Supporting Information

Protein Expression and Purification

iNOS heme domain was expressed and purified as previously described[1] with the following exceptions. Cells expressing protein were subjected to two rounds of chemical lysis. Cell pellets were resuspended in 40 mL of B-PER lysis buffer (Pierce) with 10 μg/mL benzamidine, 5 μg/mL leupeptin, 1 μg/mL each pepstatin, antipain, and chymotrypsin, ~500 μM Pefabloc (Roche), 100 μg/mL DNAse, 100 μg/mL RNAse, ~500 μg/mL lysozyme, and 20 mM imidazole per liter of cells and shaken for one hour at 4°C. The lysate was then spun down and the supernatant was loaded directly onto a nickel column (5 mL HisTrap, Amersham). The loaded column was washed with 20 column volumes of 20 mM imidazole in 50 mM NaP_i/300 mM NaCl/pH 8. The protein was eluted with 150 mM imidazole, concentrated to ~3 mL over an Amicon Ultra filtration device (10,000 MWCO, Millipore) and loaded onto a gel filtration column as previously described.[1] The anion exchange column was omitted when ≥ 95% purity was confirmed by UV-visible spectroscopy and gel electrophoresis.

Film Preparation and Voltammetry

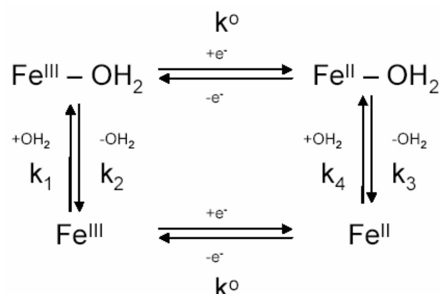
Electrodes for voltammetry (0.07 cm^2) were made using the basal plane of pyrolytic graphite. The surfaces were prepared by sanding briefly with 600-grid sandpaper, followed by polishing with 0.3 and 0.05 μm alumina slurries. The electrodes were then sonicated and dried in air. DDAB films were formed by placing 5 μL of 10 mM DDAB in water on the surface of the electrodes, followed by slow drying in air overnight. iNOS was incorporated into the film by soaking the DDAB-filmed electrode in a solution of enzyme (~ 20 μM in 50 mM KP_i pH 7 buffer) for 30 minutes, followed by gentle rinsing with ddH_2O. Films with cytochrome c were made using the same protocol.

A CH Instruments Electrochemical Workstation system was used for the reactions. Voltammetry experiments were performed in a 3-compartment cell, using a platinum wire auxiliary and a Ag/AgCl reference electrode (BAS). All experiments were performed under argon in thoroughly degassed buffer (50 mM KP_i, 50 mM KCl, pH 7) unless otherwise stated.

iNOS DDAB films for absorption spectroscopy were prepared on 3.5 x 40 mm glass slides. The slides were made hydrophobic by coating with (3-mercaptopropyl)trimethoxysilane using the following protocol. The glass slides were cleaned by boiling in piranha solution (4 : 1 H_2SO_4 : 50% H_2O_2) for 20 minutes followed by rinsing with ddH_2O. The following procedure was then performed three times: the slides were placed in a refluxing solution of 40 : 1 : 1 2-propanol : ddH_2O : (3-mercaptopropyl)trimethoxysilane for 10 minutes, washed with 2-propanol and then baked in a 100 – 107 °C oven for 10 minutes. iNOS DDAB films were cast onto the slides by placing 20 μL of 100 μM imidazole-bound iNOS and 50 μL of 10 mM DDAB in ddH_2O onto the glass slide, followed by drying in air overnight. Slides for negative controls were filmed with DDAB only. Absorption spectra were recorded with an HP spectrophotometer by placing the glass slides in a cuvette filled with 50 mM KP_i pH 7 buffer. The absorption spectrum shown below (Figure S4) is the difference spectrum of slides with and without iNOS.

