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Electrochemical activity of cytochrome P450 1A2 – the relevance of O₂ control and the natural electron donor

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Abstract

The direct electrochemical response of the membrane-bound human cytochrome P450 1A2 (CYP1A2) was studied on pyrolytic graphite electrodes, while encapsulated on a sol-gel matrix. The enzymatic reduction of O₂ was evaluated in the presence and absence of its electron donor partner, cytochrome P450 oxidoreductase (CPR). When used without CPR, CYP1A2 was shown to be highly sensitive to O₂ even in the presence of residual amounts. Under aerobic conditions (air-saturated solutions) the catalytic signal due to the reaction with O₂ was lost, suggesting the enzyme was inactivated. In contrast, the CYP1A2/CPR complex retained O₂ reductase activity with high O₂ concentration in solution. The results demonstrated a crucial role for CPR in stabilizing the immobilized CYP1A2 enzyme and in the preservation of O₂ electrocatalysis, in this electrochemical set-up. Though the enzyme's monooxygenase activity towards caffeine was not detected, this study highlights the complexity in coupling CYP1A2 reduction currents with substrate turnover, due to the simultaneous electrochemical measurement of the O₂ reduction reaction.

Keywords

Cytochrome P450; Cytochrome P450 oxidoreductase; CYP electrochemistry; O₂ reduction; sol-gel

1. Introduction

Cytochromes P450 (CYPs) constitute a large family of *b*-type heme-containing enzymes belonging to the group of monooxygenases^[1] that are present in both eukaryotic and prokaryotic organisms. They catalyze a wide variety of biological oxidation reactions, including the biosynthesis of hormones and of structural components of cells, as well as the metabolism of exogenous compounds. In mammals CYPs are membrane associated enzymes, either located on the inner membrane of mitochondria or on the cytoplasmic side of the endoplasmic reticulum (ER), where they play an important role in the biotransformation of xenobiotics and endobiotics.^[2] *In vivo*, the heme iron utilizes two protons, O₂ and two electrons, which derive from NADPH via a reductase enzyme, to oxidize organic substrates with stereo- and regio-selectivity.^[2a] In the case of ER-located CYPs, electron donation occurs through the activity of cytochrome P450 oxidoreductase (CPR). Because of CYPs versatility in i) recognition of different biological and xenobiotic compounds and ii) type of chemical reactions it can mediate, there is great interest in their application in biosensors^[3] and in biocatalysis,^[4] respectively. A major strategy of exploitation is through immobilization of the enzymes on electrodes that function as a controllable source of electrons to drive CYP catalytic activity.^[5] However, interfacing these enzymes on electrode surfaces to sustain CYP's reaction cycle electrochemically has proven to be challenging.^[6]

In direct electron transfer (DET) based systems, CYP is wired to the interface, directly exchanging electrons with the electrode.^[3a] Therefore, the enzyme needs to be immobilized in a manner that enables this exchange, but simultaneously does not hinder substrate binding or product release. The preservation of CYP's structural properties is a concern, particularly due to the relative ease with which it can be converted into the catalytically inactive

