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Voltammetric studies of the catalytic electron-transfer process between the *Desulfovibrio gigas* hydrogenase and small proteins isolated from the same genus

Cristina MORENO[1], Ricardo FRANCO[1], Isabel MOURA[1], Jean LE GALL[2] and José J. G. MOURA[1]

[1] Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Portugal
[2] Department of Biochemistry, University of Georgia, USA

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The kinetics of electron transfer between the *Desulfovibrio gigas* hydrogenase and several electron-transfer proteins from *Desulfovibrio* species were investigated by cyclic voltammetry, square-wave voltammetry and chronoamperometry. The cytochrome c_3 from *Desulfovibrio vulgaris* (Hildenborough), *Desulfovibrio desulfuricans* (Norway 4), *Desulfovibrio desulfuricans* (American Type Culture Collection 27774) and *D. gigas* (NCIB 9332) were used as redox carriers. They differ in their redox potentials and isoelectric point. Depending on the pH, all the reduced forms of these cytochromes were effective in electron exchange with hydrogenase. Other small electron-transfer proteins such as ferredoxin I, ferredoxin II and rubredoxin from *D. gigas* were tentatively used as redox carriers. Only ferredoxin II was effective in mediating electron exchange between hydrogenase and the working electrode. The second-order rate constants k for the reaction between reduced proteins and hydrogenase were calculated based on the theory of the simplest electrocatalytic mechanism [Moreno, C., Costa, C., Moura, I., Le Gall, J., Liu, M. Y., Payne, W. J., van Dijk, C. & Moura, J. J. G. (1993) *Eur. J. Biochem. 212*, 79–86] and the results obtained by cyclic voltammetry were compared with those obtained by chronoamperometry. Values for k of 10^5–10^6 M^{-1} s^{-1} (cytochrome c_3 as electron carrier) and 10^4 M^{-1} s^{-1} (ferredoxin II as the electron carrier) were determined. The rate-constant values are discussed in terms of the existence of an electrostatic interaction between the electrode surface and the redox carrier and between the redox carrier and a positively charged part of the enzyme.

Hydrogenase catalyzes the simple and reversible reaction $H_2 \rightleftharpoons 2H^+ + 2e^-$ in the presence of suitable electron donors/acceptors. This enzyme, present in sulfate-reducing bacteria, plays a central role in hydrogen metabolism [1]. A number of hydrogenases have been obtained from various sources, exhibiting different properties with respect to specific activity, oxygen stability, activation behavior, metal content, subunit structure and electron-carrier specificity [1–5].

The hydrogenase from the sulfate-reducing anaerobic bacterium *Desulfovibrio gigas* (molecular mass of 89 kDa with subunits of 26 kDa and 63 kDa [6, 7]) contains four redox centers; one nickel center, one [3Fe-4S] and two [4Fe-4S] clusters, whose existence was proven by electron paramagnetic resonance and Mossbauer spectroscopic studies [7, 8]. Hydrogenase enzymic activity induces both hydrogen production and consumption. The nickel atom is involved in the hydrogen activation and the Fe-S clusters, that act as secondary electronic carriers, are probably involved in electron transfer to the physiological partner. The enzyme is isolated in an inactive oxidized form that can be activated in a reversible way [6, 8–14].

The tetrahemic cytochrome c_3 has been considered as a natural carrier of this enzyme. The presence of cytochrome c_3 has been indicated to be necessary for the occurrence of the electronic transfer between hydrogenases of the *Desulfovibrio* genus and low-redox-potential carriers, such as ferredoxin (Fd) and flavodoxin [15].

The interaction between hydrogenase and several redox proteins, namely cytochrome c_3, rubredoxin, ferredoxin and flavodoxin, has been investigated by spectroscopic and electrochemical (kinetic) methods [16–18]. However, the doubt persists about the specificity of this enzyme in relation to cytochrome c_3 in *Desulfovibrio* species.

A direct electrochemical investigation of the electron-transfer process in which hydrogenase is involved is complicated by the fact that hydrogenase, like most other redox proteins, does not show electroactivity at electrodes under normal circumstances. Van Dijk et al. have demonstrated that direct, unmediated electrochemistry is possible at a dropping mercury electrode for *D. vulgaris* hydrogenase (an enzyme that contains only Fe–S clusters as redox centers), provided that charges opposite to the net charge of the protein (supplied by synthetic polypeptides or detergents) are added to

Correspondence to J. J. G. Moura, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, P-2825 Monte de Caparica, Portugal

Fax: +351 1 2954461.

Abbreviations. CA, chronoamperometry; CV, cyclic voltammetry; Fd, ferredoxin; NHE, normal hydrogen electrode; SCE, saturated calomel electrode.

Note. Desulfovibrio desulfuricans Norway 4 was renamed *D. baculatus* Norway 4. The organism was recently reclassified as *Desulfomicrobium baculatus* Norway 4 [62].

the solution [19, 20]. These substances adsorb at the mercury surface, preventing the irreversible adsorption and denaturation of hydrogenase. Additionally, the charge of the electrode surface is changed in a favorable way. It was concluded that electrostatic interactions have an important role in oxidation/reduction behavior of most proteins at electrodes [21–28]. Differences in electron-transfer rates are also expected in the reaction between proteins with a different isoelectric point and *D. gigas* hydrogenase.

The exact mechanism of hydrogenase-catalyzed hydrogen oxidation and proton reduction is now known, although several models based on proton/deuterium exchange experiments, EPR data and electrochemical studies have been discussed in other studies [3, 12, 29–34].

This study describes the results of an electrochemical investigation of the electron transfer rates between hydrogenase from *D. gigas* and different small-sized redox proteins (used as mediators) obtained from the same genus. Cyclic voltammetry (CV) and chronoamperometry (CA) were used to compare the obtained electron-transfer rate constants.

