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Kinetic studies of electron transfer between hydrogenase and cytochrome c_3 from *Desulfovibrio gigas*. Electrochemical properties of cytochrome c_3

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The second-order rate constant of the electron transfer between cytochrome c_3 (M_r 13000) and hydrogenase from *Desulfovibrio gigas* has been determined by spectrophotometry and cyclic voltammetry. Both of these methods gave the same value of $6.5 \cdot 10^7$ $M^{-1} \cdot s^{-1}$. Comparative measurements for other protein–protein interactions show that the foregoing value is relatively high and comparable with that obtained for two other physiological partners, cytochrome c_3 and hydrogenase from *Desulfovibrio desulfuricans* Norway. In addition, the redox potentials of the four hemes of *D. gigas* cytochrome c_3 have been measured by polarography. These values were estimated to be −195, −295, −315 and −330 mV.

Introduction

Sulfate-reducing bacteria exhibit a strictly anaerobic mode of growth based on reduction of sulfate as the terminal electron acceptor [1]. Hydrogenase which catalyzes the reversible oxidation of molecular hydrogen ($H_2 \rightleftharpoons 2H^+ + 2e^-$) plays a central role in hydrogen metabolism [2–5] and is involved in the metabolism of organic substrates by these bacteria [6,7]. Two types of hydrogenase have been characterized in different species of sulfate-reducing bacteria of the genus *Desulfovibrio*: a nickel-containing iron-sulfur protein and an exclusively iron-sulfur-containing protein [7]. The two hydrogenases can be differentiated on the basis of molecular weight, metal content, specific activity and effect of inhibitors [7].

The natural electron carrier of both types of enzyme is the tetrahemic low-redox-potential cytochrome c_3 (M_r 13000) [6–11]. This protein is required in *Desulfovibrio*-type hydrogenases for electron transfer to occur with low-redox-potential carriers such as ferredoxin and flavodoxin [9,10]. In contrast, clostridial-type hydrogenase readily reduces ferredoxin under a hydrogen atmosphere [12]. Comparison of the requirement for cytochrome c_3 (M_r 13000) between ferredoxin and *Desulfovibrio* hydrogenase reveals a high specificity between these oxidoreduction partners. Kinetic and structural studies on *D. desulfuricans* Norway cytochrome c_3 (M_r 13000) – ferredoxin and cytochrome c_3 (M_r 13000) – hydrogenase electron-transfer complexes illustrate this specificity [13–16]. EPR spectroscopy of *D. gigas* cytochrome c_3 in the presence and absence of ferredoxin has been investigated [17], but no studies have been reported on the interaction between cytochrome c_3 (M_r 13000) and hydrogenase from this microorganism in terms of kinetic data.

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The periplasmic hydrogenase from *D. gigas* has been extensively studied [18–24]. The enzyme consists of two subunits of M_r 62000 and M_r 26000. It contains one nickel atom, one [3Fe-*x*S] cluster and two [4Fe-4S] clusters as shown by EPR and Mössbauer measurements [19–25]. The nickel atom is involved in the activating site of hydrogen, whereas the [4Fe-4S] clusters, which act as secondary electron carriers [22–24], are probably involved in the electron transfer with the physiological carrier. The primary structure of *D. gigas* hydrogenase has been recently determined [26] and the three-dimensional structure of this enzyme has been undertaken [27].

Hydrogenases lose most of their activity under oxidizing conditions [28]. However, this loss of activity is a reversible process in the case of the nickel-containing type hydrogenases of *Desulfovibrio* [29–35]. Some hydrogenases, including the *D. gigas* enzyme [29–31,33], are unusual in that the reactivation of the enzyme as usually prepared is a very slow process, requiring several hours incubation in reducing conditions. By contrast, the soluble hydrogenases of *D. desulfuricans* Norway [32] and *D. africanus* [34] can be activated relatively rapidly. In the *D. gigas* enzyme, the existence of the activated and deactivated states can be correlated with different ESR-detectable forms of nickel [23,33].

