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Direct Electrochemistry of Immobilized Human Cytochrome P450 2E1

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Cytochrome P450 2E1 from human liver is a member of the super family of heme-thiolate monooxygenases involved in the phase I metabolism of pharmaceuticals and other xenobiotics such as paracetamol, chlorzoxazone, tamoxifen, ethanol, and phenolic compounds.[1] The general reaction catalyzed utilizes molecular oxygen as the ultimate electron acceptor and includes the energetically demanding insertion of an O-atom into a nonactivated C−H bond.[2]

In this communication we report the first electrochemical study of the human P450 2E1 either absorbed or covalently linked to different electrode surfaces. No reports on the electrochemistry of human P450 of family 2 have been published to date. In general, direct electrochemistry of P450 enzymes on unmodified electrodes has proven very difficult due to the deeply buried heme cofactor and instability of the biological matrix upon interaction with the electrode surface. Most efforts have been focused on characterization of the electrochemistry of P450cam using either a bare edgeplane graphite electrode[3] or glassy-carbon electrodes modified with lipid, polyelectrolyte film, or sodium montmorillonite.[4]

More recently, direct electrochemistry and catalysis of P450 BM3,[5] human 1A2,[6] and 3A4[7] has been shown on polyelectrolyte-modified electrodes. Furthermore, Shumyantseva and collaborators showed that the immobilization of the semi-synthetic form of two rabbit flavocytochrome P450's, 2B4 and 1A2, improved the catalytic performance when immobilized, underlining the importance of the regulation of the electron transfer to the heme iron.[8]

Immobilization is an important aspect in the production of nanodevices, as sensitivity may strongly depend on both concentration at the surface and specific orientation. Control over these two requirements can be achieved in a number of ways, depending on the nature of the support, properties, and stability of the biomolecule. Nonspecific adsorption to a metal-oxide surface and carbon-electrode surface and/or inclusion in polyelectrolyte/conducting polymers lead to the formation of a randomly oriented layer. Differently, alkane-thiol or other thiol-terminated chains covalently bound to a noble-metal surface and functionalized at the other end with a group able to interact specifically with a unique group on the protein surface will generate an oriented, tightly packed monolayer covalently bound to the gold surface.[9]

The human liver P450 2E1 used in these studies was N-terminally modified and expressed in *Escherichia coli* and purified according to the literature.[10] The direct electrochemistry of P450 2E1 was investigated at 25 °C using cyclic voltammetry and chronoamperometry on glassy carbon (GC) and gold electrodes. (Electrochemical characterization was performed under strictly anaerobic conditions (<1.5 ppm oxygen) to prevent formation of the Fe^{II}−dioxygen complex of a second electron transfer. Catalysis experiments were perfomed in fully oxygenated buffer. All potentials indicated are vs NHE.)

The cyclic voltammogram (CV) of P450 2E1 adsorbed onto bare GC shows two waves with a midpoint potential of −90 mV (Figure 1A). The peak current is linear with the scan rate up to 1 V s^{-1}, indicating that the protein is adsorbed to the electrode surface. The coverage, calculated from surface charge transfer was 0.96×10^{13} molecules per cm^2, indicating monolayer coverage. The electron-transfer rate was calculated[11] from the peak position in the function of the scan rate, and it was found to be 5 s^{-1} (Table 1). This value is close to what has been previously observed for other P450's[12] but is not as high as that found for the highly efficient and coupled P450 BM3.[5] When a surfactant, like didodecylammonium bromide (DDAB), was used to help the interaction with the GC electrode, the CV showed two waves with a midpoint potential of −85 mV and two additional waves with lower intensity centered at about +60 mV (Figure 1B). The peak current is linear with the scan rate up to 1 V s^{-1} for the first redox couple, while the second tends to disappear at scan rates higher than 100 mV s^{-1}. The quality of the signal prevented an accurate measurement of the k_{ET}. Similarly, when the polymer poly(diallyldimethylammonium chloride) (PDDA) was used, the CV showed two waves with a midpoint potential of −85 mV and two additional waves with lower intensity centered at about +60 mV (Figure 1C). The peak current is linear with the scan rate up to 30 Vs^{-1} for the first redox couple, while the second tends to disappear at scan rates higher than 1 V s^{-1}. The coverage is similar to that observed with the bare GC. The electron-transfer rate was found to be 2 and 1 s^{-1} for the first and second redox couple, respectively.

