Electroenzymatic Reactions. Investigation of a Reductive Dehalogenase by Means of Electrogenerated Redox Cosubstrates

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Electroenzymatic Reactions. Investigation of a Reductive Dehalogenase by Means of Electrogenerated Redox Cosubstrates

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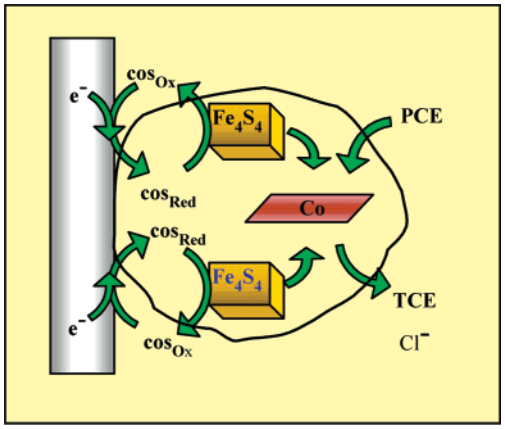
Abstract: As an illustration of how cyclic voltammetry can be used to unravel the mechanisms and kinetics of redox enzymes, the reductive dechlorination of trichloroethylene and tetrachloroethylene by a typical reductive dehalogenase, the tetrachloroethene reductive dehalogenase of *Sulfurospirillum multivorans* (formerly called *Dehalospirillum multivorans*), was investigated by means of several electrochemically generated cosubstrates. They comprised the monocation and the neutral form of methylviologen, the neutral form of benzylviologen, and cobaltocene. Cyclic voltammetry is used to produce the active form of the cosubstrate under controlled potential conditions. It shows large plateau-shaped catalytic responses, which are used to measure the kinetics of the enzymatic reaction as a function of the substrate and cosubstrate concentrations. The variation of the rate constant for the cosubstrate reaction with its standard potential shows the transition between two asymptotic behaviors, one in which the reaction is under diffusion control and the other in which it is under counter-diffusion control. Simple fitting of this plot allows an estimation of the standard potential of the electron acceptor center in the enzyme ($E^\circ = -0.57$ V vs NHE).

Introduction

In recent years, several reductive dehalogenases that couple the reductive dehalogenation of tetrachloroethylene and other chlorinated hydrocarbons to energy conservation (dehalorespiration)[1] have been isolated from anaerobic bacteria.[2,3] Interesting biological strategies are thus offered for remediation of systems polluted by halohydrocarbons.[4] Most of the reductive dehalogenases that mediate dehalogenation reactions contain a corrinoid as a cofactor, as well as two FeS clusters (Scheme 1). This is the case for the tetrachloroethylene reductive dehalogenase of *Sulfurospirillum multivorans*.[2a,5] This enzyme catalyzes the reductive dehalogenation of tetrachloroethylene (PCE) and trichloroethylene (TCE) to (*Z*)-1,2-dichloroethene with exceptionally high specific activities. It uses as corrinoid cofactor norpseudovitamin B12, rather than the usual vitamin B12.[2d] The mechanism by which the cobalt(I) corrinoid effects the dechlorination reaction currently arouses an active interest, giving rise to several recent biomimetic studies.[6]

Scheme 1



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The main kinetic characteristics of the enzyme have been determined in solution using the monocation of methylviologen (MV^+) in experiments where MV^+ was obtained by reduction of MV^{2+} by titanium(III) citrate.[2a,c] The overall kinetics exhibits a Michaelis−Menten type behavior as defined by eq 1,

$$\frac{1}{k_{ap}} = \frac{1}{2k_{substrate}[substrate]} + \frac{1}{k_{cosubstrate}[cosubstrate]} + \frac{1}{2k_i} \quad (1)$$

with $k_{PCE} = 8.5 \times 10^5\ M^{-1}\ s^{-1}$, $k_{TCE} = 7 \times 10^5\ M^{-1}\ s^{-1}$, and $k_{MV^+} = 2 \times 10^5\ M^{-1}\ s^{-1}$, while k_i is of the order of 50 s^{-1}.[7] The global rate constant, k_i, combines the rate constants of the first-order steps in the substrate and the cosubstrate half-reactions. The factors 2 in the first and third terms account for the fact that two equivalents of cosubstrate are used for each substrate equivalent consumed (Scheme 1) and that the reaction is followed by observing the disappearance of the cosubstrate.

The purpose of the study reported in the following sections was to take advantage of the capabilities of cyclic voltammetry to extend the analysis of the enzyme kinetics, with particular attention to its responses to several other cosubstrates of increasing reductive power.

