Protein Film Voltammetry Reveals Distinctive Fingerprints of Nitrite and Hydroxylamine Reduction by a Cytochrome c Nitrite Reductase*

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The cytochrome c nitrite reductases perform a key step in the biological nitrogen cycle by catalyzing the six-electron reduction of nitrite to ammonium. Graphite electrodes painted with *Escherichia coli* cytochrome c nitrite reductase and placed in solutions containing nitrite (pH 7) exhibit large catalytic reduction currents during cyclic voltammetry at potentials below 0 V. These catalytic currents were not observed in the absence of cytochrome c nitrite reductase and were shown to originate from an enzyme film engaged in direct electron exchange with the electrode. The catalytic current-potential profiles observed on progression from substrate-limited to enzyme-limited nitrite reduction revealed a fingerprint of catalytic behavior distinct from that observed during hydroxylamine reduction, the latter being an alternative substrate for the enzyme that is reduced to ammonium in a two electron process. Cytochrome c nitrite reductase clearly interacts differently with these two substrates. However, similar features underlie the development of the voltammetric response with increasing nitrite or hydroxylamine concentration. These features are consistent with coordinated two-electron reduction of the active site and suggest that the mechanisms for reduction of both substrates are underpinned by common rate-defining processes.

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An important class of nitrite reductases is composed of the penta-heme containing cytochrome c nitrite reductases. One sub-group of cytochrome c nitrite reductases is represented by *Escherichia coli* cytochrome c nitrite reductase. This enzyme draws electrons from the quinol pool via the nrfDCB gene products predicted to be a membrane-bound quinol dehydrogenase, an iron-sulfur protein, and a penta-heme c-type cytochrome, respectively (1, 2). The enzymes from *Wolinella succinogenes* and *Sulfurospirillum deleyianum* that draw electrons from the quinol pool via a tetra-heme quinol dehydrogenase, NrfH, tipify a second sub-group of cytochrome c nitrite reductases (3–5). Both the NrfDCB- and NrfH-dependent nitrite reductases catalyze the six-electron reduction of nitrite to ammonium.

These enzymes also catalyze ammonium production through the five-electron reduction of nitric oxide and the two-electron reduction of hydroxylamine in processes that may contribute to cellular detoxification (6, 7).

The crystal structures of cytochrome c nitrite reductases from *E. coli*, *W. succinogenes*, and *S. deleyianum* have recently been reported (4, 8–10). The arrangement of active site residues and heme groups is conserved across all three structures (see Fig. 1 below). A novel c-type heme, Heme 1, with lysine and water or hydroxide iron coordination forms the site for substrate binding, and a nearby Ca(II) ion bridges two regions of polypeptide containing key residues for catalysis. Four c-type hemes, Hemes 2 through 5, with *bis*-histidine iron coordination accompany Heme 1 and presumably participate in delivering electrons to the active site during catalysis. The hemes are packed in motifs common to a number of proteins, and all nearest neighbor Fe–Fe distances lie below 13 Å. The electrostatic surface of the *E. coli* enzyme around Heme 2 differs from those of the *W. succinogenes* and *S. deleyianum* enzymes and may reflect the distinct redox partners of these enzymes (8).

Spectropotentiometric titrations of the cytochrome c nitrite reductases from *E. coli*, *Desulfovibrio vulgaris* Hildenborough and *Desulfovibrio desulfuricans* have established that their redox transformations occur across a wide range of potential (2, 8, 11, 12). For *E. coli* cytochrome c nitrite reductase the redox transformations have been interpreted within a structural framework by virtue of the information contained within electron paramagnetic resonance (EPR)* spectra (Fig. 1) (8). A rhombic signal having resonances at $g = 2.91, 2.3$, and $1.5$ titrates with a mid-point potential at pH $7.0$ ($E_{1/2}^{*}$) of $-37$ mV. This signal is characteristic of a *bis*-histidine-coordinated heme Fe(III) having a near parallel alignment of its imidazole rings and is most likely to arise from Heme 2. Broad signals at $g = 10.8$ and $3.5$ characteristic of a weakly exchange coupled pair of S = 5/2 and S = 1/2 paramagnets titrate with $E_{1/2}^{*} = -107$ mV. The S = 5/2 center is most likely to be the high spin Fe(III) of Heme 1 whereas the S = 1/2 paramagnet may be nearby Heme 3 or a radical species. A signal with $g = 3.17$ characteristic of a *bis*-histidine-coordinated heme Fe(III) with near perpendicular imidazole ring planes titrates with $E_{1/2}^{*} = -323$ mV and has been attributed to either or both of Hemes 4 and 5.

