

# Redox chemistry of cobalamin and iron-sulfur cofactors in the tetrachloroethene reductase of *Dehalobacter restrictus*

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**Abstract** Respiration of *Dehalobacter restrictus* is based on reductive dechlorination of tetrachloroethene. The terminal component of the respiratory chain is the membrane-bound tetrachloroethene reductase. The metal prosthetic groups of the purified enzyme have been studied by optical and EPR spectroscopy. The 60-kDa monomer contains one cobalamin with  $E_m(\text{Co}^{1+/2+}) = -350$  mV and  $E_m(\text{Co}^{2+/3+}) > 150$  mV and two electron-transferring  $[\text{4Fe-4S}]^{(2+;1+)}$  clusters with rather low redox potentials of  $E_m \approx -480$  mV. The cob(II)alamin is present in the base-off configuration. A completely reduced enzyme sample reacted very rapidly with tetrachloroethene yielding base-off cob(II)alamin rather than trichlorovinyl-cob(III)alamin.

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**Key words:** Tetrachloroethene reductase; Reductive dechlorination; Cobalamin; Iron-sulfur; *Dehalobacter restrictus*

## 1. Introduction

The xenobiotic solvent tetrachloroethene (or: perchloroethene, PCE) belongs to the most frequently detected halogenated contaminants in the environment. Under anaerobic conditions it is reductively dechlorinated. Recently, two anaerobic bacterial strains, *Dehalobacter restrictus* [1,2], and *Dehalospirillum multivorans* [3], were isolated which grow by oxidation of  $\text{H}_2$  coupled to reductive dechlorination of PCE to cis-dichloroethene and chloride. A chemiosmotic mechanism of energy conservation with PCE as terminal electron acceptor was demonstrated for *D. restrictus* [2]. The PCE reductase, the terminal component of the respiratory chain of *D. restrictus*, is located in the cytoplasmic membrane, facing to the cytoplasm. The photoreversible inactivation of PCE reduction by 1-iodopropane indicated the involvement of a Co(I)-cobamide in reductive dechlorination catalyzed by *D. multivorans* [4] and *D. restrictus* [2]. Recently, a PCE reductive dehalogenase from *D. multivorans* was isolated as a soluble cytoplasmic protein [5]. It was shown to be a corrinoid iron-sulfur protein. Cob(I)alamin was suggested to be the active site. Reductive dechlorination of PCE was also shown to be mediated by free cobalamin in homogeneous aqueous solution with the low potential electron donor Ti(III) citrate [6,7]. The latter study suggested that the first step during reductive dechlorination of PCE is a dissociative single electron transfer.

The catalytic mechanism of enzymatic reductive dechlorination of PCE is not known. It is necessary for the development

of a mechanistic model to determine the structure and the redox potentials of the redox active cofactors. Here, we report on the optical and EPR spectroscopic and redox equilibrium properties of the transition metal cofactors of the PCE reductase from *D. restrictus*.

## 2. Materials and methods

### 2.1. Cultivation and protein purification

*Dehalobacter restrictus* (DSMZ 9455<sup>T</sup>) was cultivated with PCE as electron acceptor and  $\text{H}_2$  as electron donor in a two-liquid-phase system as previously described [2]. All subsequent steps were carried out at 4°C under exclusion of oxygen. Cell harvest was done with a Padberg continuous centrifuge (40 000 rpm, 25 l/h, 4°C). Washed cells (400 g wet weight  $\text{l}^{-1}$ ) in 25 mM Tris-HCl buffer, pH 7.8, were broken by ultrasonication as previously described [2]. The resulting crude extract was incubated (15 min, 20°C) in the presence of 0.5 M KCl and 0.1% (w/v) octyl-D-glucopyranoside and subsequently fractionated by centrifugation (200 000 × g, 1 h, 4°C). The pellet was resuspended in 25 mM Tris buffer, pH 8, and extracted (30 min, 20°C) by addition of Triton X-100 to a final concentration of 1.2% (w/v). After centrifugation (200 000 × g, 1 h, 4°C) the membrane extract (supernatant) was loaded on a Q-Sepharose column (2.6 × 8 cm) connected to a Jasco HPLC system and eluted (2 ml/min) with 300 ml of a linear NaCl gradient (0–0.35 M) in 25 mM Tris buffer, pH 8, 0.1% Triton X-100. The purity of the enzyme was followed by SDS-PAGE after silver staining and photometric activity measurement with methyl viologen as previously described [2]. Protein was determined with bicinchoninic acid [8]. Cobamide extraction from the as isolated enzyme and reversed phase HPLC analysis was done as in [9]. Identically treated cyanocobalamin (vitamin B<sub>12</sub>, Sigma) was the standard. Optical absorption spectra were recorded on a Hitachi U-2000 spectrophotometer using 1-cm quartz cuvettes.