There are two electrochemical approaches to redox enzyme kinetics using cyclic voltammetry. One is applicable when the enzyme, immobilized onto the electrode surface, may exchange electrons directly with the electrode.[8] This was not the case with our enzyme, where all attempts to promote direct electron transfer failed, presumably because of its large size (57.3 kDa). The other approach, consisting of using a one-electron reversible mediator that serves as redox cosubstrate to the enzyme, proved successful. This method has been applied satisfactorily to several homogeneous and immobilized enzymatic systems,[9−12] and rigorous theoretical treatments are available to extract the enzyme kinetics from the cyclic voltammetric raw data.[9,13]

Concerning immobilized systems, there are very few examples where the cyclic voltammetric data have been treated rigorously so as to obtain reliable kinetic and mechanistic information.[9,12b] Aside from the determination of the reductive dehalogenase characteristics, the present study thus offers another illustrative example of the strategies that can be used with immobilized redox enzymes when direct electron transfer with the electrode is not possible. It shows how, under such unfavorable conditions, the redox characteristics of the electron-exchanging center of the enzyme may be nevertheless determined.

Results and Discussion

TCE with MV^0 as Cosubstrate. Typical cyclic voltammetric responses obtained in the presence of the enzyme at a fixed concentration of MV^{2+} with increasing concentrations of TCE are shown in Figure 1a. In the absence of substrate, MV^{2+} exhibits two reversible waves that correspond to the successive formation of MV^+ and MV^0. The corresponding standard potentials are −0.44 and −0.77 V vs NHE, respectively. Upon addition of TCE, a strong catalytic increase of the current is observed at the level of the second wave, while there is a slight but definite increase of the current on the first wave, though it is insufficient for meaningful rate determinations.

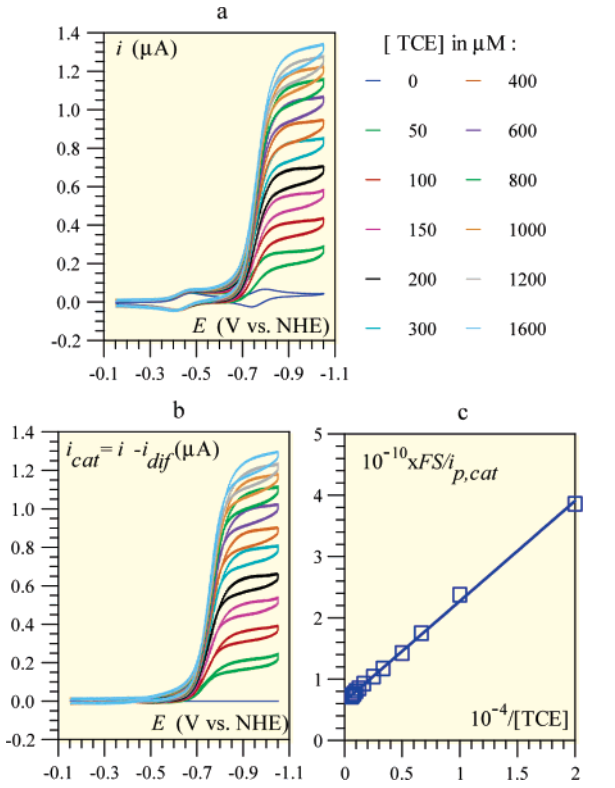

Figure 1. Cyclic voltammetry of 5 μM MV^{2+} in TRIS buffer, in the presence of 10 nM dehalogenase, as a function of increasing TCE concentrations. Scan rate: 0.03 V/s. (a) Raw voltammetric data. (b) After subtraction of the cosubstrate diffusion current. (c) Linear analysis according to eq 3.

The second wave is plateau-shaped, as expected in the case where the steady-state approximation applies for all forms of the enzyme and the consumption of the substrate by the enzymatic reaction is small as compared to its bulk concentration.[13] It soon became apparent that the cyclic voltammetry response involves essentially the enzyme adsorbed onto the electrode surface rather than the enzyme in the solution, as will be proved later on. Analysis of the raw data of Figure 1a accordingly follows the theory developed for the case where the enzyme is immobilized on the electrode.[13b] The current flowing through the electrode is the sum of two contributions, where i_{dif} is the diffusion current of the cosubstrate in the

