Electrochemical studies of the hexaheme nitrite reductase from Desulfovibrio desulfuricans ATCC 27774

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The electron-transfer kinetics between three different mediators and the hexahematin enzyme nitrite reductase isolated from Desulfovibrio desulfuricans (ATCC 27774) were investigated by cyclic voltammetry and by chronoamperometry. The mediators, methyl viologen, Desulfovibrio vulgaris (Hildenborough) cytochrome c3 and D. desulfuricans (ATCC 27774) cytochrome c3 differ in structure, redox potential and charge. The reduced form of each mediator exchanged electrons with nitrite reductase. Second-order rate constants, k, were calculated on the basis of the theory for a simple catalytic mechanism and the results, obtained by cyclic voltammetry, were compared with those obtained by chronoamperometry. Values for k are in the range 106–108 M−1 s−1 and increase in the direction D. desulfuricans cytochrome c3 → D. vulgaris cytochrome c3 → methyl viologen. An explanation is advanced on the basis of electrostatic interactions and relative orientation between the partners involved.

Chronoamperometry (computer controlled) offers advantages over cyclic voltammetry in the determination of homogeneous rate constants (faster, more accurate and better reproducibility).

Direct, unmediated electrochemical responses of the hexahematin nitrite reductase were also reported.

The study of electron-transfer processes in biological systems by electrochemical methods has recently received increasing attention. For a number of a small-sized redox proteins (e.g. cytochrome c, cytochrome c3 and ferredoxins) a direct electron transfer at metal oxide electrodes [1] or at carbon electrodes [2,3] has been achieved. The electrode kinetics of these systems can be investigated by such standard techniques as cyclic voltammetry, rotating-disk electrode or impedance measurements. Still, for larger-sized redox enzymes, no direct electron transfer at electrodes has yet been described. Reports of direct electron transfer between redox enzymes and electrodes remain scarce. Some examples are studies of hydrogenase at the dropping mercury electrode in the presence of polylysine [4], covalently modified glucose oxidase at metal electrodes [5] and cytochrome c peroxidase at tin oxide electrodes [6].

Coupling an enzyme to an electrochemical reaction by use of redox mediators offers an alternative approach. Voltammetric and chronoamperometric 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. Cass et al. [7] described such studies for the reactions between cytochrome c and flavocytochrome b2 (l-lactate dehydrogenase) and between the artificial mediator ferrocenium carboxilate and flavocytochrome b2 (l-lactate dehydrogenase). Frew et al. [8] investigated the reaction between several artificial mediators and peroxidase; Hill and Walton [9] the systems cytochrome c/azurin/cytochrome oxidase and cytochrome c/cytochrome c550/cytochrome oxidase; Hoogvliet et al. [10] the reaction between viologens and hydrogenase; Niviere et al. [11] the reaction between cytochrome c3 and hydrogenase. Although cyclic voltammetry proved to be a valuable technique for kinetic studies of electron transfer reactions of redox enzymes [7], there are disadvantages, especially when the reaction mechanism cannot be described by the theory of the simple electrocatalytic mechanism [12,13] (pseudo first-order and irreversible chemical reactions) without involving significant simplifications [9,10]. The simplifications made in order to apply the theory of a simple catalytic mechanism to more complicated reactions (second-order kinetics, reversible chemical reactions) imply the extrapolation of measured data towards an infinite scan rate. The main disadvantage of this approximation is that cyclic voltammetry cannot be used for accurate measurements at high scan rates (> 200 mV/s) with solid electrodes, unless impractical high enzyme concentrations

Abbreviation. DPV, differential pulse voltammetry.
are employed. The large number of measurements required at different scan rates and enzyme concentrations to complete a kinetic study represents another disadvantage.

In a previous application of chronoamperometry for kinetic studies of redox proteins, Ryan et al. [14] compared chronoamperometry with stopped-flow spectrophotometry for the determination of homogeneous second-order rate constants of the reaction between some Fe(II) complexes and both horse heart cytochrome c and Rhodospirillum rubrum cytochrome c₃ and Hoogvliet et al. [15] used the technique for a kinetic study of the reduction of Desulfovibrio vulgaris (Hildenborough) hydrogenase by viologens.