### 2.2. Titrations and spectroscopy

The enzyme was redox-titrated at 22°C in the presence of a mixture of 13 dye mediators as previously described [10,11]. The potential was measured at a platinum wire versus an Ag/AgCl reference electrode. All potentials reported are versus NHE, i.e. obtained by adding +195 mV to the measured potential. By substoichiometric addition of sodium dithionite the protein was stepwise reduced, and EPR samples were drawn at redox equilibrium. The protein concentration was typically 4 mg/ml in 25 mM Tris buffer, pH 8 and each mediator was present in a concentration of 35 μM. Complete reduction was obtained by the method of Massey and Hemmerich [12] by light irradiation of an EPR sample to which 10 μM 5-deazaflavin and 2 mM EDTA was added. The light was from a 150 W tungsten lamp and was directed onto the EPR tube through glass fiber optics.

EPR spectroscopy was carried out on a Bruker 200 D spectrometer equipped with cryogenics, peripheral equipment, and data acquisition/manipulation facilities as described previously [11]. Cobalt(II) spectra were simulated with our FORTRAN PC-program 'KOPER' (cf. [13–15]), which was recently used for the simulation of cobalamin spectra from a methyltransferase [16]. Under the assumption of tensor colinearity the program incorporates second-order metal hyperfine interaction, first-order ligand hyperfine interaction, linewidth varying with metal nuclear orientation. Iron-sulfur spectra were simulated with our FORTRAN PC-program 'G4', which is a generator for multi component, effective  $S = 1/2$  spectra broadened by  $g$ -strain [17–19].

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### 3. Results

#### 3.1. Protein analytical data

The PCE reductase activity of the membrane fraction was anaerobically purified by anion exchange chromatography to 98% electrophoretic homogeneity (silver stained for proteins; not shown) in a large scale procedure. The enzyme migrated under denaturing conditions in SDS-PAGE with an apparent molecular mass of 60 kDa. The specific activity was determined to be  $11.9 \mu\text{mol Cl}^- \text{ produced mg}^{-1} \text{ min}^{-1}$  in the methyl viologen linked assay [2]. The electronic spectrum of the as isolated enzyme (Fig. 1) exhibited a broad absorbance between 400 and 500 nm. The absorbance maximum at 412 nm, as identified by a relative minimum in the second derivative of the spectrum, is indicative for iron-sulfur proteins of the  $[4\text{Fe-4S}]^{(2+;1+)}$  type. Cyanolysis of the PCE reductase and subsequent analytical reversed phase HPLC of the extract revealed the presence of a cobamide in the enzyme. The relative retention time of the cobamide during HPLC as well as the absorbance maxima at 279, 361, 520 and 550 nm (inset Fig. 1) were identical to purchased 5,6-dimethylbenzimidazole cobamide (cobalamin, vitamin B<sub>12</sub>) that was treated analogously. A stoichiometry of  $0.59 \pm 0.02$  cobalt atoms per 60-kDa protein was determined for three different preparations by means of inductively coupled plasma mass spectrometry.

#### 3.2. Cobalt EPR spectroscopy

When the as isolated PCE reductase buffered at pH 8.0 was poised at a redox potential of  $\approx 0$  V, a single  $S=1/2$  EPR signal was observed from low-spin Co(II),  $3d^7$ , with hyperfine splitting from the  $I=7/2$  nucleus of  $^{59}\text{Co}$  (Fig. 2A). A simulation of the spectrum is given in Fig. 2B, and the simulation parameters are listed in Table 1. The spectrum is characteristic for the base-off form of a cobaltous corrinoid with its well resolved central hyperfine splitting both in the  $xy$ - and the  $z$ -direction, and with its sharp lines in the  $z$ -direction unsplit by ligand hyperfine interaction [16,20,21]. Quantification versus a Cu(II) standard gave  $0.9 S=1/2$  per cobalt atom.

