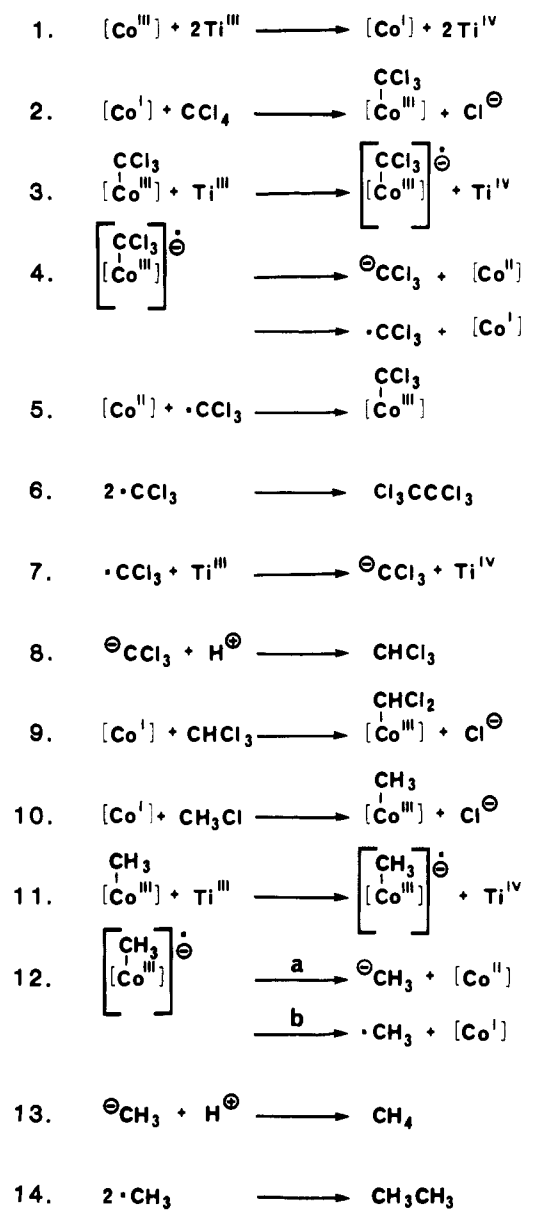
catalyst. However, the rate was only approximately 10% of that obtained in the presence of (cyanoaquo)cobinamide. It is interesting to note that, at pH 2.5 (Figure 4), CCl_4 (2.2 μmol) rapidly disappeared when incubated with Ti(III) citrate and (cyanoaquo)cobinamide. However, only small amounts of $CHCl_3$ (5 nmol), CH_2Cl_2 (96 nmol), CH_3Cl (184 nmol), and CH_4 (16 nmol) were detected after a 60-min incubation at 60 °C. These results suggest that at low pH mainly nonvolatile products are formed.

Reductive Dehalogenation Catalyzed by Methylcobalamin. Several years ago, Schrauzer et al. (1972) demonstrated that the carbon–cobalt bond of alkylcobaloximes and alkylcobalamins can be cleaved reductively by thiols and dithiols. In accord with their observations, we find that methylcobalamin is also reduced to methane by Ti(III) citrate. However, as shown in Table II, the rates of methane formation from CCl_4, $CHCl_3$, and CH_2Cl_2 with methylcobalamin as the catalyst are much higher than the rate of methane formation from methylcobalamin alone. These observations demonstrate that methylcobalamin cannot be the only intermediate in the reductive dehalogenation of these chlorinated hydrocarbons by Ti(III) citrate.