MATERIALS AND METHODS

Protein isolation and purification

The hydrogenase was isolated from *D. gigas* and purified as previously described [35]. *D. vulgaris* (Hildenborough), *D. desulfuricans* (American Type Culture Collection 27774), *D. desulfuricans* (Norway 4) and *D. gigas* cytochrome c_3 were purified as described in [36]. *D. gigas* Fd I and Fd II were purified as in [37] and *D. gigas* rubredoxin as in [38].

Chemicals

All chemicals were of analytical grade (Merck). All solutions were prepared with distilled water and flushed with purified argon (99.996%, Air Liquide).

Electrochemical measurements

The electrochemical cell consisted of a chamber with a 3-mm-diameter glassy-carbon or gold working electrode (Metrohm), a small calomel reference electrode separated from the main cell compartment by a Vycor frit and a platinum auxiliary electrode. The cell was closed by a metallic cap through which the solution could be flushed with purified argon.

Cyclic voltammetric and cronoamperometric measurements were performed using a PAR-270 potentiostat. This system was controlled by a personal computer (Zenith). All measurements were performed in buffer A consisting of 0.1 M Tris/HCl, 0.1 M acetate, 0.1 M maleate, pH 7.6. *D. gigas* [NiFe] hydrogenase is inactive as isolated [8, 9, 13, 14]. The reactivation of the enzyme involves two steps that have been throughly studied [8, 13] and electrochemical activation was described [39]. The enzyme used in the experiments described below was in the 'ready state' [9] and with a specific activity of approximately 400 μmol H_2 produced/min/mg protein. The enzyme was added shortly before the measurements were made. The solution in the cell was purged with argon (30 min) before every measurement. All measurements were performed at 25 ± 0.5°C.

For each series of measurements, the working electrode was polished for approximately 5 min with 0.3-μm (particle size) alumina (BDH) on a polishing cloth and washed with distilled water.

Before initiating the CV measurements, the potential was cycled at 100 mV/s or more between −0.2 V and −0.9 V against a saturated calomel electrode (SCE) in buffer A until a stable voltammogram was obtained. Subsequently single-scan voltammograms at scan rates of 5–100 mV/s were recorded of (a) 0.1 ml oxygen-free buffer A, (b) the same solution after the addition of the redox carrier (120 μM) and (c) the solution used in (b) with different concentrations of hydrogenase (0.25, 0.5, 1 and 2 μM).

For the CA measurements, the working electrode was kept at a conditioning potential E_1 (0.1 V) for 1 min before the potential was stepped to a measuring potential E_2 (−0.6 V). Immediately following the potential increase, the current was sampled during 5 s at a frequency of 100Hz. In this way, intensity/time curves were obtained for control solutions, for solutions containing the mediator, and for solutions containing the mediator and increasing amounts of the enzyme. All experiments were performed in triplicate and the reported data represent the mean of these results.

Data acquisition

For the computer-controlled chronoamperometric and cyclic voltammetric experiments and for some standard data processing tasks (data reduction, smoothing and subtraction of files) a software package for the electrochemical measurements (M270) was used.

The second-order electron transfer constants were obtained using the mathematical procedure outlined in [40] (see Appendix).

RESULTS AND DISCUSSION

Electron transfer between hydrogenase from *D. gigas* and several cytochromes c_3 from the same genus (using glassy-carbon and gold electrodes)

The four cytochromes c_3 used, isolated from *D. vulgaris*, *D. desulfuricans* (Norway 4), *D. desulfuricans* (ATCC 27774) and *D. gigas* (NCIB 9332), show electrochemical quasi reversible behavior at both glassy-carbon and gold electrodes.

The electrochemical effect (and pH dependence) of hydrogenase addition to a solution of cytochrome c_3 from *D. gigas* is shown in Fig. 1, for cyclic voltammetry, and in Fig. 3, for cronoamperometry. For experiments without hydrogenase, the current is controlled only by diffusion. For experiments in the presence of this enzyme, the current is controlled by the combined action of diffusion and the chemical reaction by which the oxidized mediator is reproduced.

Voltammograms of solutions of hydrogenase without the mediator do not differ from the voltammograms of control solutions. In all cases, when hydrogenase was added to the mediator solution, catalytic voltammograms were obtained indicating a steady-state condition of reduced hydrogenase in the layer near the electrode surface.

As discussed in the Appendix, the simple model of Nicholson and Shain has been used for the calculation of the second-order rate constants. Cyclic voltammograms were recorded of 120 μM cytochrome c_3 without hydrogenase at scan rates of 5, 10, 20, 50 and 100 mV/s. From these voltammograms, the diffusion-controlled peak current, $i_{d,p}$, was determined. The same series of measurements was repeated af-

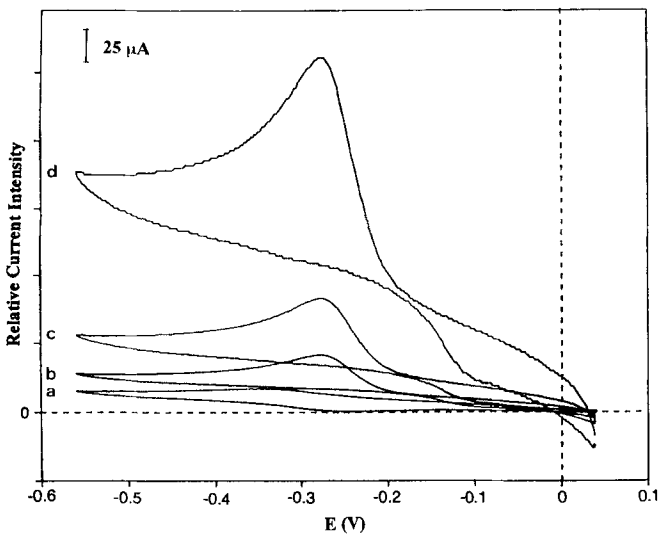

Fig. 1. The catalytic effect of *D. gigas* hydrogenase on the electrochemical response of cytochrome c_3 using cyclic voltammetry. Cyclic voltammograms (v = 5 mV/s) at a glassy-carbon electrode for *D. gigas* cytochrome c_3 (120 μM) in the absence (a) and presence of 2 μM *D. gigas* hydrogenase at pH 8.5 (b), pH 7.6 (c) and pH 6.5 (d). Other experimental conditions are described in the Materials and Methods section.