Reduction of the protein with excess dithionite resulted in the disappearance of the cobalt signal, consistent with a reduction to the Co(I) state, concomitant with the appearance of an Fe/S signal (see below). Oxidation by incubation for 5 min at ambient temperature with excess of the oxidant di-

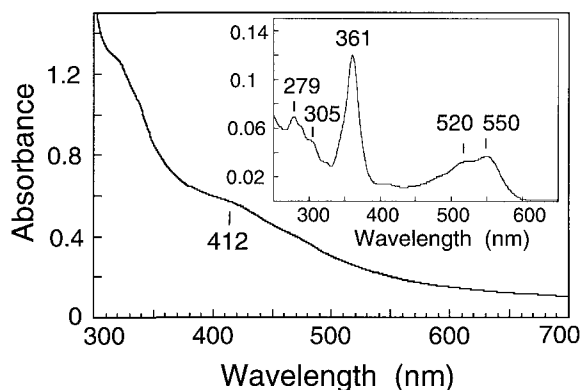


Fig. 1. Optical absorption spectrum of the as isolated *D. restrictus* tetrachloroethene reductase, 0.5 mg/ml, in 25 mM Tris buffer, 0.1% Triton X-100, pH 8.0 and of the cob(III)amide extracted from the enzyme (inset). The cobamide was extracted by cyanolysis and subsequently purified by HPLC.

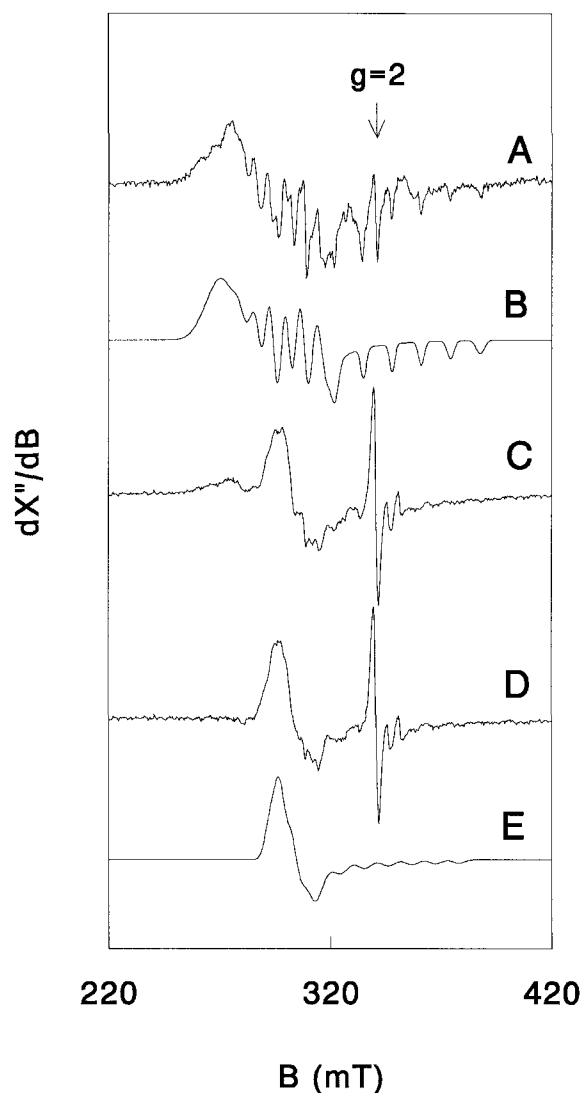


Fig. 2. Cob(II)alamin EPR spectra of *D. restrictus* tetrachloroethene reductase as a function of pH. Trace A, spectrum of 4 mg/ml enzyme in 25 mM Tris-HCl buffer, pH 8.0, poised at a redox potential of  $-27$  mV. Trace B, simulation of A (see Table 1 for parameters). Trace C, spectrum of enzyme in 125 mM Ches buffer, pH 9.6, poised at redox potential of  $-293$  mV. Trace D, difference spectrum of C minus A. Trace E, simulation of D. EPR conditions: microwave frequency, 9416 MHz; microwave power, 1.3 mW; modulation frequency, 100 kHz; modulation amplitude, 1 mT; temperature, 20 K. The radical signals at  $g=2$  are from redox mediators.

chlorophenol indophenole (DCIP,  $E_{m,8} = +150$  mV at  $30^\circ\text{C}$  [22]) did not significantly affect the cobalt signal indicating a high  $E_m$  for the oxidation to Co(III). DCIP-oxidation also resulted in the appearance of a near-isotropic signal peaking at  $g=2.015$  typical for  $[3\text{Fe-4S}]^{1+}$  (not shown). This latter signal is of low intensity,  $0.06 S=1/2$  per 60 kDa, and is presumably a minor breakdown product.