cytochrome P420 conformation.^[3a,6-7] Several approaches have been used for protein immobilization, either by direct attachment to electrodes,^[8] binding in self-assembling monolayers,^[9] immobilization in polymers and lipid membranes,^[10] or the use of additives and binding-promoters, such as surfactants and nanomaterials.^[6,11] Despite success in achieving DET and monitoring the $\text{Fe(III)} + 1\text{e}^- \leftrightarrow \text{Fe(II)}$ reversible conversion, turnover is often low or absent,^[4b,6,10c,10d,11a] which attests to the difficulties in reproducing the complex electron transfer (ET) processes in CYP catalytic activity. Aside from possible inactivation/denaturation of the enzyme due to the immobilization procedures, side reactions that consume electrons, but do not lead to substrate conversion, may confound data.^[6-7,12] Reduction of O_2 is at the basis of these uncoupling reactions that lead to the production of reactive oxygen species (Fig. S1). The auto-oxidation pathway involves the slow release of O_2^- , returning the heme iron to its ferric resting state (CYP-Fe(III)). In the peroxide shunt, the ferrous O_2 bound complex, CYP-Fe(II)- O_2 , is reduced to yield H_2O_2 . Finally, the oxidase shunt pathway bypasses substrate conversion steps after the formation of the CYP-(Fe(IV)=O)^{+•} catalytic species by reducing it, releasing H_2O and regenerating CYP-Fe(III).^[13] The efficiency of the electrochemically driven catalytic reaction may be limited by these uncoupling pathways, which may also occur under physiological conditions.^[13] Moreover, the formed reactive oxygen species may react with CYP either inactivating the enzyme or activating alternative reactions mechanisms. For example, the peroxide shunt pathway has been proposed as an electrochemical activation mechanism for CYPs, which is based on H_2O_2 reaction with CYP-Fe(III) to form the catalytic species CYP-(Fe(IV)=O)^{+•}.^[5,7a,10c]

Another potential issue for the electrochemical response of CYPs, particularly in the development of biosensor devices, lies in the measurement of catalytic activity. The CYP's

catalytic cycle involves binding and reduction of O₂, as described above. But non-bound O₂ is also electroactive in most potential windows used to monitor redox processes and therefore, it may be difficult to assign a cathodic current response to the electrocatalytic enzyme reaction. Consequently, measurement of products, following electrolysis reactions, has been used in various works to confirm substrate turnover.^[6,14]

This current report describes the study of the direct electrochemical response of human cytochrome P450 1A2 (CYP1A2) alone or in combination with its oxidoreductase (CYP1A2/CPR). The proteins were immobilized in a hybrid sol-gel thin film, based on a water-based sodium silicate, and polyethylene glycol (PEG).^[15] The DET reaction of the enzyme was characterized by cyclic voltammetry in the absence and in the presence of CYP1A2 substrate, caffeine, and with different concentrations of the co-substrate, O₂. The effects of O₂ on CYP's stability and catalytic response, as well as, the experimental issues encountered during the evaluation of the bioelectrochemical system are discussed.

2. Experimental

2.1. Chemicals

Sodium silicate solution (14 % NaOH, 27 % SiO₂), caffeine, glucose, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, nicotinamide adenine dinucleotide phosphate (NADP⁺), and resorufin were acquired from Sigma-Aldrich. Polyethylene glycol (PEG) 400 was purchased from Prolabo and hydrochloric acid solution was from Riedel-de Haën. Ethoxy-resorufin was obtained from BD Biosciences. Phosphate buffers were prepared from 0.5 M stock solutions of NaH₂PO₄·2H₂O (Prolabo) and Na₂HPO₄·2H₂O (Fluka). All solutions

were prepared with deionized water (18 M Ω cm) obtained from a Millipore MilliQ water purification system.

2.2. Enzymes

Membrane-bound recombinant human CYP1A2 (1.22 μ M in 75 mM Tris-HCl, 10% (v/v) glycerol and 25 mM EDTA, pH 7.5) was obtained by expression in *E.coli* strain DH5 α . Culturing, microsome preparations and CYP-content determinations were performed as previously described.^[16] Recombinant CYP1A2/CPR membrane-bound complex (co-expressed in *E.coli*, 1:1 stoichiometry) was acquired from Cypex and used as provided (1 μ M CYP1A2, in 50 mM Tris-acetate buffer, pH 7.6, 250 mM sucrose and 0.25 mM EDTA), except for the ethoxy-resorufin dealkylation activity measurements of CYP1A2 for which a different recombinant preparation was used (see section 2.5). Catalase [Cat] (bovine liver, 2000 – 5000 U mg⁻¹) and glucose oxidase [GOx] (Type II *Aspergillus niger*, 19.3 U mg⁻¹) were purchased as lyophilized powders from Sigma-Aldrich and prepared in 0.05 M phosphate buffer, pH 7.0. Cat and GOx were used in the O₂ scavenging system at final concentrations of 2000 and 15 U mL⁻¹, respectively. Cat was used in the H₂O₂ scavenging system also at 2000 U mL⁻¹.

