Efficient Bioelectronic Actuation of the Natural Catalytic Pathway of Human Metabolic Cytochrome P450s

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Cyt P450 catalytic cycle. The catalytic cycle of microsomal cyt P450s (Fig. S1) operates by a complex pathway.[1] Briefly, initial substrate (RH) binding to a hydrophobic pocket above the cyt P450-Fe^{III} heme is followed by a one electron reduction donated from NADPH via CPR resulting in the cyt P450-Fe^{II} heme. Dioxygen binding to this species followed by a second electron reduction by NADPH-CPR and protonation yields cyt P450-Fe^{III}-hydroperoxo complex **6**, Fig. S1. For some cyt P450s, cytochrome b_5 acts as the second electron donor and can stimulate the cyt P450 catalytic activity.[2] Protonation of complex **6** and the associated elimination of a water molecule produces active $^{+\bullet}$(P450-Fe^{IV}=O) **7**. This ferryloxy radical cation **7** is considered to be the active oxidant form of cyt P450s that transfers oxygen to the bound substrate (RH) forming the product (ROH). Reduced cyt P450-heme (**3)** upon complexing with CO (**9)** gives characteristic difference absorbance band near 450 nm (Fig. 2), or a positive shift in midpoint potential by voltammetry of about 50 mV from that observed in anaerobic buffer (Table 1).

In artificial systems, cyt P450-hydroperoxo complex **6** can be generated using peroxides without the need for NADPH, CPR, and molecular oxygen. In the case of bacterial cyt P450 101 or cyt $P450_{cam}$, the iron-sulfur protein (putidaredoxin) acts as the redox partner protein. Some mitochondrial cyt P450s rely on a non-heme iron sulfur redox protein as electron donor instead of CPR.[1]

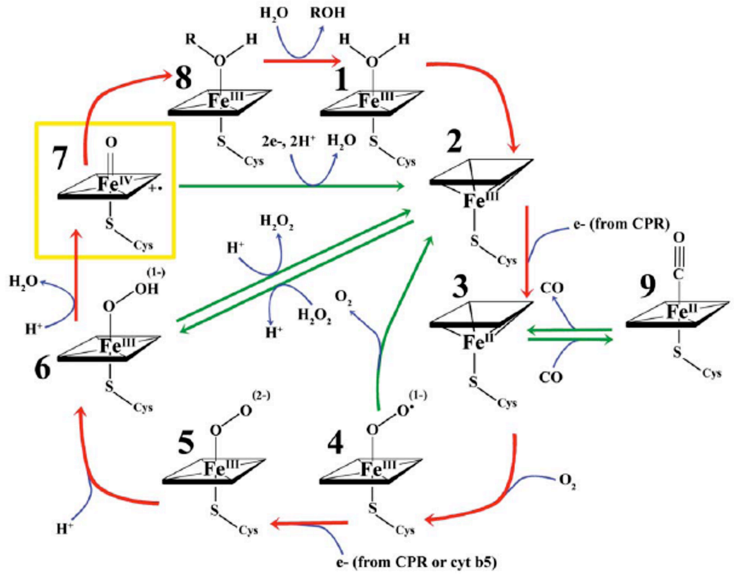

Figure S1. Proposed mechanism of cytochrome P450 catalytic cycle involving CPR as electron donor (or cyt b_5 in some cases as second electron donor) shown along with peroxide mediated catalytic pathway.[1,3]

The biocatalytic activity of cyt P450s depends strongly on formation of a transient complex with CPR to undergo reduction and subsequent steps in the catalytic cycle.[4,5] Several pioneering biochemical studies involving site directed mutagenesis to identify key amino acids/structural

elements of cyt P450 involved in the interaction with CPR, as well as the use of various reconstituted systems and different stoichiometric ratio of cyt P450 to CPR and lipid content on the resulting cyt P450-CPR interaction and the associated cyt P450 activity have been reported.[4,6-10] Researchers observed the existence of charge-pairing[11-13] as well as hydrophobic interactions between cyt P450 and its reductase.[14,15] On the other hand, in the case of cyt P450 2B4, charge neutralization was shown to favor complexation with CPR.[16] In summary, driving-force interactions between cyt P450s and CPR to form a transient complex prior to electron transfer are diverse and complex in nature and seem to vary significantly among different cyt P450s. Studies are still underway to understand interactions that allow limiting reductase present on membranes to reduce the large excess of cyt P450 in the catalytic process.[1,15,17]

