Electron transfer between the hydrogenase from *Desulfovibrio vulgaris* (Hildenborough) and viologens

1. Investigations by cyclic voltammetry

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The electron transfer kinetics between the hydrogenase from *Desulfovibrio vulgaris* (strain Hildenborough) and three different viologen mediators has been investigated by cyclic voltammetry. The mediators methyl viologen, di(n-aminopropyl) viologen and propyl viologen sulfonate differ in redox potential and in net charge. Dependent on the pH both the one- and two-electron-reduced forms or only the two-electron-reduced form of the viologens are effective in electron exchange with hydrogenase. Calculations of the second-order rate constant \( k \) for the reaction between reduced viologen and hydrogenase are based on the theory of the simplest electrocatalytic mechanism. Values for \( k \) are in the range of \( 10^6 \) to \( 10^7 \) M\(^{-1}\) s\(^{-1}\) and increase in the direction propyl viologen sulfonate \( \rightarrow \) methyl viologen \( \rightarrow \) di(n-aminopropyl) viologen. An explanation is based on electrostatic interactions. It is proposed that the electron transfer reaction is the rate-determining step in the catalytic mechanism.

In recent years the study of electron-transfer processes in biological systems by means of electrochemical methods has received increasing attention. For a number of small-sized redox proteins (e.g. cytochrome \( c \), ferredoxines, and azurine) a direct electron transfer at metal oxide electrodes [1] or at gold electrodes modified with organic non-electroactive organic 'promotors' [2] has been achieved. The electrode kinetics of these systems can be investigated by standard techniques like cyclic voltammetry, rotating-disk electrode or impedance measurements. Still, for most (larger-sized) redox enzymes no direct electron transfer at electrodes has been described yet. The literature on the direct electron transfer between redox enzymes and electrodes is still scarce. Some examples are hydrogenase at the dropping mercury electrode in the presence of polylysine [3], covalently modified glucose oxidase at metal electrodes [4], cytochrome \( c \) peroxidase at tin oxide electrodes [5], and possibly also lysyl oxidase at glassy carbon [6].

An alternative way to couple an enzymatic to an electrochemical reaction is the use of redox mediators. Electrochemical methods can be used to measure the homogeneous electron-transfer rates between redox enzymes and artificial mediators or between redox enzymes and small redox proteins that show electroactivity at electrodes. Such studies have been described by Cass et al. for the reactions between cytochrome \( c \) and flavocytochrome \( b_2 \) (L-lactate dehydrogenase) and between the artificial mediator ferrocyanide carboxylate and flavocytochrome \( b_2 \) (L-lactate dehydrogenase) [7]; by Frew et al. for the reaction between several artificial mediators and peroxidase [8]; and by Hill and Walton for the systems cytochrome \( c \), cytochrome oxidase, and cytochrome \( c \) /cytochrome \( c_{551} \) /cytochrome oxidase [9].

This paper describes the results of an electrochemical investigation of the electron-transfer rates between the low-potential redox enzyme hydrogenase from *Desulfovibrio vulgaris* (strain Hildenborough) and a number of differently charged viologen mediators.

Hydrogenases catalyze the reversible reaction:

\[
\text{H}_2 \rightleftharpoons 2 \text{H}^+ + 2 \text{e}^{-}
\]

in the presence of suitable electron donors/acceptors. A number of hydrogenases have been obtained from various sources, exhibiting different properties with respect to specific activity, oxygen stability, metal content, subunit structure and electron-carrier specificity. They are all iron-sulfur proteins and they share the ability to utilize 4,4'-bipyridinium salts (viologens) as artificial electron donors/acceptors [10].

The hydrogenase from the sulfate-reducing anaerobic bacterium *D. vulgaris*, strain Hildenborough, belongs to the most active enzymes in this group, both in hydrogen production and consumption and it is oxygen stable in the oxidized (as
isolated) form. To our present knowledge, this hydrogenase consists of two subunits (46 and 13.5 kDa, respectively) of which the largest one contains two [4Fe-4S] clusters which are probably involved in the transfer of electrons to and from the electron donor/acceptor, and a third cluster (possibly of another type, e.g. [6Fe-6S]) which is thought to be part of the (hydrogen-binding) catalytic site [11]. The primary structure of the D. vulgaris hydrogenase has recently been reported [12, 13] but the tertiary structure has still to be determined.