![EPR spectra](http://www.jbc.org)

### Reaction 1

$$\text{NO}_2^- + 6\text{e}^- + 5\text{H}^+ \rightarrow \text{NH}_4^+ + 2\text{H}_2\text{O}$$
The structure and electrochemical properties of the cytochrome c nitrite reductases prompt questions whose answers will underpin an understanding of the mechanism(s) of substrate reduction in these enzymes and about which relatively little is known. For example, can a change of oxidation state at each redox center in the enzyme modulate activity, and if so, is this achieved through changes in electronic or structural properties? Also, given the arrangement of close-packed hemes and in particular the near parallel configuration of Hemes 1, 3, and 4, how are electrons “packaged” for delivery to the substrate and reaction intermediates? Hydroxylamine could be reduced in sequential one-electron steps or a single, concerted two-electron transfer. The possible routes through which the six-electron reduction of nitrite could be achieved are clearly more numerous. There would be an opportunity for the pathways of hydroxylamine and nitrite reduction to overlap if the reduction of nitrite occurs in a series of one- or two-electron transfers (10). However, the initial kinetic study of the E. coli enzyme found the Michaelis constant describing hydroxylamine reduction to be more than two orders of magnitude greater than that for nitrite reduction (7). This led to the proposal that hydroxylamine is not a free intermediate in the reduction of nitrite by this enzyme.

Protein film voltammetry (PFV) is a relatively new addition to the range of techniques used to study redox enzymes (13). In this technique the enzyme is immobilized as a (sub-)monolayer, or film, on an electrode surface. Within the film the enzyme undergoes direct electron exchange with the electrode and ideally retains the catalytic properties of the “native” enzyme. When the film contacts a solution of its substrate, the rate at which electrons flow through the enzyme and into the substrate is quantitated by the flow of catalytic current. This current is most informative in the absence of limitations imposed by electron exchange across the electrode:enzyme interface or transport of substrate to the enzyme film. In this situation the current provides a direct measure of the enzyme’s intrinsic rate of product formation. This rate will therefore vary in a precise manner in response to defined variation of the electrode potential. For a reductase, variations in activity are defined by changes in the population of reduced, catalytically competent enzyme and the rate-defining step of catalysis. Changes in either of these parameters will give rise to sigmoidal variations of the catalytic current magnitude as the electrochemical potential is varied. As a consequence the catalytic current-potential profiles or waveforms arising from enzyme films frequently show multiple sigmoidal features; i.e. features in which the activity may increase or decrease as the electrode potential is lowered with a concomitant increase in driving force for the reaction being catalyzed (13–18). It is by visualizing such modulations of activity across the electrochemical potential domain that PFV is able to provide unique insight into the mechanisms of redox enzyme catalysis.

Here we present the first PFV study of a cytochrome c nitrite reductase. The catalytic behavior of E. coli cytochrome c nitrite reductase has been defined on progression from substrate-limited to enzyme-limited catalysis for both nitrite and hydroxylamine reduction. Distinct fingerprints of catalytic behavior are observed in each case; such fingerprints provide important new insight into the mechanisms by which this enzyme directs substrate reduction.

**Experimental Procedures**

**Protein Purification—**E. coli cytochrome c nitrite reductase, NrfA, was purified as described previously and stored as frozen aliquots in liquid nitrogen (8). Activity was measured spectrophotometrically by substrate-dependent oxidation of dithionite-reduced methyl viologen (EM spectrum at 400–650 nm) in 1 M methyl viologen, 2 mM CaCl₂, 50 mM Hepes, pH 7.0, at 20 °C (8). The samples used in these experiments had a specific activity of 880 μmol of nitrite consumed min⁻¹ mg⁻¹, and ΔA₄₅₀nm/ΔA₃₈₀₅ nm = 3.7 for the oxidized enzyme.

**Reagent Preparation—**Buffer-electrolyte was composed of 2 mM CaCl₂, 50 mM Hepes, pH 7.0, unless stated otherwise. Dissolution of the desired mass of NaN₃ in ice-cold buffer-electrolyte gave stock solutions of 25 mM NaN₃. The pH was confirmed as 7.0 after equilibration at 20 °C. Stock solutions of 2 mM hydroxylamine were prepared by dissolution of NH₂OH·HCl in ice-cold buffer-electrolyte. The pH was adjusted to 7.0 by addition of an appropriate volume of 10 mM NaOH, and the solution was brought to the desired volume with buffer-electrolyte. The pH was confirmed as 7.0 upon equilibration at 20 °C. Reagents were of Analar quality or equivalent. Solutions were prepared with water having total nitrogen content < 0.1 ppm (Fisher).

