Interruption and Time-Resolution of Catalysis by a Flavoenzyme Using Fast Scan Protein Film Voltammetry

Anne K. Jones,[‡] Raúl Camba,[‡] Graeme A. Reid,[§] Stephen K. Chapman,[†] and Fraser A. Armstrong*,[‡]

Inorganic Chemistry Laboratory, Oxford University South Parks Road, Oxford OX1 3QR, England Department of Chemistry and Institute of Cell and Molecular Biology, University of Edinburgh West Mains Road, Edinburgh EH9 3JJ, Scotland

Received March 9, 2000
Revised Manuscript Received May 15, 2000

We have carried out cyclic voltammetry measurements on a fully active adsorbed enzyme at scan rates exceeding 100 V s^{-1}, fast enough to outrun the catalytic cycle and examine electron transfers within the enzyme−substrate complex. The studies have been made with a soluble fumarate reductase called flavocytochrome c_3 (Fcc_3), MW 64 kDa, isolated from the marine bacterium *Shewanella frigidimarina* NCIMB400.[1] Flavocytochrome c_3, the structure[2] of which is shown in Figure 1, contains four *c*-type hemes and a single noncovalently bound FAD which is in the active site. We showed previously[3] that Fcc_3 displays excellent voltammetry when adsorbed at a pyrolytic graphite "edge" (PGE) electrode: the cooperative two-electron signal due to the FAD was easily observable above the underlying one-electron signals of the hemes, since for an adsorbed redox couple cooperatively transferring *n* electrons, the theoretical half-height peak width, δ, is 86/*n* mV (10 °C), and the peak height varies with n^2.[4] (By contrast, the electronic spectrum of the FAD is obscured by intense transitions from the hemes.) This led us to predict, as we now show, that we could exploit fast scan voltammetry to study the FAD during catalysis.

Catalytic and fast scan protein film voltammetry (PFV) was carried out as described in recent articles.[5−7] Figure 2 shows cyclic voltammograms measured over 4 orders of magnitude in scan rate, either in the absence or presence of fumarate at pH 7 and 10 °C.[8] At slow scan rates, the "no-substrate" voltammograms can be deconvoluted in terms of contributions from four hemes (each one electron) and the FAD, which undergoes a cooperative two-electron transfer at $E^{\circ\prime} = -143$ mV vs SHE.[3] On the basis of a six-electron exchange, typical enzyme coverages on the electrode are $(8-9) \times 10^{-12}$ mol cm^{-2} (approximately equivalent to a monolayer on a hypothetical flat electrode). As the scan rate is increased, the essential features remain, and even at 82 V s^{-1}, the FAD is still visible as a prominent two-electron component. At 100 V s^{-1} it becomes unresolvable above the underlying component due to the four hemes: this may indicate the onset of kinetic resolution of separated one-electron steps (so that the signal

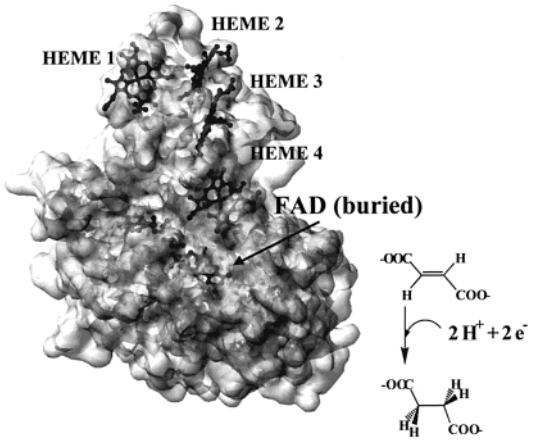

Figure 1. A view of Fcc_3 showing the van der Waals surface in translucent gray. The four hemes and the FAD are indicated.

