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Redox-triggered events in cytochrome *c* nitrite reductase

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Abstract

Escherichia coli cytochrome *c* nitrite reductase is a homodimeric enzyme whose 10 heme centres range in reduction potential from ca. −30 to −320 mV. Protein film voltammetry (PFV) was performed to assess how the reactivity of the enzyme towards a number of small molecules was influenced by heme oxidation state. The experimental approach provided a high-resolution description of activity across the electrochemical potential domain by virtue of the fact that the enzyme sample was under the precise potential control of an electrode at all times. The current potential profiles displayed by nitrite reductase revealed that heme oxidation state has a profound, and often unanticipated, effect on the interactions with substrate molecules, nitrite and hydroxylamine, as well as the inhibitor, cyanide. Thus, PFV provides a powerful route to define redox-triggered events in this complex multi-centred redox enzyme.

Keywords: Electrochemistry; Voltammetry; Cytochrome; Nitrite reductase; Cyanide

1. Introduction

While eukaryotes are restricted to aerobic environments, bacteria are able to colonise more diverse habitats by virtue of their respiratory flexibility [1]. Species such as nitrogen oxyanions and oxides, organic nitrogen oxides, sulphur oxyanions, selenate, arsenate, Fe(III) and Mn(IV) can be exploited as terminal electron acceptors of bacterial respiratory chains when the appropriate reductases are expressed. Under anoxic conditions *Escherichia coli* expresses cytochrome *c* nitrite reductase (NrfA) to perform the respiratory, six-electron reduction of nitrite to ammonium [2].

Cytochrome *c* nitrite reductase crystallises as a homodimer with 10 c-type hemes arranged such that the nearest neighbours are in close proximity, Fig. 1 [3]. In the oxidised enzyme, Heme 1 has axial iron ligands provided by the side chain of lysine 126 and a water (hydroxide) molecule. This water (hydroxide) molecule is displaced by nitrite when it binds to Heme 1 as part of the catalytic cycle. Hemes 2–5 have bis-histidine axial iron ligation. Their arrangement is proposed to facilitate electron transfer from the multi-heme cytochrome (NrfB) that delivers electrons to cytochrome *c* nitrite reductase in vivo and nitrite molecules bound at Heme 1 in the enzyme interior [4].

A combined spectroscopic, structural and potentiometric analysis of nitrite reductase has led to the assignment of heme mid-point potentials ($E_{m,7}$) indicated in Fig. 1 [3]. The lysine coordinated Heme 1 is reduced together with Heme 3 in a cooperative two-electron process with $E_{m,7} = -107$ mV. The other hemes act as one-electron centres. Heme 2 has $E_{m,7} = -37$ mV, while Hemes 4 and 5, located towards the dimer interface, are reduced only by the application of much lower potentials and have $E_{m,7} \sim -320$ mV.

The results described above illustrate a relatively detailed but *static* picture of the machinery that transforms nitrite to ammonium. Ultimately, we would like to understand how the structure coordinates long-range electron transfer, substrate binding, bond breakage, bond formation and perhaps conformational change to direct the pathway of nitrite reduction. Essentially, we would like to animate the present description of nitrite reductase.

As a first step to elucidate the functional properties of nitrite reductase, we have asked how the interactions between nitrite reductase and small molecules, both substrates and inhibitors, are modulated by change of oxidation state at the heme centres. To provide a high-resolution

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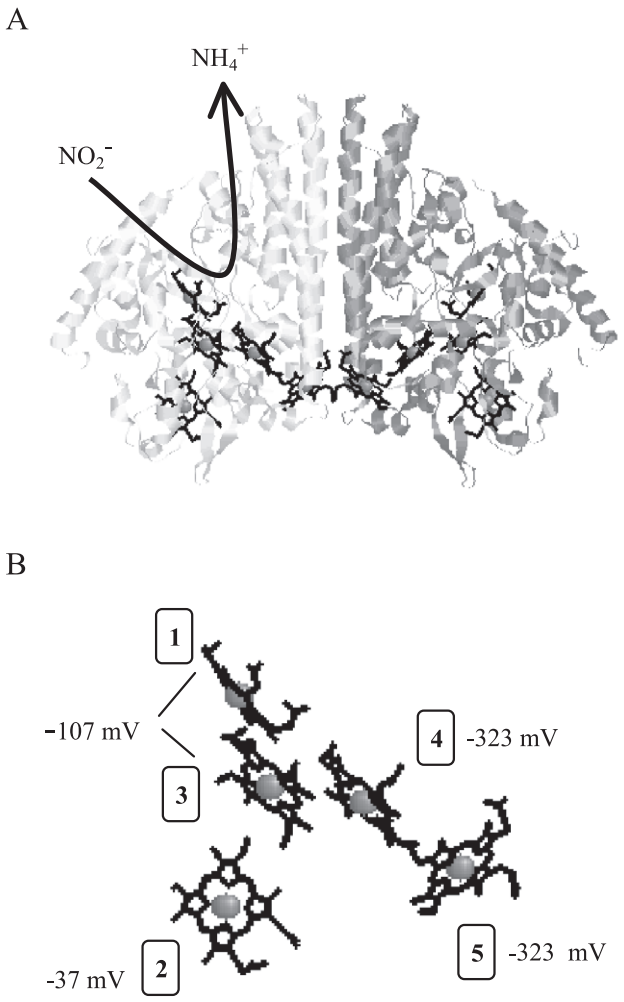


