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 surfactant2-3 or polyelectrolyte4-5 films, modifying the electrode surface covalently (mercaptans on gold6) or through adsorption (clay on carbon7) 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 and biocatalysis.9,10 Notably, Sevrioukova et al. achieved rapid heme system for this enzyme, including studies of both fundamental ET specificity have stimulated interest in developing a bioelectronic the fatty acid hydroxylase flavocytochrome P450 from Bacillus.10218

is assigned to the Fe III/II redox couple of the heme. 5,7 Compared to enzyme nor Py alone produced a similar couple. The observed electronically connect a photosynthetic reaction center to a basal N also yield high electron tunneling rates. Previously, Katz utilized wiring the enzyme in this way creates a system where the electrode and successfully achieved rapid ET with the use of a BPG electrode. 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 surfactant2-3 or polyelectrolyte4-5 films, modifying the electrode surface covalently (mercaptans on gold6) or through adsorption (clay on carbon7) 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 x 10^6 s^-1 and 4.6 x 10^9 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}/Fe^{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.9

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 ± 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.5,15

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 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.
dioxygen were generated (Figure 4). RDE experiments were theoretical lines for the one-, two-, and four-electron reduction of dioxygen. Cyclic voltammetry experiments on HOPG (0.25 cm<sup>2</sup>) surface coverage. Figure 2a suggests that there is submonolayer coverage. Figure 4.

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).

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. Conceivably, dioxygen reduction to water can occur if ET is fast enough such that, after initial reaction to form a peroxo 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: nitro-reduction 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<sup>5</sup> is so high that applying a potential of −0.5 V apparently leads to rapid reduction of dioxygen to water.

Acknowledgment. We thank J. S. Magyar and J. R. Winkler (Caltech), T. L. Poulos (U.C. Irvine), and E. M. Spain (Occidental College) for helpful discussions; C. P. Collier (Caltech) for assistance with the AFM; NSF (H.B.G.), NSERC (Canada) (A.K.U.), and David and Lucile Packard Foundation (M.G.H.) for research support.

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.

References

17. See Supporting Information for details on the calculation.
18. The variables for air saturated water are: limiting current (i<sub>L</sub>), electrode area (A, cm<sup>2</sup>), diffusion coefficient (D<sub>0</sub>, 1.7 × 10<sup>−3</sup> cm<sup>2</sup>/s for O<sub>2</sub> in water), angular velocity (ω, s<sup>−1</sup>), kinematic viscosity (ν, 0.01 cm<sup>2</sup>/s for water), bulk concentration (C<sub>0</sub>, 2.5 × 10<sup>−3</sup> mol/cm<sup>3</sup> for O<sub>2</sub> in air saturated buffer at 25 °C).

JA0466560