2.3. Bioelectrode preparation

The working electrodes (3 mm diameter disks) were made of basal plane pyrolytic graphite [PG] (GE Healthcare). The surfaces were treated by abrasion with sandpaper and polished with alumina slurry (0.3 μ m, Buehler), followed by sonication in deionized water. Finally, the electrodes were washed with deionized water and dried with compressed air.

The hybrid sol-gel was prepared as previously described.^[15] The starting sol solution contained 55 mM sodium silicate and 6 % PEG 400; this mixture was thoroughly mixed and adjusted for pH 7.0 with 1 M HCl.

Electrodes were modified by placing a 7 μ L drop of a 1:1 mixture containing membrane preparations of CYP1A2 or CYP1A2/CPR complex (each with 1 μ M CYP1A2) and sol-gel onto the clean surface. Electrodes were then air dried for 1 h. Control experiments were performed with sol-gel modified electrodes (without CYP1A2), and with CYP1A2 simply adsorbed on a clean electrode surface or immobilized in 1:1 mixtures with either 55 mM sodium silicate or 6 % PEG 400.

2.4. Electrochemical measurements

A conventional three-electrode electrochemical cell was used with an Ag/AgCl reference electrode, a Pt counter electrode (both from Radiometer) and the enzyme/sol-gel modified PG electrode. The electrochemical cell contained 10 mL of 0.05 M phosphate buffer, pH 7.0, as supporting electrolyte. Unless stated otherwise, the cell was maintained under an argon atmosphere by thoroughly purging the solution before the experiments (20 min – 1 h) and maintaining the argon flow on top during measurements. Assays in the presence of O₂ were performed with air-saturated supporting electrolyte. For non-catalytic assays, an O₂ scavenging system composed of GOx, Cat and glucose was added to the cell previously purged with argon.

Measurements were performed using a potentiostat Autolab PSTAT 12 (Eco-Chemie), using the NOVA 1.6 software (Eco-Chemie) for control and data acquisition. In the cyclic voltammetry experiments, a 50 mV s⁻¹ scan rate was used, unless stated otherwise. The

catalytic response was tested by adding small volumes (0.1 – 0.4 mL) of argon-purged or air-saturated water and caffeine (stock solution 60 mM) to the electrochemical cell, as detailed in the results section. The supporting electrolyte was magnetically stirred after each injection. Final caffeine concentrations in the cell ranged from 0.6 to 4.8 mM. A Clark-type O₂ electrode (Tekcoplus) was used to monitor the dissolved O₂ concentrations in the solutions added to the electrochemical cell and in the supporting electrolyte prior to starting the assays. All potentials are quoted against the Ag/AgCl reference electrode (+207 mV vs SHE).

2.5. CYP1A2-mediated ethoxy-resorufin dealkylation assays

The catalytic activity of CYP1A2 [EthR-*O*-deethylation, EROD] in presence or absence of sol-gel, was evaluated using membrane fractions isolated from *Escherichia coli* cell model BTC (heterologous co-expression of membrane-bound human CYP1A2 together with human full-length CPR), as reported previously.^[17] A range of EthR concentrations (0 – 5 μM) and 8 nM CYP1A2 (with or without an equal volume of sol-gel solution) were used. Initial reaction rates were measured in triplicate. The Michaelis-Menten equation was fitted to the plots of initial reaction rate vs substrate concentration ($r^2 > 0.95$) allowing the determination of steady-state kinetic parameters, using GraphPad Prism 5.01 Software.