Fig. 1. *E. coli* cytochrome *c* nitrite reductase. (A) Ribbon representation of the crystal structure; the monomer backbones are shown in shades of grey, the hemes in black. (B) Heme numbering and mid-point potentials ($E_{m,7}$) for the left-hand monomer of the representation in panel (A) [3].

picture of enzyme chemistry in the electrochemical potential domain, we have adsorbed the sample on an electrode as a sub-monolayer film within which there is (i) direct electron exchange between heme centres and the electrode and (ii) facile substrate access to the enzyme active sites. In this configuration, simultaneous stimulation and quantitation of enzyme activity are possible; stimulation through variation of the electrode potential and quantitation through measurement of the catalytic current that flows if electrons move from the electrode via the enzyme to substrate molecules in solution [5–7]. This approach to the resolution of enzyme chemistry is known as protein film voltammetry (PFV).

Cytochrome *c* nitrite reductase is amenable to PFV since it forms electrocatalytic films when adsorbed on graphite 'edge' electrodes [6]. The films exhibit turnover numbers that are at least equivalent to those measured in classical biochemical assays of enzyme activity. When current is measured as the electrode potential is swept linearly between two limits the cyclic voltammetry provides ready and intuitive visualisation of enzyme chemistry across the potential domain. In this contribution we describe PFV that reveals the profound influence of heme oxidation state on the interactions of cytochrome *c* nitrite reductase with substrate and inhibitor molecules.

2. Experimental procedures

Cytochrome *c* nitrite reductase (specific activity 1500 μmol nitrite consumed min^{-1} mg^{-1}) was purified from *E. coli* by the previously published procedure and stored as aliquots frozen in liquid nitrogen [3]. Voltammetry was performed in a three-electrode configuration glass cell housed in a N_2-filled chamber with atmospheric O_2 < 5 ppm [6,7]. Pyrolytic graphite 'edge' working electrodes were polished with an aqueous 0.3 μm Al_2O_3 slurry immediately prior to each experiment. Films of nitrite reductase were formed when freshly polished electrodes were coated with a few microlitres of ice-cold, 0.65 μM nitrite reductase in 50 mM HEPES, pH 7.0. Control experiments confirmed that there was no detectable Faradaic current in the absence of nitrite reductase. All reagents were Analar quality or equivalent and solutions were prepared with water of resistivity 18 MΩ cm. Potentials measured against a saturated KCl, Ag/AgCl reference electrode were converted to the Standard Hydrogen Electrode scale by addition of 197 mV.

3. Results and discussion

3.1. PFV of cytochrome c nitrite reductase in nitrite and hydroxylamine

A typical cyclic voltammogram recorded with a film of nitrite reductase placed in 1 μM nitrite and rapid electrode rotation is illustrated in Fig. 2A. The catalytic current, and thus enzyme activity, shows a maximum in the potential window from ca. −160 to −240 mV on the forward and reverse potential sweeps. At this nitrite concentration the response is nitrite-limited (K_M ~ 28 μM) and the modulations of activity can be correlated with the reduction potentials of heme centres where a change of oxidation state results in a change of activity [6].