ter the addition of hydrogenase to determine the catalytic peak current, $i_{k,p}$, at each enzyme concentration (0.25, 0.5, 1 and 2 μM). Similar experiments were performed at several pH values. From the quotient $i_{k,p}/i_{d,p}$ a value of λ_1 could be calculated. If all the conditions of the Nicholson and Shain model [41] are met, plots of λ_1 versus $1/v$ should yield straight lines.

For several reasons, however, some of the conditions could not be fulfilled (low enzyme and substrate concentrations, reversible instead of irreversible reactions and different diffusion coefficients for cytochrome c_3 and hydrogenase) [42]. Therefore, a typical set of λ_1 versus $1/v$ [where v = potential scan rate (mV/s)] plots yielded curved lines (Fig. 2A). From a fit of the data points obtained, the initial slope at $1/v = 0$ was determined. The initial slope values were plotted against hydrogenase concentration (Fig. 2B). From the slope of the straight line drawn through these points (including the origin) the second-order rate constant, k, was calculated. The values of k are shown in Table 1.

The deviation from linearity in these plots is probably not due to the limiting effect of the consecutive reactions on the rate of recycling of hydrogenase. It would be more correct to explain the deviation from linearity by the reversibility of the reactions. As the hydrogen-evolution reaction proceeds, the influence of the reverse reactions becomes increasingly important (the lower the scan rate, the more hydrogen will be formed in the reaction layer near the electrode surface).

Cyclic voltammograms were used to select the most suitable potential-step regimes for the different mediators for the chronoamperometric experiments. In this technique, the potential is stepped from E_1 to E_2; E_1 has a value at which the reduction process to be studied can be neglected, and E_2 is in the range where the heterogenous reaction rate for this step is sufficiently high, i.e. diffusion controlled within the time scale under consideration. The resulting current is measured as a function of time.

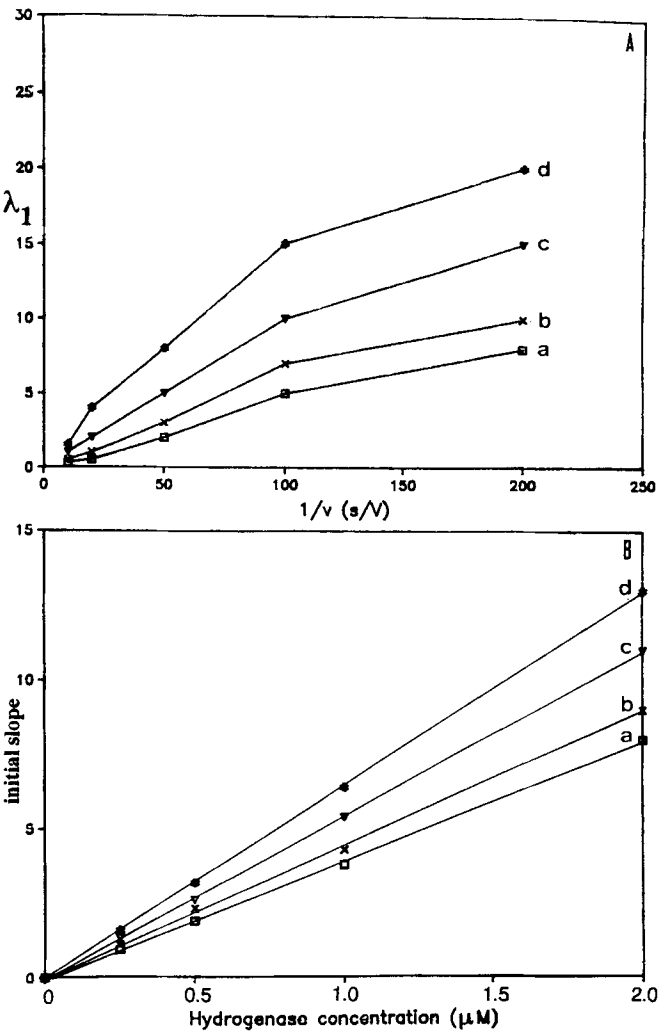

Fig. 2. Determination of the second-order rate constant parameter by cyclic voltammetry between *D. gigas* hydrogenase and *Desulfovibrio* cytochromes c_3. (A) Plots of the kinetic parameter λ_1 versus $1/v$ for increasing hydrogenase concentrations. The hydrogenase concentrations were 0.25 (a), 0.5 (b), 1.0 (c) and 2.0 μM (d). 120 μM *D. gigas* cytochrome c_3 was used at pH 6.5. (B) Initial slope (obtained from λ_1 versus $1/v$ plots) versus hydrogenase concentration (pH 7.6). *D. gigas* cytochrome c_3 (a); *D. desulfuricans* (ATCC 27774) cytochrome c_3 (b); *D. desulfuricans* (Norway) cytochrome c_3 (c); *D. vulgaris* cytochrome c_3 (d). The method used is described in [40, 41].

Due to the catalytic effect, it was observed that the current in the presence of hydrogenase decreases less rapidly than in the case of simple diffusion of the cytochrome c_3. The catalytic effect is noticeable at $t > 0.25$ s and is dependent on the enzyme concentration.