When the pH was raised to 9.6 with Ches buffer, the cobalt signal of samples poised at  $E_{m,9.6} = 0$  V remained unchanged in shape but decreased in amplitude. Upon lowering the potential to  $-0.3$  V a second cobalt signal was observed on top of the original signal (Fig. 2C). Subtracting the base-off signal from this complex spectrum resulted in the difference spectrum of Fig. 2D. Quantification of both the subtracted spectrum and the resulting difference spectrum indicates approx-

imately equal spin concentration for the two forms of Co(II). A simulation of the spectrum from the second Co species is given in Fig. 2E. The spectral parameters are characteristic for cobalamin in the base-on configuration: a significant contribution from nitrogen superhyperfine interaction in the *z*-direction and a highly anisotropic Co hyperfine interaction.

### 3.3. Iron-sulfur EPR spectroscopy

When the protein was titrated to potentials below  $-0.3$  V a signal appeared that is typical for  $[4\text{Fe-4S}]^{1+}$ : one *g*-value greater than  $g_e = 2.00$  and two *g*-values around  $g \approx 1.9$ ; onset of relaxational broadening upon heating was observed at  $T \approx 25$  K. Fig. 3A is the spectrum of a sample poised at a potential of  $-0.32$  V. In addition to the isotropic signal from mediator radicals the powder derivative spectrum exhibits more than three extrema (peaks and derivative features). This indicates that the spectrum is not from a single component. When the protein was reduced with excess dithionite at high pH the spectrum of Fig. 3B was obtained. The powder shape is significantly different from the spectrum in Fig. 3A. It is characteristic for 8 Fe ferredoxins, i.e. two  $S = 1/2$   $[4\text{Fe-4S}]^{1+}$  cubanes at a distance of approximately 1 nm in mutual dipolar interaction [23,24].

However, spin quantification afforded only approximately one spin per protein molecule. This indicates that the two cubanes are only partially reduced. Further reduction of the clusters with the stronger reductant titanium citrate (cf. [14]) was not informative because the Ti(III),  $3d^1$ , spectrum completely obscured the iron-sulfur spectrum. When the protein was further reduced with the very strong reductant system provided by light illumination of deazaflavin/EDTA, the spectrum of Fig. 3C was obtained. Comparison of the integrated intensity to that of the cob(II)alamin signal in oxidized enzyme, above, gave a  $[4\text{Fe-4S}]$  to Co ratio of 2 to 0.8. Prolonged illumination did not lead to a further increase in signal amplitude.

The integrated intensity of trace A in Fig. 3 is only 16% of that of trace C. This means that the statistical chance for an enzyme molecule in this preparation to have both cubanes reduced is maximally  $(0.16)^2$ , namely, when the two reduction