Nitrite reductase isolated from D. desulfuricans (ATCC 27774) belongs to the ammonia-forming respiratory class [16]. It is membrane bound and its synthesis can be substantially increased by growing the bacteria with nitrate rather than sulfate as terminal oxidant. The purified enzyme has a molecular mass of 66 kDa and exhibits an optical absorption spectrum of a typical c-type cytochrome. Based on its absorption coefficient and iron content, this nitrite reductase contains six c-type hemes/molecule [16, 17] and catalyses the six-electron reduction step of nitrite to ammonia.

\[
\text{NO}_2^- + 8 \text{H}^+ + 6 \text{e}^- \rightarrow \text{NH}_2\text{H} + 2 \text{H}_2\text{O}.
\]

EPR and visible redox titrations indicated that the hemes have a span of redox potentials from +150 to −400 mV; some, therefore, susceptible to reduction by ascorbate but others reduced only by diithionite ([17] and our unpublished data). A combination of EPR and Moessbauer measurements indicated that one high-spin (s = 5/2) and five low-spin (s = 1/2) hemes are present. The system is complex and two pairs of magnetically interacting hemes were detected (low spin/low spin and low spin/high spin). The high-spin heme was found to bind NO, suggesting that this heme could be an intermediate present in an enzyme-bound form [17].

The current study describes the results of an electrochemical investigation of the electron transfer rates between nitrite reductase isolated from D. desulfuricans (ATCC 27774) and the mediators methyl viologen, D. vulgaris (Hildenborough) cytochrome c₃ and D. desulfuricans (ATCC 27774) cytochrome c₃. Both cyclic voltammetry and chronoamperometry were used to determine the homogeneous rate constants. Direct, unmediated electrochemical responses of the enzyme, with and without the substrate, are also reported.

**MATERIALS AND METHODS**

**Isolation and purification of the enzyme and the redox proteins**

*D. desulfuricans* (ATCC 27774) was grown with nitrate rather than sulfate as terminal electron acceptor to promote the production of nitrite reductase, and the enzyme was purified as previously described [16,17]. *D. desulfuricans* (ATCC 27774) cytochrome c₃ and *D. vulgaris* (strain Hildenborough) cytochrome c₃ were purified as described in [18].

**Chemicals**

Methyl viologen was obtained from Sigma as the dichloride salt. All other chemicals were of analytical grade. All solutions were prepared with distilled water.

**Electrochemical measurements**

The electrochemical cell consisted of a perspex house with a 3-mm diameter glassy carbon working electrode (Metrohm), a small Ag/AgCl 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 nitrogen.

Cyclic voltammetric and chronoamperometric measurements were performed using an Autolab-110 system (ECO-Chemie) consisting of a potentiostat, a 16-channel 12-bit ADC equipped with a programmable gain amplifier to yield increased resolution, and a four-channel 12-bit DAC module. The whole system was controlled by a personal computer (Olivetti M24). All measurements were performed in 0.1 M Bistris, pH 7.0. Each solution could contain, additionally, 10 mM sodium nitrite. The enzyme was added shortly before the actual measurements were started. The solution in the cell was purged with nitrogen before and between the measurements. All measurements record activity at 25 ± 0.5°C.

For each series of measurements, the working electrode was polished during about 5 min with 0.3 µm alumina (BDH) on a polishing cloth, then washed with distilled water. Before initiating the actual cyclic voltammetric measurements, the potential was cycled at 50 mV/s between −0.2 and −0.9 V against Ag/AgCl in blank buffer until a stable voltammogram was obtained. Subsequently single-scan voltammograms at scan rates varying over 5–200 mV/s were recorded for (a) 0.1 ml oxygen-free buffer containing 10 mM sodium nitrite, (b) the same solution after addition of mediator and (c) of the solution used in (b) with different concentrations of nitrite reductase.

For the chronoamperometric measurements, the working electrode was kept at a conditioning potential *E*, for 1 min before the potential was stepped to a measuring potential of *E*. Immediately following the potential increase, the current was sampled during 5 s at a frequency of 100 Hz. In this way, intensity/time (*i*/*t*) curves were obtained for blank solutions, for solutions containing mediator, and for solutions containing mediator and increasing amounts of enzyme, respectively. All experiments were performed in triplicate.

**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 electrochemical measurements (GPES) was used (ECO-Chemie). GPES also served in preparation of Cottrell plots (*i* versus *t*<sup>1/2</sup>). In all experiments, only one of each ten sampled points was stored on disk.