**Protein Film Voltammetry—**Cyclic voltammetry was performed using a three-electrode cell configuration housed in a N₂-filled chamber (atmospheric O₂ < 2 ppm) as described previously (14). Pyrolytic graphite edge (PGE) and polycrystalline gold working electrodes of 3-mm diameter were polished immediately prior to use with an aqueous slurry of 0.3-μm Al₂O₃, sonicated, rinsed, and dried with a tissue. Frozen 5-μl aliquots of 0.6 μM nitrite reductase in 50 mM Hepes, pH 7.0, were taken into the glove box, thawed, and immediately placed on the surface of a freshly prepared working electrode. After approximately 15 s the enzyme solution was removed from the electrode surface, which was then rinsed with room temperature buffer-electrolyte to remove any loosely bound enzyme. Excess buffer-electrolyte was carefully removed from the electrode with a tissue taking care not to dry the electrode surface. The electrode was then placed in the electrochemical cell, and voltammetry commenced. Voltammetry was performed with an Autolab electrochemical analyzer under the control of GPES software. Electrode rotation was with an EG&G model 636 electrode rotator. Potentials are reported with respect to the standard hydrogen electrode by addition of 197 mV to the potential measured with an Ag/AgCl (saturated KCl) reference electrode.

**Voltammetric Analysis—**The experimentally observed catalytic current magnitude, iₓ, was determined at ~550 mV as the difference between the measured current and the non-catalytic current, the latter estimated by linear extrapolation of the non-catalytic part of the voltammogram. The rate at which electrons passed through the enzyme film during catalysis was obtained on division of iₓ by the Faraday constant (96,484 Coulombs mol electrons⁻¹).

It was found that the catalytic response observed during hydroxylamine reduction was limited only by the properties of the enzyme. In this situation, iₓ could be equated to the flow of current through the enzyme, iₓ = i_{cat}, anticipated from a Michaelis-Menten analysis of redox enzyme kinetics,

$$i_{cat} = \frac{i_{max} C_e}{C_e + K_m}$$  

(Eq. 1)

where i_{max} is the catalytic current observed at the maximum turnover.
rate, $C_s$ the substrate concentration, and $K_m$ the Michaelis constant. Variation of $i_{\text{cat}}$ with hydroxylamine concentration was determined in three independent experiments. For each experiment, values of $i_{\text{cat}}$ were corrected for the first order loss of signal amplitude with time. $K_m$ was then determined from the variation of the time-corrected catalytic current magnitudes with $C_s$. Data were fitted to Equation 1 in Origin using the Levenberg-Marquardt algorithm for non-linear least-squares curve fitting. The value of $K_m$ reported in the text is the average of the three independent experimental determinations. The error reported is that which encompasses the experimentally determined values of $K_m$.

The catalytic currents observed during nitrite reduction were not limited solely by the properties of the enzyme. The influence of electrode rotation rate on $i_{\text{cat}}$ indicated that the catalytic response is limited in part by substrate transport to the enzyme film. To estimate the value of $i_{\text{cat}}$ that would be recorded in the absence of mass transport limitations, i.e. at infinitely high electrode rotation rates, the method of Sucheta et al. was adopted (18). The value of $i_{\text{cat}}$ was related to the electrode rotation rate through the relationship,

$$\frac{1}{i_{\text{cat}}} = \frac{1}{i_{\text{cat(max)}}} + \frac{1}{0.62\pi FADH_2^{N\alpha} D^{1/2} v^{1/2} C_s^{1/2}}$$  \hspace{1cm} (Eq. 2)

where $\omega$ is the electrode rotation rate expressed as an angular velocity, $\omega = (2\pi \times \text{revolutions per minute})/60 \text{ s}^{-1}$. From Equation 2, a plot of $i_{\text{cat}}^{-1}$ versus $\omega^{-1/2}$ at a given substrate concentration yields $i_{\text{cat(max)}}^{-1}$ upon extrapolation to $\omega^{-1/2} = 0$. The gradient of this plot is defined by the additional terms in Equation 2, where $n$ is the number of electrons transferred in reduction of the substrate to the product, $F$ is the Faraday constant, $A$ the electrode area, $D$, the diffusion coefficient of the substrate, and $v$ the kinematic viscosity of the solution. For a cytochrome $c$ nitrite reductase film, values of $i_{\text{cat}}$ were corrected for loss of magnitude over the time of each experiment, and the subsequent plots of $i_{\text{cat}}$ against $\omega^{-1/2}$ were linear at each nitrite concentration investigated (Fig. 2, insets). Values of $i_{\text{cat(max)}}$ were determined from Equation 2, and $K_m$ was determined from Equation 1 as described above.

## RESULTS

### Establishing PFV of Cytochrome $c$ Nitrite Reductase—A series of initial experiments established that *E. coli* cytochrome $c$ nitrite reductase is amenable to study by PFV. Freshly polished, pyrolytic graphite edge (PGE) electrodes were covered with solutions containing various concentrations of the enzyme, and the electrodes were then rinsed to remove any loosely bound material and placed in an electrochemical cell containing a solution of nitrite. Cyclic voltammetry showed that electrodes exposed to a dilute solution ($< 2 \mu M$) of the enzyme gave rise to large, negative currents at potentials below approximately 0 V (Fig. 2). These currents indicated that catalytic reduction was occurring at negative potentials. Control experiments provided no evidence for catalytic reduction in the absence of the enzyme (Fig. 2, dotted lines). Cytochrome $c$ nitrite reductase is clearly capable of utilizing direct electron exchange with PGE electrodes to drive nitrite reduction. To confirm that the catalytic response originated from an enzyme film, enzyme-coated electrodes were transferred to fresh nitrite-containing solutions where the catalytic response was found to persist with only slight loss of magnitude.