Film Characterization by Quartz Crystal Microbalance (QCM). The frequency of a QCM resonator decreases in proportion to the mass on its gold coating.[18] Drying before measurement minimizes bias from interfacial viscoelasticity changes. *In-situ* QCM on MPA-coated gold/quartz resonators showed that adsorption saturation for protein and polyion layers occurs in 15-20 min. For 9 MHz quartz resonators, mass per unit area M/A (g cm^{-2}) of the film layer is related to the QCM frequency shift ΔF (Hz) by [19]

$$M/A = -\Delta F/(1.83 \times 10^{8}) \qquad \text{(S1)}$$

where A is the area of the gold disk on the quartz resonator in cm^2. Eq S2 gives the previously validated relation between ΔF and nominal film thickness (d) for proteins and polyions:[19]

$$d(nm) \approx -(0.016 \pm 0.002)\Delta F(Hz) \qquad \text{(S2)}$$

QCM frequency changes after each layer of the film is adsorbed and dried are illustrated for an LbL assembly cyt P450 1A2 and microsomes containing CPR and cyt b_5 (CPR+b_5) on a MPA-Au surface (Figure S2). Frequency of the dried film decreased linearly and reproducibly for the adsorption steps as expected for reproducible layer formation. Nominal thicknesses of the films were estimated from eq S2. Results of QCM experiments for all films are summarized in Table S1.

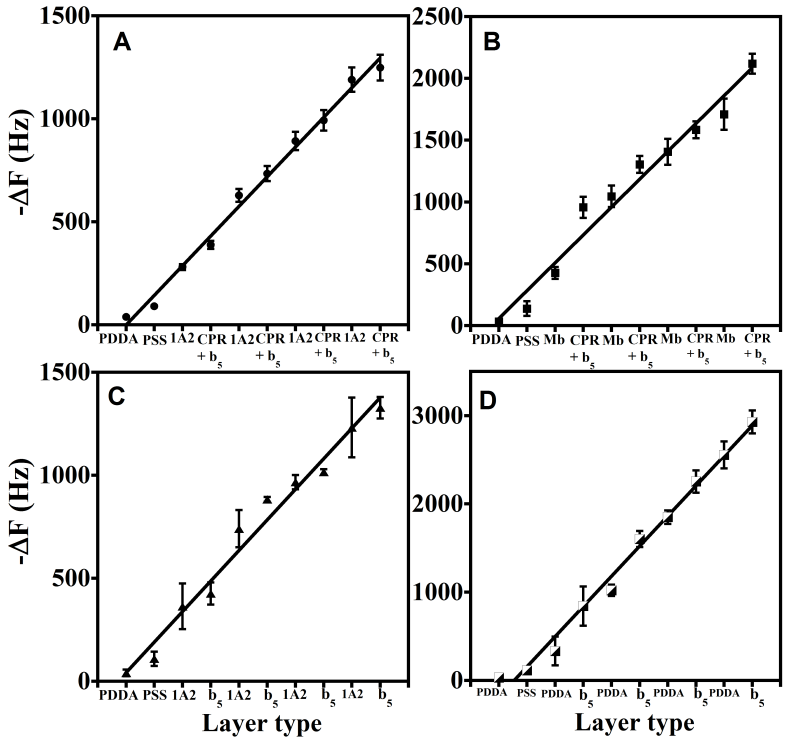

***Figure S2*. QCM of LbL films.** Frequency decreases at each fabrication step utilizing alternate LbL adsorption of purified enzymes and microsomes containing CPR and cyt b_5 (CPR+b_5) on gold resonators coated with a monolayer of 3-mercaptopropionic acid (MPA). QCM plots for LbL films of (A) P450 1A2 and CPR+b_5; (B) Mb and CPR+b_5; (C) P450 1A2 and cyt b_5 ; and (D) PDDA and cyt b_5. Each film has PDDA/PSS as first two layers and additional layers as labeled on x-axis. Resonators were rinsed in water between adsorption steps, and then dried in nitrogen before measuring frequency changes.