D. gigas cytochrome c_3 (M_r 13000) is characterized by the presence of four low-redox-potential hemes per molecule [17,36], but the role of the four hemes and the biological significance for their E_M values are not well understood. The primary structure of *D. gigas* cytochrome c_3 (M_r 13000) has been determined [37] and the obtention of crystals has led to preliminary crystallographic data on this protein [38]. *D. gigas* hydrogenase readily reduces the periplasmic tetraheme cytochrome c_3 (M_r 13000) and the cytoplasmic octaheme cytochrome c_3 (M_r 26000) [9]. However, the cellular localization of both hydrogenase and cytochrome c_3 (M_r 13000) in the periplasmic space indicates that these two redox proteins act as physiological partners [6,39].

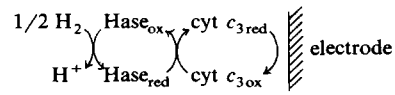
As shown in a previous work on the interaction between cytochrome c_3 (M_r 13000) and hydrogenase from *D. desulfuricans* Norway [16], electrochemical techniques offer a convenient way for measuring fast rates of electron transfer between proteins. In the present work, the interaction of *D. gigas* hydrogenase with cytochrome c_3 (M_r 13000) has been investigated using electrochemical and spectrophotometric methods in view of kinetic parameters determination. This study includes the electrochemical determination of the four redox potentials of *D. gigas* cytochrome c_3 (M_r 13000).

Materials and Methods

Hydrogenase and cytochrome c_3 (M_r 13000) from *Desulfovibrio gigas* were purified as previously reported [18,40].

Electrochemical determinations: principle and apparatus

The hydrogenase–cytochrome c_3 electron transfer chain can be simulated on the basis of the following electrochemical model:



(Hase symbolizes hydrogenase)

Exchanges between hydrogenase and the electrode are very slow, but they are fast in the case of cytochrome c_3 and do not need mediators. In the presence of an excess of substrate, here hydrogen, cytochrome c_3 enzymatically reduced by hydrogenase is electrochemically reoxidized and an enhanced anodic current is detected. Cyclic voltammetry is a convenient way to monitor the advance of the catalytic reaction and to measure its homogeneous rate constants.

Differential pulse polarography at the dropping mercury electrode and cyclic voltammetry at the hanging mercury drop electrode were used for the determination of redox potentials [41]. Kinetics data were obtained from cyclic voltammetry at a basal-plane pyrolytic graphite electrode [16]. Differential pulse polarograms were performed using a PAR 174 A Polarographic Analyzer equipped with an M 174/70 drop timer. The drop time was of 5 s and the pulse amplitude of −50 mV. Cyclic voltammograms were performed with a PAR 173 Potentiostat coupled to a PAR 175 Universal Programmer. Curves were recorded on a Sefram *X-Y* chart recorder.

All potential values are given vs. the normal hydrogen electrode. U-grade hydrogen was purified by bubbling through reduced methylviologen (MV) solution to eliminate residual oxygen. Oxygen was purged from solutions by bubbling purified U-grade hydrogen which was the substrate of the enzymatic redox reaction. All experiments were performed in 10 mM Tris-HCl buffer at pH 7.6, which served as the supporting electrolyte in electrochemistry experiments. The temperature was maintained constant at $25 \pm 0.1°C$.

Spectrophotometric measurements

D. gigas hydrogenase was first reactivated by incubation under pure hydrogen before performing kinetics measurements. For this purpose, a sample of enzyme (5 γ/ml) in 50 mM Tris-HCl (pH 7.6) was kept in a 1 ml serum-stoppered flask and gently bubbled with hydrogen grade N 55 (from Air Liquide) for 3 h at 25°C. Traces of cytochrome c_3 were added to facilitate the reactivation process. Reactivation of hydrogenase was checked spectrophotometrically by following the reduction of methyl viologen by hydrogen as described in Ref. 31. The specific activity of the reactivated enzyme was around 800 μmol reduced MV/min per mg enzyme. When a sample of hydrogenase in the unready state [31] was added to a mixture containing reduced cytochrome c_3 and reactivated hydrogenase under hydrogen the rate of reactivation process increased significantly and took only 1 h at 25°C. These observations made during the electrochemical experiments were confirmed by spectrophotometric measurements.

