Opportunities for mesoporous nanocrystalline SnO$_2$ electrodes in kinetic and catalytic analyses of redox proteins

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Abstract

PFV (protein film voltammetry) allows kinetic analysis of redox and coupled-chemical events. However, the voltammograms report on the electron transfer through a flow of electrical current such that simultaneous spectroscopy is required for chemical insights into the species involved. Mesoporous nanocrystalline SnO$_2$ electrodes provide opportunities for such 'spectroelectrochemical' analyses through their high surface area and optical transparency at visible wavelengths. Here, we illustrate kinetic and mechanistic insights that may be afforded by working with such electrodes through studies of *Escherichia coli* NrfA, a pentahaem cytochrome with nitrite and nitric oxide reductase activities. In addition, we demonstrate that the ability to characterize electrocatalytically active protein films by MCD (magnetic circular dichroism) spectroscopy is an advance that should ultimately assist our efforts to resolve catalytic intermediates in many redox enzymes.

Introduction

Interprotein electron transfer forms an essential component of many cellular pathways and one that is frequently facilitated by positioning redox-active cofactors close to the surface of the proteins involved. Such cofactors are brought into close proximity within the electron transfer complex to allow physiologically 'useful' rates of long-range interprotein electron transfer. This design feature can be exploited to achieve facile electron transfer between redox proteins and electrodes, allowing the former to be studied by dynamic electrochemical methods. Here, electron exchange between the protein and electrode is stimulated by defined variation of the electrode potential and quantified as a flow of electrical current. When the protein under investigation is adsorbed on an electrode surface, rates of electron transfer and coupled chemical processes are 'viewed' with high fidelity because the experiment is free from the limitations caused by the relatively slow process of protein diffusion that dominates the electrochemistry of proteins in solution. Consequently, such experiments have proved popular for studies of redox enzymes in addition to proteins that act as 'wires' or 'shuttles' when moving electrons between other proteins in an approach known as PFV (protein film voltammetry; Figure 1A). Here, we provide a brief overview of PFV as a tool for kinetic analysis and use our studies of a multihaem cytochrome to illustrate the possibilities afforded when PFV is performed with simultaneous spectroscopy.

Opportunities for kinetic analysis by PFV

A major strength of PFV is the range of accessible timescales that allows access to equilibrated and transient states in studies of a single sample. Cyclic voltammetry is usually the first technique employed in a PFV study because the results are intuitively interpretable as arising from reversible, irreversible or catalytic redox events. Here, the electrode potential is swept linearly between two limits in a saw-tooth fashion (Figure 1B). The experimental timescale is defined by the rate of change of electrode potential, more usually referred to as the scan rate, and typically varied from approx. 10^{-3} to 10^3 V·s^{-1}. An electrical current is plotted as a function of electrode potential, providing an immediate visualization of redox events resolved across the electrochemical potential and time domains as described in several excellent reviews (e.g. [1]). Here, we restrict ourselves to highlighting two results that illustrate the types of information uniquely available through fast-scan PFV: (i) resolution of the rate constants describing electron and coupled-proton transfer that provide precedent for resolving such events in enzymes that generate transmembrane proton gradients [2]; and (ii) definition of active site reduction potential in a Michaelis complex made possible by the ability to move electrons in, and out, of the complex before chemical events associated with catalysis could occur [3].

A possible drawback of cyclic voltammetry is that change of potential is intrinsically associated with progression of time. When clear distinction between the consequences of these parameters is required pulse, or 'step', voltammetry is appropriate. Here, the electrode is held at a defined potential and then stepped, or pulsed, to a second potential with the corresponding electrical current recorded as a function of