A table of *i*<sub>c</sub>/*i*<sub>a</sub> values as a function of *λ*<sub>c</sub> [where *λ*<sub>c</sub> = *kc*<sub>c</sub>/(RT/nF)(1/ν)] was generated by the numerical solution of the integral equations for the dimensionless functions of the catalytic and the diffusion currents [12,13]. This table, equivalent to the working curve [12] was used to determine the corresponding *λ*<sub>c</sub> values for the experimentally measured *i*<sub>c</sub>/*i*<sub>a</sub> figures. For large values of *i*<sub>c</sub>/*i*<sub>a</sub> (e.g. *λ*<sub>c</sub> > 2), the value for *λ*<sub>c</sub> was calculated with the aid of Eqn (2) (see below). The calculated *λ*<sub>c</sub> values were plotted versus the reciprocal of the scan rate. The initial slopes of the resulting curves were determined by fitting the data with a third-order
The slope of a plot of the 'initial slope' versus nitrite reductase concentration finally delivered a value for the second-order constant, \( k \) (standard linear regression program).

**Theoretical considerations; the catalytic mechanism**

In the presence of a suitable mediator, e.g. methyl viologen, nitrite reductase is able to catalyse both the reduction of nitrite to ammonia and the oxidation of ammonia to nitrite. Addition of nitrite reductase to a solution of methyl 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 1, where MV is methyl viologen (for the oxidation process the arrows should be in the reverse direction).

![Scheme 1](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 regenerated, is called a catalytic mechanism. It can, in principle, be used to measure homogeneous electron-transfer rates. The simplest catalytic mechanism is represented by

\[
P + e^- \rightarrow Q
\]

\[
Q + Z \rightarrow P,
\]

Scheme 2.

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 \( k' = k_{e^2_i} \); (c) the homogeneous chemical reaction is irreversible; (d) the diffusion coefficients of all species (P, Q and Z) are the same.

The equations applicable to linear-sweep voltammetry for this particular mechanism have been described by Savéant and Vianello [13] and by Nicholson and Shain [12]. 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 \( i_{p} \) using a working curve (or table) calculated from an integral equation given by Savéant and Vianello [13]. This integral equation describes the dimensionless function for the catalytic current, which contains \( \lambda_1 \) 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 \( \lambda_1 \) vs \( 1/v \) plot. The dimensionless variable \( \lambda_1 \) is defined as:

\[
\lambda_1 = k \frac{C_i^*}{(RT/nF)} \frac{1}{(1/v)},
\]

where \( C_i^* = \text{bulk concentration of enzyme Z(M)} \).

The \( \lambda_1 \) value is derived from the value of \( i_{k,p}/i_{p} \) with help of the computer-generated working table. For small scan rates and/or large rate constants \( (\lambda_1 > 1.5) \), 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, \( E' \), sufficiently cathodic. In this region, there is a simple relationship between \( i_{k,p}/i_{p} \) and \( \lambda_1 \) [[10],

\[
(i_{k,p}/i_{p}) = (1/0.4463)(\lambda_1)^{1/2}.
\]

The mathematics describing a chronoamperometric experiment in the case of a catalytic mechanism have been published by Delahay and Stiehl [19], who obtained the following expression for the time dependence of the catalytic current \( i_k \):

\[
i_k = nFA D^{1/2}C^0 \left\{ (k C_i^*)^{1/2} \right. \left[ \exp \left( -k C_i^*(nt)^{1/2} \right) \right] + \left[ \exp \left( -k C_i^* \right) \right] \right\}.
\]

where \( \text{erf} \text{ (x)} = (2/\sqrt{n}) \int e^{-y^2} \text{ dy} \). Combining this equation with the expression for the diffusion-controlled current, \( i_d \) (the Cottrell equation)

\[
i_d = nFA D^{1/2}C^0 (nt)^{-1/2},
\]

and defining a dimensionless variable, \( \lambda_2 \) as

\[
\lambda_2 = \frac{kC_i^*}{\text{t}},
\]

a relation between \( i_{k,p}/i_d \) and \( \lambda_2 \) is obtained:

\[
(i_{k,p}/i_d) = \lambda_2^{1/2} \left[ \pi^{1/2} \text{ erf} (\lambda_2^{1/2}) + \text{exp} ( -\lambda_2^{1/2})\right].
\]