3. Results and Discussion

3.1. Immobilization of CYP1A2 in a sol-gel film

The direct electrochemical response of CYP1A2 was evaluated on PG electrodes upon immobilization in a sodium silicate/PEG 400 based sol-gel.^[15] Cyclic voltammograms (CVs) obtained in argon-purged solutions reveal a broad and intense cathodic wave centered at -

0.335 V (Fig. 1D). In contrast, only small reduction peaks (between -0.3 to -0.5 V) are observed when CYP1A2 is directly adsorbed on the bare PG, or immobilized on either a PEG 400 or a sodium silicate film, respectively (Fig. 1A-C). This demonstrates that the hybridization of the sol-gel film with the organic polymer, PEG 400, enables the immobilization of the protein in an electrochemically active form, with a sufficient fraction properly orientated towards the electrode. The use of PEG 400, in particular, has been described as highly beneficial for the stabilization of membrane proteins.^[15]

The signal decreases rapidly in the first minutes after introduction of the CYP1A2/sol-gel/PG electrodes in the electrochemical cell, but stabilizes after approximately 20 cycles. This signal decay is reproducible and may be attributed to the reduction of traces of O₂ in the electrochemical cell by the encapsulated CYP1A2.^[11b] Further evidence of O₂ catalytic reduction is the absence of anodic signal, which is a characteristic voltammetric signature of electrochemical catalysis.^[18] Note that the cathodic signal decrease is also observed with the sodium silicate and PEG 400 films, but is significantly less pronounced in the protein-only films, which is likely to retain considerably less O₂ (Fig. 1A). Results obtained with sol-gel control electrode (without CYP1A2) confirm the presence of small amounts of O₂ in the argon-purged electrochemical cell and its permeation through the sol-gel film, as the direct reduction of dissolved O₂ at the electrode surface is observed below -0.5 V (Fig. S2A).

Therefore, we conclude that under the applied experimental conditions, and due to the high affinity of the ferrous heme to O₂, the electrochemical response of CYP1A2 in the sol-gel electrodes was dominated by the catalytic reduction of O₂.