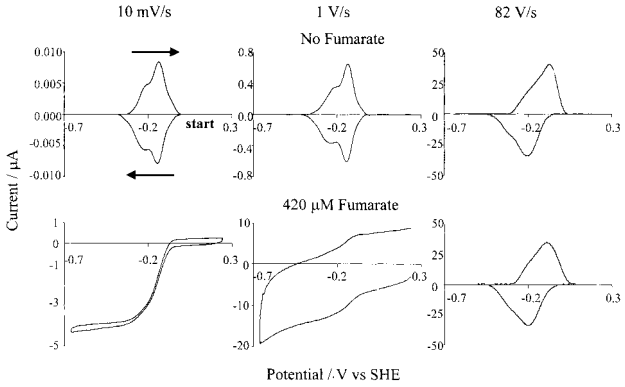

Figure 2. Cyclic voltammograms of Fcc_3 adsorbed on a PGE electrode, at various scan rates, all at pH 7, 10 °C and commenced from an oxidative poise. Top row shows voltammograms of enzyme without substrate in the solution, and arrows on first example give direction of cycle. Bottom row shows voltammograms in the presence of 420 μM fumarate: those at 10 mV s^{-1} and 1 V s^{-1} have been measured with electrode rotating at high frequency (1500 rpm) to maintain substrate transport to the electrode surface[10] (increasing the rotation rate further did not increase the catalytic current). With the exception of those in the presence of fumarate at 10 mV s^{-1} and 1 V s^{-1}, voltammograms displayed no rotation rate dependence and have been baseline corrected for non-Faradaic current (see ref 5c).

‡ Inorganic Chemistry Laboratory, Oxford University.
§ Institute of Cell and Molecular Biology, University of Edinburgh.
† Department of Chemistry, University of Edinburgh.

(1) (a) Morris, C. J.; Black, A. C.; Pealing, S. L.; Manson, F. D. C.; Chapman, S. K.; Reid, G. A.; Gibson, D. M.; Ward, F. B. *Biochem. J.* **1994**, *302*, 587−593. (b) Easter, M. C.; Gibson, D. M.; Ward, F. B. *J. Gen. Microbiol.* **1983**, *129*, 3689−3696. (c) Pealing, S. L.; Cheeseman, M. R.; Reid, G. A.; Thomson, A. J.; Ward, B.; Chapman, S. K. *Biochemistry* **1995**, *34*, 6153−6158.

(2) Taylor, P.; Pealing, S. L.; Reid, G. A.; Chapman, S. K.; Walkinshaw, M. D. *Nat. Struct. Biol.* **1999**, *6*, 1108−1112.

(3) Turner, K. L.; Doherty, M. K.; Heering, H. A.; Armstrong, F. A.; Reid, G. A.; Chapman, S. K. *Biochemistry* **1999**, *38*, 3302−3309.

(4) Laviron, E. *Electroanalytical Chemistry*; Bard, A. J., Ed.; Marcel Dekker: New York, 1982; Vol. 12, pp 53−157.

(5) (a) Sucheta, A.; Cammack, R.; Weiner, J.; Armstrong, F. A. *Biochemistry* **1993**, *32*, 5455−5465. (b) Armstrong, F. A.; Heering, H. A.; Hirst, J. *Chem. Soc. Rev.* **1997**, *26*, 169−179. (c) Heering, H. A.; Weiner, J. H.; Armstrong, F. A. *J. Am. Chem. Soc.* **1997**, *119*, 11628−11638.

(6) Protein film fast scan cyclic voltammetry is described in: (a) Hirst, J.; Armstrong, F. A. *Anal. Chem.* **1998**, *70*, 5062−5071. (b) Hirst, J.; Duff, J. L. C.; Jameson, G. N. L.; Kemper, M. A.; Burgess, B. K.; Armstrong, F. A. *J. Am. Chem. Soc.* **1998**, *120*, 7085−7094. (c) Hirst, J.; Jameson, G. N. L.; Allen, J. W. A.; Armstrong, F. A. *J. Am. Chem. Soc.* **1998**, *120*, 11994−11999. For fast scan cyclic voltammetry of small-molecule monolayers, see, for example: (d) Weber, K.; Creager, S. E. *Anal. Chem.* **1994**, *66*, 3164−3172.

