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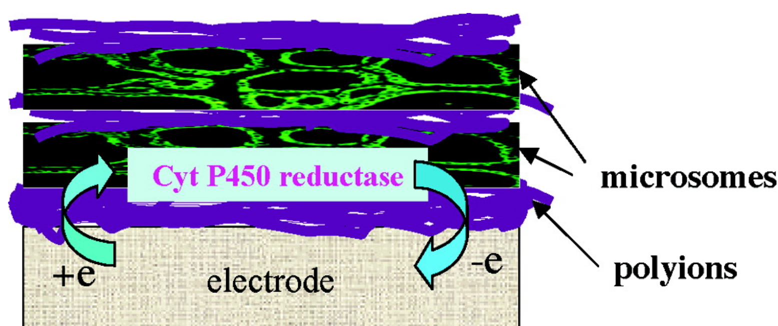

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Protein Film Electrochemistry of Microsomes Genetically Enriched in Human Cytochrome P450 Monooxygenases

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Monooxygenases metabolize lipophilic substrates in the liver via vectorial electron transfer from NADPH to reductase to iron heme enzyme cytochrome (cyt) P450 in a complex pathway involving reaction of reduced cyt P450 with dioxygen and subsequent conversion to an active oxidant.[1] This metabolic pathway can activate pollutants and drugs to genotoxic substances that damage DNA.[2,3] Cyt P450s are uniquely regio- and stereospecific and have also generated considerable interest as synthetic catalysts.[4−7]

Electrons can be delivered to dissolved cyt $P450_{cam}$ by the electrochemically reduced redox partner, putidaredoxin[5] or ferredoxin.[6] Direct voltammetry of purified cyt $P450_{cam}$ in solution has been achieved.[8] Reversible voltammetry was reported for cyt P450s in lipid,[9] clay,[10] and polyion films.[11] Cyt P450 in films was activated for catalysis by electrochemically generated H_2O_2;[11] this differs from the natural pathway, but gave identical products.

In a 1977 report, catalytically active rabbit liver microsome dispersions gave no response for cyt P450 at mercury electrodes.[12] More recently, genetically engineered bacteria containing cyt P450s were developed for biocatalysis.[13] Microsomes genetically enriched in human cyt P450 (CYP) monooxygenases are now commercially available and widely used to study drug metabolism.[14] We felt that these microsomes containing lipid, cyt P450 enzyme, and cytochrome P450 reductase might be electrochemically activated along the same pathway utilized in human liver. Herein, we demonstrate direct voltammetry of cyt P450 reductases in stable polyion films of genetically enriched CYP1A2 and CYP3A4 microsomes. Electrochemically driven substrate oxidation was supported by these microsome films.

The microsomes (Gentest, Woburn, MA) are expressed from human cDNA using a baculovirus−insect system. We assembled films of microsomes on 9 mHz gold quartz crystal microbalance (QCM) resonators and on rough pyrolytic graphite (PG, $A = 0.16$ cm^2) electrodes using alternate layer-by-layer adsorption.[11] Microsome (ms) dispersions with ∼4 mg mL^{-1} protein, sodium poly(styrene sulfonate) (PSS, MW 70 000, 3 mg mL^{-1} + 0.5 M NaCl), and polyethylenimine (PEI, avg. MW 25 000, 2 mg mL^{-1}) were used. Initial layers of PEI/PSS/PEI were constructed on the surfaces, followed by alternate adsorption of microsomes and PEI to make six bilayer films, denoted as $[CYP1A2ms/PEI]_6$ and $[CYP3A4ms/PEI]_6$. Negatively charged microsomes in 0.1 M phosphate buffer, pH 7.4, were adsorbed at 4 °C. Adsorption times for microsomes and rabbit cyt P450 reductase (18.8 nmol mL^{-1}) that gave reproducible voltammetry on PG were 1 h, and 20 min for polyions.[11] Films were washed with water between steps. QCM (Figure S1, Supporting Information) showed decreases in resonator frequency at each step consistent with reproducible layer growth. QCM revealed that $[CYP1A2ms/PEI]_6$ films contained 25 $\mu g\ cm^{-2}$ microsomes and had 100 nm nominal thickness and a bilayer thickness of 15 ± 4 nm. $[CYP3A4ms/PEI]_6$ films contained 18 $\mu g\ cm^{-2}$ microsomes and were nominally 87 nm thick with a bilayer thickness of 13 ± 5 nm.