The exact mechanism of hydrogenase-catalyzed hydrogen oxidation and proton reduction is not known, although several models based on proton-deuterium exchange experiments, EPR data and electrochemical studies have been discussed in the literature [14—21]. 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, under normal circumstances does not show electroactivity at electrodes.

Van Dijk et al. have demonstrated that direct, unmediated electrochemistry for D. vulgaris hydrogenase (and several other redox proteins) is possible at a dropping mercury electrode if charges oppose to the net charge of the proteins in the form of synthetic polypeptides or detergents are added to the solution [22, 3]. Because of adsorption of these substances at the mercury surface the irreversible adsorption and denaturation of hydrogenase is prevented. Besides, the charge of the electrode surface is changed in a favourable way. It was concluded that electrostatic interactions play an important role in the oxidation/reduction behaviour of most redox proteins at electrodes. This explanation is in agreement with the results obtained by other investigators [1, 23—29]. Differences in electron-transfer rates are also expected in the reaction between mediators with a different charge and D. vulgaris hydrogenase, which has a negative charge at the site of the protein that is most likely to combine with electron carriers [12]. Cyclic voltammetry (this paper) and chronoamperometry (following paper) were used to investigate the influence of charge and redox potential of the mediator on the kinetics of the homogeneous electron-transfer reaction.

**EXPERIMENTAL PROCEDURES**

**Isolation, purification and assay of hydrogenase**

D. vulgaris (strain Hildenborough NCIB 8303) was grown and its hydrogenase was extracted and purified according to Van der Westen [30]. The purified enzyme preparations were stored under liquid nitrogen.

The hydrogenase was assayed by measuring the hydrogen evolution activity. This was done manometrically in a standard way for the assay of the purified enzyme preparation [30]. For assays of hydrogenase activity of the solution in the electrochemical cell a gas chromatograph equipped with a catharometric detector was used. In both methods hydrogen production was linear with time and the activities measured with the two methods were in good agreement with each other.

**Chemicals**

Methyl viologen (MV) was obtained from Sigma as the dichloride salt and was used as received.

Di(n-aminopropyl) viologen (DAPV) was synthesized according to Simon and Moore and was obtained as the tetrabromide salt [31]. Propyl viologen sulfonate (PVS) was synthesized as follows. An amount of 1.43 g 4,4'-bipyridine dihydride (Aldrich) was dissolved in 8.8 g warm 1,3-propane sulfonate (Aldrich). The reaction mixture was heated at 100 °C. Within a few minutes a white precipitate was formed. After addition of 15 ml methanol the mixture was refluxed for 3 h and stirred for about 16 h at room temperature. The mixture was filtered and washed five times with 10 ml methanol. The resulting precipitate was dried under vacuum over P2O5. A yield of 1.3 g (44%) of bisalkylated product was obtained as the zwitterion (as confirmed by elemental analysis).

All other chemicals were of analytical grade and were used as received. All solutions were prepared with water obtained from a nanopure water purification system (Barnstead).

**Electrochemistry**

The electrochemical cell (home made) consisted of a perspex house with a glassy carbon working electrode (a disk of 5 mm diameter, coated along the side with silicon and sealed in glass [32]), a saturated calomel reference electrode (SCE, Metrohm) and a platinum auxiliary electrode, which was separated from the solution by an agar-agar salt bridge to avoid introduction of oxygen into the main cell compartment (oxygen is formed at the auxiliary electrode during cathodic scans).

The buffers used in the electrochemical experiments were solutions of 0.25 M Bistris or Tris, adjusted to the desired pH (between 6 and 9) with HCl. The ionic strength of the solutions was adjusted with NaCl to a value of 0.7 M. This was mainly done to prevent variations in hydrogenase activity; the activity is nearly independent of salt concentration at high ionic strengths [33]. Additionally each buffer solution contained 20 mM glucose. In combination with glucose oxidase (type II, Sigma), added shortly before the actual measurements to the solution in the cell in a concentration of about 2 U/ml, this resulted in an effective removal of traces of oxygen.

Unless mentioned otherwise, viologen concentrations were 0.25 mM, low enough to be able to neglect any influence arising from dimer formation of the semiquinone form.

Because this hydrogenase in reduced condition is unstable towards oxygen [30], all measurements were performed under strictly anaerobic conditions. The solution in the cell was purged with purified nitrogen before and between the measurements and passed over the solution during the measurements. The nitrogen gas was purified by passing it through a column of BTS catalyst (BASF) at about 120°C followed by passage through a wash bottle containing a solution of photoreduced methyl viologen [34]. The glucose/glucose oxidase combination in the solution served as a final oxygen-scavenging system. All measurements were performed at 20 ± 0.5°C.