Reduction of cytochrome c_3 by reactivated hydrogenase was measured spectrophotometrically at 552 nm. The reaction mixture in a 1 ml cuvette with a serum stopper containing 50 mM Tris-HCl (pH 7.6), 1–20 μM cytochrome c_3, 20 μmol glucose, 1.5 μl ethanol, 500 units of catalase, 10 μl of a 10 mg/ml solution of glucose oxidase was bubbled with hydrogen for 15 min. The cell was subsequently incubated at 25°C. The reaction started by addition of 0.5–3 μl hydrogenase solution (5 μg/ml) and the absorbance was followed. Initial rates of cytochrome c_3 reduction were calculated using absorption coefficients of 27 and 111 $mM^{-1} \cdot cm^{-1}$ at 552 nm for the oxidized and reduced forms of cytochrome c_3, respectively. A 2 mm light path cuvette was used for assays with high concentrations of cytochrome c_3 (50 μM).

Results

Determination of the redox potentials of cytochrome c_3

The cyclic voltammogram of a 80 μM *D. gigas* cytochrome c_3 solution at the hanging mercury drop electrode is shown in Fig. 1A for a scan rate of 5 $mV \cdot s^{-1}$. Two cathodic (1c, 2c) and two anodic (1a, 2a) peaks corresponding to two successive reduction-reoxidation steps are detected. The overall appearance does indicate that the redox reactions at the mercury electrode are rather fast. The differential pulse polarogram of a 80 μM *D. gigas* cytochrome c_3 solution is given in Fig. 1B. Two reduction peaks, at $E_{p1} = -170$ mV and $E_{p2} = -295$ mV, are detected. By using the calculation method described in previous papers [41,42], the four individual redox potentials of hemes have been measured, at $E_{h1} = -195$ mV, $E_{h2} = -295$ mV, $E_{h3} = -315$ mV and $E_{h4} = -330$ mV, with an accuracy of about ± 10 mV. It is to note that one of the hemes has a noticeably less negative redox potential ($E_{h1} - E_{h2} = 100$ mV) than the others. Very similar behavior was observed when using the pyrolytic graphite electrode.

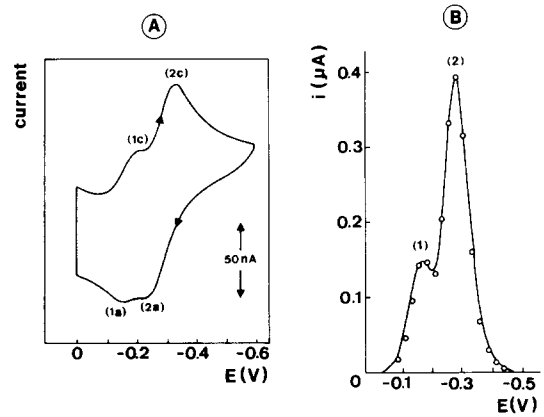


Fig. 1. (A) Cyclic voltammogram at the hanging mercury drop electrode of a 80 μM *D. gigas* cytochrome c_3 solution in 10 mM Tris-HCl (pH 7.6). Scan rate: 5 $mV \cdot s^{-1}$. (B) Differential pulse polarogram of the same solution. Drop time 5 s (———) experimental curve; (○) calculated points.