Subsequent studies established that the behavior of cytochrome $c$ nitrite reductase during nitrite reduction was distinct from that during hydroxylamine reduction. The catalytic waveforms describing reduction of each substrate are presented separately below.

### Overview of the Behavior Observed during Nitrite Reduction—Cyclic voltammograms arising from films of cytochrome $c$ nitrite reductase in 5 and 130 $\mu M$ nitrite showed a clear dependence on the rate of electrode rotation (Fig. 2). With the electrode stationary (0 rpm) the sweep toward more negative potentials showed a peak in the reduction current that was absent from the return sweep. This observation was consistent with the presence of a highly active enzyme film that rapidly lowers the concentration of substrate at the electrode surface soon after the onset of catalysis. In agreement with this suggestion, the catalytic current magnitude increased when the electrode was rotated to continually deliver substrate to the enzyme film. Two observations were made as the electrode rotation rate was increased and the rate of substrate delivery to the enzyme film became less of a limitation on the rate of catalysis. First, the catalytic current magnitudes, $i_{\text{cat}}$, increased and approached a constant value (Fig. 2, insets). Second, the values of $i_{\text{cat}}$ for the forward and reverse sweeps became increasingly superimposable. The form of the catalytic response observed at the highest electrode rotation rates is therefore dominated by the rates of processes intrinsic to the
enzyme and its interaction with substrate. At the highest electrode rotation rate used in this study (3000 rpm) it was clear that sweeping across the electrochemical potential domain gave rise to distinct modulations of activity in 5 and 130 μM nitrite. At 5 μM nitrite a peak in the rate of substrate reduction was observed at −235 mV on sweeping in both directions across the electrochemical potential domain (Fig. 2A). The catalytic waveform was unaffected by addition of the reaction product, ammonium. Therefore, product inhibition was not responsible for the decrease in activity observed at lower potentials.

Successive voltammograms recorded from a single enzyme film over a 2-h period showed a slow decrease in magnitude of the catalytic response. The half-life for signal loss was approximately 40 min. Whether the decrease in magnitude was due to film desorption or enzyme inactivation is unclear. Importantly, however, no detectable changes in the shape or position of features in the catalytic waveform were noted over the 2-h period. This is consistent with the response arising from a single form of electrocatalytically active enzyme whose population decreases over the time of the experiment without the formation of new species that contribute to the voltammetric response.

Films of cytochrome c nitrite reductase prepared on gold electrodes displayed similar catalytic waveforms to those observed at PGE electrodes. Detailed analysis of the catalytic waveform arising at gold electrodes was precluded by a rapid decrease in signal magnitude during these experiments: the half-life for signal loss was approximately 4 min. However, a clear peak in activity was observed on both the forward and reverse potential sweeps at approximately −253 mV. PGE and gold electrodes present surfaces of quite different properties for interaction with cytochrome c nitrite reductase. The observation of similar variations of activity across the electrochemical potential domain at both electrode materials demonstrates that these variations arise from intrinsic properties of the enzyme. The slight difference in peak potentials exhibited by the electrode materials could reflect the influence of the electrode: protein interface on the reduction potential of the heme center(s) responsible for the modulations of activity.

Detailed film voltammetry was performed on PGE electrodes at scan rates from 5 to 100 mV s⁻¹ to assess how effectively the catalytic cycle was coupled to electron transfer events. The catalytic waveform at a given scan rate was obtained as the difference between voltammograms recorded in the presence and absence of an enzyme film. The magnitude, shape, and position of features in the catalytic waveform were invariant across this entire range of scan rates. Interfacial electron exchange between the enzyme and electrode is therefore facile. The rates of chemical and intramolecular electron transfer events of catalysis are also sufficiently rapid not to become uncoupled from interfacial electron transfer across this range of scan rates. Indeed it is known that this enzyme exhibits a turnover number of 770 s⁻¹ of dithionite-reduced methyl viologen, which corresponds to a turnover number of approximately 30 s⁻¹ in assays of nitrite-dependent oxidation of dithionite-reduced methyl viologen, which corresponds to a flux of 4620 electrons s⁻¹ (8). For comparison, Paracoccus pantotrophus nitrate reductase, NarGH, which exhibits a turnover number of approximately 30 s⁻¹ in assays of nitrate-dependent oxidation of dithionite-reduced methyl viologen corresponding to a flux of 60 electrons s⁻¹, displayed catalytic waveforms that are attenuated at scan rates greater than 40 mV s⁻¹ (15).