For large values of \( \lambda_2 \) (\( t \rightarrow \infty \) or \( k \) very large), the error function approaches unity and the exponential expression approaches zero, in which case Eqn (6) can be simplified to

\[
(i_{k,p}/i_d) = (\pi \lambda_2^{1/2}) = 1.7724 \lambda_2^{1/2}.
\]

In the presence of the error function in Eqn (6), it is impossible to calculate \( \lambda_2 \) directly from \( i_{k,p}/i_d \) data. Therefore, just as in the cyclic voltammetry case, a working table is used to calculate \( \lambda_2 \) (and thus \( k \)) from experimentally obtained \( i_{k,p}/i_d \) values. For large \( \lambda_2 \) values \( (\lambda_2 > 1.5) \) \( i_d/i_{k,p} \) can be calculated with Eqn (7) [15].

The reaction mechanism for the intermolecular electron-transfer reaction between nitrite reductase and mediators can be complex. The kinetic information may be based on the theory for the simple catalytic mechanism (Scheme 1) if the chosen time domain is small. In a small enough time scale, there will be a linear relation between \( \lambda_2 \) and \( t \). Therefore, initial slopes of \( \lambda_2 \) versus \( t \) plots should be used.

**RESULTS AND DISCUSSION**

The electron transfer kinetics between \( D. \text{ desulfuricans} \) (ATCC 27774) nitrite reductase and three different electron carriers [methyl viologen, \( D. \text{ vulgaris} \) (Hildenborough) and \( D. \text{ desulfuricans} \) (ATCC 27774) cytochrome c5] was studied using cyclic voltammetry and chronoamperometry methods.

The reactivity of the enzyme towards the electrode system is first considered, as an essential step for the kinetic study performed.
Independent visible and Moessbauer static redox titrations (our unpublished results) indicate that one of the hemes has a reduction potential quite high (150 ± 15 mV versus normal hydrogen electrode). Apparently, this contribution is absent in our experimental voltammogram. Then, the DPV results were simulated using only five heme redox transitions, at reduction-potential range values that agree with our independent measurements. The fact that one of the hemic contributions is not detected by electrochemical measurements can be speculated to be due to a specific structural characteristic, i.e. the heme can be quite protected in the interior of the polypeptide chain, having a difficult access to the electrode surface. However, no evidence excludes the heme being reduced by a intramolecular step controlled kinetically and not thermodynamically, as we should forsee using the relative values of the reduction potentials involved.

The results obtain in this multi-redox system are an extension of our previous work on tetraheme cytochrome c₃ [2], now applied to situations where spin equilibria and catalytic mediated processes occur. Also, new perspectives for the substrate binding to hemes can be considered. In particular, the reactivity of the nitrite reductase active site (high-spin heme) with NO will be one of the aspects to be explored, now that the necessary methodology has been established.

Kinetics of the reaction between reduced mediators and nitrite reductase

The three mediators used show electrochemically reversible (or quasireversible) behaviour at glassy carbon electrodes ($\Delta E_P = 60$ mV, $i_{\text{p}}/i_{\text{p}}^{1/2} = \text{constant}$, $E_{\text{p}}$ independent of the scan rate). The effect of addition of nitrite reductase to a solution of mediator is shown in Fig. 2A for methyl viologen and in Fig. 2B for D. vulgaris cytochrome c₃. Cyclic voltammograms of solutions of nitrite reductase without mediator did not differ from those of blank solutions (the direct electrochemistry of this enzyme could only be detected using differential pulse voltammetry and square wave voltammetry). In all cases where nitrite reductase was added to mediator solution, sigmoid-shaped voltammograms were obtained at scan rates up to 200 mV/s, indicating a steady-state condition of reduced nitrite reductase in the reaction layer near the electrode surface. If one compares Fig. 2A and B, a clear difference can be observed; in the presence of methyl viologen, the catalytic current is much higher than that observed in the presence of cytochrome c₃.