Electrochemical study of hydrogenase–cytochrome c_3 electron transfer

Cyclic voltammetry experiments at the graphite electrode were performed in hydrogen-saturated solutions using the following procedure: first the voltammograms of a 42 μM cytochrome c_3 solution were recorded at different scan rates ($v = 5 - 100$ mV $\cdot$ s^{-1}) over the potential range 100 to -600 mV. Upon a first addition of hydrogenase to the solution, a progressive change in the voltammograms occurred. Peaks disappeared and a catalytic anodic current flowed. This behavior was particularly apparent at the slower scan rates, indicating a catalytic regeneration of reduced cytochrome c_3 by hydrogenase in the presence of hydrogen. In fact, an evolution of the catalytic current upon the addition of hydrogenase was observed over a relatively long time. A steady-state cycle was obtained only after repeated cycling for approx. 3 h. The dependence of the enhanced anodic current on time is given in Fig. 2A and a steady-state voltammogram relative to the solution containing 42 μM cytochrome c_3 and 15 nM hydrogenase is shown in Fig. 2B. The reaction sequence can be schematized as follows:

$$1/2\ H_2 + Hase_{ox} \rightarrow H^+ + Hase_{red} \quad (1)$$

$$Hase_{red} + cyt\ c_{3ox} \xrightarrow{k} Hase_{ox} + cyt\ c_{3red} \quad (2)$$

$$cyt\ c_{3red} \rightleftharpoons cyt\ c_{3ox} + 4\ e^- \quad (3)$$

The rate constant k of the homogeneous reaction (reaction 2) was obtained under conditions of hydrogen excess using the analysis of Nicholson and Shain [43] for catalytically coupled reversible electron transfer reactions. The values of i_k/i_d corresponding to the ratio of the kinetically controlled to diffusion-controlled current shown in Fig. 2B (curves 2 and 1, respectively) were studied as a function of scan rate, v. By using the working curve given in the Fig. 14 of Ref. 43 we have obtained the kinetic parameter k_f/a (where $a = nFv/RT$, F, R and T having their usual meaning), then k_f by plotting k_f/a vs. $1/v$; k_f represents the pseudo-first-order rate constant. By using several concentrations of hydrogenase, the second-order homogeneous rate constant k was obtained by plotting k_f vs. hydrogenase concentration (Fig. 3). We found $k = (6.5 + 1) \cdot 10^7$ M^{-1} s^{-1}.

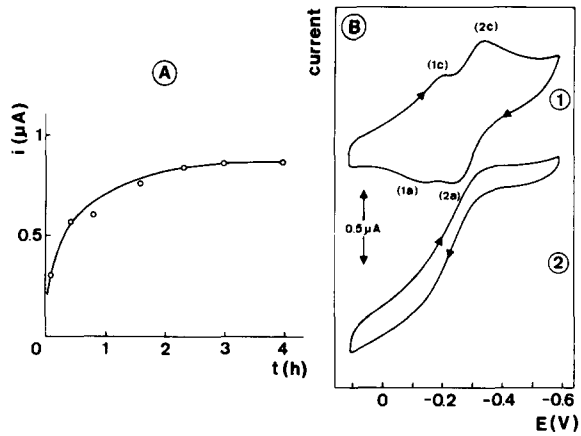


Fig. 2. (A) Dependence of kinetic current on time. Current was measured at $E = 0$ for a solution containing 42 μM *D. gigas* cytochrome c_3 and 15 nM *D. gigas* hydrogenase in 10 mM Tris-HCl, using the graphite electrode. (B) Cyclic voltammograms at the graphite electrode of 42 μM *D. gigas* cytochrome c_3 in 10 mM Tris-HCl (pH 7.6). (1) Without hydrogenase; (2) after the addition of 15 nM hydrogenase. This steady-state cycle is obtained after repeated cycling for 3 h. Scan rate: 5 mV $\cdot$ s^{-1}.

We have verified that when substituting hydrogen by nitrogen, the catalytic current was suppressed and the cyclic voltammogram given in Fig. 2B (curve 1) was restored.