The catalytic waveform recorded in 130 μM nitrite under conditions of least limitation from substrate mass transport was clearly distinct from that recorded in 5 μM nitrite (Fig. 2). At both PGE and gold electrodes two reversible, sigmoidal increases of activity centered on approximately −184 and −312 mV accompanied the lowering of the electrode potential. These features are more clearly resolved in a plot of the first derivative of the catalytic current with respect to applied potential described below (Fig. 3A).

There were negligible differences between the catalytic waveforms recorded at scan rates from 20 to 200 mV s⁻¹. However, unexpected behavior was observed at scan rates below 20 mV s⁻¹. Here, as the potential was swept to more negative values, the activity became progressively smaller than that recorded at the higher scan rates. On returning to more positive potentials the activity of the film continued to decrease relative to that obtained on the forward sweep. At these slower scan rates prolonged exposure of the film to more negative potentials was causing inactivation of the enzyme film and an inequality in the catalytic current magnitudes measured at a given potential on the forward and reverse sweeps. The inequality became more pronounced when CaCl₂ was omitted from the experiments. Optimum equality of the forward and reverse catalytic currents required the presence of >0.5 mM CaCl₂, and experiments were routinely performed in the presence of 2 mM CaCl₂. Interestingly there was no detectable influence of MgCl₂ on the catalytic voltammetry. In the presence of 2 mM MgCl₂ the catalytic voltammetry was indistinguishable from that observed in the absence of CaCl₂.

A possible origin for the specific role of Ca(II) ions in determining the voltammetry of cytochrome c nitrite reductase lies in the presence of a structurally conserved Ca(II) ion associated with the enzyme’s active site (4, 8, 10). The importance of this
Ca(II) ion in the catalytic process was illustrated in studies of the *S. deleyianum* enzyme where Ca(II) ion dissociation resulted in a decrease in activity that was reversed on re-association of Ca(II) (6). The slow inactivation of films of *E. coli* cytochrome c nitrite reductase may then reflect an absence of the active site Ca(II) ion, perhaps resulting in a failure of the enzyme to efficiently process reaction intermediates and leading to a subsequent deviation from the “normal” catalytic cycle. However, the structure of the *E. coli* enzyme reveals a second Ca(II) ion, coordinated between Hemes 3 and 4, and it cannot be excluded that dissociation of this Ca(II) ion contributes to the voltammetric behavior (8). Further experiments established that the activity lost on poising the enzyme film at −450 mV for 60 s was recovered after poising the film at 100 mV for 30 s. Thus, although it was not possible to conclusively identify the cause of the decrease in activity triggered by exposure to low potentials (although see below), the effect is clearly reversed by exposure to more positive electrode potentials and presumably oxidation of centers within the enzyme.

Estimation of the Kinetic Parameters Describing Nitrite Reduction by the Enzyme Film—As described fully under “Experimental Procedures,” the method of Sucheta *et al.* was employed to estimate the activity of the enzyme film in the absence of limitations from substrate delivery to the film, i_{cat, enzyme}, i.e. at infinitely high electrode rotation rates (18). A scan rate of 30 mV s\(^{-1}\) was employed to ensure the catalytic current magnitudes of the forward and reverse potential sweeps were close to unity across the range of nitrite concentrations investigated. A typical plot illustrating the variation of i_{cat, enzyme} with nitrite concentration for a single film of cytochrome c nitrite reductase is shown in Fig. 4A.

The value of K\(_m\), 25 ± 2 \(\mu\)M (average of three determinations), is in excellent agreement with the value of 28 ± 3 \(\mu\)M determined in spectrophotometric assays of the enzyme under comparable conditions (8). These results indicate that the enzyme has undergone minimal, if any, perturbation upon incorporation into the electrode-immobilized film.

To define the turnover number of the electrode-immobilized enzyme, the population of electrocatalytically active enzyme must be known. In favorable cases this can be determined by integration of the voltammetric response observed in the absence of substrate, and which will be comprised of a series of wave pairs corresponding to reduction and oxidation of centers in the enzyme (13, 17, 18). Unfortunately, it has not yet been possible to detect such signals from cytochrome c nitrite reductase, even when enzyme films that give rise to clear catalytic currents in the presence of nitrite were transferred into solutions lacking substrate or containing inhibitors. Rather, the voltammograms were indistinguishable from those obtained in the absence of enzyme (Fig. 2, broken lines). From the dimensions of the cytochrome c nitrite reductase monomer, which approximates to a sphere of radius 30 Å, an enzyme monolayer is predicted to contain \(\approx 5 \times 10^{-12}\) mol cm\(^{-2}\). Voltammetry of an electrode covered to greater than 25% of a monolayer should give rise to clearly visible signals due to reduction and oxidation of the enzyme’s redox centers (15). The surface population of electroactive cytochrome c nitrite reductase in these experiments should be significantly less than a monolayer. If it is assumed that the population of electroactive enzyme comprises 25% of a monolayer, then from the typical current densities observed during nitrite reduction at the enzyme-limited maximal rate (approximately 0.3 mA cm\(^{-2}\) at \(-550\) mV) a lower limit for the turnover number of molecules in the film is calculated to be 370 s\(^{-1}\). Molecules of cytochrome c nitrite reductase within the film therefore support a flux of at least 2230 electrons s\(^{-1}\) at the maximum turnover rate. This compares favorably with the flux of 4620 electrons s\(^{-1}\) measured in spectrophotometric assays (8).