As indicated, the simple model proposed by Nicholson and Shain [12] was used for the calculation of the second-order rate constants. Cyclic voltammograms of 0.3 mM methyl viologen and 0.6 mM cytochrome c₃ solutions, without nitrite reductase, at scan rates of 5, 10, 20, 50, 100 and 200 mV/s were recorded, and from these the diffusion-controlled peak current, $i_{\text{p}}$, was determined. The same series of measurements was repeated after addition of nitrite reductase to determine the catalytic peak/plateau current, $i_{\text{p}}$, at each enzyme concentration. All reaction mixtures were at pH 7.0. From the quotient $i_{\text{p}}/i_{\text{p}}$, a value of $\lambda_c$ could be calculated. If all the conditions given before in the discussion of the Nicholson and Shain model are met, plots of $\lambda_c$ versus $1/\nu$ should yield straight lines. For several reasons in this particular case, however, some of those conditions could not be fulfilled (low enzyme and substrate concentrations, reversible instead of irreversible reactions and a difference in the diffusion coefficients of mediator and enzyme). Therefore, a typical set of $\lambda_c$ vs $1/\nu$ plots yielded curved lines (Fig. 3A).
Fig. 2. Catalytic effect of *D. desulfuricans* (ATCC 27774) nitrite reductase on the electrochemical response of methyl viologen and cytochrome c₃. (A) Cyclic voltammograms (1) 0.3 mM methyl viologen in Bistris, pH 7.0, and 10 mM NaNO₃; (2) as in (1) with addition of 50 µM nitrite reductase. Potential scan rate, 5 mV/s. (B) Cyclic voltammograms (1) 0.6 mM cytochrome c₃ from *D. vulgaris* in Bistris, pH 7.0, and 10 mM NaNO₃; (2) as in (1) with addition of 50 µM nitrite reductase. Potential scan rate 5 mV/s.

From a fit of the points obtained, the initial slope at 1/ν = 0 was determined. The initial slope values were plotted against nitrite reductase concentration (Fig. 3 B). 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.

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 *E₁* to *E₂*; *E₁* has a value at which the reduction process to be studied can be neglected, and *E₂* is in the range where the heterogeneous reaction rate for this reduction step is sufficiently high, i.e. diffusion-controlled within the time scale under consideration. The resulting current is measured as a function of time. For all the mediators, only one potential step regime was employed. The potential was stepped from −0.1 V to −0.9 V against Ag/AgCl. To eliminate disturbance due to convection, in all experiments the potential was held at *Eₙ* during 1 min after homogenization of the solution and before the potential step was applied.

For the experiments without nitrite reductase, the current is controlled only by diffusion. For experiments in the presence of nitrite reductase, the current is controlled by the combined action of diffusion and the chemical reaction by which the oxidized mediator is reproduced.

To prevent disturbing effects from convection, no measurements beyond *t* = 5 s were used. The value of data sampled at short times is limited by the magnitude of the residual (mainly double-layer charging) current. Its effect is largely reduced by the subtraction of *i(t)* data measured in blank buffer from *i(t)* data measured in solutions containing mediator or both mediator and nitrite reductase. All measurements were performed in triplicate and all further data processing was with averaged and residual current-subtracted data (data not shown). Due to the catalytic effect, it was
Table 1. Second-order rate constants for the reaction between nitrite reductase and mediators.

<table>
<thead>
<tr>
<th>Mediator</th>
<th>( k ) calculated by</th>
<th>( k ) calculated by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cyclic voltammetry</td>
<td>chronoamperometry</td>
</tr>
<tr>
<td>Methyl viologen</td>
<td>1.6 \pm 0.1 \times 10^8</td>
<td>2.2 \pm 0.1 \times 10^8</td>
</tr>
<tr>
<td><em>D. vulgaris</em> cyt. <em>c</em>_3</td>
<td>1.1 \pm 0.1 \times 10^7</td>
<td>1.2 \pm 0.1 \times 10^7</td>
</tr>
<tr>
<td><em>D. desulfuricans</em> cyt. <em>c</em>_3</td>
<td>2.0 \pm 0.1 \times 10^6</td>
<td>3.2 \pm 0.1 \times 10^6</td>
</tr>
</tbody>
</table>

Fig. 4. Cottrell plot of 0.3 mM methyl viologen in 0.1 M Bistris, pH 7.0, and 10 mM NaNO₃.

observed that the current in the presence of nitrite reductase decreases less rapidly than in the case of simple diffusion of mediator. The catalytic effect is noticeable at \( t > 0.25 \) s and is dependent on the enzyme concentration.