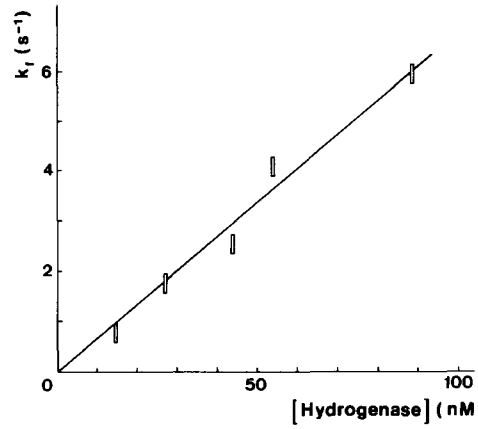


Fig. 3. Pseudo-first-order rate constant, k_f, as a function of hydrogenase concentration. Points result from intermediary plots and from the working curve given by Nicholson and Shain (Fig. 14 in Ref. 43).

Spectrophotometric studies of hydrogenase-cytochrome c_3 electron transfer

The reaction scheme for the reduction of cytochrome c_3 by hydrogenase (Eqn. 2) can be also expressed as follows:

$$\text{cyt } c_{3ox} + \text{Hase}_{red} \xrightarrow{K_m} (\text{cyt } c_3 - \text{Hase}) \xrightarrow{k_{cat}} \text{cyt } c_{3red} + \text{Hase}_{ox} \quad (4)$$

where K_m is the Michaelis constant and k_{cat} the catalytic constant. The Michaelis constant value was found to be 7.4 μM at pH 7.6 and 25 °C from Eadie-Hofstee plot (Fig. 4). For the first-order rate constant determination, a saturating concentration of cytochrome c_3 (50 μM) was used. In these conditions, the rate equation becomes $V = k_{cat}$ [Hase]. The value of k_{cat} deduced from Fig. 5 was 494 s^{-1}. At low cytochrome c_3 concentration ($\ll K_m$), the equation of Michaelis can be turned into:

$$V = \frac{k_{cat}}{K_m}[\text{Hase}][\text{cyt}c_3]$$

Under these conditions, we define an analogous second-order rate constant for the reaction of cytochrome c_3 reduction by hydrogenase: $k = k_{cat}/K_m$. This gives a value of $k = 6.6 \cdot 10^7$ $M^{-1} \cdot s^{-1}$, in excellent agreement with the result of the electrochemical study.

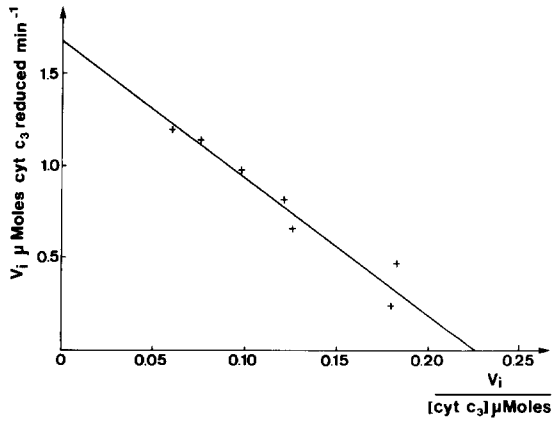


Fig. 4. Effect of cytochrome c_3 concentration on initial reaction rate. The results are presented as an Eadie-Hofstee plot. Reduction of cytochrome c_3 was followed at 552 nm in 50 mM Tris-HCl (pH 7.6); hydrogen pressure, 1 atm; temperature, 25 °C. For each concentration of cytochrome c_3, 4.5 ng of activated hydrogenase was injected in the 1 ml cuvette assay. The slope yielded $-1/K_m$.