Figure 1 | Simultaneous voltammetry and optical spectroscopy of adsorbed protein

(**A**) Schematic diagram of the spectroelectrochemical experiment with protein adsorbed on an optically transparent SnO_2 electrode. (**B**) Excitation profiles for cyclic voltammetry at relatively slow (broken line) and fast (solid line) scan rates. (**C**) One of the many possible excitation profiles for pulse voltammetry.
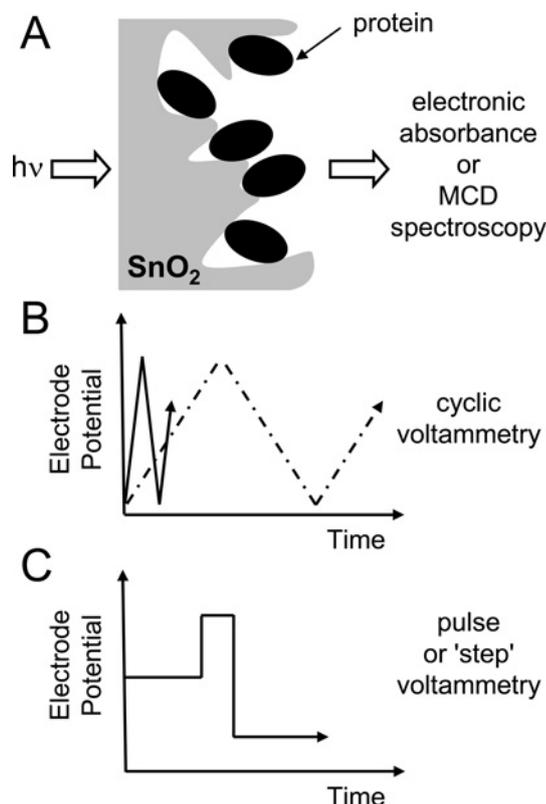
time, e.g. Figure 1(C). The potential step occurs within a fraction of a second and serves to initiate or 'trigger' electron transfer in a manner analogous to methods used to initiate reactions for fast reaction kinetics, e.g. laser photolysis and steps of pH or temperature. Applied potentials are chosen from a broad and continuous range, removing the restriction to start from fully oxidized or reduced samples, and multiple pulses can also be applied to explore reversibility and/or the consequences of distinct triggers within a single sample [4].

PFV with simultaneous optical spectroscopy using mesoporous nanocrystalline SnO₂ electrodes

Voltammetry measures electron transfer as electrical current, with the consequence that no direct information is available on the chemical nature of events occurring within the protein. Such deductions may be trivial when considering equilibrium studies of proteins containing a single redox site. However, parallel spectroscopic insights are clearly desirable when seeking to identify transient intermediates and describe the

behaviour of multicentred proteins, and for this, a number of strategies are possible. Here, we focus on the use of mesoporous nanocrystalline SnO_2 electrodes that allow for PFV with simultaneous optical spectroscopy [5–7]. These electrodes have good conductivity below +300 mV when compared with SHEs (standard hydrogen electrodes) and combine transparency in the visible region of the electromagnetic spectrum with a high-surface area accessible to molecules from a surrounding solution. In favourable cases, this allows adsorbed proteins to achieve the densities required for informative electronic absorption spectroscopy.

Spectroelectrochemistry of a multihaem nitrite reductase adsorbed on SnO₂ electrodes: proof of principle

Escherichia coli NrfA is a pentahaem-containing protein that can facilitate anaerobic respiration through nitrite reduction or host invasion by detoxification of exogenously produced NO. The structure of NrfA has been resolved and its spectroscopic properties are well documented [8,9]. The active site contains a Lys–water (hydroxide) co-ordinated high-spin haem to which electrons are delivered by four low-spin haems with His–His ligation. PFV with graphite electrodes has defined the nitrite, hydroxylamine and NO reductase activities of this enzyme although it has not yet been possible to detect redox activity from an NrfA film in the absence of substrates [10,11].

Given that NrfA is amenable to PFV and that its haems give rise to strong optical transitions in the visible region, we hope that spectroelectrochemical studies of NrfA adsorbed on SnO_2 electrodes will provide new insights into the catalytic mechanism about which relatively little is known. Our first aim was to establish that NrfA could adsorb as an electroactive film on SnO_2 electrodes with minimum perturbation of the properties displayed in solution. We used electrodes consisting of a 4 μm SnO_2 layer formed from particles of 15 nm diameter deposited on a conducting glass slide [5–7]. After these electrodes had been soaked in solutions of NrfA and rinsed with buffer-electrolyte to remove loosely bound protein, their electronic absorption spectra showed features typical of oxidized NrfA superimposed on a background arising from scattering by the SnO_2 layer [8]. The spectra did not change when the electrodes were held at +366 mV, but features typical of fully reduced NrfA were detected when electrodes were poised at −634 mV (e.g. Figure 2A). Significantly these spectra established that all the adsorbed protein responded to the applied electrode potential.

Cyclic voltammetry of the NrfA-coated electrodes showed peaks of current, absent from voltammetry of 'bare' SnO_2, that could be assigned to reduction and oxidation of centres in NrfA (Figure 2C). Simultaneous measurement of the electronic absorbance at 552 nm showed cycles of increasing and decreasing intensity, as expected for cycles of reduction and oxidation respectively of the low-spin haems. Taking a first derivative of the absorbance change with respect to the electrode potential (dA_{552}/dE) allowed direct correlation of

