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Reduction of Dioxygen Catalyzed by Pyrene-Wired Heme Domain Cytochrome P450 BM3 Electrodes

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Cytochromes P450 (P450s) catalyze oxygenations of inert substrates under physiological conditions.[1] Exploiting this activity in vitro would be greatly facilitated if reductants other than NADPH could be found. While an electrode is perhaps the most attractive source of electrons, direct electrochemistry of P450 has been elusive, owing to poor electronic coupling to the deeply buried heme and inactivation through surface adsorption. Investigations of electrochemical reduction of P450 have led to clever techniques for effecting electron transfer (ET). These methods include confining the protein within surfactant[2,3] or polyelectrolyte[4,5] films, modifying the electrode surface covalently (mercaptans on gold[6]) or through adsorption (clay on carbon[7]) and modifying the enzyme with molecular electronic relays.[8]

We are working on electrochemical methods for reduction of the fatty acid hydroxylase flavocytochrome P450 from *Bacillus megaterium* (BM3). BM3's high turnover rates and broad substrate specificity have stimulated interest in developing a bioelectronic system for this enzyme, including studies of both fundamental ET and biocatalysis.[9,10] Notably, Sevrioukova et al. achieved rapid heme reduction photochemically (2.5×10^6 s^{-1} and 4.6×10^5 s^{-1} with and without substrate) by covalently tethering a ruthenium diimine to an engineered cysteine (N387C) on the heme domain of BM3 (hBM3).[11] The positioning of the Ru complex was meant to mimic the interaction between hBM3 and its reductase: indeed, the rapid rates suggest that the complex was attached at a position that was well coupled to the heme. It occurred to us that "wiring" the N387C hBM3 mutant to an electrode through the engineered cysteine could also yield high electron tunneling rates. Previously, Katz utilized *N*-(1-pyrene)iodoacetamide (Py) (thiol specific) to anchor and electronically connect a photosynthetic reaction center to a basal plane graphite (BPG) electrode.[12] Thus, we made the hBM3 single surface cysteine mutant at position 387, attached Py to the cysteine, and successfully achieved rapid ET with the use of a BPG electrode. Wiring the enzyme in this way creates a system where the electrode mimics the reductase, leaving the active site accessible to molecules in solution.

Protein integrity after labeling with Py was confirmed by observing the Soret band of the reduced heme at 448 nm in CO-saturated buffer. Labeled protein (Py-hBM3) was verified by observing fluorescence of Py (~50% labeled). The protein film was prepared by suspending a BPG electrode in a ~20 μM Py-hBM3 solution. Cyclic voltammetry on the resulting film (Figure 1) revealed a couple centered at −340 mV. Neither unmodified enzyme nor Py alone produced a similar couple. The observed $E_{1/2}$ is assigned to the $Fe^{III/II}$ redox couple of the heme.[5,7] Compared to the native enzyme in its resting state (six-coordinate heme, low spin) as measured by redox titration, this potential is shifted approximately +230 mV.[9] As previously suggested, local electrostatic effects (e.g., solvation, surface interactions) likely contribute to the altered potential.[5]

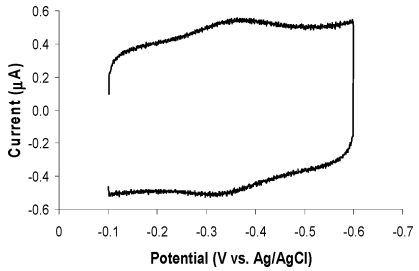

Figure 1. Cyclic voltammogram of the Py-hBM3 conjugate on BPG (0.07 cm^2) at 200 mV/s in 50 mM KP_i/20 mM KCl/pH 7.

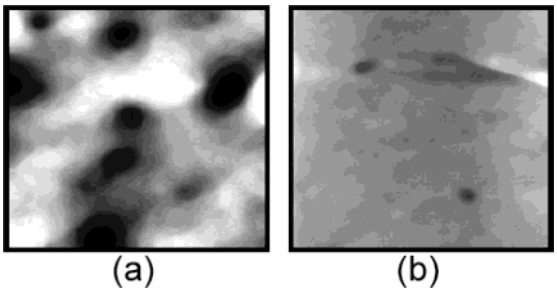

Figure 2. AFM images (800 nm × 800 nm) of HOPG soaked in (a) Py-hBM3 and (b) hBM3.

The cathodic to anodic peak-current ratio in Figure 1 is approximately 1.05, indicating a chemically reversible system.[13] A plot of the cathodic peak current versus the scan rate is linear, characteristic of a surface-confined species.[13] This plot also indicates the number of electrons transferred: the slope of the line divided by the area under the voltammogram at any sweep rate (39 nC) is equal to nF/4RT.[14] Performing this operation yields $n = 1.2 \pm 0.1$, fully consistent with one-electron transfer.