Digital Simulations

Simulations were performed with software from the CH Instruments Electrochemical Workstation, which utilizes a fast finite difference algorithm. A square scheme was used for the model:



k^o and E^o for the 5-coordinate heme were experimentally determined to be 370 s^{-1} and -150 mV. For the 6-coordinate heme, k^o and E^o were estimated to be 10 s^{-1} (consistent with previous ET studies with NOS[2]) and -250 mV (based on $E_{p,c}(2)$). A surface-confined system was modeled with the following parameters: capacitance = 4 μF, surface coverage = $5x10^{-11}$ mol/cm^2, electrode area = 0.07 cm^2. Values for k_1, k_2, and k_3 were entered into the simulator, which calculated a corresponding value for k_4 and produced a simulated CV. Values for $k_3 < 100$ s^{-1} resulted in CVs that were irreversible for the five-coordinate heme at all scan rates, thus setting a lower limit on this value. Using this procedure, values for k_1, k_2, and k_3 of 1 s^{-1}, 0.5 s^{-1}, and > 100 s^{-1} were able to accurately model the voltammograms in Figure 2a (see Figure S3). The simulated CVs were found to be very sensitive to variations in k_1 and k_2: approximately, k_1 and k_2 values greater than 5-fold different from 1 and 0.5 s^{-1} resulted in CVs that did not reproduce the experimental data.

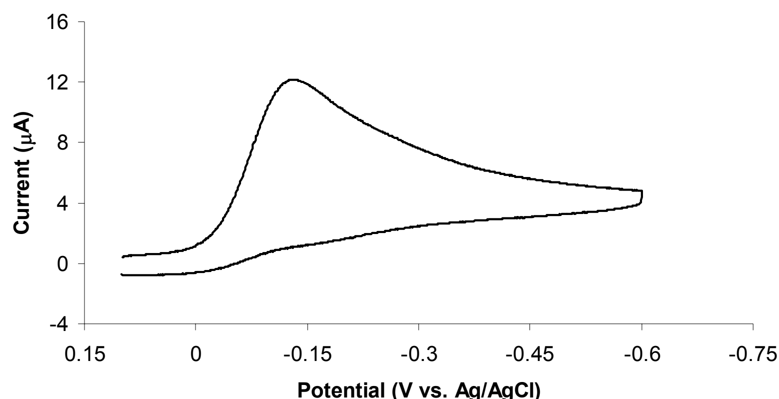


Figure S1. Cyclic voltammogram of iNOS in DDAB on BPG at 200 mV/s in 50 mM KP_i/50 mM KCl/pH 7 and 94 μM O_2.

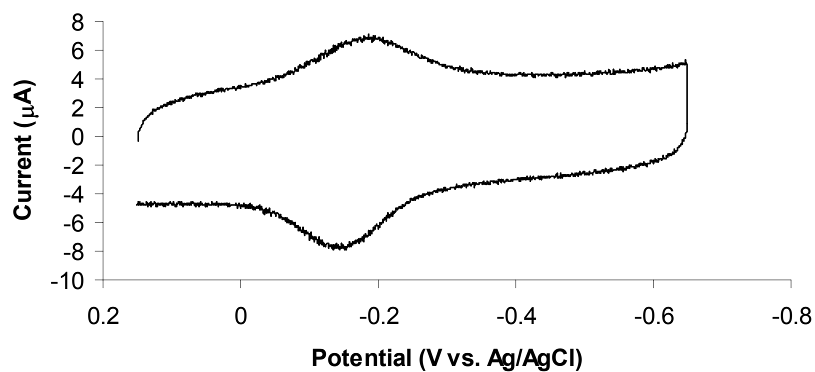


Figure S2. Cyclic voltammogram of iNOS in DDAB on BPG in 50 mM KP_i/50 mM KCl/pH 7 and 500 mM imidazole at 1 V/s. Note that only a single cathodic process is observed (cf. Figure 2a).