$$i = i_{dif} + i_{cat} \quad (2)$$

absence of enzymatic reaction. At the plateau of the wave,

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(7) The kinetic rate constants were obtained from the reciprocal plots of Figure 4 in ref 2a, giving the dehalogenase activity as a function of PCE, TCE, or MV^+ concentration.
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$$\frac{FS\Gamma_E^0}{i_{p,cat}} = \frac{1}{2k_{substrate}[substrate]_{x=0}} + \frac{1}{k_{cosubstrate}[cosubstrate]_{x=0}} + \frac{1}{2k_i} \quad (3)$$

where F is the faraday constant, S the electrode surface area, and Γ_E^0 the surface concentration of the enzyme immobilized on the electrode. $[substrate]_{x=0}$ symbolizes the concentration of the substrate at the electrode surface. Since substrate consumption is negligible, $[substrate]_{x=0} = C_S^0$, its bulk concentration. $[cosubstrate]_{x=0}$ stands for the concentration of the active form of the cosubstrate (here its reduced form) at the electrode surface. At the plateau, $[cosubstrate]_{x=0} = C_C^0$, the bulk concentration of the oxidized form.

Conversion of Figure 1a into Figure 1b constitutes the first step of data analysis according to eq 2. The second step is illustrated by Figure 1c, which shows the application of eq 3. From the slope of the resulting straight line, $1/(2k_{TCE}\Gamma_E^0) = 1.6 \times 10^6$ (k_{TCE} in M^{-1} s^{-1}, Γ_E^0 in mol cm^{-2}), it follows that the surface concentration of the enzyme is $\Gamma_E^0 = 0.4 \times 10^{-12}$ mol cm^{-2}, taking $k_{TCE} = 7 \times 10^5$ M^{-1} s^{-1}.[7]

We then examined how the catalytic currents vary with the concentration of cosubstrate for a fixed concentration of TCE. The raw data are shown in Figure 2a. Subtraction of the cosubstrate diffusion current leads to the curves in Figure 2b. For the four lowest MV^{2+} concentrations, the expected plateau-shaped curves are observed.[14] The slope of the resulting $FS/i_{p,cat}$ vs $1/[MV^{2+}]$ straight line, $1/(k_{MV^0}\Gamma_E^0) = 2.25 \times 10^4$ (k_{MV^0} in M^{-1} s^{-1}, Γ_E^0 in mol cm^{-2}), leads, using the previously determined value of Γ_E^0, 0.4×10^{-12} mol cm^{-2}, to $k_{MV^0} = 9 \times 10^7$ M^{-1} s^{-1}.

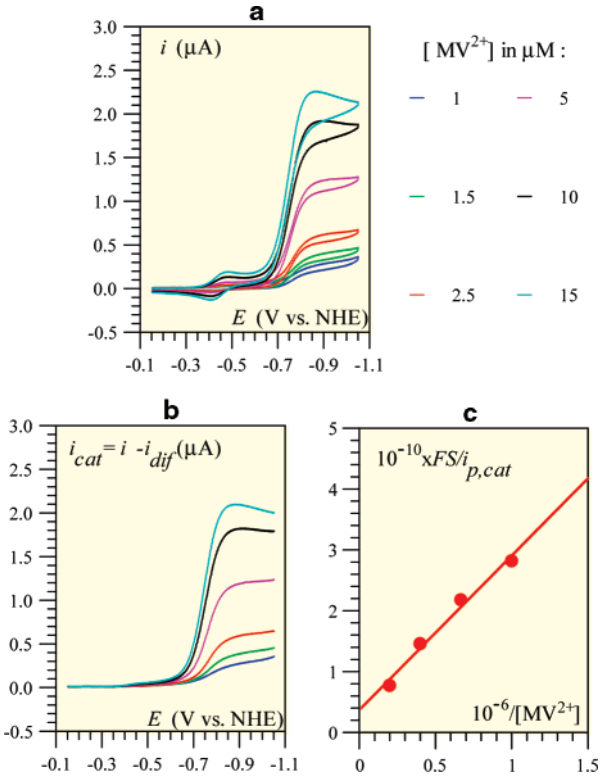

Figure 2. Cyclic voltammetry of MV^{2+} in TRIS buffer, in the presence of 10 nM dehalogenase and 1 mM TCE, as a function of increasing MV^{2+} concentrations. Scan rate: 0.03 V/s. (a) Raw voltammetric data. (b) After subtraction of the cosubstrate diffusion current. (c) Linear analysis according to eq 3.