From arrays containing the diffusion-controlled currents, i_d versus $t^{-1/2}$ plots were examined to check the validity of the Cottrell equation in this particular experimental setup and it was concluded that on the time scale 0.25–5 s the current showed Cottrell behavior and was completely diffusion-controlled.

The ratio of the catalytic and diffusion currents (i_k/i_d) was converted into λ_2 values and plots of this kinetic parameter versus time showed, in all cases, a similar behavior; namely linearity in the region 0.25–2.0 s and deviation from linearity at larger times (Fig. 4A). Reasons for deviation from lin-

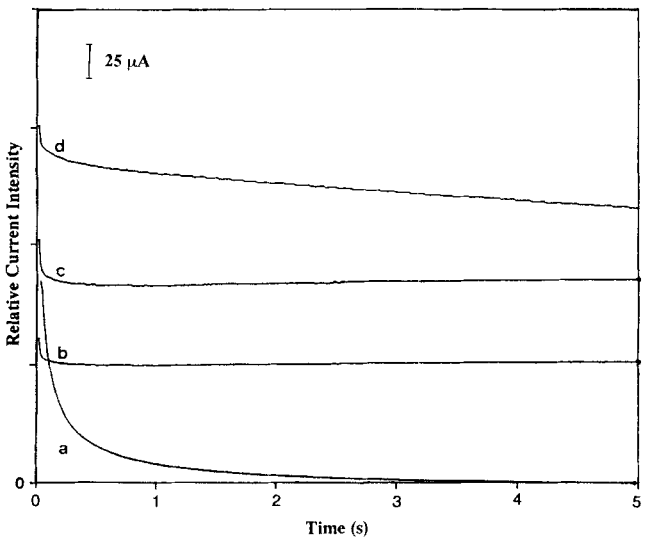

Fig. 3. The catalytic effect of *D. gigas* hydrogenase on the electrochemical response of cytochrome c_3 using chronoamperometry. Chronoamperometric voltammograms at glassy-carbon electrode for *D. gigas* cytochrome c_3 (120 μM) in the absence (a) and presence of 2 μM *D. gigas* hydrogenase at pH 8.5 (b), pH 7.6 (c) and pH 6.5 (d). The experimental conditions are described in the Materials and Methods section.

earity discussed for cyclic voltammetry are equally valid for the chronoamperometry results. Initial slopes were obtained by linear-regression analysis of the data within the range of 0.25−2.0 s and were plotted versus the hydrogenase concentration (Fig. 4B). From the slope of the straight line drawn through the points (including the origin), the second-order rate constant k was calculated (Table 1).

Table 1 shows an increase in k at decreasing pH values in the glassy-carbon electrode experiments, for all the c_3-type cytochromes studied. This was expected, as protons are the substrate for hydrogenase and the specific activity for the hydrogen production of this enzyme has a maximum at low pH values [29]. However, there is no simple relationship between the catalytic current and the substrate concentration, as has been found for enzymes like glucose oxidase [43] and peroxidase [44]. This can partly be explained by the fact that the measurements could not be performed under excess substrate conditions. Additionally, in the case of hydrogenase the substrate concentration (pH) also determines the formal potential, the charge of the enzyme, the conformation and the stability.

Analysis of Table 1 shows also that the second order rate constant for the electron transfer between the *D. gigas* hydrogenase and several cytochromes c_3 decreases in the following order: *D. vulgaris* (Hildenborough) (pI = 10) > *D. desulfuricans* (Norway) (pI = 7) > *D. desulfuricans* (ATCC 27774) (pI = 7) > *D. gigas* (pI 5), at pH 7.6, at glassy-carbon electrodes. These data show that the rate constant decreases with the decrease of the protein isoelectric point. At low pH values (pH = 6.5), this relation is no longer maintained and a similar rate constant k is determined both for the protein with the higher isoelectric point (*D. vulgaris* cytochrome c_3) and for the protein with the lowest isoelectric point (*D. gigas* cytochrome c_3). The determined rate constants were higher when cytochrome c_3 and hydrogenase were purified from the same species. At the gold electrode, this relation is observed for the pH range 6.5−9.5.

The glassy carbon surface has a pK_a value of approximately 5.6 [45], which implies that the surface is negatively charge at the working pH.

D. vulgaris cytochrome c_3 has a pI of 10, presenting a global positive charge in this pH range. In former studies, it was determined that this protein shows a quasi-reversible electrochemical response, usually with adsorption signals, at carbon surfaces [46]. If the interacting surfaces (electrode plus protein) present opposite charges, an adequate directional approximation will be allowed to produce an effective electron transfer (with facile formation of the protein-electrode complex) and it could also make the reversible

Table 1. Second-order rate constants (k) for the electron transfer between hydrogenase from *D. gigas* and several redox proteins from *Desulfovibrio* species. The error for the rate constants was estimated as ± 0.1. CV, cyclic voltammetry; CA, chronoamperometry; n.d., not determined.

Redox carrier	Organism	Electrode	Method	k ($\times 10^{-6}$) at pH			
				6.5	7.6	8.5	9.5
				$M^{-1} s^{-1}$			
Cytochrome c_3	*D. vulgaris*	Glass-carbon	CV	6.2	6.3	3.8	0.6
			CA	6.4	6.4	3.6	0.9
Cytochrome c_3	*D. desulfuricans* (Norway)		CV		5.3		
			CA		5.1		
Cytochrome c_3	*D. desulfuricans* (ATCC 27774)		CV		4.1		
			CA		3.9		
Cytochrome c_3	*D. gigas*		CV	6.1	3.7	1.2	0.3
			CA	6.9	3.2	1.1	0.1
Fd II	*D. gigas*		CA		0.013		
Fd I	*D. gigas*		–		n. d.		
Rubredoxin	*D. gigas*		–		n. d.		
Cytochrome c_3	*D. vulgaris*	Gold	CV	5.7	6.1	1.7	0.5
			CA	5.8	6.3	1.2	0.3
Cytochrome c_3	*D. gigas*		CV	6.3	7.7	2.1	0.8
			CA	5.9	8.3	2.4	0.7