Discussion

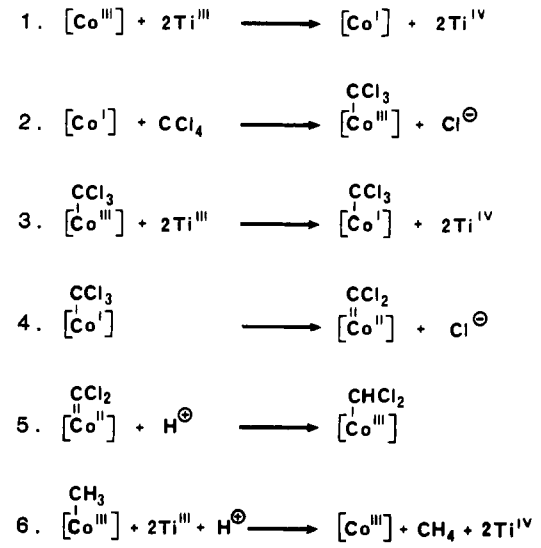
The results presented here demonstrate that corrinoids such as aquocobalamin, methylcobalamin, and (cyanoaquo)cobinamide are effective catalysts in the reductive dehalogenation of chlorinated hydrocarbons by Ti(III) citrate or DTT. Our results show that CCl_4, $CHCl_3$, CH_2Cl_2, and CH_3Cl are

Scheme I



successively reduced to their lower homologues and finally to methane. The identification of methyl- or (chloromethyl)-corrinoids as intermediates in the dehalogenation suggests a reaction mechanism outlined in Scheme I. The initial reaction involves the two-electron reduction of the Co(III) corrinoids to the strong nucleophilic Co(I) form. Reaction of the latter with CCl_4 yields a (trichloromethyl)corrinoid. Further one-electron reduction of this (trichloromethyl)corrinoid yields an unstable radical anionic species that undergoes either homolytic or heterolytic cleavage of the carbon–cobalt bond. Heterolytic cleavage would generate a trichloromethyl anion and a Co(II) corrinoid, while homolytic cleavage would yield a trichloromethyl radical and a Co(I) corrinoid. Scheffold et al. (1987) have pointed out that alkylcobinamides with strong electron-withdrawing groups such as (trichloromethyl)cobinamide, should be readily reduced to their corresponding radical anions. The trichloromethyl anions formed in the heterocyclic cleavage react with the aqueous solvent to generate $CHCl_3$, and the trichloromethyl radicals could either couple to hexachloroethane, react with Co(II) corrinoid to re-form the (trichloromethyl)corrinoid, or undergo a one-electron reduction to the trichloromethyl anion.

Scheme II



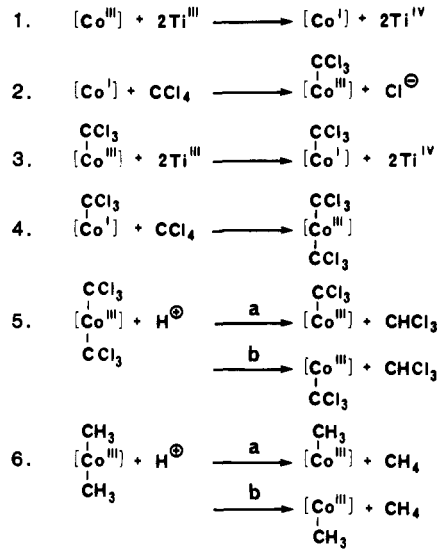
This series of reactions yields $CHCl_3$ and some less volatile compounds derived from the radical coupling. The sequence is repeated with $CHCl_3$, which is reduced to CH_2Cl_2, and finally with CH_3Cl, which reacts with the Co(I) corrinoid to form a methylcorrinoid. Reduction of this methylcorrinoid to the radical anion and its decomposition yield a methyl anion and a methyl radical. The methyl anion produces methane upon reaction with the solvent, and the radicals combine to give ethane. Under our reaction conditions, only traces of ethane are detected, suggesting that radical coupling is not important in CH_3Cl reduction.