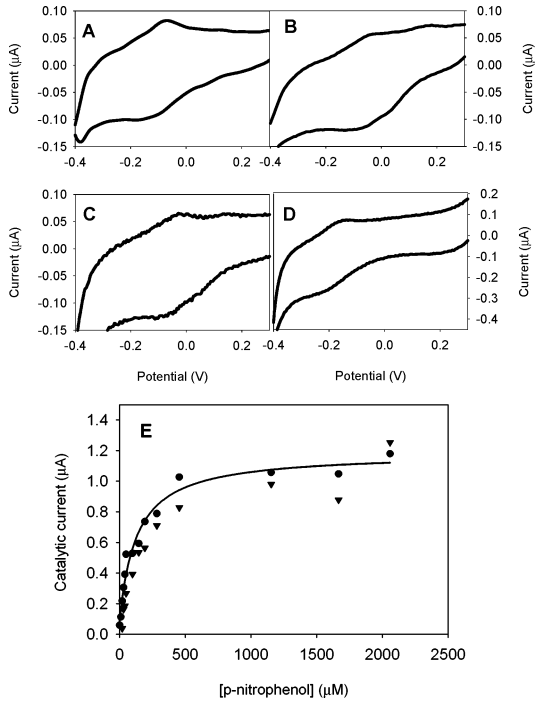

Figure 1. Cyclic voltammograms of P450 2E1 on different electrode surfaces. (A) bare glassy carbon; (B) GC/DDAB; (C) GC/PDDA; (D) Au/CYSAM/MALIM. Scan rate 50 mV/s in 500 mM potassium phosphate pH 7.0. (E) *p*-Nitrophenol titration of Au/CYSAM/MALIM/P450 2E1. Circles and triangles: *p*-nitrophenol in 500 mM potassium phosphate pH 7.0 for two different electrodes modified with Au/CYSAM/MALIM/P450 2E1.

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Table 1. Redox and Catalytic Parameters for P450 2E1 Immobilized on Different Electrode Surfaces

surface	E_m (mV)	k_s(s^{-1})	[*p*-nitrocatechol] (μM)
bare GC	-90 ± 5	5 ± 0.5	$-^b$
GC/DDAB	-85 ± 5	$-^a$	$-^b$
	$+60 \pm 5$		
GC/PDDA	-85 ± 5	2 ± 0.5	1.4
	$+60 \pm 5$	1 ± 0.5	
Au/MPA/PDDA	$+191 \pm 5$	2 ± 0.5	0.1
Au/CYSAM/MALIM	-177 ± 5	10 ± 0.5	2.2

[a] Not determined. [b] Not detectable.

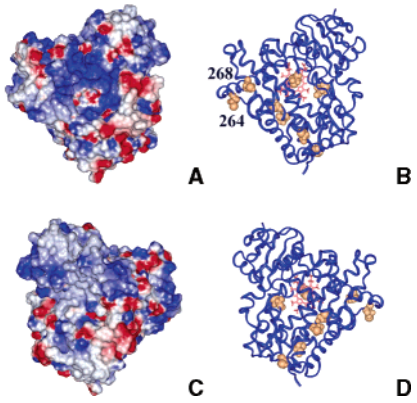

Figure 2. Delphi surface (A, C) and α-carbon ribbon (B, D) representation of P450 2E1. (A, B) reductase binding face (proximal); (C, D) substrate entrance face (distal). Electrostatic potentials are indicated in blue (positive) and red (negative), and the cysteine residues are represented in yellow, space-filled.