Non-turnover signals for cytochrome *c* nitrite reductase that would allow direct determination of the reduction potentials of heme centres in the enzyme film have yet to be detected by PFV. However, the K_M values displayed by nitrite reductase films are similar to those obtained from solution phase assays and suggest that the enzyme experiences little perturbation when adsorbed on the electrode surface [6]. The reduction potentials defined by potentiometric titration can then be used as a basis for interpretation of the catalytic voltammetry. Since the modulations of catalytic activity with potential displayed by nitrite reduc-

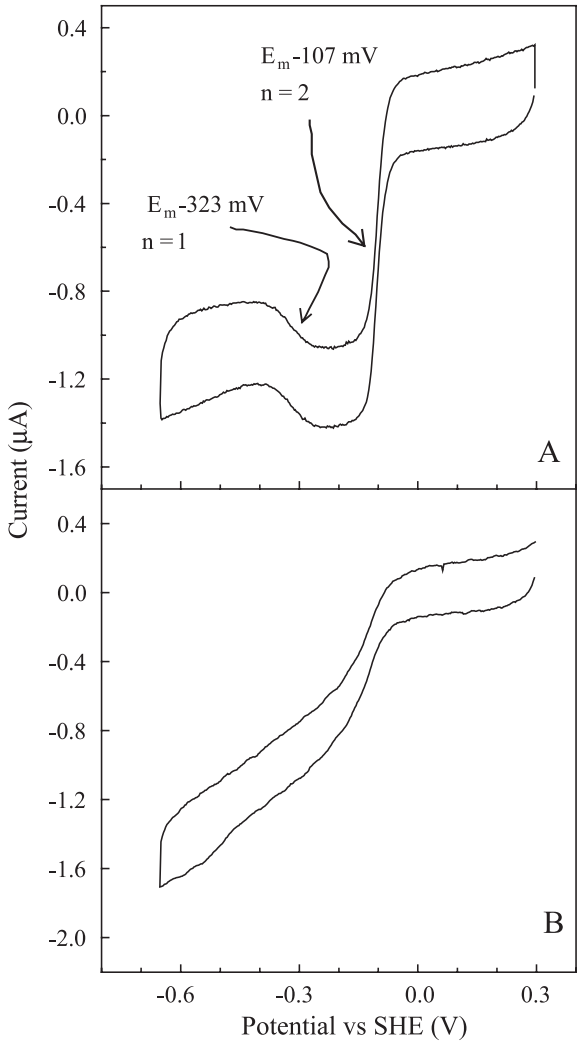


Fig. 2. Protein film voltammetry from *E. coli* cytochrome *c* nitrite reductase under conditions for substrate-limited turnover. Typical cyclic voltammograms in 1 μM nitrite (A) and 1 mM hydroxylamine (B). Voltammetry was performed in 50 mM HEPES, 2 mM $CaCl_2$, pH 7.0, 25 °C with scan rate 30 mV s^{-1}, electrode rotation rate 3000 rpm.

tase are separated by a plateau of maximal current each component can be fitted to independent Nernst equations.

The region of the wave between ca. −80 and −160 mV is well described by an $n=2$ process with $E_m=-107$ mV [6]. Thus, the activity of the enzyme is turned on by reduction of Hemes 1 and 3, where the former provides the nitrite coordination site. This is readily explained if reduction of Hemes 1 and 3 triggers an electron cascade that transforms substrate to product and it may indicate that reduction of these hemes is a pre-requisite for nitrite coordination in the inner coordination sphere of the Heme 1 iron. It is interesting to note that at the potentials where activity is 'switched on' Hemes 1, 3 and 2 in each monomer are reduced and the dimer holds the six electrons required to reduce a nitrite molecule bound in one active site to ammonium. It is possible that facile inter-monomer electron exchange within a functional dimer could be utilised to prevent the build up of potentially harmful reaction intermediates.

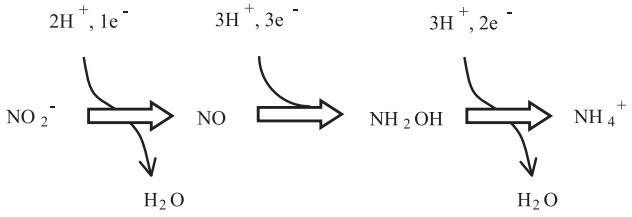
Why then does the enzyme show an attenuation of activity at lower potentials when the driving force for the reaction being catalysed is actually increased? The region of the catalytic wave between ca. −240 and −400 mV is well described by an $n=1$ process with $E_m=-323$ mV and the trigger responsible for the attenuation of activity can be ascribed to reduction of Hemes 4 and 5 [6].