A Wenking POS 73 potentiostat connected to a Philips PM 8043 x-y recorder was used for the cyclic voltammetry experiments.

**Procedure**

Before each series of measurements the working electrode was polished during about 5 min with 0.3 μm alumina (Buehler) on a polishing cloth, after which the electrode was cleaned in an ultrasonic bath filled with nanopure water. Before the actual measurements were done the potential was cycled at 50 mV/s between −0.2 and −1.2 V vs SCE in blank buffer until a stable voltammogram was obtained. Subsequently single-scan voltammograms at scan rates varying
over 5–100 mV/s were recorded of (a) 2.0 ml oxygen-free blank buffer containing 2 U/ml glucose oxidase, (b) of the same solution after addition of 10–50 µl of a 10 mM viologen solution, and (c) of the last obtained solution after addition of one or more 10-µl aliquots of a hydrogenase preparation.

After every scan the solution in the cell was stirred for a short while. Before a new amount of hydrogenase was introduced into the cell, a 10-µl sample was drawn from the solution and the enzyme activity was determined. Peak and plateau currents were measured manually from the recorded voltammograms and corrected for the background currents. The resulting data were analyzed with the help of appropriate computer programs (written in Fortran-77).

The calculated enzyme activities (in U/ml) were converted into concentrations by using a maximum specific activity for the hydrogenase in the H2 production reaction of 4600 U/mg [30] and the old value of 50 kDa for the molecular mass. An activity of 1 U/ml then equals a hydrogenase concentration of 4.4 nM.

The pKₐ of DAPV (8.3) was determined from titration with NaOH. Dimerization constants of the viologens were determined in 50 mM Tris buffer, pH 8, according to Mayhew and Müller [35].

**Computer programs**

A table of $i_{k,p}/i_{a,p}$ values as a function of $\lambda$ was generated by the numerical solution of the integral equations for the dimensionless functions of the catalytic and the diffusion currents given by Savvant and Vianello [36] and by Nicholson and Shain [37], respectively. This table (equivalent to the working curve of Nicholson and Shain) was used to determine the corresponding $\lambda$ values for the experimentally measured $i_{k,p}/i_{a,p}$ data. For large values of $i_{k,p}/i_{a,p}$ ($\lambda > 2$), the value for $\lambda$ was calculated with help of Eqn (2) (see below). The calculated $\lambda$ values were plotted versus the reciprocal of the scan rate. The initial slope of the resulting curves was determined by fitting the data with a third-order polynomial function. The regression coefficient ($r^2$) for these fits was in all cases better than 0.99. The slope of a plot of ‘initial slope’ versus hydrogenase concentration finally delivered a value for the second-order rate constant $k$ (standard linear regression program).

**RESULTS**

**The catalytic mechanism**

In the presence of a suitable mediator, e.g. methyl viologen, hydrogenase is able to catalyze both the reduction of protons to hydrogen and the oxidation of hydrogen to protons. Addition of hydrogenase to a solution of viologen, which is reversibly reduced at an electrode, results in enhanced cathodic and anodic currents. Considering only the reduction process, the reaction sequence can schematically be represented by Scheme I (for the oxidation process the arrows should be in the reverse direction).

![Scheme I](image)

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 reproduced, is called a catalytic mechanism. It can in principle be used to measure homogeneous electron-transfer rates. The simplest catalytic mechanism is represented by:

$$\text{P} + \text{e} \rightleftharpoons \text{Q}$$

$$\text{Q} + \text{Z} \rightarrow \text{P}$$

Scheme II

with the following assumptions:

- a) the heterogeneous electron transfer of the redox couple P/Q is a one-electron, reversible reaction, i.e. the electron transfer is very fast and uncomplicated;
- b) species Z is present in large excess, i.e. the homogeneous chemical reaction is pseudo-first-order, with the corresponding reaction rate constant $k' = k c^*_z$;
- c) the homogeneous chemical reaction is irreversible;
- d) the diffusion coefficients of all species (P, Q and Z) are the same.

The mathematics of linear sweep voltammetry for this particular mechanism has been described by Savvant and Vianello [36] and by Nicholson and Shain [37]. The pseudo-first-order reaction rate constant $k'$ can be obtained from the ratio of catalytic peak current ($i_{k,p}$) to diffusion-controlled peak current ($i_{a,p}$) using a working curve (or table) calculated from an integral equation given by Savvant and Vianello [36]. This integral equation describes the dimensionless function for the catalytic current, which contains $\lambda$ as the dimensionless parameter for the second-order rate constant $k$.