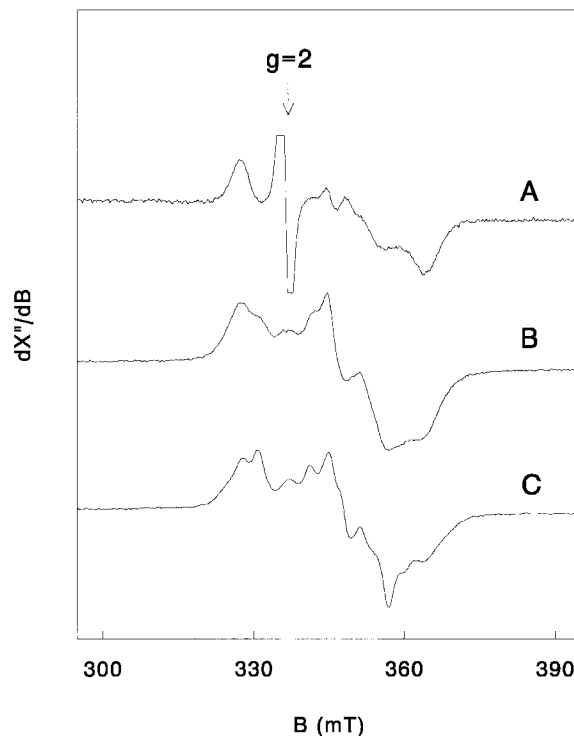


Fig. 3. EPR spectra of reduced iron-sulfur cubanes in *D. restrictus* tetrachloroethene reductase as a function of redox potential. Trace A, spectrum of 4 mg/ml enzyme in 25 mM Tris-HCl buffer, pH 8.0, poised at a potential of  $-319$  mV. Trace B, enzyme in 100 mM Ches buffer, pH 9.6, reduced by incubation for 10 min at ambient temperature with 20 mM sodium dithionite (estimated end potential  $\approx -0.55$  V). Trace C, enzyme in Tris-HCl buffer, pH 8.0, plus 10  $\mu\text{M}$  deazaflavin and 2 mM EDTA after light irradiation for 5 min (estimated end potential  $\approx -0.7$  V). EPR conditions: microwave frequency, 9.41 GHz; microwave power, 1.3 mW; modulation frequency, 100 kHz; modulation amplitude, 1 mT; temperature, 14 K.

potentials are equal. Therefore, the spectrum in Fig. 3A is dominated by the 'pure' spectra of non-interacting cubanes from enzyme molecules in which only either one of the cubanes is reduced. This conclusion forms the premise for the analysis presented in Fig. 4cf in which the spectrum was deconvoluted by simulation as a sum of two *g*-strain broadened  $S = 1/2$  spectra. The resulting spectral parameters are reported in Table 1. The ratio of the two component spectra indicated that the cubane-2 with the sharp spectrum of Fig. 4D has a significantly lower reduction potential.

### 3.4. Redox titrations and substrate effect

The valency of the prosthetic groups as a function of the applied potential was determined by monitoring the EPR signals of the cob(II)alamin and the  $[4\text{Fe-4S}]^{1+}$  in reductive titrations with dithionite. The results of titrations carried out in Tris-HCl buffer, pH 8.0, and Ches buffer, pH 9.6, are presented in Fig. 5. All data have been fit to single-electron Nernstian processes. The base-off cobalamin signal showed maximal amplitude from the highest potential downwards, and the cobalt reduced to the Co(I) state at a relatively high potential of  $-350 \pm 20$  mV. This redox potential may have a slight pH-dependence, however, much more protein and experimenting would be required to quantitatively establish this dependence.

Table 1  
Simulation parameters of EPR signals from *D. restrictus* tetrachloroethene reductase

	x-value	y-value	z-value
Co(II)amide base-off			
g-value	2.305	2.305	1.968
$A_{\text{Co}}$ (mT)	6.5	6.5	12.9
$W_0$ (mT)	9.5	9.5	4.0
B	-0.07	-0.07	-0.03
C	0.025	0.025	0.03
Co(II)amide base-on			
g-value	2.24	2.18	1.98
$A_{\text{Co}}$ (mT)	1.0	1.0	10.5
$A_{\text{N}}$ (mT)	0	0	1.8
$W$ (mT)	3.5	3.5	3.5
Cubane-1			
g-value	1.846	1.908	2.056
$\Delta$	0.016	0.020	0.016
Cubane-2			
g-value	1.919	1.946	2.052
$\Delta$	0.006	0.005	0.006

$W$  is the width of a Gaussian in field space with  $W = W_0 + Bm_1 + Cm_1^2$  (cf. [13–16]);  $\Delta$  is the width of a Gaussian in frequency space (cf. [17–19]).

A base-on form of the cob(II)alamin was found by EPR at pH 9.6 but not at pH 8.0. The Co(I/II) reduction potential for the two forms was not significantly different, however, the Co(II/III) potential for the base-on form was much lower than for the base-off form:  $E_{m,9.6} = -240 \pm 20$  mV. The two potentials for the two subsequent one-electron reduction steps of the base-on form are too close for the intermediate Co(II) form to reach 100% intensity (cf. Fig. 5A). Since quantification at  $E = -0.3$  V indicated the spectra of the two forms to have equal intensity, the base-on form must be the dominant configuration at pH 9.6. In mediated titrations of the cubane clusters it proved impossible to get full development of the EPR intensity compared to that found with light/deazaflavin reduction. The potential for the (1+/2+)-transition was estimated to be  $-480$  mV (Fig. 5B). Again, there may be a slight pH dependence of the  $E_m$  but the present data set is insufficient to determine this dependence. Due to the incomplete titration curve no significant difference in  $E_m$  for the two cubanes was identified although the plotted intensity is that of integrated spectra.