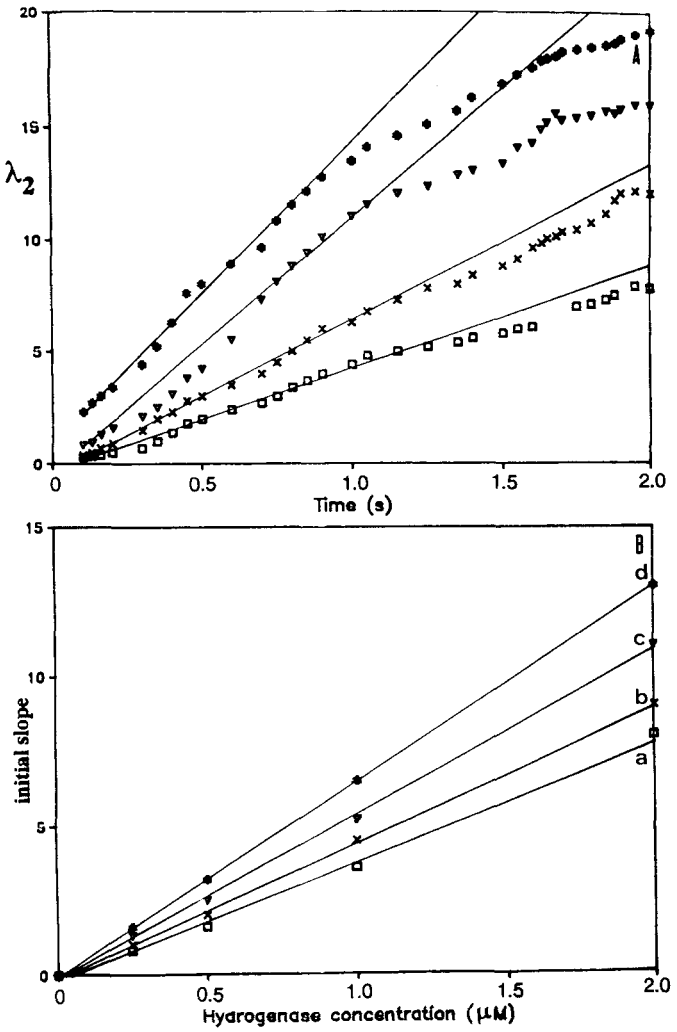

Fig. 4. The determination of the second-order rate constant parameter by chronoamperometry between *D. gigas* hydrogenase and *Desulfovibrio* cytochromes c_3. (A) Plots of the kinetic parameter λ_2 versus time for increasing hydrogenase concentrations. The hydrogenase concentrations were 0.25 (□), 0.5 (×), 1.0 (▼) and 2.0 μM (*). 120 μM *D. gigas* cytochrome c_3 was used at pH 6.5. (B) Initial slope (obtained from λ_2 versus t plots) versus the hydrogenase concentration (pH 7.6). *D. gigas* cytochrome c_3 (a); *D. desulfuricans* (ATCC 27774) cytochrome c_3 (b); *D. desulfuricans* (Norway) cytochrome c_3 (c) and *D. vulgaris* cytochrome c_3 (d). The method used is indicated in [40, 41].

dissociation of this complex difficult. Thus, the small difference observed between the rate constants k determined for the glassy-carbon electrode and for the gold electrode, in the case of *D. vulgaris* cytochrome c_3, can be justified by the fact that the gold surface does not present charged groups, avoiding adsorption problems. Only at pH 6.5 is a more significant increase of k observed at the gold electrode. This result was expected since the hydrogen production activity of the studied hydrogenase is higher at low pH values [29].

D. gigas cytochrome c_3 has a pI of 5, showing a global negative charge in the pH range studied. This protein shows a quasi-reversible electrochemical behavior at the gold electrode but a very weak one at the carbon surface [46]. It is difficult to observe a good electrochemical response for this protein at the glassy-carbon electrode. This fact can be explained because both surfaces show a negative charge, resulting in mutual repulsion. For these conditions, it would be very difficult to produce an adequate relative orientation between the protein and electrode surface to produce an effective electron transfer.

The second-order rate constants for the reaction between *D. gigas* cytochrome c_3 and hydrogenase, using glassy carbon electrodes, are smaller than the ones obtained for the same reaction with *D. vulgaris* cytochrome c_3 at pH values higher than 7. This result could be due to the limiting effect of the slow heterogeneous electron-transfer reaction between the first protein and the carbon surface. However, at pH 6.5, and also considering that the surface charge of the cytochrome approaches neutrality at this pH value, the determined rate constant k is higher than the rate constant for the second cytochrome.

The electrochemical experiments conducted with the gold electrode support this explanation, since the *D. gigas* cytochrome c_3 yielded higher k values than the values obtained with the *D. vulgaris* cytochrome, at all the pH values measured.

These last results could lead to the conclusion of the existence of an electrostatic interaction between the redox carrier and a neutral or positive region of the hydrogenase molecule, since after the elimination of the carbon surface influences, with the use of the gold electrode, hydrogenase shows higher electron transfer rates with the cytochrome that presents a global negative charge (*D. gigas* cytochrome c_3). This explanation is only valid assuming that the several cytochromes present the same type of structure, including the same relative orientation of the hemes.

The two *D. desulfuricans* cytochromes have pI values of approximately 7 and show an intermediate behavior relative to those described before.

Electron transfer between *D. gigas* Fd II and hydrogenase

An effective electronic transfer between *D. gigas* Fd II and hydrogenase, at glassy-carbon electrodes, is observed at pH 7.6, by square-wave voltammetry and chronoamperometry.