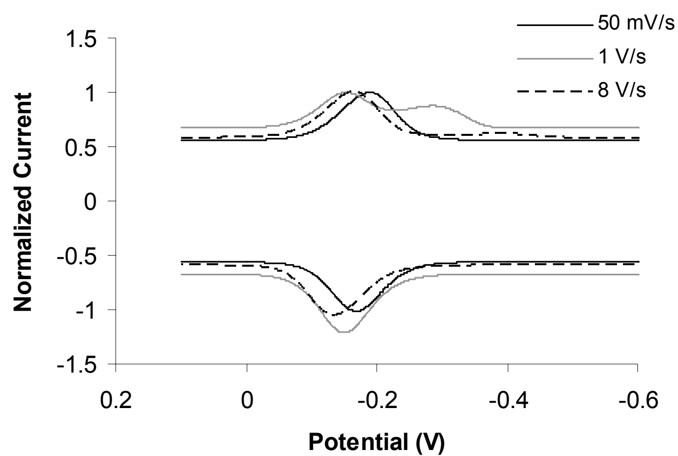


Figure S3. Digitally simulated voltammograms at a) 0.05 (black), b) 1 (gray), and c) 8 (dashed) V/s for iNOS in DDAB films. Simulation details are listed in the experimental section (*vide supra*).

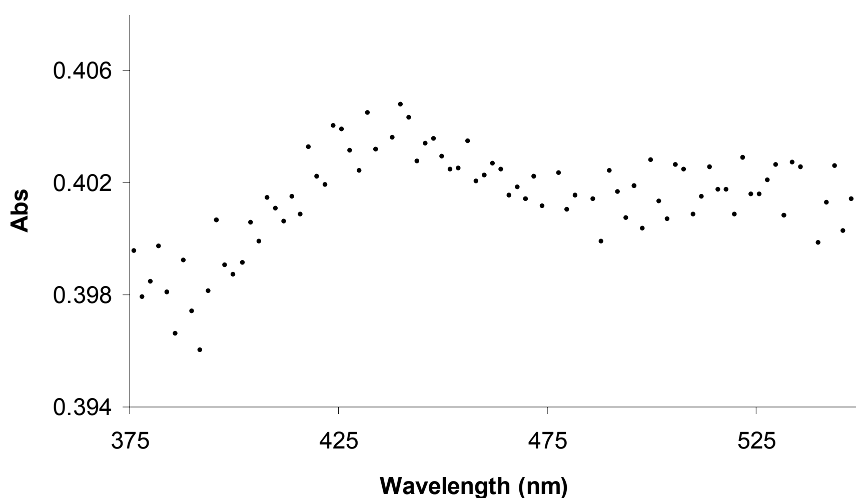


Figure S4. Absorption spectrum of an imidazole-bound iNOS DDAB film cast onto a glass slide. λ_{max} = 430 nm.

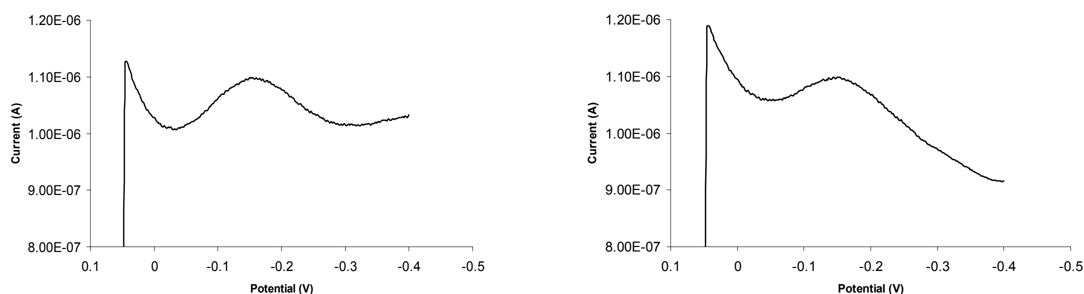


Figure S5. Square wave voltammograms of cytochrome c in DDAB films on graphite electrodes in the (a) absence and (b) presence of dioxygen. E_p = -155 mV vs. Ag/AgCl. Note that cytochrome c does not catalyze dioxygen reduction, in contrast to iNOS (Figure S1).

References

(1) Hurshman, A. R.; Marletta, M. A. *Biochemistry* **2002**, *41*, 3439-3456.

(2) Stuehr, D. J.; Santolini, J.; Wang, Z.; Wei, C.; Adak, S. *J. Biol. Chem.* **2004**, *279*, 36167-36170.