From arrays containing the diffusion-controlled currents, Cottrell plots (\( i_q \) vs \( t^{-1/2} \)) were examined to check the validity of Eqn (4) in this particular experimental setup. A typical plot is shown in Fig. 4. Only data in the region 0.25–5 s were used for linear regression analysis, because data sampled at shorter times deviates from linearity as a result of not completely compensated residual current and the large uncertainty of the subtracted data in this region. Correlation coefficients were, in all cases, greater than 0.99 and the intercepts in the Cottrell plots were small (<0.1 \( \mu A \)). Therefore, it was concluded that on the specified time scale the current showed Cottrell behaviour and was completely diffusion-controlled.

The ratio of the catalytic and diffusion currents (\( i_{cat}/i_d \)) was converted into \( \lambda_2 \) values with the help of the table containing the calculated \( \lambda_2 \) and \( i_d/i_{cat} \) data. Plots of \( \lambda_2 \) versus time showed, in all cases, a similar behaviour; linearity in the region 0.25–2.0 s and deviation from linearity at larger times (Fig. 5 A). Reasons for deviation from linearity 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 nitrite reductase concentration (Fig. 5 B). From the slope of the straight line drawn through the points (including the origin), the second-order rate constant \( k \) was calculated (Table 1).

The measured second-order rate constants for the reaction between methyl viologen or *D. vulgaris* cytochrome *c*_3 or *D. desulfuricans* (ATCC 27774) cytochrome *c*_3 and nitrite reductase were in the range \( 10^6–10^8 \) M\(^{-1}\) s\(^{-1}\). The relatively high values seen here are consistent with a high turnover rate for this enzyme. The second-order rate constants determined in the presence of methyl viologen are compatible with the activity measurements performed using methyl viologen and dithionite as electron donor system [20]. More important are the differences observed in the values of \( k \) for the three mediators. At pH 7.0, the value of \( k \) increase in the direction *D. desulfuricans* (ATCC 27774) cytochrome *c*_3 \( \rightarrow D. vulgaris \)
cytochrome $c_5 \rightarrow$ methyl viologen. It is known that electrostatic interactions play a significant role in the electron-transfer reactions of proteins [9]. The observed differences confirm that interaction between nitrite reductase and positively charge electron carriers should be promoted.

According to van Leeuwen et al. [21], the maximal diffusion-controlled reaction rate constant between methyl viologen and D. vulgaris cytochrome $c_5$ is (not taking into account monopolar and dipolar effects) given by the following expression:

$$k_{\text{max}} = 1.652 \times 10^{10} \times A_1 \times A_2 \times [(r_1 + r_2)^2/(r_1 r_2)],$$  

where $A_n (n = 1, 2)$ is the surface area of the molecule $n$ (nm$^2$) and $r_n (n = 1, 2)$ is the radius of the molecule $n$ (nm). If $A_1 = A_2 = 1$ and $r_1 = 0.4$ nm (radius of the methyl viologen [21]) and $r_2 = 1.6$ nm (radius of the cytochrome $c_5$ from D. vulgaris [21]) the rate constant will be $k_{\text{max}} = 1.03 \times 10^{10}$ M$^{-1}$ s$^{-1}$. However, the observed value is about $10^9$ M$^{-1}$ s$^{-1}$. Since 100% of the surface area of methyl viologen is reactive, apparently only 10% of the surface area of the cytochrome $c_5$ is reactive. Considering that the total surface area of this protein is 32 nm$^2$ [21], this implies that, on average, 3.2 nm$^2$ is the reactive area of cytochrome $c_5$. For each heme, on average, there is a reactive area of 0.80 nm$^2$ and a circular area with a radius of 0.36 nm.

Performing the same calculations (radius of the nitrite reductase equal to 2.7 nm and surface area equal to 92 nm$^2$ [21]), the maximal diffusion-controlled rate constant between methyl viologen and nitrite reductase will be $k_{\text{max}} = 1.47 \times 10^{10}$ M$^{-1}$ s$^{-1}$. However, the observed values were $1.6 \times 10^9$ for cyclic voltammetry, and $2.2 \times 10^8$ for chronamperometry (Table 1), showing that only 1.09% (1.00 nm$^2$) and 1.50% (1.38 nm$^2$) of the surface area are reactive in the respective method of measurement.