Fig. 5 shows the square-wave voltammograms obtained for this reaction. The electrochemical behavior of *D. gigas* Fd II has been recently detailed [47, 48]. Fd II presents an electrochemical response resulting in two broad waves, one centered at −130 mV and another centered at −690 mV versus NHE. These transitions have been interpreted as being due to the +1/0 (3-Fe cluster) redox transition and the negative redox step being reminiscent of a further $2e^-$ reduced state, as proposed for *Desulfovibrius africanus* Fd III by Armstrong et al. [49].

After the addition of 2 μM hydrogenase to the Fd II solution, the current intensity and the shape of the voltammograms are modified, resulting in only one well-defined wave, centered at −130 mV and associated with a threefold-higher current intensity. The second-rate constant k calculated for this reaction is 1.3×10^4 M^{-1} s^{-1}, which is a smaller value than the averaged one for cytochrome c_3. However, it is interesting to note that the electrochemically reduced [3Fe-4S] Fd II cluster can transfer electrons to hydrogenase, without the presence of any other intermediate electron carrier. This result has already been shown by other methods for the reverse reaction [15].

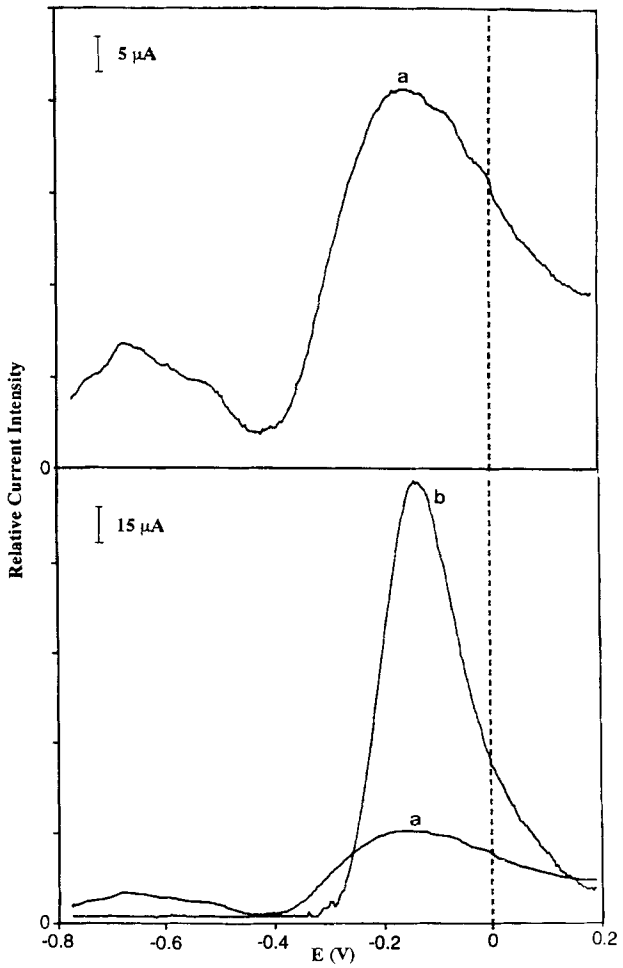

Fig. 5. The catalytic effect of *D. gigas* hydrogenase on the electrochemical response of Fd II using square-wave voltammetry. Square-wave voltammogram [frequency = 5 Hz, E_s (step potential) = 2 mV] at a glassy-carbon electrode for *D. gigas* Fd II (120 μM) before (a) and after (b) the addition of 2 μM *D. gigas* hydrogenase. Other experimental conditions are described in the Materials and Methods section.

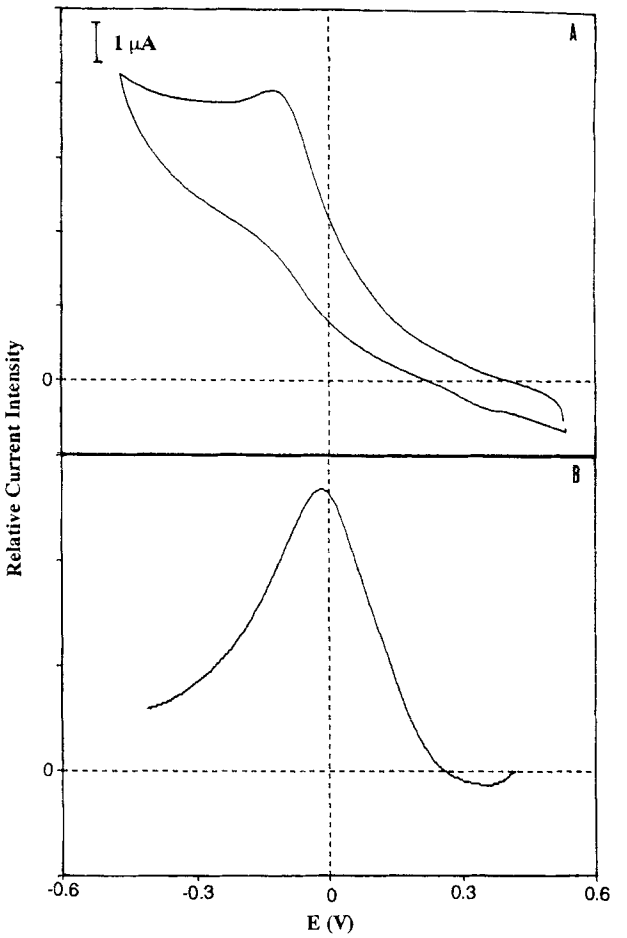

Fig. 6. The electrochemical behaviour of *D. gigas* rubredoxin using cyclic voltammetry and square-wave voltammetry. (A) Cyclic voltammogram (v = 10 mV/s) and (B) square-wave voltammogram (frequency = 5 Hz, E_s = 2 mV) at a glassy-carbon electrode for *D. gigas* rubredoxin (120 μM). Other experimental conditions are described in the Materials and Methods section.