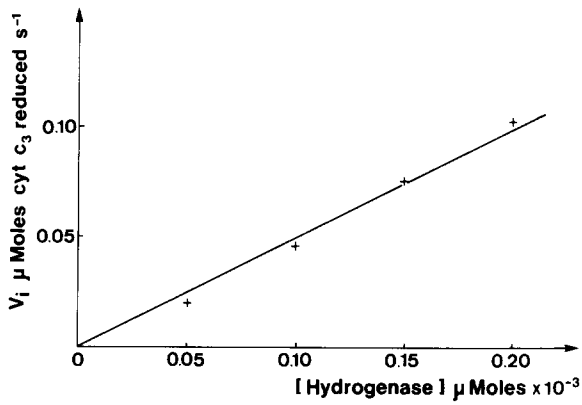


Fig. 5. Effect of hydrogenase concentration on initial reaction rate. Reduction of cytochrome c_3 was followed at 552 nm in 50 mM Tris-HCl (pH 7.6); hydrogen pressure, 1 atm; temperature, 25 °C; cytochrome c_3, 50 μM. The slope yielded the catalytic constant k_{cat}.

Discussion

The present work shows that cytochrome c_3 from *D. gigas* is a fast electrochemical system. It can act as a mediator agent for transferring electrons between hydrogenase and the graphite electrode. The four hemes of cytochrome c_3 exhibit negative redox potential values (−195, −295, −315 and −330 mV). It appears from polarographic measurements that one redox potential sharply separates from the others, whereas EPR data [17] gave values (−235, −235, −306 and −315 mV) which are in a comparatively less wide range. Such discrepancies between EPR and polarographic data can be due to the differences in experimental methods. In the EPR redox titration, mediator dyes are used to obtain equilibration at each potential, then protein samples are cooled to 26 K [17]. In the polarographic method, the protein interacts directly with the working electrode and the measurements are made at 25 °C.

The kinetics of the reduction of *D. gigas* cytochrome c_3 by its physiological partner hydrogenase in the presence of hydrogen was investigated by an electrochemical and a spectrophotometric method. The value of $6.5 \cdot 10^7$ $M^{-1} \cdot s^{-1}$

obtained from both methods is relatively high when compared with the rate constants reported for other interprotein electron-transfer reactions [44–46]. In the case of the flavocytochrome b_2-cytochrome c system, the rate constant measured in similar conditions is 10-fold lower than the value reported in the present study [45]. The comparison of the rate constant of *D. gigas* hydrogenase-cytochrome c_3 system with that of the homologous system from *D. desulfuricans* Norway [16] shows that the two values are closely related ($6.5 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ and $3 \cdot 10^7$ $M^{-1} \cdot s^{-1}$, respectively) although the two hydrogenases exhibit significant differences in their structure and activity. *D. desulfuricans* Norway soluble hydrogenase contains a selenium atom and lacks [3 Fe-xS] cluster [32,47]. It shows a rapid activation process and its EPR spectra exhibit a characteristic [4Fe-4S] signal in the reduced state and no Ni(III) signal in the oxidized state [32,33]. In contrast, *D. gigas* hydrogenase shows a slow activation process associated with modification of EPR spectra of nickel and the appearance of an unusual broad signal assigned to [4Fe-4S] cluster in the reduced form [23,24]. These differences between the two hydrogenases suggest that the catalytic mechanisms of the two enzymes are significantly different.

In the same way, cytochromes c_3 of the two species exhibit redox potentials significantly different: -195, -295, -315 and -330 mV for *D. gigas* cytochrome c_3 (this work) and -165, -305, -365 and -400 mV for *D. desulfuricans* cytochrome c_3 [42]. It results from these data that the values of the rate constants of the two systems are linked to the high specificity between hydrogenase and cytochrome c_3 from the same organism, since the quite different physicochemical and catalytic properties of the two systems do not appear to modify significantly these values. It can be assumed that favourable arrangement of both protein molecules occurs during electron transfer reaction. Weber and Tollin [44] have shown that electrostatic interactions influence considerably electron-transfer rates between protein molecules. These electrostatic interactions can assist in preorienting molecules along a productive reaction trajectory. The determination of the three-dimensional structures of hydrogenase and cytochrome c_3 from *D. gigas* which have been recently undertaken [27,38] should improve the understanding of the interaction between the two partners in terms of structural data.

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