All prosthetic groups in the enzyme were fully reduced by 5 min illumination with light in the presence of deazaflavin/EDTA. When the substrate PCE was anaerobically added and hand-mixed following by quick freezing in liquid nitrogen (i.e. dead time  $\approx 15$  s), the EPR signal of the base-off cob(II)-alamin was fully developed and EPR of the cubane was absent. Also, no radical signal was observed.

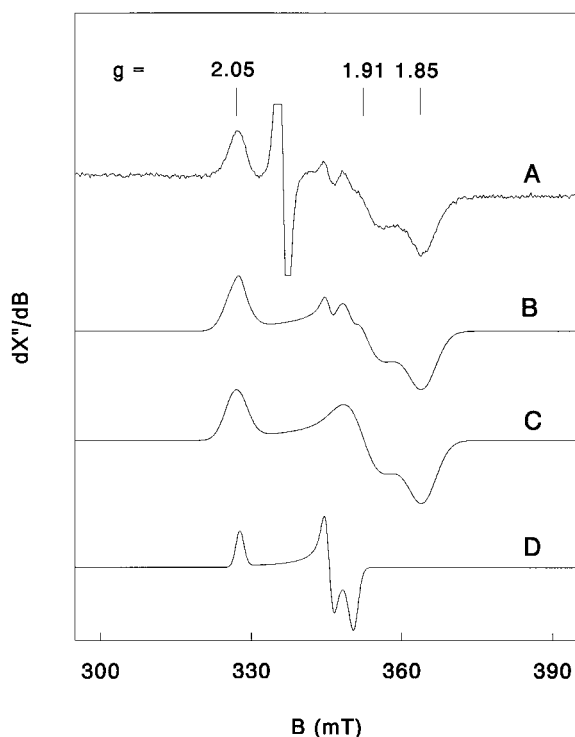


Fig. 4. Analysis by simulation of the two-cubane EPR from *D. restrictus* tetrachloroethene reductase. Trace A (identical to trace A of Fig. 3) is obtained at the onset of reduction when dipolar interaction between cubanes is still insignificant. Trace B is a simulation of A assuming two  $S = 1/2$  systems, broadened by colinear  $g$ -strain, in a ratio (integrated intensity) of 20 to 1. Traces C and D are the individual spectral components used to construct the sum-simulation in trace B (see Table 1 for parameters).