Electron transfer between *D. gigas* ferredoxin I or rubredoxin and hydrogenase

No effective electron exchange between Fd I or rubredoxin and *D. gigas* hydrogenase is observed. The addition of these proteins results in no alteration of the voltammograms. This result is not conclusive in the rubredoxin case, since this protein shows an irreversible electrochemical behavior (a very slow heterogeneous electron transfer) both at the gold and at glassy-carbon electrodes (Fig. 6). With the necessary precaution due to the electrochemical irreversibility, the redox potential determined for this protein, by square wave voltammetry at pH 7.6, is -40 ± 10 mV versus NHE.

In the Fd I case (-450 mV versus NHE at pH 7 [48]), a reversible direct electrochemistry is shown at glassy-carbon electrodes, but no catalytic currents are observed with the presence of the hydrogenase from the same organism.

Conclusion

The electrochemical study of complex protein systems is possible as a consequence of the great investment in the study of the behavior of the isolated proteins. The application of the theoretical fundamentals of the catalytic mechanism of electrochemical reactions followed by chemical reactions allowed the determination of the homogeneous kinetics of coupled electron-transfer systems.

Previous studies have shown that cytochrome c_3 is a rapid electrochemical system [46]. In this study it is shown that this protein can act as a mediator for the electron transfer between hydrogenase and glassy-carbon and gold electrodes.

The kinetics of the oxidation of cytochrome c_3, belonging to several *Desulfovibrio* species, by the considered physiological partner i.e. hydrogenase (in the hydrogen-consuming mode), was investigated by electrochemical methods (cyclic voltammetry, square-wave voltammetry and chronoamperometry). The determined homogenous second-order rate constants are in the range 10^5-10^6 M^{-1} s^{-1}. The uptake-reaction rate constant determined for the reduction reaction of the cytochrome c_3 with hydrogenase, both from *D. gigas*, in the presence of hydrogen was determined to be 6.5×10^7 M^{-1} s^{-1} [18]. Considering that the enzymic activity of this hydrogenase, for the hydrogen-uptake reaction, is several times higher than the activity for the hydrogen production reaction [29], the lower rate-constant values for this last reaction (approximately ten times smaller) were predictable.

The observed small differences between the electronic transfer constants obtained for the reaction between the hydrogenase from *D. gigas* and several cytochromes c_3 from *Desulfovibrio* species were already justified in terms of interenzymic electrostatic interactions as well as interactions between the proteins and the electrode surfaces. The fact that the cytochromes used in this study differ in their averaged reduction potential should be taken into consideration, although they are all sufficiently low to enable hydrogenase reduction and hydrogen production activity. It can be concluded that the rate-constant values determined for the different systems are similar in magnitude and could be related with the high specificity between hydrogenase and this type of protein, since differences in the catalytic and physicochemical properties of the various c_3 cytochrome systems do not seem to significantly affect these values.

However, it can also be concluded that a favorable spatial arrangement should occur between both molecules, enzyme and electron carrier, during the electron-transfer reaction. Weber and Tollin have shown that the electrostatic interactions have a considerable influence on the electron-transfer rates between proteins [50]. These electronic interactions can be determined as prerequisites of molecular orientation along a productive reactional pathway. Recently, there has been a renewed interest in the study of binary complexes of electron-transfer proteins (flavodoxin/cytochrome c_3, rubredoxin/cytochrome c_3 [51, 52]) and evidence has been obtained for the electrostatic nature of these interactions. With recent developments in the three-dimensional structure resolution of many of these proteins (namely cytochromes c_3, flavodoxins, ferredoxins; [15]) and work in progress for hydrogenase [53], it should be possible to reach a better definition of the recognition sites, allowing a more detailed discussion of this topic.

In several bacterial hydrogenases, the applied redox potential was revealed to be the determinant in the hydrogen evolution activity. In the *D. gigas* hydrogenase, the determined potential at which half of the maximal activity is observed was -360 mV at pH 7.0 with reference to the NHE [13]. Accumulated evidence leads to the conclusion that the nickel atom is the active center in this hydrogenase [4]. The reduction potential for the disappearance of the Ni-C signal, originating from an EPR-silent active species, was determined to be approximately -400 mV against NHE at pH 7.0 [9, 13].

Fd II acts as a mediator system between hydrogenase and the solid electrode. The enhancement of the redox transition at -130 mV suggests that the one-electron reduction of the 3Fe cluster is the electron transfer step. We should emphasize that our data cannot indicate the actual reduction state of *D. gigas* hydrogenase under these mediated conditions. The evolution of hydrogen will be used in future studies to elucidate the reduction state under these conditions. This redox process is not mediated by Fd I. Two main reasons for the unreactivity between *D. gigas* hydrogenase and Fd I might be proposed. Either no favorable precursor complex is formed in these conditions or this hydrogenase requires at least one high redox potential on its redox partner.

Recently, electrochemical techniques have also been used to investigate the reactivity of [Fe] and [Ni-Fe-Se] hydrogenases with their oxido-reduction partner, namely the tetraheme cytochrome c_3 [16]. In this study, the reactivity of [Fe] hydrogenase from *D. vulgaris* (Hildenborough) and the [Ni-Fe-Se] hydrogenase from *D. desulfuricans* (Norway) was compared. The value of $3 \times 10^8\ M^{-1}\ s^{-1}$ was obtained for the rate constant of the *D. vulgaris* (Hildenborough) hydrogenase/*D. desulfuricans* (Norway) c_3 system. This rate constant is several times higher than the values determined in our study. This observation is in agreement with the fact that the enzymic activity of *D. vulgaris* hydrogenase is much higher than the activity of the *D. gigas* hydrogenase [29]. Noteworthy is the fact that no reactivity was observed for the *D. desulfuricans* (Norway) hydrogenase/*D. vulgaris* (Hildenborough) cytochrome c_3 system [16].