We may now use the values of k_{TCE} and k_{MV^0} to estimate k_i from the intercepts in Figures 1c and 2c according to eq 3. k_i is thus found to be between 550 and 850 s^{-1}. Having the various rate constants in hand, we may now estimate the respective contributions to the catalytic current of the enzyme in the solution and the enzyme adsorbed on the electrode surface, and see whether our assumption of a prevailing role of the adsorbed enzyme is justified. In pure kinetic conditions, with negligible consumption of the substrate in the reaction layer, homogeneous enzyme catalysis also gives rise to plateau-shaped waves. The plateau current is then given by[13a]

$$\frac{i_p^{hom}}{FS} = [MV^{2+}]\sqrt{D_{MV^{2+}} k_{MV^0} C_E^0}\sqrt{\frac{2}{\sigma}\left[1 - \frac{\ln(1+\sigma)}{\sigma}\right]}$$

where C_E^0 is the enzyme concentration and the competition parameter σ is defined by

$$\sigma = k_{MV^0} C_C^0 \left(\frac{1}{2k_{TCE}[TCE]} + \frac{1}{2k_i}\right)$$

In the adsorbed case, the current is the sum of the current given in eq 2 and the diffusion current of the cosubstrate. The ratio between the homogeneous and the immobilized contributions is thus obtained from

$$\frac{i_p^{hom}}{i_p^{imm}} = \frac{\sqrt{D_{MV^{2+}} k_{MV^0} C_E^0}\sqrt{\frac{2}{\sigma}\left[1 - \frac{\ln(1+\sigma)}{\sigma}\right]}}{0.446\sqrt{D_{MV^{2+}}\frac{Fv}{RT}} + \frac{k_{MV^0}\Gamma_E^0}{1+\sigma}} \quad (4)$$

Figure 3 shows that the relative contribution of homogeneous catalysis is below 10% in all the experiments that have been used to extract the kinetic characteristics, thus justifying its neglect in the preceding analyses.

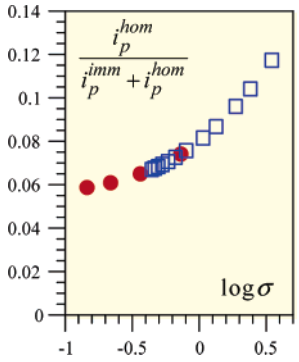

Figure 3. Relative contribution of the homogeneous enzyme catalysis in the experiments of Figures 1 and 2. The symbols are the same as in Figures 1c and 2c. A value of 5×10^{-6} cm^2 s^{-1} was used for $D_{MV^{2+}}$.

(14) The cyclic voltammetric response shows a flat peak at the highest concentration, which corresponds to the fact that substrate impoverishment at the electrode surface ceases to be negligible as it is when plateau-shaped curves are obtained.

PCE with MV^0 as Cosubstrate. Two similar series of experiments were carried out with PCE. Figure 4 shows a series of cyclic voltammetric responses obtained at a fixed concentration of MV^{2+} with increasing concentrations of PCE. The raw data are displayed in Figure 4a. After subtraction of the cosubstrate diffusion current (Figure 4b), the linear plot of Figure 4c is obtained from the plateau currents. From its slope, $1/(2k_{PCE}\Gamma_E^0) = 1.4 \times 10^6$ (k_{PCE} in M^{-1} s^{-1}, Γ_E^0 in mol cm^{-2}), the surface concentration of the enzyme is estimated as $\Gamma_E^0 = 0.4 \times 10^{-12}$ mol cm^{-2}, taking for k_{PCE}, 8.5×10^5 M^{-1} s^{-1}.[7]