Electrochemical response on gold electrode could only be observed after modification of the surface. Derivatization with mercaptopropionic acid (MPA) and subsequently with PDDA gives origin to two waves with a midpoint potential of +191 mV (data not shown). This value is in agreement with what was observed in similar conditions with human P450 3A4.[6] The peak current is linear with a scan rate up to 30 V/s, and the electron-transfer rate was found to be 2 s^{-1}. The coverage was calculated to be 5×10^{13} molecules per cm^2, indicating a multilayer formation of about five layers.

To rationalize the results, a 3D model of P450 2E1 was constructed on the basis of the structures of the P450 BM3 heme domain,[13] rabbit P450 2C5,[14] and the human P450 2C9.[15] The Delphi surface (Figure 2A, C) shows the presence of a patch of positive charges on the proximal side, which is opposite to the substrate-binding site and offers the closest distance of the heme iron to the surface. This area of positive electrostatic potential will facilitate the interaction with a negatively charged electrode surface such as the glassy carbon or the gold modified with MPA. In all the experiments described above the protein was adsorbed to the electrode surface in a nonoriented and noncovalent way. A different approach, using the property of gold to strongly bind sulfydryl groups, was used to achieve an oriented and covalent immobilization of the protein via exposed cysteine residues. The model was used to measure the solvent-accessible area of the eight cysteine residues present in the P450 2E1 sequence. Our model (Figure 2B, D) shows that Cys 437 (5th ligand of the heme iron), Cys 177, 174, and 452 are completely buried into the protein matrix. The remaining Cys 480 and Cys 488 have an exposure of about 15%, while Cys 261 and 268 have an exposure of 37 and 25% respectively. Therefore, Cys 261 and 268 are the two most probable candidates for the covalent binding to the electrode. As the exposed cysteines Cys261 and 268 are also on the proximal side, a clean gold surface was modified first with cystamine (CYSAM) and second with maleimide (MALIM). The modified electrode was dipped in the protein solution overnight at 4 °C, allowing the covalent linkage of the cysteine to the maleimide layer. The CV showed two waves with a midpoint potential of −177 mV (Figure 1D). The peak current is linear with the scan rate up to 30 V s^{-1}, and the electron-transfer rate was found to be 10 s^{-1}, indicating faster electron transfer compared to that with the other modifications. While the coverage was that of a monolayer, 1.1×10^{13} molecules per cm^2, as the one observed with the GC, the faster electron-transfer rate observed for the Au/CYSAM/MALIM/P450 2E1 is probably due to the controlled and favorable orientation achieved with this strategy. Moreover, the control over this parameter results in only one midpoint potential comparable to that of Fe^{III}/Fe^{II} of other P450 enzymes in anaerobic conditions.

To investigate the ability of the immobilized protein to catalyze the hydroxylation of a substrate marker of P450 2E1 activity, a potential bias of −300 mV was applied in the cell where the oxygenated solution was constantly stirred. The formation of the product of conversion of *p*-nitrophenol into *p*-nitrocatechol was quantified spectroscopically at 515 nm using an ϵ_{515} of 12.4 mM^{-1} cm^{-1}. The concentrations of the product formed are reported in Table 1. While no *p*-nitrocatechol was detected in the absence of the enzyme, in its presence 1.4 μM and 2.2 μM of the product were detected on GC/PDDA/P450 2E1 and Au/CYSAM/MALIM/P450 2E1, respectively. This finding supports the evidence that the electrochemical signal is associated to catalytically active immobilized P450 2E1. To test its ability to work as a biosensor, the Au/CYSAM/MALIM/P450 2E1 electrode was immersed into an oxygenated buffer and titrated with *p*-nitrophenol. The results shown in Figure 1E indicate that the immobilized enzyme responds to the presence of the substrate in a Michaelis−Menten fashion and that, when the contribution of the buffer is subtracted, the net current at saturation is about 1 μA. The plot of the catalytic current as a function of the substrate concentration shows a K_M of 130 ± 3 μM, in good agreement with the 197 μM value previously found for 2E1 in solution.[16] This report paves the way for the investigation of human P450 immobilized in an oriented format in which the catalytic properties can be tailored for specific biosensing purposes.

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