Based on the values calculated above for the reactive areas of the proteins and with the Eqn (8), the $k_{\text{max}}$ value for the reaction between the D. vulgaris cytochrome $c_5$ and the nitrite reductase can be calculated, $k_{\text{max}}$(cyclic voltammetry) = $7.7 \times 10^9$ M$^{-1}$ s$^{-1}$ and $k_{\text{max}}$(chronamperometry) = $1.06 \times 10^8$ M$^{-1}$ s$^{-1}$. The experimental measured values were $1.1 \times 10^9$ M$^{-1}$ s$^{-1}$, for cyclic voltammetry, and $1.2 \times 10^8$ M$^{-1}$ s$^{-1}$, for chronamperometry (Table 1). In these calculations the following assumptions were made: (a) no ionic interactions; (b) just one electron transfer/collision; (c) the electron transfer is thermodynamically driven, i.e. the averaged reduction potential of the cytochrome $c_5$ is at least 60 mV more negative than that of the nitrite reductase.

No values for the reaction between methyl viologen and D. desulfuricans (ATCC 27774) cytochrome $c_5$ are available. However, the observed $k$ values for D. desulfuricans cytochrome $c_5$ are about one order of magnitude smaller than those of D. vulgaris cytochrome $c_5$. The reason the D. desulfuricans (ATCC 27774) cytochrome $c_5$ exhibits such a sluggish electron transfer might be the consequence of an improper orientation of this cytochrome towards the enzyme, due perhaps to a preferred orientation of a heme, with a rather positive reduction potential, towards the nitrite reductase, causing slow electron transfer.

The different catalytic efficiencies ($i_3/i_2$) exhibited by the three mediators exist also under steady-state conditions (cyclic voltammogram at 5 mV/s scan rate), perhaps as an indication that the electron-transfer reaction between electron carrier and nitrite reductase, and not some further stage in the mechanism, is important in determining the rate of the reaction.

A second conclusion that can be drawn from the results from cyclic voltammetry is that the deviation from linearity in the $\lambda$, versus $1/\nu$ plots is probably not due to the limiting effect of the following reactions on the rate of recycling of nitrite reductase. It would be more correct to explain the deviation from linearity as a consequence of the reversibility of the reactions.

From this study, it appears that cyclic voltammetry may be a useful technique in kinetic studies of nitrite reductase. However, the necessity to extrapolate the measured values to infinitely high scan rates restricts its usefulness. Scan rates over 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 chronamperometric technique has advantages over cyclic voltammetry (measurements at a smaller time scale are possible).

The results from chronamperometric show that in all $\lambda$, versus $t$ plots, linearity can be observed in the small time-scale region ($0.25 < t < 2$ s). This is an indication that the approximation of simple, pseudo-first-order kinetics in this region is correct. Deviations from the theory of the simple catalytic mechanism become only apparent at $t > 2$ s. The advantage of chronamperometry over cyclic voltammetry is clear. In the cyclic voltammetric experiments, there are only six measured data points in a $\lambda$, versus $1/\nu$ plot (at $\nu = 5$, 10, 20, 50, 100 and 200 mV/s). In the chronamperometric experiments, 50 experimentally obtained data points appear in a $\lambda$, versus $t$ plot and the initial slope has been calculated by linear regression analysis of some 15 points actually measured.

If one compares the $k$ values obtained with this technique (Table 1) with the values obtained by cyclic voltammetry at the same pH, there is a reasonable agreement between the two data sets. Due to the way the initial slope was obtained, however, a relatively large systematic error may be seen in the values of $k$ determined by cyclic voltammetry. Based on these arguments, the values obtained by chronamperometry seem the more reliable ones.

**Conclusions**

The information available on the natural electron donors of nitrite reductase is scarce. Our unpublished observations indicate that [NiFe] hydrogenases [D. gigas and D. desulfuricans (ATCC 27774)] can use dihydrogen to reduce this enzyme. Also, D. vulgaris (Hildenborough) [Fe] hydrogenase was used to reduce this enzyme in the presence of FAD [22]. These systems are potentially interesting to be studied in the presence of tetraheme cytochrome $c_5$ (considered a natural cofactor of hydrogenase) and will allow facilitated electron transfer from electrodes to hydrogenase and hexaheme nitrite reductase.

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