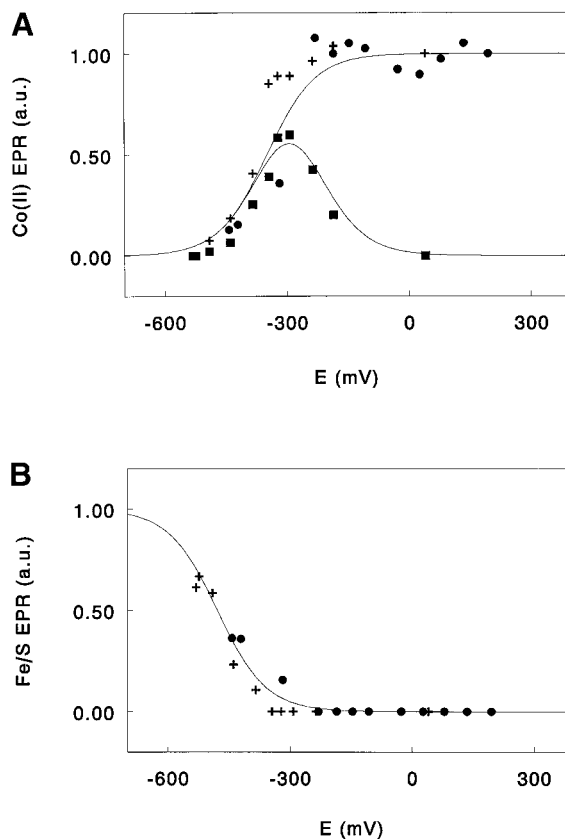


Fig. 5. Mediated redox titrations of tetrachloroethene reductase from *D. restrictus*. Relative intensities of EPR signals from 4 mg/ml enzyme have been plotted as a function of the equilibrium redox potential in the presence of mediators (35  $\mu$ M each). Trace A is from Co(II) signal amplitudes. The base-off form was measured at  $g = 2.3$ , pH 9.6 ( $\blacksquare$ ); the base-on form was measured at  $g = 2.2$ , pH 8.0 ( $\bullet$ ) or 9.6 ( $+$ ). Trace B is from integrated intensities of  $[4Fe-4S]^{1+}$  signals at pH 8.0 ( $\bullet$ ) or 9.6 ( $+$ ). The solid traces are least-square fits to the Nernst equation for  $n = 1$  with the following  $E_m$ -values: A,  $-240$  mV,  $-350$  mV; B,  $-480$  mV.

#### 4. Discussion

The PCE reductase of *D. restrictus* is the final enzyme of the PCE respiratory chain that catalyzes the reductive cleavage of a carbon chlorine bond. The enzyme was extracted from the cytoplasmic membrane and purified in a soluble, active form as a 60-kDa polypeptide containing approximately one cobamide and two iron-sulfur cubane cofactors. Growth of *D. restrictus* strictly depended on the supplementation of the culture medium with vitamin B<sub>12</sub> (our unpublished results). Therefore, the cobamide present in the PCE reductase is probably a dimethylbenzimidazole cobamide, i.e. the cobalamin vitamin B<sub>12</sub>. Chromatographic and optical spectroscopic properties of the extracted cobamide confirmed this assumption.

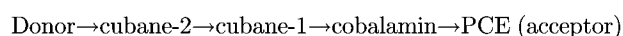
The redox potential of  $-350$  mV for the base-off and the base-on Co(I/II) redox couple is relatively high compared to the redox potentials of other corrinoid enzymes:  $E_m = -504$  mV for the base-off corrinoid/Fe-S protein of *Clostridium thermoaceticum* [25],  $E_m = -526$  mV for the base-on and  $E_m = -572$  mV for the base-off cobalamin of *Escherichia coli* methionine synthase [21], and  $E_m = -426$  mV for the base-on cobamide of N<sup>5</sup>-methyltetrahydromethanopterin:

coenzyme M methyltransferase of *Methanosarcina mazei* [26]. The unusually high redox potential makes the cobalamin easily reducible; it is not yet known which factor confers this phenomenon to the cobalamin. The PCE reductase can be reduced in vitro to the Co(I)-state with reduced methyl viologen; in vivo it has probably not to be initially activated by nucleoside triphosphates [26] or by methylating substrates [21] like other cobalamin proteins.

The iron-sulfur clusters are of the  $[4\text{Fe-4S}]^{(2+;1+)}$  cubane type. Their redox potentials are rather low, which indicates that the clusters do not function as a storage of reducing equivalents but only as electron-transfer devices. The two low potentials are quite different from the potentials tentatively postulated by Neumann et al. for the iron-sulfur clusters of the *D. multivorans* reductive dehalogenase [5]. The reduced cubanes can readily reduce the cobalamin to the Co(I) state, which is consistent with the suggestion that no activation of this enzyme is required to allow full reduction of the cobalamin active site.

Incubation of the Co(I) enzyme with PCE yielded the Co(II) form quantitatively. Combined with the photoreversible inactivation of PCE dechlorination by 1-iodopropane reported for *D. restrictus* [2] and *D. multivorans* [4] this indicates that the cob(I)alamin is the active site of the PCE reductase. In analogy to the dechlorination mechanism by free cobalamin (Glod et al., 1997 [7]) the reaction mechanism of PCE reductase presumably involves a dissociative single electron transfer. This implies that Co(III) is not a physiologically relevant redox state in the enzyme. Our findings do not support the formation of a trichlorovinyl-cob(III)alamin as the product of an addition-elimination reaction as claimed for PCE dechlorination by *D. multivorans* [5]. However, the vinyl radical that should have been formed intermediately upon dissociative single electron transfer has not been observed yet in EPR. Kinetic (rapid mixing/rapid freezing) experiments monitored with EPR should corroborate the proposed electron transfer mechanism.