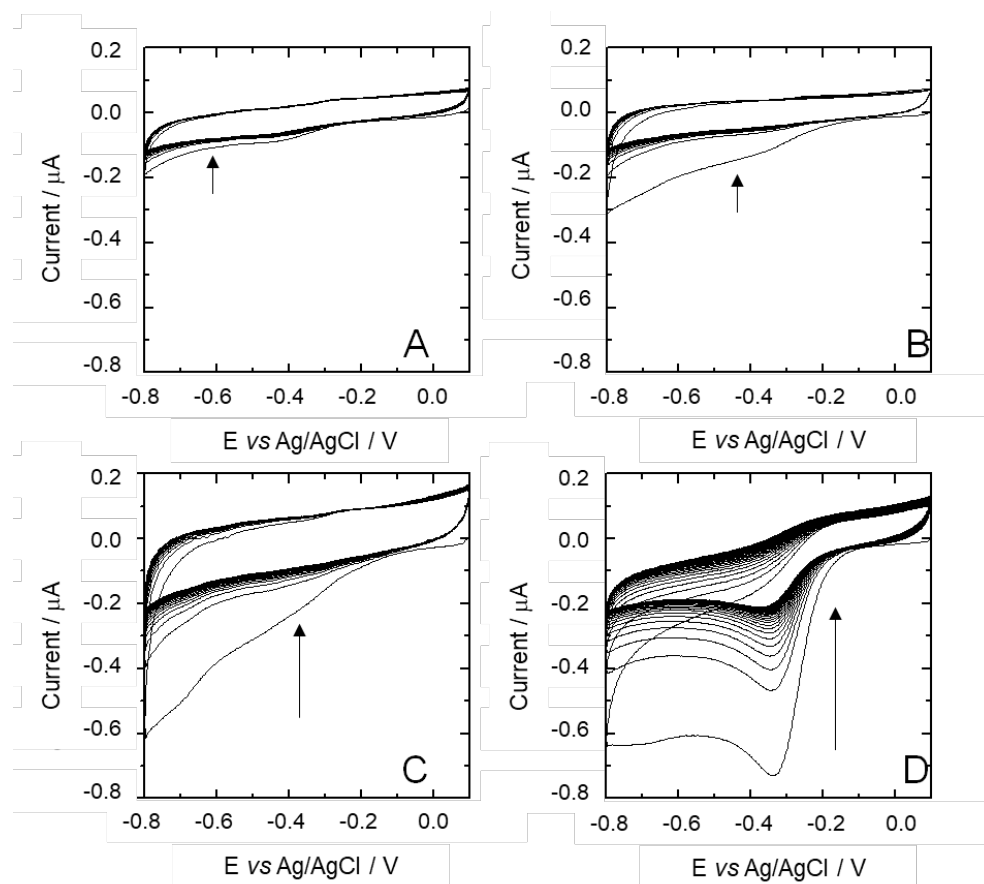


Fig. 1. Cyclic voltammetry response of CYP1A2, either (A) directly adsorbed on the PG surface or immobilized in thin-films of (B) PEG 400, (C) sodium silicate and (D) sol-gel based on sodium silicate and PEG 400. The first 40 successive scans are represented (arrows indicate time progression). All measurements were performed in 0.05 M phosphate buffer, pH 7.0, after purging the solution with argon.

3.2. Direct electrochemistry of CYP1A2 in the presence of a O_2 scavenging system

Cathodic processes are often subject to interference from dissolved O_2 , since it can be reduced directly at the electrode surface or react with enzymes, analytes or electron donors

(e.g. redox mediators, reducing agents) involved in the electrochemical reaction. To avoid the resulting background currents, O₂ is typically removed from the electrolyte solution by purging it with an inert gas before measurements. Alternatively, different chemical and enzyme-based O₂ scavenging systems have been explored for maintaining anaerobic conditions (often in open systems) without the need for laboratory-based equipment.^[19] To further characterize the electrochemical response of the CYP1A2/sol-gel electrodes we employed the GOx/Cat system in order to study the direct electrochemical response of the CYP1A2/sol-gel electrodes without interference of residual O₂. The system is based on the catalytic oxidation of glucose by GOx with concomitant reduction of O₂ (eq. S1); the H₂O₂ generated from this reaction is dismutated into H₂O and O₂ by Cat (eq. S2). The resulting O₂ is further reduced by GOx. In the net cyclic reaction, two glucose molecules are used for the reduction of one O₂ molecule (eq. S3). The high turnover rates of the two enzymes leads to a rapid decrease of O₂ content in solution.^[19]

Upon addition of the enzymatic O₂ scavenging system to the argon-purged electrolyte solution, the cathodic currents assigned to the direct reduction of O₂ at the sol-gel control electrode (without CYP1A2), are quickly eliminated (within 3 minutes, Fig. S2A). This indicates that even after continued purging with argon (up to 1 h), traces of O₂ can remain in the electrochemical cell and diffuse through the sol-gel film. The two enzymes can thus remove the residual O₂ more efficiently than the physical purging with argon. A reversible electrochemical signal, centered at -0.41 V, is observed in the CVs after prolonged incubation with the O₂ scavenging system (20 min) (Fig. 2SB). This was attributed to the system components, possibly the free flavin cofactor, a common impurity in commercial GOx preparations, and suggests the small cofactor may be adsorbing to the sol-gel film.^[20]

The CVs of the CYP1A2/sol-gel/PG electrodes obtained in the presence of GOx, Cat and glucose, revealed two current peaks (Fig. 2) in addition to the signals of the O₂ scavenging system, previously observed with the control electrode. The peaks can be assigned to CYP's Fe(III)/Fe(II) conversion. The anodic and cathodic peak currents are identical and proportional to the scan rates, while peak separations vary between 30 and 90 mV in the range of scan rates investigated. These characteristics are consistent with a quasi-reversible diffusion-less electrochemical process. The formal reduction potential, -301 ± 17 mV vs Ag/AgCl, is constant over the whole scan rate range and in agreement with the reported value for CYP1A2 immobilized in polystyrene sulfonate films.^[18]