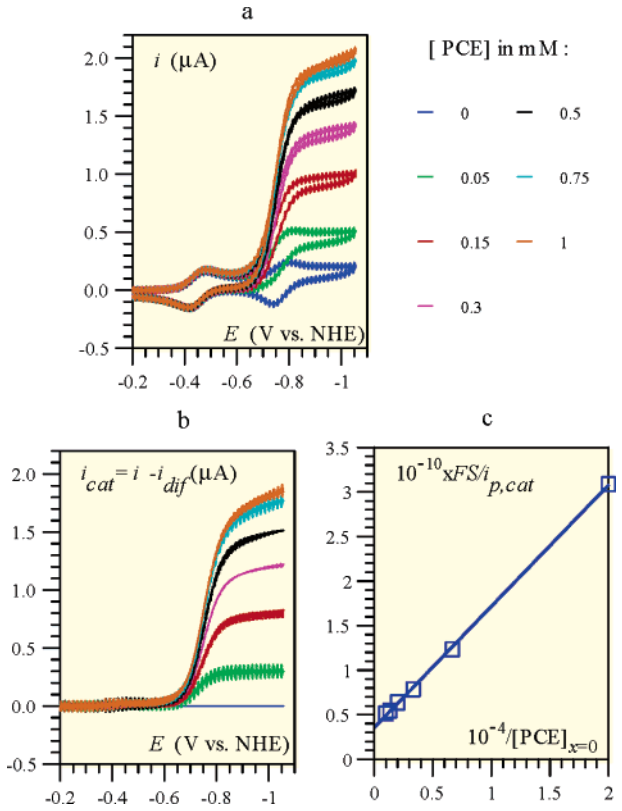

Figure 4. Cyclic voltammetry of 2.5 μM MV^{2+} in TRIS buffer, in the presence of 10 nM dehalogenase, as a function of increasing PCE concentrations. Scan rate: 0.03 V/s. (a) Raw voltammetric data. (b) After subtraction of the cosubstrate diffusion current. (c) Linear analysis according to eq 3.

We now examine how the catalytic currents vary with the concentration of cosubstrate for a fixed concentration of PCE. The raw data are shown in Figure 5a. The slope of the resulting $FS/i_{p,cat}$ vs $1/[MV^{2+}]$ straight line (Figure 5c), $1/(k_{MV^0}\Gamma_E^0) = 2.8 \times 10^4$ (k_{MV^0} in M^{-1} s^{-1}, Γ_E^0 in mol cm^{-2}), leads to $k_{MV^0} = 8 \times 10^7$ M^{-1} s^{-1}, practically the same as the rate constant derived from the TCE experiments (9×10^7 M^{-1} s^{-1}), when one uses the previously determined value of Γ_E^0, 0.4×10^{-12} mol cm^{-2}. From the intercepts in Figures 4c and 5c, k_i is found to be between 1500 and 2000 s^{-1}.

Coming now to the justification of the neglect of homogeneous enzyme catalysis in the experiments involving PCE and MV^0, we see in Figure 6 that this is indeed the case, the relative contribution of homogeneous catalysis remaining below 15% in all experiments.

PCE with Cobaltocene and Benzylviologen as Cosubstrates. A series of experiments was carried out for comparing electrochemically generated MV^0 to cobaltocene (generated from

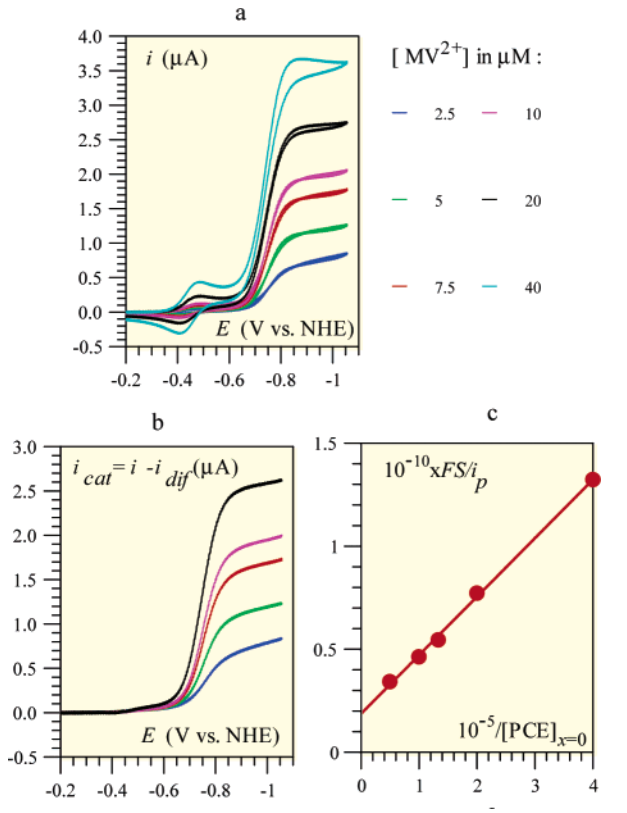

Figure 5. Cyclic voltammetry of MV^{2+} in TRIS buffer, in the presence of 10 nM dehalogenase and 1 mM PCE, as a function of increasing MV^{2+} concentrations. Scan rate: 0.03 V/s. (a) Raw voltammetric data. (b) After subtraction of the cosubstrate diffusion current. (c) Linear analysis according to eq 3.