A second reaction mechanism, which also involves the intermediacy of (chloromethyl)- and methylcorrinoids, is outlined in Scheme II. Such a mechanism has been postulated by Ricroch et al. (1971), who found that (trifluoromethyl)cobaloxime was dehalogenated by $NaBH_4$ to methylcobaloxime, presumably via the difluoromethyl and monofluoromethyl derivatives. They also showed that during these reactions the carbon–cobalt bond was not cleaved and that (trideuteriomethyl)cobaloxime was formed when the reduction was carried out in a deuterated solvent (Brown et al., 1984). In this reaction scheme, the (trichloromethyl)corrinoid is reduced by two electrons (rather than by one electron) to the Co(I) (trichloromethyl)corrinoid. This Co(I) corrinoid loses one of the chlorine atoms as a chloride anion, yielding a Co(II) (dichloromethyl)corrinoid, which protonates to the Co(III) (dichloromethyl)corrinoid. This series of reactions is repeated until all the chlorine atoms are removed and a methylcorrinoid is formed. In the final reaction, this methylcorrinoid is reduced to methane.

It should be noted that, in these two reaction schemes, a Co methylcorrinoid is the penultimate intermediate of methane formation. However, our observations presented in Table II indicate that the rate of methane formation from methylcobalamin is much lower than the rate of methane formation from the chlorinated hydrocarbons catalyzed by methylcobalamin. Thus, these results clearly preclude methylcobalamin as the only intermediate in these reactions.

An alternative series of reactions that can account for our observations are outlined in Scheme III. The initial reactions in this series also involve a two-electron reduction of the Co(III) corrinoid by Ti(III) citrate or DTT, the formation of a (trichloromethyl)corrinoid, and an additional two-electron reduction of this Co(III) (trichloromethyl)corrinoid to the Co(I) form. Subsequent reaction of this nucleophilic species

Scheme III



with CCl_4 generates a Co(III) bis(trichloromethyl)corrinoid, which reacts with protons of the solvent to yield $CHCl_3$ and a (trichloromethyl)corrinoid. This sequence of reactions is repeated with $CHCl_3$, CH_2Cl_2, and finally CH_3Cl. The final reaction cycle involves the two-electron reduction of methylcorrinoid, reaction with CH_3Cl to generate Co(III) dimethylcorrinoid, and its reaction with the solvent to yield CH_4.

Thus far, dialkyl derivatives of corrinoids have not yet been detected. However, Costa et al. (1969, 1971) and Farmery and Bush (1970) have reported the preparation of *trans*-dialkyl–cobalt(III) complexes of several model compounds of vitamin B_{12}. [For a review of these complexes, see Witman and Weber (1977).] For instance, Costa and co-workers prepared the dimethyl derivative of [*N*,*N*′-trimethylenebis(biacetyl oxime imine)]cobalt(III) and found that this complex reacts with protons to give CH_4. Farmery and Bush (1970) reported that reduction of a similar vitamin B_{12} model compound, (2,12-dimethyl-3,7,11,17-tetraazabicyclo[11.3.1]heptadeca-1(17),2,11,13,15-pentaene)methylcobalt by $NaBH_4$ in methanol or by sodium amalgam in acetonitrile gave a methyl–cobalt(I) complex, which was stable under anaerobic conditions. Addition of an alkyl halide, such as CH_3Cl, resulted in the oxidative alkylation and produced the *trans*-dimethyl complex.

It is surprising that $NaBH_4$, stannous chloride, and sodium dithionite did not serve as reducing agents in the reductive dehalogenation of CCl_4. Of the reducing systems tested, only Ti(III) citrate and DTT were effective in the dehalogenation reactions.

As shown in Figure 1, the rates of reductive dehalogenation by Ti(III) citrate decreased in the series $CCl_4 > CHCl_3 > CH_2Cl_2 > CH_3Cl$. These results indicate that the methylcorrinoid intermediates carrying three electron-withdrawing chlorine atoms are most readily reduced. The electrophilic character of the cobalt atom decreases as these chlorine atoms are removed.

The pronounced effect of pH on the rate of dehalogenation (Figure 4) is puzzling because the redox potential of the simple Ti(III)–Ti(IV) couple should not be affected by pH (Zehnder & Wuhrmann, 1976). However, Strubl (1938) reported that, in the presence of citric acid, the half-wave potential of the redox couple lies at −480 mV only in acidic or neutral solutions. At alkaline pH, the redox behavior became anomalous, with the cathodic wave shifting to more negative potential. Furthermore, Latimer (1956) formulated the redox reaction as $H_2O + Ti^{3+} = TiO^{2+} + 2H^+ + e^-$; this reaction involving protons would certainly be affected by the pH.