For the simple catalytic mechanism described above, the second-order rate constant can be calculated from the slope of a $\lambda$ vs $1/v$ plot. The dimensionless variable $\lambda$ is defined as:

$$\lambda = k c^*_z (RT/nF) 1/v.$$  \hspace{1cm} (1)

(The symbols are defined in the footnote on the first page.)

The $\lambda$ value is derived from the value of $i_{k,p}/i_{a,p}$ with help of the computer-generated working table. For small scan rates and/or large rate constants ($\lambda > 1.5$) a steady state is established by mutual compensation of diffusion and chemical reaction rates. The voltammogram becomes sigmoid-shaped, showing a limiting current region at potentials sufficiently cathodic of $E^\text{"c}$ in this region there is a simple relationship between $i_{k,p}/i_{a,p}$ and $\lambda$ [36]:

$$\frac{i_{k,p}}{i_{a,p}} = \frac{1}{0.4463 \sqrt{\lambda}}.$$  \hspace{1cm} (2)

However, the reaction mechanism is often more complicated than is shown in Scheme II. The case where species Z represents hydrogenase will be briefly discussed below.

As the exact mechanism of the hydrogenase-catalyzed reaction is not known, the complete reaction scheme cannot be written down with certainty. 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 ultimately hydrogen [14–17, 19, 21]. Most universally, the reaction scheme for the methyl-viologen-mediated and hydrogenase-catalyzed proton reduction can be proposed as:
The random mechanism implies that the sequence of the above given set of reactions may as well be ab, a'b, ab' or a'b'. It will be clear that this reaction scheme is far more complicated than the simple catalytic mechanism used by Savéant and Vianello and by Nicholson and Shain in their calculations. A more general treatment of catalytic currents in cyclic voltammetry has been given by Andrieux et al. [38] and by Savéant and Su [39], considering second-order effects, consecutive reversible reactions, and two-electron processes (ECE and SET mechanisms). Still, their treatment does not cover the particular mechanism for hydrogenase and a solution of the set of differential equations describing this mechanism would become very complex. Therefore, for the investigation of the intermolecular electron transfer reaction between hydrogenase and viologens an approach was considered which was also followed by Hill and Walton for azurine and cytochrome c [31]. It is based upon the idea that the actual reaction mechanism may be approximated by the simple mechanism in Scheme II if the chosen time domain is very small (i.e. large scan rates in cyclic voltammetry). In that case, especially with regard to hydrogenase:

a) first-order conditions are met, even if there is no large excess of enzyme over mediator (in practice often difficult to achieve) because the reaction between viologen and 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 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 (which can lead to appreciable deviations from former theoretical treatments [40]) can be neglected, because the concentration profile for the enzyme in the reaction layer has hardly been developed.

Note also that there is a strong analogy between the use of the current at $v \to \infty$ (or $t \to 0$) and enzyme activity measurements, where the initial velocity of the enzymatic reaction is measured to minimize the influence of product inhibition and depletion of (co-)substrate.

**Viologens as mediators for hydrogenase**

The three viologens used, MV, DAPV and PVS, show electrochemically reversible behaviour at glassy carbon electrodes ($E_p \approx 60$ mV; $i_{0}/v$ = constant, $E_p$ independent of scan rate). They are reduced in two one-electron steps. In the employed concentration ($\leq 0.25$ mM) dimer formation of the semiquinone form can be neglected. The structure of the viologens and a typical cyclic voltammogram are shown in Fig. 1. Formal potentials, net charge and dimerization constants ($K_d$) are summarized in Table I. The influence of the pH on the redox potentials in the pH range of 6-9 can be neglected for MV and PVS; for DAPV there is a shift of about $15$ mV in cathodic direction on changing the pH from 6 to 9.

The effect of addition of hydrogenase to a solution of viologen is shown in Fig. 2 for MV and in Fig. 3 for DAPV. In the case of PVS the voltammogram had the same shape as shown in Fig. 3. Voltammograms of solutions of hydrogenase without viologen did not differ from voltammograms of blank solutions. In all cases where hydrogenase was added to a viologen solution sigmoid-shaped voltammograms (plateaus instead of peaks) were obtained at scan rates up to $100$ mV/s indicating a steady-state condition of reduced hydrogenase in the reaction layer near the electrode surface.