Table S1. Average characteristics of enzyme films from QCM.

Assembly	Cyt P450 (pmol)	Cyt b_5 (pmol)	Mb (pmol)	CPR (pmol)	Nominal Thickness (nm)
PDDA/PSS/(1A2/CPR+b_5)$_6$	19 ± 2	1.5 ± 0.2		0.2 ± 0.03	29 ± 3
PDDA/PSS/(2E1/CPR+b_5)$_6$	20 ± 3	1.6 ± 0.3		0.22± 0.03	30 ± 4
PDDA/PSS/(2E1/msCPR)$_6$	42 ± 5			0.9 ± 0.1	67 ± 6
PDDA/PSS/(Mb/CPR+b_5)$_6$		5.7 ± 1	59 ± 9	0.6 ± 0.1	45 ± 4
PDDA/PSS/(1A2/b_5)$_6$	30 ± 4	41 ± 5			34 ± 2
PDDA/PSS/(PDDA/b_5)$_6$		287 ± 30			73 ± 7

Measurement of heterogeneous electron transfer rate constants (k_s) from CVs. For the estimation of electrochemical rate constant k_s (Table 1), we used CVs of enzyme and/or CPR LbL films with increasing scan rates in the range 0.005 to 1.2 V s^{-1} (Fig. 3A) where good quality data were obtained. At scan rates above 1.2 V s^{-1}, domination of charging current and peak broadening interfered with the accurate peak potential measurements. As per the suggestion of Hirst and Armstrong,[20] we first subtracted the constant non-kinetic oxidation-reduction peak separation ($\Delta E_{p,corr}$) at lower scan rates from that at higher scan rates and fit the corrected peak separations to Butler-Volmer theoretical peak separation vs. scan rate theory for a confined surface electrochemical layer (Fig. S3) for surface confined voltammetry.[21] Average standard hET rate constants (k_s) were estimated using these corrected ΔE_p following Laviron's method.[21] Good fits of theory to experiment (Fig. S3) indicated that reliable estimates of k_s for the enzyme and CPR films used in this study were obtained within the confines of the Laviron method and simple Butler-Volmer theory.

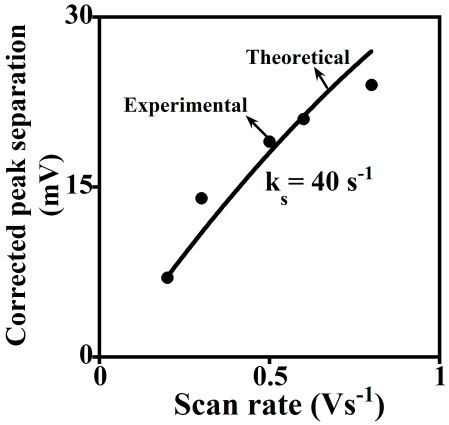

Figure S3. **Agreement of experimental vs. theoretical peak separation with scan rates for cyt P450/CPR LbL films for k_s = 40 s^{-1}.** Influence of cyclic voltammetry scan rates for PDDA/PSS (/cyt P450 1A2/CPR+b_5)$_6$ films on experimental (●) peak separation (ΔE_p) corrected for scan rate independent non-kinetic contribution. The theoretical lines were computed for Butler-Volmer theory for the rate constant (k_s) value shown.[21]