(7) Flavocytochrome c_3 was prepared according to refs 1c and 3. All electrochemical experiments were undertaken in a glovebox under nitrogen. The electrochemical cell and the PGE rotating disk electrode (0.03 cm^2 geometrical area) have been described previously (see ref 5a). Fast scan voltammetry (scan rates >1 V/s) with *iR* compensation was carried out as described in refs 6a−c) using an Autolab Electrochemical Analyzer (Eco-Chemie, Utrecht, Netherlands) equipped with PGSTAT 30, Scangen, and ADC750 modules. Voltammograms were Fourier smoothed and non-Faradaic background currents were subtracted as described by Heering et al. (ref 5c). For each experiment, the working electrode was polished, and the film was formed as described in ref 3, at 10 °C. The mixed buffer/electrolyte system used as in ref 3 included 200 μg ml^{-1} Polymyxin B sulfate (Sigma) as coadsorbate to stabilize the film. Solutions of fumaric acid (Fluka, 99.5%) were prepared in the mixed buffer system, and the pH was adjusted using NaOH. All potentials are reported with reference to the standard hydrogen electrode (SHE) based on a potential of 242 mV for SCE at 20 °C.

(8) 10 °C is a physiological temperature for *Shewanella frigidimarina* (habitat, North Sea).

10.1021/ja000848n CCC: $19.00
Published on Web 06/21/2000

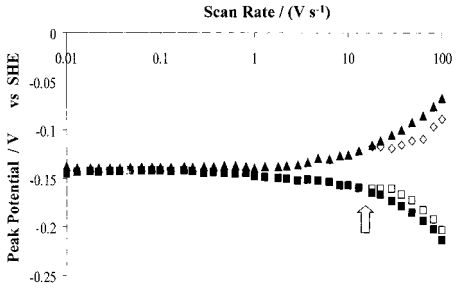

Figure 3. Voltammetric peak positions as a function of scan rate for Fcc_3 at pH 7, 10 °C. Symbols represent: (■), (▲) reductive and oxidative peaks respectively, in the *absence* of fumarate; (□), (△) reductive and oxidative peaks in the *presence* of 420 μM fumarate. At slowest scan rates, there is an uncertainty in peak position of 1−2 mV, whereas at highest rates the uncertainty is 5−7 mV. Arrow indicates slowest scan rate (14 V s^{-1}) at which peaks should become visible[12] based on a model assuming k_{cat} is the rate of transfer of reducing equivalents from FAD to substrate. It employs a finite difference calculation to predict voltammograms based on the parameters $k_{cat} = 120\ s^{-1}$, $k_0 = 500\ s^{-1}$ (Butler−Volmer electrochemical rate constant), and $n_{app} = 1.6$ (see ref 3).

is no longer distinguishable) or that electrons are unable to reach the FAD in the period of the cycle. On the latter note, the peak area (normalized with respect to scan rate) decreases by only about 25% as the scan rate is increased from 1 mV s^{-1} to 100 V s^{-1}, compared to 33% if the FAD no longer contributes.[9] The voltammograms are not affected by whether the cycle is commenced from an oxidative (0.242 V vs SHE) or a reductive poise (−0.658 V vs SHE).

In the presence of fumarate, cycling at slow scan rates produces a steady-state catalytic wave. Analysis[5a] of the fumarate concentration dependence for currents measured with a rotating disk electrode[10] yields Michaelis parameters $k_{cat} = 120\ s^{-1}$ and $K_m = 26\ \mu$M at 10 °C, which agree well with values ($k_{cat} = 151\ s^{-1}$ and $K_m = 21\ \mu$M) obtained under the same conditions by a conventional solution assay[3] with methyl viologen as electron donor. The catalytic activity of the enzyme is thus unaffected by confinement on the electrode. As the scan rate is increased, the sigmoidal waveform converts to peak-like signals similar to those obtained in the absence of substrate. This shows that electrons are "re-called" to the electrode before they can be passed to the substrate, that is, the catalytic cycle is outrun. The fumarate concentration, 420 μM, is well above K_M so that 94% of the active sites are occupied by substrate; yet the shape of the voltammogram is very similar to that obtained in its absence, and once again, at 82 V s^{-1} the FAD is still detectable above the heme envelope.