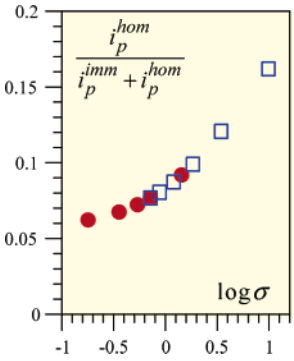

Figure 6. Relative contribution of the homogeneous enzyme catalysis in the experiments of Figures 4 and 5. The symbols are the same as in Figures 4c and 5c. A value of 5×10^{-6} cm^2 s^{-1} was used for $D_{MV^{2+}}$.

cobalticinium, Cc^+) and doubly reduced benzylviologen cation BV^0 in terms of rate constants (see Figures 7 and 8). Cobalticinium is a more reducing cosubstrate (the standard potential of the Cc^+/Cc couple is -0.93 V vs NHE), whereas BV^0 is less reducing. The interest of doubly reduced benzylviologen, BV^0, is that its standard potential, -0.62 V vs NHE, stands between those of the MV^{2+}/MV^+ and MV^+/MV^0 couples.

The rate constants are inversely proportional to the slopes of the linear plots in the right-hand diagrams of Figure 8 (13, 10, and 13 cm^{-1} s from top to bottom respectively), leading to the results summarized in Table 1.

Standard Potential of the Electron-Accepting Centers. It is remarkable that the rate constants are practically the same, and close to 10^8 M^{-1} s^{-1}, for the three most reducing

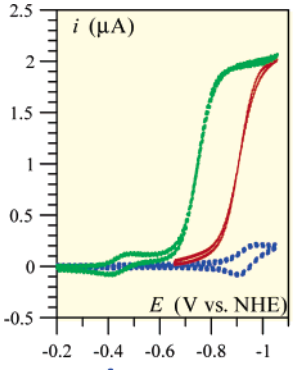

Figure 7. Cyclic voltammetry of cobalticinium and MV^{2+} (10 μM) in TRIS buffer, in the presence of 50 nM dehalogenase. Scan rate: 0.03 V/s. Blue dashed line, cobalticinium alone; red solid line, cobalticinium in the presence of 1 mM PCE; green dotted line, MV^{2+} in the presence of 1 mM PCE.

cosubstrates (Figure 9). It is also interesting to note that the same rate constant has also been found with TCE as substrate and MV^0 as cosubstrate. By comparison with the diffusion limit (10^9 M^{-1} s^{-1}) corresponding to the diffusion of the cosubstrate toward any point on the enzyme surface, this value is compatible with control by the diffusion of the cosubstrate to a portion of enzyme surface, corresponding to a solid angle of $4\pi/10$. This is a quite reasonable conclusion in view of the fact that the domain where the electron-accepting centers are likely to be located is close to the enzyme surface, thus defining a cone for the access of the cosubstrate.

Using the value found previously for MV^+ (data point on the left of the plot in Figure 9), one can estimate the standard potential of the electron-accepting centers according to the following procedure.[15a]

The rate constant for electron transfer between the cosubstrate and the enzyme redox center may be expressed as[15b]

$$\frac{1}{k_{\text{cosubstrate}}} = \frac{1}{k_{\text{dif}}} + \frac{1}{k_{\text{dif}} \exp\left[-\frac{F}{RT}(E - E^0)\right]} + \frac{k'_{\text{dif}}}{k_{\text{dif}} k_{\text{act}}} \quad (5)$$

The first term involves the bimolecular diffusion limit, k_{dif} (horizontal dotted green straight line in Figure 9). The second term is the "counter-diffusion" term (ascending dotted red straight line in Figure 9). k'_{dif} is a first-order rate constant (in s^{-1}), equal to the second-order diffusion limit (in M^{-1} s^{-1}). E^0 is the enzyme standard potential and E the standard potential of the redox cosubstrate. k_{act} is the activation-controlled rate constant that may be expressed in the vicinity of the standard potential (symmetry factor equal to 0.5) as

$$k_{\text{act}} = k_0 \exp\left[-\frac{F}{2RT}(E - E^0)\right]$$

where k_0 is the standard rate constant (rate constant at zero driving force).

As a crude approximation, E^0 can be obtained from the intersection of the first two terms of eq 5 taken separately. A value of -0.60 V vs NHE is thus obtained. A better approximation consists of taking the first two terms of eq 5 together, still ignoring the third term. A more correct value of -0.57 V vs NHE is then obtained. Introducing nonzero values for the third term does not lead to any improvement of data fitting. This simply means that the standard activation rate constant, k_0, is very large, larger than the diffusion limit.

