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Direct Electrochemistry of Tetraheme Cytochrome c_{554} from *Nitrosomonas europaea*: Redox Cooperativity and Gating

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We have carried out protein film voltammetry (PFV) of the tetraheme cytochrome c_{554} (cyt c_{554}) from *Nitrosomonas europaea*, resolving all four of the heme potentials. Additionally, we find that cyt c_{554} is specifically tuned to engage in a two-electron reduction of the high potential hemes. The monomeric protein cyt c_{554} contains four *c*-type hemes (labeled **I** through **IV**, Figure 1) that are organized as two pairs of stacked hemes. Previous structural[1,2] and spectroscopic studies of cyt c_{554}[3−7] have established a considerable level of detail regarding the electronic and magnetic structure of the four heme groups of the protein, including an estimation of reduction potentials using redox potentiometry (+47, +47, −147, and −276 mV, as indicated in Figure 1).[4,7] Cyt c_{554} is the natural redox partner of hydroxylamine oxidoreductase (HAO), a homotrimeric enzyme containing eight heme groups per monomer, and which oxidizes hydroxylamine to nitrite, a four-electron oxidation.[4,8−10] In the course of HAO catalysis, cyt c_{554} is reduced by two electrons only,[5,11] so that the generation of a one- or three-electron reduced form of cyt c_{554} is prevented. To date, assessing the redox chemistry of cyt c_{554} has been challenging due to the complexity associated with the multiple heme cofactors. We have used PFV to probe the redox reactions of cyt c_{554} and have found evidence for previously unobserved cooperativity and redox directionality, in comparison to other studies of multiheme protein electrochemistry.[12,13]

We have conducted PFV of cyt c_{554} with multiple types of electrode materials and surface modifications (edge-plane pyrolytic graphite as well as gold modified with carboxylic acid-terminated alkanethiols).[14] Typical data are shown in Figure 2A, collected at a mercaptobenzoic acid (MBA)-modified polycrystalline gold electrode, at pH 7.0. Three reversible features are clear from the cyclic voltammogram itself: two at significantly lower potential (−182 and −283 mV), and an additional feature at higher potential (∼+40 mV), as predicted by the previous characterization of cyt c_{554}. The electrochemical response is highly reversible, the cyt c_{554} films are stable throughout hours of experiment, and the values of the peak current (i_p) are linearly dependent upon the scan rate (υ), as should be the case for an adsorbed electrochemical reaction.[15] For all of the signals, the observed values of E_m are essentially pH invariant: a modest pH dependence of −12 to −15 mV/pH unit is observed (Figure 2B), which is much less than the −59 mV/pH unit expected for a $1H^+$:1e′ process.

The electrochemical data allow us to probe the previously suggested degeneracy of the reduction potentials of **I** and **II**.[4,7] Figure 2A shows the deconvolution of the complex voltammogram into a series of voltammetric features, following the subtraction of the background current. By considering the electroactive surface coverage of each of the four redox cofactors to be identical,

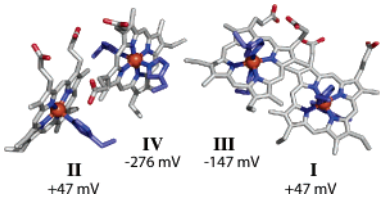

Figure 1. The four hemes of cyt c_{554}, with estimated potentials from ref 4, as assigned in ref 7.

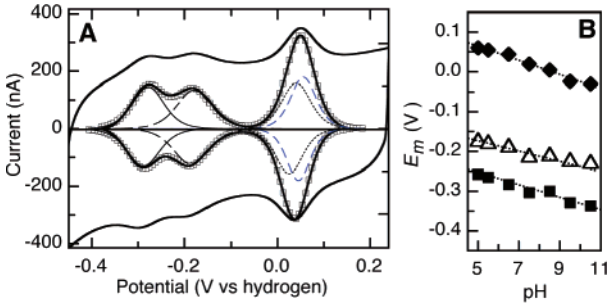

Figure 2. (A) Voltammetric response of cyt c_{554} at mercaptobenzoic acid-modified gold at pH 7.0. Baseline-subtracted data are the inset (solid line), along with the deconvolution of the data (dashed lines) and their sum (□). (B) The midpoint potentials observed for **I** and **II** (◆), **III** (△), and **IV** (■), as a function of pH, with dashed trend lines.

deconvolution of the voltammetric response was performed by a least-squares fit to the sum of four voltammetric shapes where the stoichiometric number of electrons was identical ($n = 1$) but the apparent number of electrons (n_{app}) was fit for each cofactor.[16] This procedure results in data shown in Table 1, indicating (1) the potentials of all four hemes are resolvable; (2) **I** and **II** display distinct, though similar, potentials; and (3) n_{app} corresponds to $1e^-$ for the low-potential hemes **III** and **IV**, yet $n_{app} > 1$ for both **I** and **II**. The pair of hemes do not display a perfect degree of cooperativity, in which case the two features would be best fit by a single redox couple, where $n_{app} = 2$. The breakdown of ideality may be caused by the dispersion of molecular conformations upon the electrode surface, leading to broadening of individual peaks, and artificially lowered values of n_{app}. Our direct electrochemical measurements also agree with recent potentiometry[4] and E_m calculations,[17] suggesting that the previous structure/potential assignments are correct.[4,7]

The source of the cooperativity is of great interest. It does not appear to be related to *inter*molecular interactions, as there is no dependence of the apparent n_{app} values as a function of surface coverage. Alternatively, cooperativity could be achieved by the relative kinetics of interfacial and *intra*molecular electron transfer events. Cyt c_{554} most likely binds to HAO via an acidic patch, orienting **I** to be closest to the enzyme.[1] We believe that the