E_rCE_o-simulations. Digital simulations of the CVs provided a much more detailed analysis of the mechanistic pathway than is possible with simple Butler-Volmer theory, which only considers electrochemical electron transfer steps. Simulations employed the CH Instruments surface

voltammetry software package, and we added the constant non-kinetic oxidation-reduction peak separation ($\Delta E_{p,corr}$) described above to all simulations, as this factor is not explicitly considered by the simulation package. Initial simulations of CVs using single (E) or tandem electron transfer (EE) models, with the first E for cyt P450 1A2 and the second E for CPR, gave very poor fits to the experimental data. The simulations of CVs using the E_rCE_o model (eqs 1-3) allowed us to consider the influence of the known redox equilibrium between cyt P450 and CPR (eq 2) on the CV data. The best fit parameters are presented in the paper in Scheme 1, and here we briefly summarize results for some parameter choices that did not fit the data. Ratios of k_f to k_b had a critical influence on peak shape and evolution of CVs with increasing scan rates. Using $k_f=k_b$ for the chemical step (eq 2) yielded a decreasing reduction-oxidation peak separation with increasing scan rates that clearly did not fit experimental results. Simulations with $k_f > k_b$ where the forward rate is greater than the reverse rate (eq 2) yielded catalytic CVs with larger reduction peaks than oxidation peaks, again inconsistent with the experimental CVs (Fig. 3A). Only when combining $k_b \geq 5k_f$ using the individual electrochemical rate constant parameters in eqs 1-3, did the simulations reproduce experimental peak shapes for cyt P450 1A2/CPR+b_5 (Fig. 3A,C) and cyt P450 2E1/CPR+b_5 (Fig. S4 A) films, and provide good fits to the experimental formal potentials in the trumpet plots (Figs. 3D & S4 B).

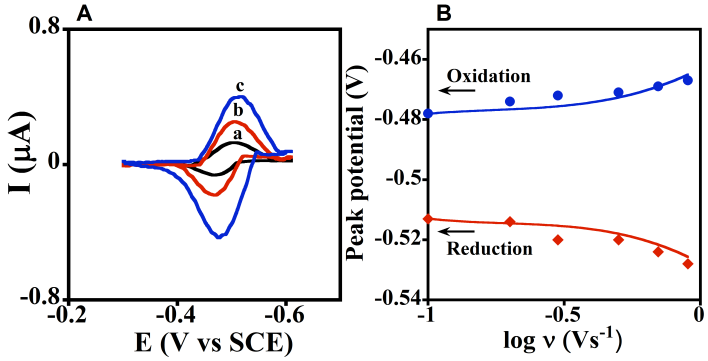

Figure S4. **Experimental CVs and trumpet plots with simulation for cyt P450 2E1/CPR films. A.** Experimental background-subtracted CVs: **a-c,** PDDA/PSS (/cyt P450 2E1/CPR+b_5)$_6$ films at scan rates (b) 0.1, (c) 0.2, and (d) 0.3 Vs^{-1}. **B.** Influence of scan rate on oxidation (blue circles) and reduction (red diamonds) peak potentials for PDDA/PSS(/cyt P450 2E1/CPR+b_5)$_6$ films plotted with the peak potentials (lines) simulated using the E_rCE_o model in Eqs 1-3.

Table S2. Electrochemical parameters of control myoglobin films with reductase in anaerobic 50 mM phosphate buffer plus 0.1 M NaCl, pH 7.0.

Film Assembly	$E^{o'}$ vs. NHE with N_2 (mV)	+ $E^{o'}$ shift with CO (mV)[a]	k_s (s^{-1}) [b]
PEI(/PSS/Mb)$_4$[c]	-100 ± 5	49 ± 4	26 ± 5
PDDA/PSS(/Mb/CPR+b_5)$_6$	-140 ± 6	49 ± 5	37 ± 5

a. av ± SD for n = 3 electrodes.
b. k_s was estimated using a thin-film Butler-Volmer model.[21]
c. From ref. 22. Where PEI = poly(ethyleneimine).

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