The above can be summarized in the following single-electron transfer scheme:



The acceptor is PCE; the physiological donor has yet to be identified. The electron flow from the periplasmically oriented hydrogenase to the PCE reductase is mediated by menaquinone. However, menaquinone appears not to be the direct electron donor since the analogue 2,3-dimethyl-1,4-naphthoquinone failed to directly react with the isolated PCE reductase (unpublished results). Elucidation of the complete respiration chain is in progress; a detailed biochemical characterization of the PCE reductase of *D. restrictus* will be published elsewhere.

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## References

- [1] Holliger, C., Schraa, G., Stams, A.J.M., Zehnder, A.J.B., Appl. Environ. Microbiol. 59 (1993) 2991–2997.
- [2] Schumacher, W., Holliger, C., J. Bacteriol. 178 (1996) 2328–2333.
- [3] Scholz-Muramatsu, H., Neumann, A., Messmer, M., Moore, E., Diekert, G., Arch. Microbiol. 163 (1995) 48–56.
- [4] Neumann, A., Wohlfahrt, G., Diekert, G., Arch. Microbiol. 163 (1995) 276–281.
- [5] Neumann, A., Wohlfahrt, G., Diekert, G., J. Biol. Chem. 271 (1996) 16515–16519.
- [6] Gantzer, C.J., Wackett, L.P., Environ. Sci. Technol. 25 (1991) 715–722.
- [7] Glod, G., Angst, W., Holliger, C., Schwarzenbach, R.P., Environ. Sci. Technol. 31 (1997) 253–260.
- [8] Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., Anal. Biochem. 150 (1985) 76–85.
- [9] Stupperich, E., Steiner, I., Rühlemann, M., Anal. Biochem. 155 (1986) 365–370.
- [10] Dutton, P.L., Methods Enzymol. 54 (1978) 411–435.
- [11] Pierik, A.J., Hagen, W.R., Eur. J. Biochem. 195 (1991) 505–516.
- [12] Massey, V., Hemmerich, P., J. Biol. Chem. 252 (1977) 5612–5614.
- [13] Jetten, M.S.M., Pierik, A.J., Hagen, W.R., Eur. J. Biochem. 202 (1991) 1291–1297.
- [14] Holliger, C., Pierik, A.J., Reijerse, E.J., Hagen, W.R., J. Am. Chem. Soc. 115 (1993) 5651–5656.
- [15] Arendsen, A.F., Verhagen, M.F.J.M., Wolbert, R.B.G., Pierik, A.J., Stams, A.J.M., Jetten, M.S.M., Hagen, W.R., Biochemistry 32 (1993) 10323–10330.
- [16] Daas, P.J.H., Hagen, W.R., Keltjens, J.T., van der Drift, C., Vogels, G.D., J. Biol. Chem. 271 (1996) 22346–22351.
- [17] Hagen, W.R., Hearsen, D.O., Sands, R.H., Dunham, W.R., J. Magn. Reson. 61 (1985) 220–232.
- [18] Hagen, W.R., Hearsen, D.O., Harding, L.J., Dunham, W.R., J. Magn. Reson. 61 (1985) 233–344.
- [19] Hagen, W.R. (1989) in: Advanced EPR: Applications in Biology and Biochemistry (Hoff, A.J., Ed.) pp. 785–812, Elsevier, Amsterdam.
- [20] Pilbrow, J.R. (1982) in: B<sub>12</sub> (Dolphin, D., Ed.) Vol. 1, pp. 939–430, John Wiley and Sons, New York.
- [21] Banerjee, R.V., Harder, S.R., Ragsdale, S.W., Matthews, R.G., Biochemistry 29 (1990) 1129–1135.
- [22] Clark, W.M. (1960) Oxidation-Reduction Potentials of Organic Systems, Williams and Wilkins, Baltimore, MD.
- [23] Mathews, R., Charlton, S., Sands, R.H., Palmer, G., J. Biol. Chem. 249 (1975) 4326–4328.
- [24] Grande, H.J., Dunham, W.R., Averill, B., van Dijk, C., Sands, R.H., Eur. J. Biochem. 136 (1983) 201–207.
- [25] Harder, S.R., Lu, W.-P., Feinberg, B.A., Ragsdale, S.W., Biochemistry 28 (1989) 9080–9087.
- [26] Lu, W.-P., Becher, B., Gottschalk, G., Ragsdale, S.W., J. Bacteriol. 177 (1995) 2245–2250.