Our observation that corrinoids are able to catalyze the reductive dehalogenation of chlorinated hydrocarbons by reducing agents suggests that the reported dehalogenation of CCl_4 and $CHCl_3$ by anaerobic bacteria may be catalyzed by the corrinoids or corrinoid enzymes present in these organisms.

Registry No. DTT, 3483-12-3; $CHCl_3$, 67-66-3; CH_2Cl_2, 75-09-2; CH_3Cl, 74-87-3; CH_4, 74-82-8; CCl_4, 56-23-5; Ti, 7440-32-6; ethane, 74-84-0; aquocobalamin, 13422-52-1; (cyanoaquo)cobinamide, 13963-62-7; methylcobalamin, 13422-55-4.

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Energetics of Complementary Side-Chain Packing in a Protein Hydrophobic Core†

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ABSTRACT: The energetics of complementary packing of nonpolar side chains in the hydrophobic core of a protein were analyzed by protein engineering experiments. We have made the mutations Ile → Val, Ile → Ala, and Leu → Ala in a region of the small bacterial ribonuclease barnase where the major α-helix packs onto the central β-sheet. The destabilization resulting from the creation of cavities was determined by measuring the decrease in free energy of folding from reversible denaturation induced by urea, guanidinium chloride, or heat. The different methods give consistent and reproducible results. The loss in free energy of folding for the mutant proteins is 1.0–1.6 kcal/mol per methylene group removed. This exceeds by severalfold the values obtained from model experiments of the partitioning of relevant side chains between aqueous and nonpolar solvents. Much of this discrepancy arises because two surfaces are buried when a protein folds—both the amino acid side chain in question and the portions of the protein into which it packs. These experiments directly demonstrate that the interior packing of a protein is crucial in stabilizing its three-dimensional structure: the conversion of leucine or isoleucine to alanine in the hydrophobic core loses half the net free energy of folding of barnase with a concomitant decrease in yield of the expressed recombinant protein.

It is of fundamental importance to understand the laws that govern the three-dimensional conformations adopted by proteins. This knowledge will enable the design of novel proteins, the rational alteration of existing proteins, and the deduction of the tertiary structure of proteins from their primary structure. In addition, understanding the precise molecular basis of protein structure–function relationships, such as ligand binding, conformational change, and protein–protein interactions, depends on this knowledge. These goals are far from realization, although progress toward solving the problem of protein folding is being made rapidly from a variety of approaches. Examples of theoretical approaches are the phenomenological, which range from the classification of single amino acids by their tendency to be found in a given type of secondary structure (Chou & Fasman, 1974; Garnier et al., 1978) to the classification of combinations of secondary structural motifs which constitute overall tertiary folding patterns (Levitt & Chothia, 1976; Janin & Chothia, 1980; Sternberg, 1983; Chothia, 1984), and the ab initio and computational methods (Nemethy et al., 1983; Weiner et al., 1984). There are, however, two apparently overwhelming barriers to calculating the conformation of a protein with the lowest free energy. The free energy of folding is the difference in the free energies of the folded and unfolded states and for smaller proteins is generally only 5–20 kcal/mol. As the noncovalent interaction energies in each state are some 10^3 kcal/mol or so, calculation of free energies of folding requires an accuracy of better than ±0.1–1%, which is far beyond the precision of present energy functions. Further, although the structures of many folded proteins are known to high resolution, structures of unfolded proteins are unknown, and so one of the states involved in the calculations is ill-defined.

The advent of protein engineering has provided a direct experimental attack on the protein folding problem by allowing the systematic production and analysis of mutant proteins. The initial applications are typified by the studies on staphylococcal nuclease (Shortle & Meeker, 1986) and bacteriophage T4 lysozyme (Alber et al., 1987). Mutant proteins differing in thermal stability were selected after spontaneous or random mutagenesis and then subjected to thermodynamic and

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