In the above procedure, we have assumed that the characteristic rate constants of the enzyme are the same in solution (corresponding to the first point on the plot of Figure 9) and in the adsorbed state (corresponding to the other points on the plot of Figure 9). Several examples have been given of denaturation

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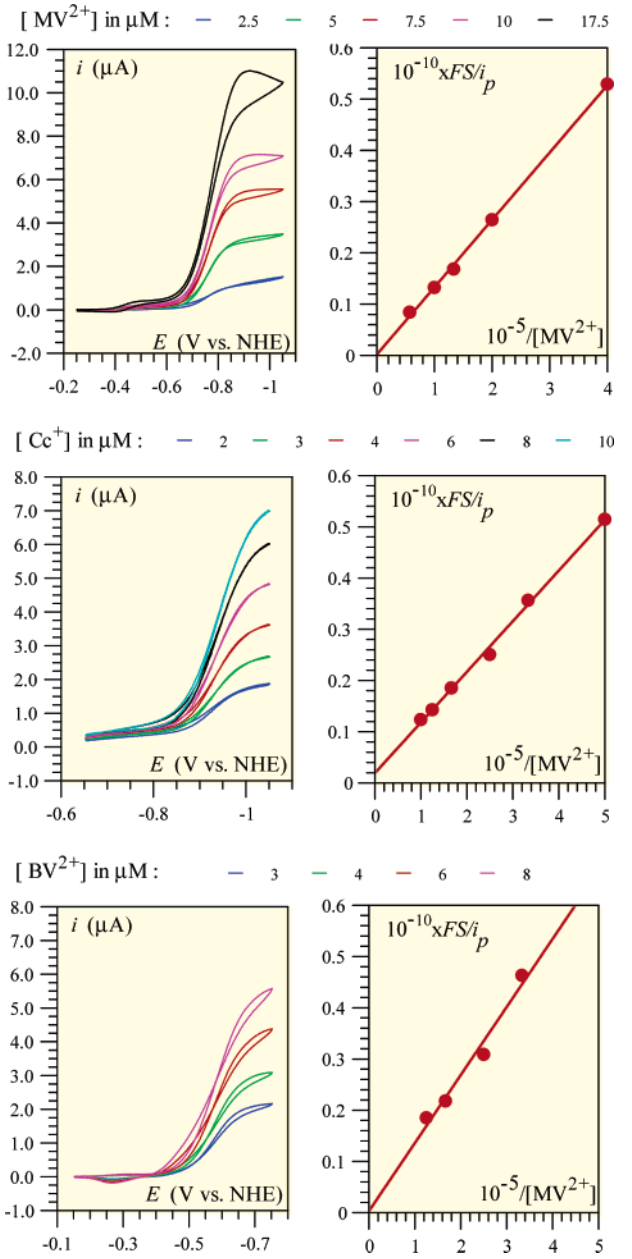

Figure 8. Cyclic voltammetry of MV^{2+}, Cc^+, and BV^{2+} in TRIS buffer, in the presence of 50 nM dehalogenase and 1 mM PCE, as a function of increasing cosubstrate concentrations. Scan rate: 0.03 V/s. Left: voltammetric data. Right: linear analysis according to eq 3.

Table 1. Cosubstrate Rate Constants with PCE as Substrate

	cosubstrate			
	MV^+	BV^0	MV^0 [a]	Cc
E^0 (V vs NHE)	−0.44	−0.62	−0.77	−0.93
$k_{\text{cosubstrate}}$ (M^{-1} s^{-1})	2×10^5	0.8×10^8	0.8×10^8	10^8

[a] With TCE as substrate the rate constant is 9.0×10^7.

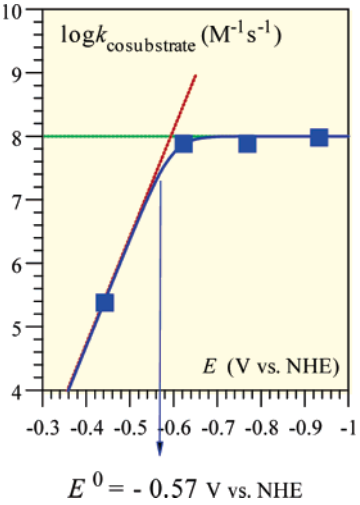

Figure 9. Variations of the cosubstrate rate constant with its standard potential. Determination of the standard potential of the electron-accepting centers (see text).

of enzymes upon adsorption onto electrode surfaces. It is thus conceivable that the rate constants corresponding to the three right-hand points are underestimated. What would be the consequence on E^0 determination? The maximal value these rate constants can take is the unrestricted diffusion limit, i.e., 10^9 M^{-1} s^{-1}. Figure 10 shows an attempt to fit the data in these conditions. The best fit corresponds to an E^0 value -0.60 V vs NHE, but it is seen that the fit is clearly poorer than in the preceding case. We may thus conclude that -0.57 V vs NHE is a more exact value, probably known with an accuracy better than ± 10 mV.