Polyion−protein films assembled layer-by-layer feature extensive interlayer mixing that facilitates in-film electron transfer.[11] $[CYP1A2ms/PEI]_6$ and $[CYP3A4ms/PEI]_6$ films on PG gave reversible reduction−oxidation peaks at a midpoint potential −0.49 V vs SCE at 0.1 V s^{-1} (Figure 1a,c). Films of rabbit cyt P450 reductase gave reversible CVs (Figure 1b) with peak potentials nearly the same as that of the microsomes. Control microsomes with no cyt P450 and smaller amounts of reductase gave smaller CV peaks (Figure 1d). Integrations of CVs revealed amounts of electroactive protein in nmol cm^{-2}: 0.087 for CYP1A2ms, 0.025 for CYP3A4ms, 0.008 for control ms, and 0.07 for rabbit reductase. Values are roughly consistent with amounts of reductase in microsomes (Gentest, as pmol/mg total protein: CYP1A2 = 90; CYP3A4 = 20; control ms = 0.5)

Peak current versus scan rate for microsome films was linear (10−1000 mV s^{-1}). Reduction peak potentials shifted negative in the higher scan rate range, and oxidation−reduction peak ratios were ∼1. Data are consistent with quasireversible thin film voltammetry.

Upon reduction with CO present, heme proteins form Fe^{II}−CO complexes that shift midpoint potentials ca. 50−100 mV via the influence of complexation following electron transfer.[9a] Cyt P450 reductases have flavin cofactors[1] and are not influenced by CO. When CO was added to buffers, microsome peaks did not shift potentials (Figure 2). Even after 18 h, small changes in reverse CVs were found, but no peak shifts.

CYP1A2ms and CYP3A4ms CVs had midpoint potentials of −0.49 V vs SCE at 0.1 V s^{-1}. In polyion films, the midpoint potential for pure cyt P450 1A2[11e] is −0.31 V at pH 7.0 and for cyt P450 3A4 is −0.32 V at pH 7.4.[11e] These comparisons, correspondence of microsome and rabbit reductase potentials (Figure 1), and lack of CO peak shifts (Figure 2) suggest that microsome CVs can be assigned to *cyt P450 reductases*. Additional CVs showed that the microsome films did not electrocatalytically reduce O_2 or H_2O_2 (Figures S1 and S2, Supporting Information), which are characteristic reactions of cyt P450 enzymes.[9,11] Thus, all evidence points to cyt P450 reductases rather than cyt P450 enzymes as the initial electron acceptors in CV.

If electrons enter films via the reductase, oxidations might be driven electrochemically by the pathway utilized in human liver.[1] Subsequent reductase-to-cyt P450 electron transfer would have to be slow, as it was not observed by voltammetry. To test this hypothesis, electrolyses were done at −0.6 V, where the reductase is reduced (cf. Figure 1). Electrolyses of styrene using microsome films produced styrene oxide (Table 1). Control microsomes formed

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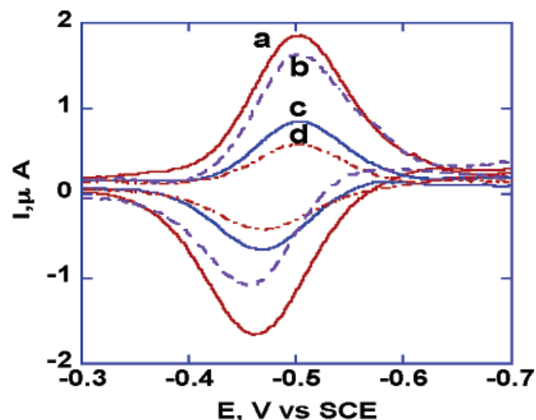

Figure 1. Background subtracted cyclic voltammograms (CV) of films on PG electrodes at 0.1 V s^{-1} in 0.1 M phosphate buffer pH 7.4 + 0.1 M KCl at 4 °C: (a) $[CYP1A2ms\text{-}PEI]_6$; (b) [rabbit cyt P450 reductase-$PEI]_6$; (c) $[CYP3A4ms\text{-}PEI]_6$; and (d) [control ms-$PEI]_6$.