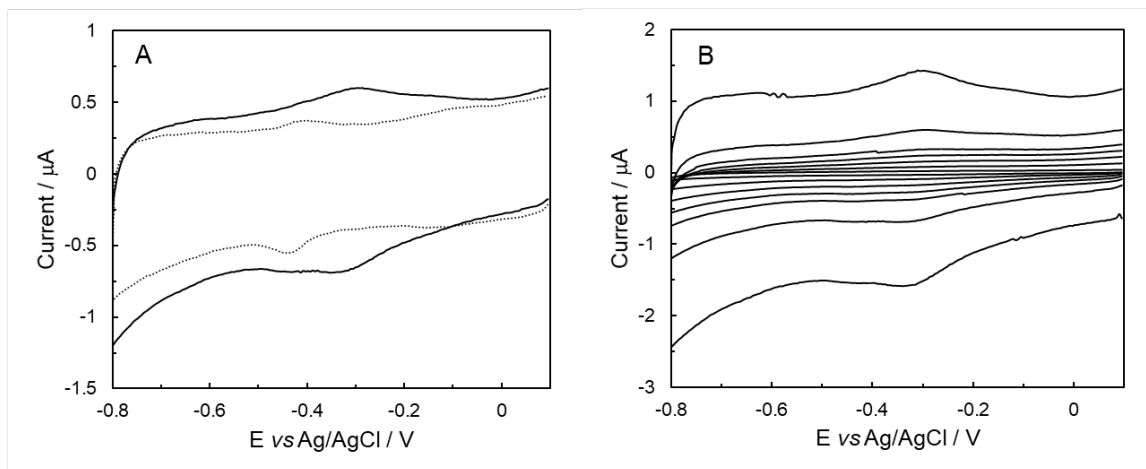


Fig. 2. (A) Cyclic voltammetry response of the control sol-gel/PG (dotted line) and CYP1A2/sol-gel/PG (solid line) electrodes in the presence of the enzymatic O₂ scavenging system. (B) Scan rate variation for the CYP1A2/sol-gel/PG electrode, 20 – 500 mV s⁻¹. Measurements were performed in 0.05 M phosphate buffer, pH 7.0, purged with argon and upon addition of GOx (15 U mL⁻¹), Cat (2000 U mL⁻¹) and glucose (50 mM).

The results obtained with the argon-purged supporting electrolyte *versus* the additional use of the enzyme-based O₂ scavenging system, confirm that the electrochemical activity with the immobilized CYP1A2 is highly sensitive to the presence of O₂. In contrast to argon purging alone, addition of GOx and Cat can remove trace amounts of O₂, probably trapped in the enzyme film, revealing the direct electrochemical response of the sol-gel immobilized CYP1A2 free from O₂ interference.

3.3. Electrocatalytic activity of CYP1A2

The O₂ dependence of CYP1A2/sol-gel/PG electrochemical response is depicted on Fig. 3A. The experiment was initiated with argon-purged electrolyte solution to which small volumes of air-saturated water (O₂ 7.23 ppm/0.226 mM) were added, corresponding to an increase in O₂ concentration up to 17.4 μM. Monitoring of the O₂ content in the solutions injected into the electrochemical cell was performed with a Clark-type dissolved O₂ electrode. CYP1A2's peak currents increased proportionally with added water volumes, as a result of the electrocatalytic reduction of the added O₂ (Fig. S3). However, when the electrode was exposed to a higher concentration of O₂ namely, to air-saturated electrolyte (approximately 0.22 mM O₂), the cathodic signal, although intense in the first scan, quickly disappeared during subsequent continuous cycling (Fig. 3B). The peak shifted to lower potentials (approximately -0.7 V), which is consistent with direct reduction of O₂ on the PG electrode.