Although cytochrome c_3 has been considered as the general physiological partner of hydrogenase, since it shows high homogeneous electronic-transfer rates both in hydrogen uptake [18] and hydrogen-production reactions (this study), it was observed that this enzyme can efficiently transfer electrons to another electron carrier, namely Fd II. This result, demonstrating that direct electron transfer is possible between hydrogenase and some redox partners in the absence of cytochrome c_3, does not agree with the concept that this hemoprotein is the obligate physiological partner or cofactor for hydrogenase.

APPENDIX

The catalytic mechanism

The kinetics of electron transfer between hydrogenase and several small electron-transfer proteins were investigated by electrochemical methods using the following assumptions. In the presence of a suitable mediator (e.g. cytochrome c_3), hydrogenase is able to catalyze both the reduction of protons to hydrogen and the oxidation of hydrogen to protons. The addition of hydrogenase to a solution of cytochrome c_3, which is reversibly reduced at an electrode, results in enhanced cathodic and anodic currents, and the methodology described in [40] can be applied.

The reaction mechanism involving an initial heterogeneous electron transfer reaction at an electrode followed by a homogeneous chemical reaction, in which the original compound is regenerated, is called a catalytic mechanism. This mechanism can, in principle, be used to measure homogeneous electron-transfer rates. The simplest catalytic mechanism is represented by

$$M_{ox} + e^- \rightleftharpoons M_{red}$$

$$M_{red} + E \xrightarrow{k} M_{ox},$$

with the following assumptions: (a) the heterogeneous electron transfer of the redox couple M_{ox}/M_{red} is a one-electron reversible reaction, i.e. the electron transfer is very fast and uncomplicated; (b) species E is present in a large excess, i.e. the homogeneous chemical reaction is a pseudo-first-order reaction, with the corresponding reaction rate constant $k' = kc_z^o$ (where c_z^o is the molar bulk concentration of enzyme E); (c) the homogeneous chemical reaction is irreversible; (d) the diffusion coefficients of all species (M_{ox}, M_{red} and E) are the same.

The rate constant k of the homogeneous reaction was obtained using the analysis of Nicholson and Shain [41] for the 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 were studied as a function of the scan rate. The pseudo-first-order reaction rate constant k' can be obtained from the catalytic peak current ($i_{d,p}$) to diffusion-controlled peak current ($i_{d,p}$) ratio using a curve calculated from an integral equation given by Sa-

véant and Vianello [54]. This integral equation describes the dimensionless function for the catalytic current, which contains λ (λ_1 for cyclic voltammetry and λ_2 for chronoamperometry) as the dimensionless parameter for the second-order rate constant, k [40]. By using several concentrations of hydrogenase, the second-order homogeneous rate constants k were obtained from the plot of the pseudo-first-order rate constants versus hydrogenase concentration.

As the exact mechanism of the hydrogenase-catalyzed reaction is not known, the complete reaction scheme cannot be established. For several hydrogenases it has been proposed that one proton and two electrons are added to the enzyme in random order. The enzyme-hydride thus formed should react subsequently with a second proton to yield hydrogen [25, 55–58]. The reaction scheme for the cytochrome c_3 (M) mediated and hydrogenase-(E) catalyzed proton reduction can be proposed as:

$$
\begin{aligned}
&M_{ox} + e^- \rightleftharpoons M_{red}\\
&M_{red} + E \rightleftharpoons E^- + M_{ox}\\
&E^- + H^+ \rightleftharpoons EH \text{ (hydride)}\\
&EH + M_{red} + H^+ \rightleftharpoons EH_2 + M_{ox}\\
&EH_2 \rightleftharpoons E + H_2,
\end{aligned}
$$

with the overall reaction

$$2H^+ + 2e^- \rightleftharpoons H_2$$

in which two electrons are supplied by the mediator. The above mechanism is the most plausible from previous observations [4, 12, 32]. It is clear that this reaction scheme is far more complicated than the simple catalytic mechanism used by Nicholson and Shain in their calculations. A more general treatment of catalytic currents in cyclic voltammetry was provided by Andrieux et al. [59] and Savéant and Su [60], considering second-order effects, consecutive reversible reactions and two-electron processes. A solution of the set of different equations describing this mechanism would become very complex. Therefore, for the investigation of the intermolecular electron-transfer reaction between hydrogenase and redox proteins, the approach followed by Hill and Walton for azurin and cytochrome c_{551} [61] and by Niviére et al. for hydrogenase and cytochrome c_3 was followed [18]. This approach is based upon the idea that the actual reaction mechanism may be approximated by the simple mechanisms if the chosen time domain is small. In this case, especially with regard to hydrogenase, the following points are relevant: (a) first-order conditions are met, even if there is no large excess of enzyme over mediator, because the reaction between the redox protein and the oxidized hydrogenase has not proceeded far enough to cause a significant depletion of hydrogenase; (b) the rate of recycling of oxidized enzyme by the consecutive reactions will not limit the catalytic current; (c) the reaction between the enzyme and mediator can be considered to be irreversible, because the rate of the reverse reactions is very small compared to the rate of the forward reactions, due to the difference in concentrations; (d) the difference in diffusion coefficients of the reacting species can be neglected, since the concentration profile for the enzyme in the reaction layer has hardly been developed.

It should be noted that the calculation of the rate constants uses a formalism that has a strong analogy with the use of the current (at $t \rightarrow 0$) and enzyme activity measurements, where the influence of product inhibition and the depletion of substrate is avoided by measuring the initial velocity of the enzymic reaction [42].

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