If one compares Figs 2 and 3 a clear difference can be observed: for MV both cathodic waves are increased, for DAPV and PVS only the second cathodic wave is increased. This can be explained by the difference in redox potentials of the three viologens. The formal potential of MV is lower than those of DAPV and PVS (Table I). If the formal potential of hydrogenase is equal to the potential of the hydrogen electrode as has been found by Grande et al. [33], its value is $-686$ mV vs SCE at $pH = 7.5$ and at $20^\circ$C. Comparing the formal potentials of MV, DAPV and PVS with this value it will be clear that at pH 7.5 only the radical cation of MV will be able
Table 1. Summary of parameters of the employed viologens

\[ E^0 = \frac{(E_{pc} + E_{pa})}{2}; V^{2+}, V^+, \text{ and } V^0 \]  indicate the oxidized, one-electron reduced and two-electron reduced forms, respectively. All parameters were determined at pH 7.5 and 20°C. \( K_d \) values were determined by C. van Dijk et al. (unpublished results).

<table>
<thead>
<tr>
<th>Structure (Fig. 1)</th>
<th>( E^0 ) (1)</th>
<th>( E^0 ) (2)</th>
<th>Net charge</th>
<th>( K_d )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mV</td>
<td>mV</td>
<td>( V^{2+} )</td>
<td>( V^+ )</td>
</tr>
<tr>
<td>MV R = -CH₃</td>
<td>-695</td>
<td>-1015</td>
<td>2+</td>
<td>1+</td>
</tr>
<tr>
<td>DAPV R = -(CH₂)₃NH₃</td>
<td>-625</td>
<td>-950</td>
<td>4+</td>
<td>3+</td>
</tr>
<tr>
<td>PVS R = -(CH₂)₃SO₃</td>
<td>-635</td>
<td>-980</td>
<td>0</td>
<td>1-</td>
</tr>
</tbody>
</table>

Apart from the reduction of protons, the reverse reaction (the oxidation of hydrogen) is also catalyzed by *D. vulgaris* hydrogenase. In the voltammograms shown in Figs 2 and 3 this is indicated by an increase of the anodic current that corresponds with the oxidation of the radical cation to the oxidized form. A limited amount of reduced viologen is formed upon reduction of the hydrogenase by the hydrogen gas produced during the cathodic scan. The reduced viologen, which is only present in a high concentration in the vicinity of the electrode surface, is quickly depleted by reoxidation to reduce the hydrogenase. The radical cations of DAPV and PVS can only reduce hydrogenase at pH values lower than 6.5. Voltammograms recorded at pH 6.0 showed two cathodic waves which were both increased compared to the currents in the voltammograms of solutions of DAPV or PVS without hydrogenase present (not shown). At a pH of 9.0 also the radical cation of MV is unable to reduce hydrogenase in the electrochemical experiments (only the second cathodic wave was increased).

From a fit of the points obtained the initial slope at \( 1/v \) should yield straight lines if all conditions given before in the discussion of the Nicholson and Shain model are met.

\[ k = \frac{\lambda}{1 - \lambda} \]

It has been pointed out, however, that there are several reasons why in this particular case most of those conditions are not fulfilled (low enzyme and substrate concentrations, reversible instead of irreversible reactions, a difference in the diffusion coefficients of viologen and hydrogenase). Therefore, a typical set of \( \lambda \) vs \( 1/v \) plots shows curved lines (Fig. 4). From a fit of the points obtained the initial slope at \( 1/v = 0 \) was determined. The initial-slope values were plotted against hydrogenase concentration (Fig. 5). From the slope of the straight line drawn through these points (including the origin) the second-order rate constant \( k \) was calculated. The values at the electrode surface. This is shown by the pronounced oxidation peak in the case of DAPV (Fig. 3).