(**A**) Electronic absorption spectra with the electrode poised at +366 (broken line) and −634 mV (light solid line) in the absence of nitrite and −634 mV in a stirred solution of 1 mM nitrite (heavy solid line). The response of the SnO₂ layer has been subtracted from these spectra. (**B**) MCD spectra with the electrode poised at +280 and −440 mV in the absence of nitrite. (**C**) Results from a sweep to positive potentials during cyclic voltammetry (5 mV · s⁻¹) with simultaneous electronic absorption spectroscopy in the absence of nitrite. Faradaic current (line) and first derivative of the absorbance with respective to potential (dA/dE) for 552 nm (open circles) and 442 nm (filled squares). Buffer-electrolyte: 2 mM CaCl₂ and 50 mM Hepes (pH 7) at 24°C.
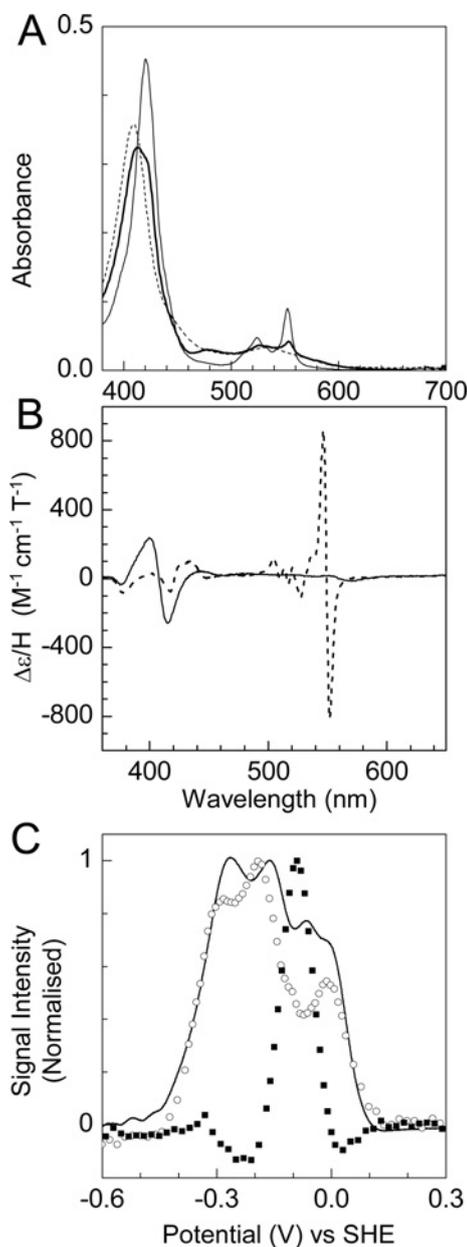
−200 mV. In the latter region, the voltammetric current reported on redox events with no counterpart in redox transitions of the low-spin haems. Repeating the voltammetry while monitoring the electronic absorbance at 442 nm to detect redox transitions of the high-spin haem showed a narrow window of redox activity centred on −100 mV that accounted for the 'missing' contribution to the voltammetric peak (Figure 2C). Thus spectroelectrochemistry of NrfA on SnO₂ electrodes was able to define the total redox activity of the enzyme and resolve the contributions from its distinct types of cofactor. Further analysis showed that each dataset could be well described by Nernstian analyses, giving reduction potentials for the NrfA haems in good agreement with those displayed by solutions of the enzyme [8].

Exploring opportunities for kinetic resolution of NrfA adsorbed on SnO₂ electrodes

The similar spectroscopic and redox properties displayed by solutions of NrfA and NrfA adsorbed on SnO₂ electrodes establish that the enzyme experiences little perturbation on adsorption and provide the platform for studies of NrfA catalysis and electron transfer kinetics that are now in progress. The active-site high-spin haem can be singled out for study through its optical properties. Pulse voltammetry with electronic absorbance monitored at 442 nm suggests that high-spin haem reduction 50 mV below its reduction potential occurs at a rate of approx. $0.5 \cdot s^{-1}$ and oxidation 100 mV above the reduction potential occurs at a rate of approx. $1 \cdot s^{-1}$ (Figure 3A). Monitoring the 442 nm electronic absorbance during cyclic voltammetry at a range of scan rates produces dA_{442}/dE plots with an increased peak separation at increased scan rate (Figure 3B). Such a behaviour is typical of situations where interfacial electron transfer becomes sluggish on the voltammetric timescale. Comparison with the predictions for a Butler–Volmer description of interfacial electron transfer provides a starting point to consider these data and yields an apparent 'heterogeneous rate constant' of the order of $0.5 \cdot s^{-1}$ at the high-spin haem reduction potential and of the same order of magnitude as the rates deduced from pulse voltammetry. Future work will aim to extend the mechanistic framework for interpreting kinetic data such as these by establishing the role of the low-spin haems in mediating electron exchange between the electrode and high-spin haem.