**FIG. 4. Representative variations of the catalytic current magnitude with substrate concentration for films of *E. coli* cytochrome c nitrite reductase.** A. nitrite concentrations 3.4, 7, 14, 28, 63, 130, 265, 532, and 1050 \(\mu\)M. The solid line describes the catalytic current arising from a Michaelis-Menten description of enzyme kinetics with K\(_m\) = 24 \(\mu\)M and i_{max} = 17 \(\mu\)A. B. hydroxylamine concentrations were 0.6, 1.25, 1.9, 3.7, 7.5, 15, 30, 58, 113, 214, 305, and 387 \(\mu\)M. The solid line describes the catalytic current arising from a Michaelis-Menten description of enzyme kinetics with K\(_m\) = 137 \(\mu\)M and i_{max} = 243 \(\mu\)A. Experimental conditions are as given in Fig. 2.
The insensitivity of the hydroxylamine reductase activity to changes of Ca(II) ion concentration contrasted with the behavior observed during nitrite reduction. The structures of substrate-bound W. succinogenes cytochrome c nitrite reductase provide a possible explanation for these different behaviors (9). The iron of Heme 1 coordinates the substrate nitrogen atom in both the nitrite- and hydroxylamine-bound enzymes. The oxygen atoms of nitrite coordinate Arg-114 and His-277, the latter being located on a loop of polypeptide positioned by the structurally conserved Ca(II) ion. The hydroxylamine oxygen atom coordinates only Arg-114. As a consequence, hydroxylamine has no direct interaction with that region of the active site positioned by the structurally conserved Ca(II) ion. The observation that Ca(II) ion concentrations influence only the nitrite reductase activity of the E. coli enzyme suggests that dissociation of Ca(II) from the active site, rather than from the binding site located between Hemes 3 and 4, gives rise to the observed effects.

Estimation of the Kinetic Parameters for Hydroxylamine Reduction—A typical plot showing the variation of $i_{\text{catal}}$ with hydroxylamine concentration arising from a film of cytochrome c nitrite reductase is presented in Fig. 4B. Control experiments performed with freshly polished PGE electrodes and hydroxylamine concentrations above 60 mM showed the presence of broad waves corresponding to non-enzymatic reduction and oxidation processes. Inhibition of the cytochrome c nitrite reductase film with cyanide showed that the non-enzymatic contribution to the voltammetry was suppressed by the presence of the enzyme film. The non-enzymatic contribution to the total catalytic current was found to be less than 2% of that measured in 400 mM hydroxylamine. Experiments were restricted to hydroxylamine concentrations below this level to ensure minimal impact of the non-enzymatic response on the PFV of cytochrome c nitrite reductase. Several independent experiments produced results similar to those of Fig. 5B and from which a value of $K_m = 127 \pm 25 \text{ mM}$ was defined.

Transfer of an enzyme film from a solution containing 400 $\mu$M nitrite to one containing 40 mM hydroxylamine caused a 3.5-fold increase in catalytic current magnitude. The maximum electron flux during hydroxylamine reduction can be calculated to be at least 14-fold greater than that observed during nitrite reduction. For an enzyme film comprised of 25% of a monolayer, this corresponds to a flux of 31,220 electrons s$^{-1}$ and a turnover number of 15,610 s$^{-1}$. By contrast spectrophotometric assays of hydroxylamine turnover yielded $K_m = 30 \pm 4 \text{ mM}$ and a turnover number of 2382 s$^{-1}$, corresponding to a maximum electron transfer rate of 4764 electrons s$^{-1}$, the latter value being only slightly greater than that measured for nitrite reduction (8). The different enzyme activities reported by the two methods are readily explained if the PFV response is limited by intrinsic properties of cytochrome c nitrite reductase whereas the spectrophotometric assays are limited by the assay conditions.