A route to begin to understand the consequence(s) of reduction at Hemes 4 and 5 is afforded by the proposed pathway of nitrite reduction by cytochrome *c* nitrite reductase, Scheme 1 [4,8,9]. The pathway contains the intermediates nitric oxide and hydroxylamine. These molecules are also substrates of the enzyme and both are reduced to ammonium. PFV performed under hydroxylamine-limited turnover conditions (1 mM hydroxylamine, K_M ~ 127 mM) displays a clear catalytic response, Fig. 2B [6]. The position of the catalytic wave is consistent with reduction of Hemes 1 and 3 'switching on' the hydroxylamine reductase activity. Although the current does not reach a constant value at low potentials there is no indication that this activity is attenuated. Thus, reduction of Hemes 4 and 5 attenuates a step in the process of nitrite transformation to hydroxylamine. Such an affect may arise from redox-triggered conformational changes communicated to the active site and to which nitrite is more sensitive than hydroxylamine. Alternatively, a buildup of charge in the enzyme when all hemes are reduced may attenuate intra-molecular electron exchange.

3.2. Cyanide inhibition of the hydroxylamine reductase activity of cytochrome c nitrite reductase

Cyanide was previously reported as a high-affinity inhibitor of cytochrome *c* nitrite reductase [9]. The relative simplicity of the catalytic response observed during hydroxylamine turnover provided an opportunity to resolve any influence of heme oxidation state on the interaction of cyanide with cytochrome *c* nitrite reductase.

Experiments were performed in 20 mM hydroxylamine to ensure a reasonable catalytic response after the addition of inhibitor, Fig. 3A. At this hydroxylamine concentration, enzyme turnover is not substrate-limited and the catalytic waveform reflects the rate-defining events relevant to catalysis under the experimental conditions [5–7,10]. Introduc-



Scheme 1.

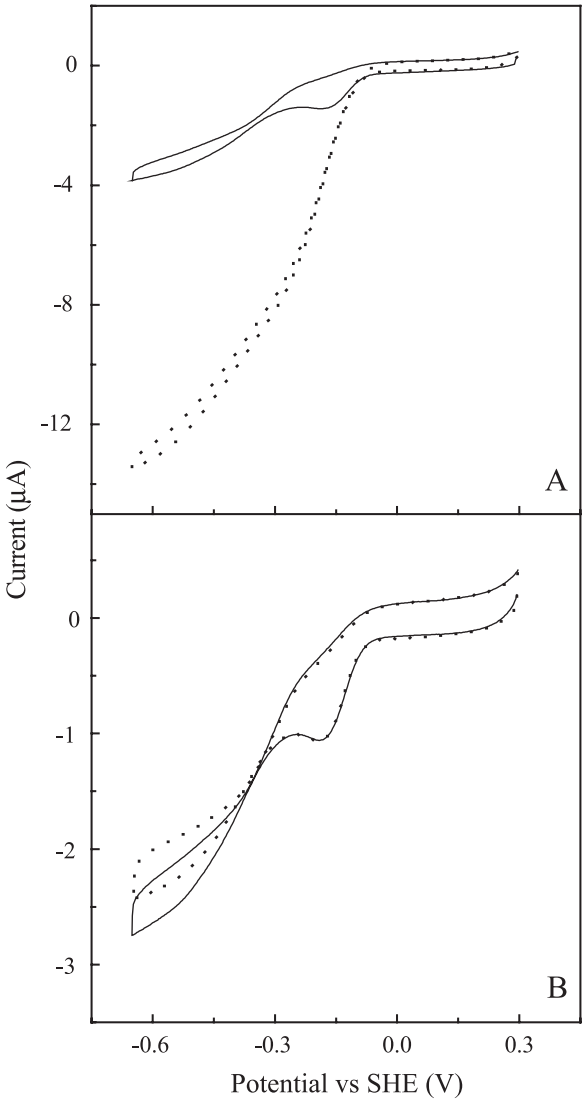


Fig. 3. Cyanide induced perturbation of the hydroxylamine reductase activity of *E. coli* cytochrome *c* nitrite reductase. (A) PFV of nitrite reductase placed in 20 mM hydroxylamine. Consecutive scans were recorded immediately prior to (broken line) and after (solid line) the addition of 2.8 μM potassium cyanide. Voltammetry was performed at 30 mV s^{-1} with electrode rotation at 3000 rpm in 50 mM HEPES, 2 mM $CaCl_2$, pH 7.0 at 25 °C. (B) PFV of nitrite reductase in 2.8 μM cyanide and 20 mM hydroxylamine recorded at 30 mV s^{-1} with electrode rotation at 200 rpm (broken line) and 3000 rpm (solid line) other conditions as above.

tion of 2.8 μM potassium cyanide resulted in a decrease of the catalytic current magnitude by ca. 75%. The catalytic current was restored on transfer of the film to 20 mM hydroxylamine solution to indicate that cyanide inhibition is a reversible process.