To establish that catalytic properties of NrfA could be probed by spectroelectrochemistry, nitrite was introduced into the experiment. Cyclic voltammetry defined a catalytic reduction wave, not detected in the absence of NrfA, that reflected NrfA-catalysed nitrite reduction [8]. Electronic absorption spectroscopy during turnover with the electrode poised at −600 mV showed no evidence of the features characteristic of reduced NrfA and consistent with all the adsorbed enzyme retaining its catalytic, i.e. functional, activity (Figure 2A). The nature of the predominant species present during turnover and its implications for the mechanism of

the optical and voltammetric data (Figure 2C). Both datasets defined NrfA redox activity between approx. +100 and −450 mV aside from the region between approx. 0 and

Figure 3 | Time-resolved perspectives of the high-spin haem redox transformation in NrfA adsorbed on SnO$_2$ electrodes

(**A**) The electronic absorbance at 442 nm for an NrfA-coated (thin line) and 'bare' SnO$_2$ (thick line) electrode during pulse voltammetry. Initially, the electrode was held 100 mV positive of the high-spin haem reduction potential. After 20 s, the potential was stepped 50 mV below the high-spin haem reduction potential and after 40 s the potential was returned to the initial value. (**B**) Scan-rate-dependence of the peak potentials for dA_{442}/dE plots derived from electronic absorption spectroscopy during cyclic voltammetry. Results for the oxidative (circles) and reductive (squares) peaks are shown together with lines illustrating the behaviour predicted by the Butler–Volmer equation for $k^o = 0.5$ s^{-1}. Buffer-electrolyte: as in Figure 2.
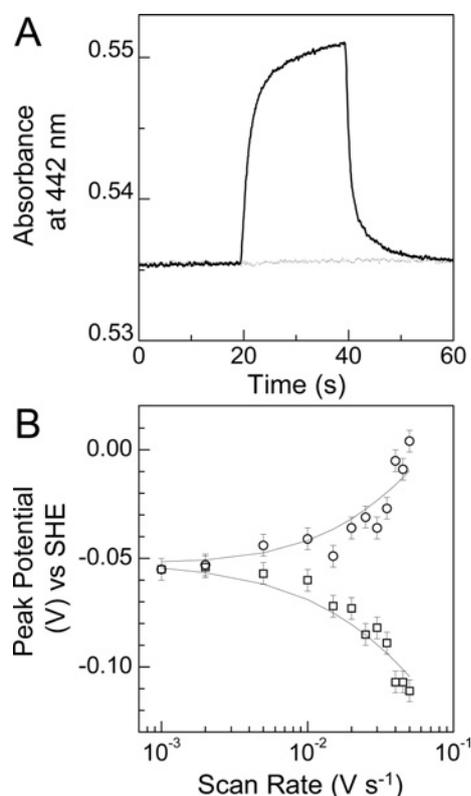
nitrite reduction and elucidation of rate-limiting events in that process are currently under investigation.

MCD (magnetic circular dichroism) spectroscopy of adsorbed protein films

Electronic absorption spectroscopy has provided useful perspectives on the NrfA haems but MCD spectroscopy affords greater chemical insights since it reports on haem oxidation states, spin states and ligation states [8]. Preliminary experiments have established that NrfA adsorbed on SnO$_2$ electrodes is amenable to MCD spectroscopy (Figure 2B). The MCD spectra show features characteristic of the oxidized and reduced proteins when poised at +280 and −440 mV respectively, opening the way to study catalytic species with a more informative spectroscopic probe than electronic

absorption spectroscopy and one that can also be used for time-resolved data collection.

Thoughts in closing

Spectroelectrochemistry of NrfA adsorbed on mesoporous nanocrystalline SnO$_2$ has illustrated how PFV with simultaneous optical spectroscopies can usefully dissect the redox behaviour of a multihaem cytochrome and, by extension, many other proteins. Indeed, we are finding that many of the proteins studied in our laboratories are amenable to this approach. This is significant because a number of these proteins have failed to yield detectable voltammetry at graphite electrodes and many of the others, despite yielding clear catalytic currents, fail to achieve an electroactive population large enough to produce detectable peaks in the absence of substrate. Previously, the chemical heterogeneity of graphite surfaces has been credited with facilitating the voltammetry of many proteins. By contrast, the surface of mesoporous nanocrystalline SnO$_2$ is chemically monotonous although negatively charged. It may be that the comparable dimensions of the proteins, SnO$_2$ nanoparticles and pores (5–20 nm) within the SnO$_2$ layer facilitate PFV by ensuring that much of the surface of any adsorbed protein is close to the SnO$_2$ surface so the chances of facile interfacial electron transfer are high. Thus we are optimistic that spectroelectrochemical studies employing PFV with mesoporous nanocrystalline SnO$_2$ electrodes will provide a powerful approach to monitor reaction kinetics and characterize catalytically relevant states.

Funding

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