Development of the Catalytic Waveform on Progression from Hydroxylamine-limited to Enzyme-limited Turnover—Catalytic current derivatives summarizing the waveforms observed in hydroxylamine concentrations ranging from ~1 to 375 mM are presented in Fig. 3B. The fingerprint of hydroxylamine reductase activity is clearly distinct from that obtained during the reduction of nitrite. The catalytic current derivatives for hydroxylamine limited turnover contain a single positive feature that reflects the increase in activity observed on scanning toward more negative potentials. For a hydroxylamine concentration of 1.2 mM the increase in activity was centered on ~98 mV and had the steepness associated with a concerted two-electron process at higher potentials but broadened at lower

![Graph](image.png)

**Fig. 5.** Baseline-subtracted protein film voltammetry from cytochrome c nitrite reductase in 1.7 $\mu$M nitrite and 1.2 mM hydroxylamine. A, solid line, the response in 1.7 $\mu$M nitrite; circles, a Nernstian plot having $n = 2$ and $E_m = -105 \text{ mV}$; diamonds, a Nernstian plot having $n = 1$ and $E_m = -320 \text{ mV}$. B, solid line, the response in 1.2 mM hydroxylamine; circles, a Nernstian plot having $n = 2$ and $E_m = -86 \text{ mV}$. Voltammograms were recorded at a scan rate of 20 mV s$^{-1}$ with electrode rotation at 3000 rpm, other details are as in Fig. 2.
potentials (Fig. 5B). On increase of the hydroxylamine concentra-
tion the catalytic wave broadened and was displaced toward
more negative potentials. At the highest hydroxylamine con-
centrations investigated the waveform displayed two promi-
inent sigmoidal increases in activity centered on approximately
−230 and −300 mV, and reflected in the peaks of the corre-
sponding catalytic current derivatives. A small increase in $i_{\text{cat}}$
centered on −500 mV was persistently revealed in the catalytic
current derivatives obtained at the higher hydroxylamine con-
centrations (Fig. 3B).

**DISCUSSION**

PFV has clearly established that the catalytic currents mea-
sured during substrate reduction by *E. coli* cytochrome c nitrite
reductase are determined not only by the identity and concen-
tration of the substrate but also the electrochemical potential
experienced by the enzyme. The distinct patterns of behavior
observed during nitrite and hydroxylamine reduction highlight
how the dependence of enzyme activity on applied potential
cannot be simply predicted from knowledge of the reduction
potentials describing centers within the enzyme. Rather, PFV
reveals the exquisite sensitivity of cytochrome c nitrite reduc-
tase to control from a variety of factors.

PFV is most informative when it reflects the intrinsic prop-
erties of an enzyme (13). These properties should be independ-
ent of the technique used to study the enzyme, and ideally the
enzyme will exhibit these properties in its natural environ-
ment. The catalytic waveforms observed during the PFV of
cytochrome c nitrite reductase do not appear to be significantly
limited by rates of electron exchange across the electrode:pro-
tein interface. The catalytic currents observed at the higher
hydroxylamine concentrations used in this study are much
greater than those observed during nitrite reduction. The
waveforms are also largely free from limitations due to sub-
strate delivery to the enzyme film at the highest electrode
rotation rates of this study. The integrity of enzyme molecules
within the film is therefore most readily assessed by compari-
son of their catalytic behavior with that observed during solu-
tion phase assays under equivalent conditions. This is made
possible by the nature of the cyclic voltammetric experiment in
which the catalytic current provides a direct measure of the
rate at which electrons flow through the enzyme film and into
substrate. The $K_m$ values exhibited by films of cytochrome c
nitrite reductase during nitrite and hydroxylamine reduction
differ by approximately three orders of magnitude, in agree-
ment with the behavior observed in parallel solution assays (8).
A lower estimate of the turnover number describing nitrite
reduction by the film is also in agreement with that found in
solution assays. However, hydroxylamine reduction by the
films occurs with a turnover number at least 6-fold greater
than that measured in the solution assay. In this case the voltammetric experiment is clearly free from a limitation im-
posed by the conditions of the solution assay.

Calculation of absolute turnover numbers for cytochrome c
nitrite reductase within the film is prevented by uncertainty in
the number of molecules that give rise to the catalytic response.
Nevertheless, a wealth of information on those factors that
determine the activity of the enzyme is provided by the distinct
variations of activity observed on sweeping across the electro-
chemical potential domain. These variations of activity are
readily interpreted in a film voltammetric experiment, because
the oxidation state of each redox center in the enzyme popula-
tion under study is under the control of the electrode potential
at all times. In the absence of substrate, the redox centers will
be switched between their accessible oxidation states in a
Nernstian fashion as the electrode potential is swept linearly
across the electrochemical potential domain. On introducing
substrate into such an experiment at a concentration well
below the $K_m$, a small percentage of the enzyme population will
interact with the substrate at any given time and therefore at
any given potential. At each potential the rate of catalysis will
therefore reflect the population of enzyme molecules primed to
pass electrons to the substrate. For a reductase in which the
only redox active group is that associated with the active site
this population will be that containing a reduced active site.
The catalytic current will therefore increase in a sigmoidal
fashion on sweeping toward more negative potentials and re-
fect a Nernstian titration of the active site (15, 17). When the
oxidation states of additional redox centers in an enzyme con-
tribute to the definition of its activity, additional sigmoidal features will appear in the voltammetry (13–18). When the
reduction potentials of those centers defining changes of activ-
ity across the electrochemical potential domain are sufficiently
well separated, the features in the voltammogram will reflect a
Nernstian titration of each center whose oxidation state exerts
regulatory control. The magnitude and direction of each sig-
moidal change in activity will be governed by the difference
between the rate of product formation when the redox center in
question is 100% oxidized and that when it is 100% reduced.