Closer inspection of the catalytic response observed in the presence of cyanide reveals a peak on the sweep to negative potentials that is absent from the return sweep. The peak describes inhibition of enzyme activity as the electrode potential is swept to more negative values and hemes within the enzyme are reduced. That the peak does not simply reflect slow association of cyanide with the enzyme film is supported by several observations. First, the form of the voltammogram is independent of the time the electrode is held at positive potentials prior to initiation of the voltammetric sweep. Second, since cyanide association with the enzyme could conceivably decrease the concentration of cyanide at the film to a level sufficient to attenuate the observed rate of cyanide binding, the voltammetric response was confirmed to be essentially independent of electrode rotation under the experimental conditions, Fig. 3B. Third, the voltammograms observed during repeated cyclic voltammetry overlaid that observed on the first potential cycle. Thus, cyanide binds to the enzyme film *each time* it is taken from the oxidised to a more reduced state. Consequently, cyanide must be *released* from the enzyme on the sweep towards more positive potentials although at a rate that is slow compared to the voltammetric timescale since no peak of activity is defined on the sweep to positive potentials.

Cyanide clearly has a lower affinity for oxidised than more reduced states of cytochrome *c* nitrite reductase. Additional evidence of the relatively slow rates of cyanide association with, and release from, the enzyme film is afforded by variation of the experimental time domain. When the scan rate is decreased from 30 mV s^{-1} the peak describing cyanide binding to the reduced enzyme becomes less pronounced. At 1 mV s^{-1}, no such peak is observed, the catalytic currents of the forward and reverse sweeps overlay, and the oxidation state-dependent equilibration of cyanide with cytochrome *c* nitrite reductase is observed under conditions of steady state.

4. Conclusions

Protein film voltammetry provides a powerful tool with which to resolve the oxidation state sensitivities of redox enzyme activity. The ability to provide simultaneous resolution of enzyme chemistry in the electrochemical potential and time domains offers the opportunity to provide unique insights into enzyme reactivity. These studies of cytochrome *c* nitrite reductase exemplify the ability of PFV to reveal new facets of enzyme chemistry.

PFV has revealed that reduction of the low potential hemes in cytochrome *c* nitrite reductase provides a trigger to attenuate the rate of nitrite- but not hydroxylamine-limited turnover. While PFV alone cannot resolve the origin of this behaviour it does provide a guide to the conditions under which spectroscopic and structural methods may uncover the origin of this phenomenon at the molecular level. The technique has the additional advantage that information is gained from minuscule amounts of sample (on the order of 10^{-12} mol of enzyme per film) compared to the requirements for most methods that provide molecular level resolution.

Cyanide-induced perturbation of the catalytic response for hydroxylamine reduction has visualised the lower affinity of cyanide for oxidised over reduced forms of the enzyme.

This behaviour is striking since a consideration of electrostatic factors leads to an expectation that cyanide will bind with higher affinity to ferric rather than ferrous heme. Indeed cyanide binds with much greater affinity to ferric than ferrous myoglobin and hemoglobin [11,12]. By contrast, *Paracoccus pantotrophus* cytochrome cd_1 nitrite reductase and the siro-heme containing sulfite reductase from *E. coli*, like cytochrome *c* nitrite reductase, display considerable affinity for cyanide in their reduced states [13,14]. The crystal structures of reduced, cyanide bound cytochrome cd_1 and siro-heme sulfite reductase show cyanide as an axial ligand to the active site heme. The site of cyanide binding in cytochrome *c* nitrite reductase cannot be resolved by PFV. However, the observation that cyanide inhibition is triggered by a reductive process in a potential window that encompasses the reduction potentials of Hemes 1 and 3 suggests that cyanide may bind to Heme 1 in a manner that parallels the behaviour displayed by the other anion reductases.

If cyanide does bind as an axial ligand to Heme 1, how might the relative affinities for the ferrous and ferric states be explained? The cytochrome *c* nitrite reductase from *Wolinella succinogenes* has been reported to have a higher affinity for nitrite in the reduced as compared to oxidised state [15]. This was rationalised by the presence of π-back bonding in the Heme 1 Fe(II)–NO_2^- complex not available to the Fe(III)–NO_2^- complex. Cyanide is a strong π-acceptor ligand and a similar description of the bonding to Heme 1 may account for the behaviour reported here. Stabilisation of a cyano-ferrous Heme 1 complex could also be achieved if reduction of Hemes 1 and 3 leads to protonation of histidine and arginine residues in the active site pocket to generate favourable electrostatic or H-bonding interactions with the exogenous ligand. It is hoped that future studies will resolve whether these factors, or a conformational change within the active site, ultimately determine the oxidation state sensitivity of cyanide binding to *E. coli* cytochrome *c* nitrite reductase.

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