Figure 3 shows how the FAD oxidative and reductive peak positions vary with scan rate in the presence and absence of fumarate. These "trumpet-shaped" plots contain information on many aspects of electron transfer, such as the thermodynamics, kinetics, and time scale of coupled reactions.[6a−c] In the absence of fumarate, data are definable from the slowest rates (10 mV s^{-1}) until the FAD becomes unresolved (above 100 V s^{-1}). The symmetry of the plots shows that there are no complications due to rate-limiting chemical processes in any particular direction.[6a] Analysis according to the Butler−Volmer model[4,11] shows that the FAD must be reduced and reoxidized with two-electron kinetics that are equivalent to a standard electrochemical rate constant of 375 s^{-1}. In the presence of fumarate, the plot cannot start until a scan rate is reached that is fast enough to outrun catalysis.[12] The results reveal that the FAD reduction potential does not shift significantly in the presence of fumarate; furthermore, the data, which now correspond to the FAD "trapped" in the ES complex, lie *within* the plot for enzyme alone and show that the interfacial or intramolecular electron-transfer rates are measurably higher (550 s^{-1}) in the enzyme−substrate complex.

The observation of signals from an active site at such high scan rates is so far unprecedented for an enzyme on an electrode and raises the question of why flavocytochrome c_3 is so electroactive. We do not know how the enzyme molecules are oriented on the electrode or indeed how much lateral or rotational motion they retain. However, referring to Figure 1, the FAD is quite centrally buried, and there is only a low probability of its undergoing direct electron exchange with the electrode.[13] By contrast, the four heme groups provide a 40 Å long wire running close to the surface of the protein and down to the active site.[14] Aside from the obvious need for fast electron transfer, which is very likely to be achieved through this heme relay, it is also important for the catalytic center to possess two-electron cooperativity so that its signal is clearly observed and distinguished. Such criteria are likely to be fulfilled with many metalloflavoenzymes.

These experiments allow us easily to isolate and examine the various electron-transfer (ET) processes in the catalytic cycle. ET to and from the FAD cannot be rate-determining because turnover is outrun at scan rates lower than those at which the FAD component disappears. The fact that signals at high scan rate have the same shape and size irrespective of the presence of substrate shows also that two additional electrons do not flow in and out of the bound substrate as would be expected if product dissociation was rate-limiting. The observation that the FAD signal retains its essential symmetry and is unshifted in potential in the presence of fumarate shows that substrate binding is not delayed until the FAD is reduced. By elimination, the rate-determining step must lie within the stage in which reducing equivalents are transferred from reduced FAD to substrate.

This type of experiment suggests new opportunities for studying mechanisms of electron-transport enzymes, namely the ability to "trap" and examine *transient* species by electrochemical rather than spectroscopic methods. It thus becomes feasible to determine, directly, reaction coordinates for catalytic electron transport in these complex systems.

Acknowledgment. Anne K. Jones thanks the Rhodes Trust for a Scholarship and Raúl Camba is grateful to CONACYT (Mexico). This research was supported by the UK BBSRC (Grants: 43/B10492, 15/B07650, and 15/C12775).

Supporting Information Available: Additional figures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA000848N

(9) This attenuation is remarkably small, considering the very large dynamic range: many smaller proteins show greater decreases in normalized peak area as the scan rate is raised. See Hirst et al. (ref 6a).

(10) For an account of the use of rotating disc electrodes, see: *Instrumental Methods in Electrochemistry;* Southampton Electrochemistry Group: Ellis Horwood, 1993; Chapter 4.

(11) Britz, D. *Digital Simulation in Electrochemistry*, 2nd ed.; Springer-Verlag: Berlin, 1988.

(12) Observability of non-catalytic electron transfer is judged from the first appearance of local extrema in the voltammogram.

(13) Page, C. C.; Moser, C. C.; Chen. X. X.; Dutton, P. L. *Nature* **1999**, *402*, 47−52.

(14) Fcc_3 may therefore be considered as an example of an enzyme that is naturally "wired" to display electroactivity at an electrode. See: Heller, A. *Acc Chem. Res.* **1990**, *23*, 128−134.