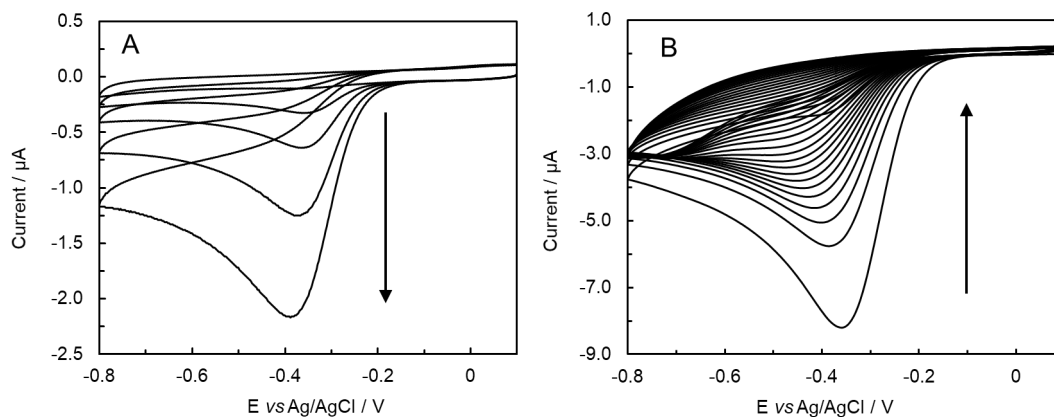


Fig. 3. Cyclic voltammetry response of CYP1A2/sol-gel PG electrodes in the presence of O₂. (A) CVs represent the response in argon-purged supporting electrolyte and 4 additions of air-saturated water to the solution (arrow indicates increasing added O₂ concentrations of 2.2 – 17.4 μM). (B) Consecutive CVs measured upon placing the electrode in air-saturated supporting electrolyte (arrow indicates time progression). All measurements were performed in 0.05 M phosphate buffer, pH 7.0.

Although loss of activity in oxygenated solutions was previously reported for both human (CYP3A4) and bacterial (CYP101, CYPcam) enzymes,^[4b,10c,21] the detrimental effects of O₂ on CYP's electrocatalytic activity have rarely been discussed. Our results show that under relatively high O₂ concentrations CYP1A2 electrochemical signal was lost, thereby compromising the measurement of its monooxygenase activity. Looking for possible explanations, we have investigated the previously proposed electrochemical activation pathways for CYPs, represented in Fig. 4.^[5,10c]