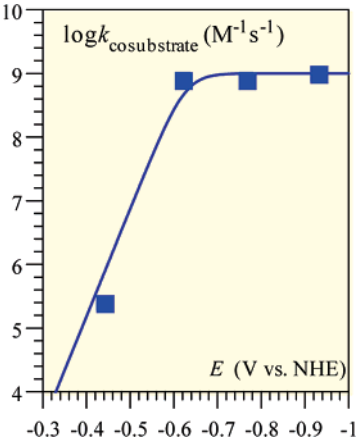

Figure 10. Alternative attempt to estimate the enzyme standard potential (see text).

A value of -0.57 V vs NHE makes the electron-accepting center sufficiently reductive to convert cobalt(II) into cobalt(I) in the corrinoid cobalt center of the enzyme and also the cobalt(III) alkyl or alkenyl derivatives that are likely to be formed as intermediates in the catalytic loop.

Concluding Remarks

Cyclic voltammetry of *Sulfurospirillum multivorans*[5] dehalogenase allows a complete kinetic characterization of the enzyme. The responses result prevalently from the enzyme adsorbed on the glassy carbon electrode rather than from the enzyme present in the solution. The rate constant for the reaction of the cosubstrate with the enzyme could thus be derived from the cyclic voltammetric responses for three couples of increasing reducing power: BV^+/BV^0 ($E° = -0.62$ V vs NHE), MV^+/MV^0 ($E° = -0.77$ V vs NHE), and Cc^+/Cc ($E° = -0.93$ V vs NHE). It is remarkable that the rate constants for the three cosubstrates are the same ($(8-10) \times 10^7$ M^{-1} s^{-1}) and do not depend on whether the substrate is PCE or TCE. This indicates that the reaction is under diffusion control. Comparing this results with the rate constant, 2×10^5 M^{-1} s^{-1}, obtained for the MV^{2+}/MV^+ ($E° = -0.44$ V vs NHE) allows the determination of a standard potential of -0.57 V vs NHE for the electron-accepting center in the enzyme. This value is sufficient for a facile electron transfer from the iron−sulfur clusters to the cobalt(II) corrinoid and the alkyl or alkenyl cobalt(III) corrinoid formed in the catalytic process.

Experimental Section

Reagents. The *Sulfurospirillum multivorans*[5] bacteria was grown anoxically on a medium containing pyruvate and fumarate as described earlier.[1a] The PCE reductive dehalogenase was extracted from the cells of bacteria and purified on a series of chromatographic columns (Q-sepharose, phenyl-superose, Superdex) as previously described.[2a]

The activity of the purified PCE reductive dehalogenase was determined spectrophotometrically under a N_2 atmosphere from the decrease in absorbance at 578 nm of the radical MV^+ ($\epsilon = 9.7$ mM^{-1} cm^{-1}) in the presence of a saturating concentration of chlorinated hydrocarbon.[2a] The MV^+ radical was chemically generated from titanium(III) citrate. The enzyme concentration was indirectly determined from the amount of cobalt quantified from ICP-AES analysis. A dehalogenase molecular weight of 57.3 kDa was selected.[2a]

The PCE, TCE, *cis*-DCE, methylviologen dichloride, and benzylviologen dichloride compounds were used as received (Aldrich). The cobalticinium was obtained from Strem Chemicals. A 0.1 M Tris buffer (pH 7.5) containing 0.5 mM NH_4Cl was systematically used. All of the aqueous solutions were prepared using water purified by a Milli-Q water purification system from Millipore.

Electrochemical Experiments. An Autolab potensiostat (EcoChemie, Utrecht, The Netherlands) interfaced to a personal computer was used for cyclic voltammetry (CV). Carbon-based screen-printed electrodes (9.6 mm^2 for the sensing disk area) were used as disposable working electrodes, and they were prepared as previously described[16] from a homemade carbon-based ink composed of graphite particles (Ultra Carbon, UCP 1M, Johnson Matthey) and polystyrene, and using a manual screen-printer (Circuit Imprimé Français, Bagneux, France) equipped with a screen-stencil of 77 threads cm^{-1}. A saturated calomel electrode (SCE) and a platinum counter electrode were employed as a reference and counter electrodes. The potentials indicated in the text are referred to the NHE. To avoid O_2 damage of the reductive dehalogenase, all experiments were carried out under N_2 in a glovebox (O_2 < 2 ppm, Jacomex, France), in which a water-jacketed electrochemical cell was maintained at 20 ± 0.5 °C with a circulating water bath.

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