The PFV of cytochrome c nitrite reductase during substrate
limited reduction of nitrite and hydroxylamine shows that ac-
tivity is initiated by reduction of a constituent of the active site
with a reduction potential in the vicinity of −100 mV. For both
substrates the steepness of the corresponding voltammetric
feature is consistent with coordinated two-electron reduction of
this redox center. Correlating these observations with the EPR
spectroscopic and equilibrium electrochemical properties of
this enzyme, it is most likely that the centers giving rise to the
spin-coupled pair ($E_{m,7} = −107$ mV) are those that define the
redox chemistry of the active site (8). The redox chemistry of
Heme 1 is effectively coupled to that of Heme 3 or a nearby
amino acid, such as Tyr-216, to provide a site for coordinated
exchange of two electrons. An active site loaded with two elec-
trons will provide sufficient reducing equivalents for the two-
electron reduction of hydroxylamine to ammonia. To perform
the six-electron reduction of nitrite to ammonia, additional
electrons must be drawn through the active site. Once the
active site is reduced and nitrite is bound, the additional elec-
trons required for ammonia formation will flow rapidly through
the enzyme from the electrode driven by the strong driving
force for product formation ($E_{m,7} \text{NO}_2^-/\text{NH}_4^+ = +340$ mV).
The flow of these additional electrons contributes to the magnitude
of the catalytic response at each potential. However, the shape
and position of the waveform remain defined by the population
of catalytically competent enzyme present at each electrode
potential.

The features describing reduction of the active site during
nitrite and hydroxylamine reduction show a slight difference in
their steepness that may reflect a different stability of the
semi-reduced active site during each catalytic process (17).
Such behavior could arise if the reduction potentials of the
active site are different in each case. For cytochrome c nitrite
reductase the possibility, that hydroxylamine is an interme-
iate on the pathway of nitrite reduction and therefore bound to
the active site in the oxidized, semi-reduced and reduced forms,
provides a possible reason for the active site exhibiting distinct
properties during each catalytic process.

PFV of cytochrome c nitrite reductase has also visualized how reduction of a one-electron center having a reduction po-
tential of approximately −320 mV leads to a decrease of activ-
ity during nitrite-limited reduction. This center is most likely
to be provided by Heme 4 and/or 5 ($E_{m,7} = −323$ mV) (8).
Reduction of the low potential heme may modify the activity of
the enzyme through modulation of its electronic properties or a conformational change; the latter has been suggested to occur on reduction of D. desulfuricans cytochrome c nitrite reductase (12). Whichever mechanism is responsible for the decrease in nitrite reductase activity triggered by exposing the enzyme to “low” potentials, it is of considerable note that such behavior is not observed during hydroxylamine reduction.

For both nitrite and hydroxylamine reduction, clear changes in the catalytic waveform are observed on progression from substrate-limited to enzyme-limited turnover. The voltammetry still reflects the population of reduced, catalytically primed enzyme present at each potential, but this population becomes increasingly defined by the rates of chemical and/or electrochemical events in the catalytic cycle as the substrate concentration is raised (13, 14). The waveforms develop in a similar manner on increase of the hydroxylamine or nitrite concentration. The response becomes displaced toward more negative potentials, broadens, and a second increase in current appears at lower potentials. The observations suggest that similar rate-defining events underlie substrate reduction in both cases. They are consistent with the rate of enzyme-limited catalysis being defined by the rate of electron delivery to the active center and most likely gated by a chemical event (17, 20). The differences between the absolute responses recorded in each substrate will reflect differences in the rate constants and reduction potentials specific to the catalytic cycle describing reduction of each substrate. Interestingly, our inspection of the solution voltammetry of D. desulfuricans cytochrome c nitrite reductase under conditions supporting a maximum nitrite reduction rate and measured with a view to development of a nitrite biosensor, shows a catalytic waveform of similar shape to that observed during PFV of the E. coli enzyme (19, 21). This suggests that the description of enzyme-limited substrate reduction uncovered by PFV will be common to the cytochrome c nitrite reductases, in agreement with the conservation of active site structure and heme packing motifs recently revealed by structural studies of this important family of enzymes.

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