**Kinetics of the reaction between reduced viologen and hydrogenase**

As has been discussed before, the simple model of Nicholson and Shain has been used for the calculation of the second-order rate constants. Cyclic voltammograms were recorded of 0.25 mM viologen solutions without hydrogenase at scan rates of 5, 10, 20, 50 and 100 mV/s. From these voltammograms the diffusion-controlled peak current \( i_{dp} \) was determined. The same series of measurements was repeated after addition of hydrogenase to determine the catalytic peak/plateau current \( i_{k,p} \) at each hydrogenase concentration. All measurements were done at pH 7.5 and at pH 9.0. From the quotient \( i_{k,p}/i_{dp} \) a value for \( \lambda \) could be calculated from a table of calculated \( i_{k,p}/i_{dp} \) values as a function of \( \lambda \). Plots of \( \lambda \) versus \( 1/v \) should yield straight lines if all conditions given before in the discussion of the Nicholson and Shain model are met.
Fig. 5. Initial slope (obtained from $\dot{I}/I$ vs $I/l$ plots) vs hydrogenase concentration. DAPV pH 7.5 (*); MV1 pH 7.5 (x); MV2 pH 7.5 (+); PVS pH 7.5 (o); DAPV pH 9.0 (o); MV2 pH 9.0 (a).
relatively high values are consistent with the known high turnover rate for this enzyme [10]. Due to the relatively high rate constants catalytic currents can be measured even at very low substrate concentrations (pH 9.0 = 10^−9 M H⁺). Other important factors are the large effective diffusion coefficient of the substrate and the fact that the substrate concentration is expected to remain at a constant level due to the buffering of the solutions.

More important are the differences observed in the values of k for the three different viologens. At pH 7.5 the value of k increases in the direction PVS → MV → DAPV. This might be explained by the differences in the charge of the reduced viologens (−2, 0, +2, respectively for the two-electron-reduced PVS, MV and DAPV). At pH 9.0 the largest part of DAPV⁺ is uncharged and MV and DAPV show the same rate constant. It is known from the nucleotide sequence of the gene encoding the hydrogenase that the part of the protein containing the two [4Fe-4S] clusters is acidic and thus carries an excess of negative charges at pH 7.5 [12]. It is also known that electrostatic interactions play an important role in the electron-transfer reactions of hydrogenase [3]. The observed differences confirm our expectation that interaction between hydrogenase and positively charged electron carriers should be promoted.

The differences in catalytic efficiency (kcat/Δν) between the three viologens exist also under steady-state conditions (Δν = 5 mV/s). This may be an indication that the electron transfer reaction between electron carrier and hydrogenase, and not some further stage in the mechanism, is important in determining the rate of the reaction. This assumption would be in agreement with the observed dependence of λ on the DAPV concentration. According to the theory of Andrieux et al. [38] there is a linear relationship between λ and the reciprocal of the DAPV concentration if the electron transfer reaction is the kinetically controlling one. If the rate determining step is in a later stage of the mechanism λ would be independent of the DAPV concentration. A similar conclusion has been drawn by Ziomek et al. in a kinetic study of D. desulfuricans hydrogenase with DAPV and MV as electron carriers [42]. They reported equally high kcat/Δνapp values in both production and uptake reactions for both mediators and concluded that the initial formation of the enzyme-viologen complex determines the rate of the reaction.

A second conclusion that can be drawn from the results is that the deviation from linearity in the λ vs 1/Δν plots is probably not due to the limiting effect of the following 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).

The calculated second-order rate constant must be the smallest one or the mean of two electron transfer steps. The pair of reactions V⁺⁺ + E/H⁺ and V⁺⁺ + EH/H₂ is simultaneously series and parallel; series with respect to the enzyme species, parallel with respect to V⁺⁺, i.e. these reactions are competitive and consecutive. Based on the present results it is impossible to assign the observed value of k to one specific reaction.

For the same enzyme as has been used in this investigation, Lespinat et al. have concluded that the limiting catalytic step in the hydrogen production activity should be either dihydrogen-bond formation, product release or proton abstraction from the solvent [21]. This seems to be contradictory to our conclusion. However, their conclusion was based on the results of proton-deuterium exchange reactions which did not involve any electron transfer step other than the reduction of the enzyme by hydrogen or deuterium in the initial activating process, i.e. their studies were performed in the absence of viologen and cannot be compared to the electrochemical studies described in this paper.

From this study it appears that cyclic voltammetry may be a useful technique in kinetic studies of hydrogenase. However, the necessity to extrapolate the measured values to infinitely high scan rates restricts its usefulness. Scan rates over 100–200 mV/s cannot be used in practice at low current densities with solid electrodes, since the increasing charging current makes it impossible to obtain accurate values for the peak/plateau currents. Besides, catalytic currents are small at high scan rates because of turnover limitations. Therefore, for quantitative measurements the chronoamperometric technique has advantages over cyclic voltammetry (measurements at a smaller time scale are possible). Additionally, this technique is very suitable for computerization, making faster and more reproducible measurements possible. Results obtained with this technique will be discussed in the following paper.

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REFERENCES