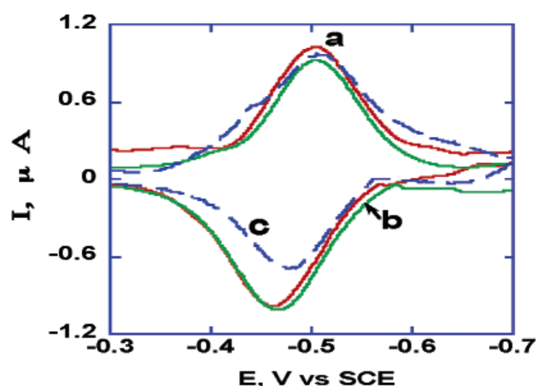

Figure 2. Background subtracted CVs of films on PG electrodes at 0.1 V s^{-1} at pH 7.4 for [CYP1A2 ms-PEI] $_6$ film at 4 °C: (a) after purging with nitrogen; (b) after bubbling CO for 10 min; and (c) after 18 h in buffer saturated with CO.

Table 1. Catalytic Oxidation of Styrene at 4 °C for 4 h at −0.6 V vs SCE[a]

entry	film	styrene[b]oxide found (nmol)	H_2O_2 found (mM)
1	$[CYP1A2ms/PEI]_6$	0.12 ± 0.03	0.3
2	$[CYP1A2ms/PEI]_6$ + 1 mg of catalase	0.17 ± 0.04	0.0
3	$[CYP3A4ms/PEI]_6$	0.39 ± 0.04	0.3
4	[control ms-$PEI]]_6$	0.02 ± 0.04	0.9
5	$(PEI/PSS)_3$ control	0.03 ± 0.01	2.9

[a] With 1.5 mL of pH 7.4 buffer (0.1 M phosphate + 0.1 M KCl) saturated with styrene at 4 °C using 4 PG electrodes, total $A = 0.64$ cm^2, avg. of three electrolyses. Oxygen bubbled for initial 20 min, then oxygen environment maintained. Gas chromatography was used for product analysis.[11] Hydrogen peroxide concentration estimated (±10%) by Quantofix Peroxide 100 test sticks (Macherey-Nagel GmbH). [b] Corrected for traces of styrene oxide in styrene reactant.

negligible styrene oxide. Entries 1 and 3 show that more styrene oxide was formed by CYP3A4 than by CYP1A2, consistent with 2-fold more cyt P450 enzyme in CYP3A4 microsomes (Gentest). Inclusion of catalase destroyed peroxide as expected (entry 2), but styrene oxide yield was similar to entry 1. Results are consistent with the natural pathway, but further work is needed for definite confirmation.

Electrolyses using active microsome films produced 0.3 mM H_2O_2 after 4 h, with controls giving larger amounts (Table 1). CVs suggested (Figure S3) that a small amount of oxygen reduction to H_2O_2 accompanies reduction of cyt P450 reductase in the films. Reduction of oxygen (−0.8 V peak) competes poorly with reduction of reductase, so less H_2O_2 is formed with reductase present (Table 1). H_2O_2 does not drive the cyt P450-catalyzed oxidation since destruction with catalase did not decrease product yield.

In summary, voltammetry of films containing genetically enriched monooxygenase microsomes delivers electrons to cyt P450 reductase. Microsome films can be activated electrochemically for substrate oxidation. Monooxygenase microsome films on electrodes should be useful for biocatalytic, metabolic, and toxicity studies.

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Supporting Information Available: Three additional figures presenting QCM results, and effects of peroxide and oxygen on CV. This material is available free of charge via the Internet at http://pubs.acs.org.

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