Voltammetry in CO-saturated buffer shifted $E_{1/2}$ by +35 mV, as found for other P450 electrochemical systems (+45 to +80 mV).[2,3,7] The $E_{1/2}$ was also found to vary linearly with pH according to $E_{1/2} = 56$ mV − 58 mV/pH, indicating proton-coupled electron transfer.[4,15]

To characterize the surface, protein films were cast onto highly oriented pyrolytic graphite (HOPG) and imaged using atomic force microscopy (AFM) in tapping mode. Figure 2a shows a section of HOPG (800 nm × 800 nm) soaked in a Py-hBM3 solution, revealing a series of small islands (dark spots) ranging from 2 to 5 nm in height. Given that hBM3 is ~65 Å in diameter, it can be inferred that these islands represent protein clusters on the surface. Figure 2b shows the corresponding image of HOPG soaked in unlabeled hBM3. Clearly, no surface features are visible; this image is identical with HOPG soaked in buffer alone and implies that only the Py-hBM3 conjugate adsorbs to the surface. Regarding

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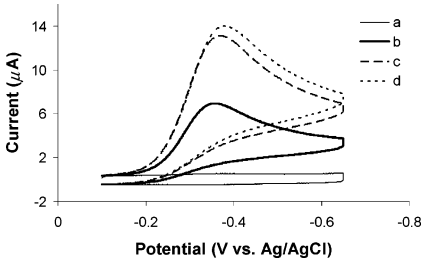

Figure 3. Cyclic voltammograms at 200 mV/s of Py-hBM3 on BPG in the presence of increasing amounts of dioxygen: (a) 0, (b) 42, (c) 71, and (d) 94 μM.

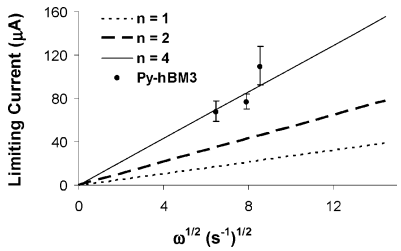

Figure 4. Solid lines represent Levich plots derived for the one-, two-, and four-electron reduction of dioxygen. The points represent the limiting current at 400, 600, and 700 rpm for Py-hBM3 films on BPG-RDE in the presence of dioxygen (250 μM).

surface coverage, Figure 2a suggests that there is submonolayer coverage. Cyclic voltammetry experiments on HOPG (0.25 cm^2) with a Py-hBM3 film (hBM3 monolayer $= 1.4 \times 10^{-12}$ mol) confirm this finding: integrating under the cathodic peak yielded 6.2×10^{-13} mol of electroactive protein, or ~44% surface coverage.

The standard rate constant ($k°$, $\Delta G° = 0$) for the BPG-Py-hBM3 system was evaluated using Laviron's theory,[16] yielding a value of 650 ± 50 s^{-1}, which is the fastest electrode kinetics reported for any P450 system (cf. 221 s^{-1} for hBM3 in DDAB films).[2] ET rates for photochemical reduction of the Fe^{III} heme[11] were used to estimate rates at zero driving force ($\Delta G° = 0$) for substrate-free and substrate-bound hBM3: these were found to be 280 and 3300 s^{-1}.[17] As can be seen, $k°$ from our electrochemical experiments falls within this range.

The BPG-Py-hBM3 system is an excellent catalyst for dioxygen reduction (Figure 3). Negative controls (Py or hBM3 on BPG only) reveal slow dioxygen reduction at more negative potentials. Possible fates of dioxygen are reduction to peroxide (directly or through superoxide decomposition) or to water. To determine the number of electrons transferred to dioxygen, Py-hBM3 films were cast onto a BPG rotated-disk electrode (RDE). Using the Levich equation for a RDE,[13]

$$i_L = 0.62nFAD_o^{2/3}\omega^{1/2}\nu^{-1/6}C$$

theoretical lines for the one-, two-, and four-electron reduction of dioxygen were generated (Figure 4).[18] RDE experiments were conducted by performing electrolysis at −0.5 V and determining the limiting current for each rotation rate. The results of these experiments (solid points, Figure 4) scatter around the theoretical line for $n = 4$, suggesting that the BPG-Py-hBM3 system converts dioxygen primarily to water. Our proposal of an efficient four-electron reduction pathway is further supported by results from an Amplex Red fluorescence assay for hydrogen peroxide, which revealed that only a small fraction of the current (<17%) was used to generate the two-electron reduction product. This is in stark contrast to other P450 electrochemical systems, where peroxide is the primary product of dioxygen reduction.[2,4] Conceivably, dioxygen reduction to water can occur if ET is fast enough such that, after initial reaction to form a peroxy complex, the final two electrons arrive at the active site before peroxide dissociation. Precedent for this can be found in previous work with ruthenium-modified cobalt porphyrins:[19] π-back-bonding by the ruthenium ligands increased the ET rate, creating a catalyst that reduced dioxygen primarily to water. For the BPG-Py-hBM3 system, the estimated $k°$ is so high that applying a potential of −0.5 V apparently leads to rapid reduction of dioxygen to water.

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Supporting Information Available: Details of electrode preparation, mutagenesis, protein purification, protein labeling, electron-transfer rate calculations, Amplex Red peroxide assay, voltammetry, and RDE methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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(17) See Supporting Information for details on the calculation.
(18) The variables for air saturated water are: limiting current (i_L, A), electrode area (A, cm^2), diffusion coefficient (D_o, 1.7×10^{-5} cm^2/s for O_2 in water), angular velocity (ω, s^{-1}), kinematic viscosity (v, 0.01 cm^2/s for water), bulk concentration (C, 2.5×10^{-7} mol/cm^3 for O_2 in air saturated buffer at 25 °C).
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