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Table 1. Potentials of Cyt c_{554}, Determined and Calculated

	This Study		Potentiometry[4]	Calcd[17]
heme	E_m (mV)	n_{app}	E_m (mV)	E_m (mV)
I and **II**	+ 50, + 32	1.4, 1.2	+ 47, + 47	+ 75, + 63
III	−183	1.0	−147	−210
IV	−283	1.0	−276	−284

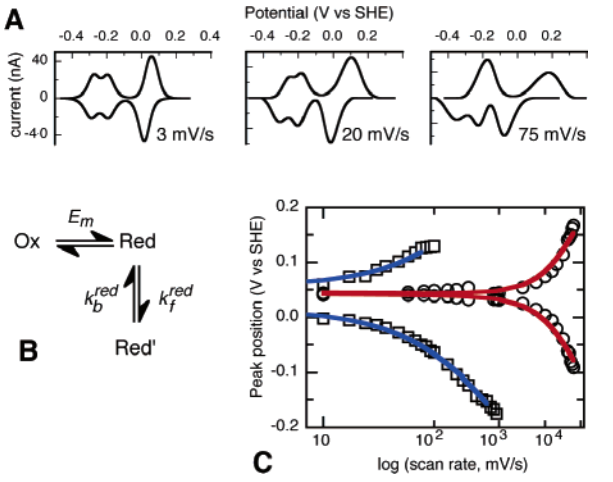

Figure 3. (A) Baseline-subtracted voltammograms for cyt c_{554} immobilized at a MUA-modified electrode, at scan rates of 3, 20, and 75 mV/s, indicative of a coupled reaction for **I** + **II** following reduction (panel B). (C) Trumpet plots generated for the peak positions of the couple associated with **I** + **II**, at either MUA- (squares) or MBA-based electrodes (circles).

ω-carboxylate-terminated electrodes mimic the HAO−cyt c_{554} orientation. Thus, if **II** is modestly higher in potential than **I**, thermodynamically favorable reduction of **II** combined with fast internal electron transfer (relative to slower intermolecular/interfacial ET) could yield the observed cooperativity. A similar kinetic model has been suggested to account for the spectroscopically detected reduction of **I** and **II** with catalytic amounts HAO and substrates, previously.[7] Our data indicate that **I** and **II** are designed to serve as a functional two-electron unit in HAO reactivity.

To examine the impact of interfacial electron transfer rate upon the observed cooperativity, we substituted the aromatic thiol-based electrode for the alkane mercaptoundecanoic acid (MUA), which resulted in electrodes yielding fundamentally slower electrokinetics. Importantly, the substitution lowers the interfacial electron transfer rate (k^0) to the time scale associated with HAO reduction of cyt c_{554}, ~25 s^{-1}.[18] By monitoring the position of the heme **III** cathodic and anodic peaks as a function of scan rate (υ) (Trumpet plot analysis[19]), we observed values of k^0 from 241 s^{-1} on MBA to 8.4 s^{-1} on MUA electrodes. Thus, on MUA, interfacial electron transfer roughly equates what might be found in vivo. In this slower regime of k^0, the reversibility of the electrochemical data dramatically changes. While the MUA-based results mimic those found with the faster MBA surface at slow scan rates (3 mV/s, Figure 3A, left), the voltammetric response for **I** + **II** distorts as the scan rate is increased (Figure 3A, middle and right). By increasing υ, the reductive peak shifts to a lower potential while the oxidative signal broadens, eventually becoming unobservable at scan rates >200 mV/s. To account for these data, a coupled reaction must follow the reduction of **I** + **II** (Figure 3B). On MUA, when the scan rate is slow (as compared to either k_f^{red} or k_b^{red}), the voltammetry of the high-potential hemes pair is reversible. As υ is increased, a slow back-reaction of Red′ → Red prevents the regeneration of the Ox form, the impact of which is clearly observed in Trumpet plots that compare the **I** + **II** feature as observed on MBA and MUA (Figure 3C). The molecular nature of Red′ can be rationalized as a distinct global cyt c_{554} conformation adopted at low potentials, which interferes with the reoxidation of the **I** + **II** heme pair. The possibility that Red′ represents a specific *intra*molecular gate cannot be excluded, though the effective gating does not appear to be directly coupled to proton transfer chemistry, as the same voltammetric behavior is observed at both acidic (4.5) and basic (9.5) pH values.

Importantly, the observation of cooperativity and redox gating occurs at time scales that are similar to the turnover of cyt c_{554} by its redox partner HAO,[18] suggesting an unforeseen contribution to catalysis. In such a scenario, the reduction of the cyt c_{554} **I** + **II** heme pair operates in a facile $2e^-$ manner, as compared to the backward reoxidation when electron transfer from HAO is rate-limiting. Thus, the system is poised to prevent the generation of odd-electron species in HAO, by unidirectional reduction of cyt c_{554} by two electrons.

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Supporting Information Available: Alternative fits to the deconvoluted voltammograms. This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) PFV was achieved using a PGSTAT 12 electrochemical analyzer (Ecochemie), with experiments conducted in a thermostated glass electrochemical cell fit with a calomel working electrode (Accumet) and a platinum wire counter electrode. Protein films were prepared by taking either freshly polished EPG electrodes or alkanethiol-modified polycrystalline gold electrodes and then soaking either electrode in a dilute (~10 μM) protein solution of cyt c_{554} (prepared as described in ref 4) in pH 7.0 buffer composed of 20 mM MOPS. Electroactive protein films could be achieved by soaking for a few hours, but the best results were attained at 4 °C, overnight. Once prepared, electrodes were then used directly by rinsing the excess protein solution from the electrode surface and then placing the electrode tip in the prechilled electrochemical cell.
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