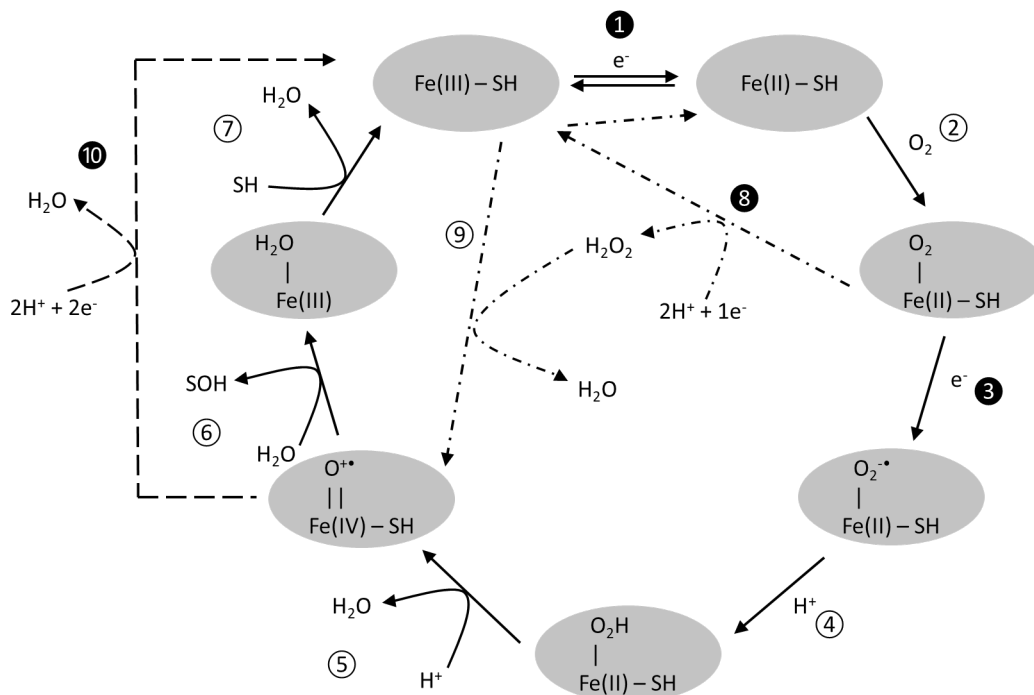


Fig. 4. Proposed CYP catalytic cycle indicating the peroxide shunt electrochemical activation pathway (dash-dotted lines). Reduction of the catalytic intermediary CYP-(Fe(IV)=O)^{+•} at the electrode (oxidase shunt) is also represented (dashed line). Electron transfer steps are marked with a black circle. SH and SOH represent substrate and product, respectively. Adapted from [5,10d].

In a DET based mechanism, the electrode replaces electron delivery by the CPR component. Thus, after the one-electron reduction of CYP-Fe(III) on the electrode (step 1) and binding of O₂ to form CYP-Fe(II)-O₂ complexes (step 2), a second electron is transferred generating CYP-Fe(II)-O₂^{•-} (step 3). Two subsequent protonation steps lead to the formation of the CYP-(Fe(IV)=O)^{+•} catalytic species, responsible for substrate turnover. An alternative pathway for DET based biocatalysis under aerobic conditions was proposed by Rusling and co-workers [5,10c]. This pathway (see Fig. 4, dash-dotted lines) is known as the electrochemical peroxide

shunt and is dependent on CYP activation by H_2O_2 formed by the electrocatalytic reduction of O_2 . This reduction (step 8) is supported by an observed sharp increase in cathodic current in the presence of minute concentrations of O_2 (*cf* section 3.1). H_2O_2 may then react with CYP-Fe(III) to form the catalytic intermediary CYP-(Fe(IV)=O)⁺ (step 9); this species can also be reduced at the electrode to regenerate CYP-Fe(III) (step 10).^[5,10c] We propose that in the presence of elevated O_2 levels, the cycle is blocked at some point. For this blockage, we suggest the following hypotheses: i) the conformational change required to convert CYP-Fe(II)- O_2 into H_2O_2 and CYP-Fe(III)/(II) recycling^[22] does not occur (blocking of step 8); ii) the accumulation of high amounts of H_2O_2 and/or the excessive cathodic current due to the reduction of O_2 to H_2O_2 leads to CYP degradation and to the consequential interruption of the cycle in step 9. To test the latter hypothesis, the electrochemical assay was performed in the presence of Cat, a H_2O_2 scavenging enzyme. No relevant differences were detected in the CVs in air-saturated solutions in the presence or absence of 2000 U.mL⁻¹ Cat, suggesting that the loss of activity of the immobilized CYP1A2 is not caused by the accumulation of H_2O_2 in the electrochemical cell (Fig. S4). However, since Cat operates in solution we cannot rule out the possibility that a high local concentration of H_2O_2 can be generated inside the sol-gel film, before diffusion to the bulk solution, which could damage the immobilized CYP1A2. To test the 1st hypothesis, O_2 reduction was evaluated in the presence of CYP1A2's electron donor protein, CPR. These results are presented and discussed in detail on section 3.4. The overall conclusion is that maintenance of catalytic O_2 reduction by CYP1A2 is favored in the presence of CPR. Accordingly, we propose that CPR is essential for the maintenance of the peroxide shunt pathway, avoiding a potential blockage during the conversion of CYP-Fe(II)- O_2 into H_2O_2 and CYP-Fe(III)/(II) (step 8 in Fig. 4).

The catalytic activity of the CYP1A2/sol-gel hybrid films towards caffeine was subsequently studied. As detailed above, air-saturated solutions cannot be used, as the protein signal is lost in these conditions. Consequently, the experiment was initiated in argon-purged supporting electrolyte and small volumes of air-saturated caffeine solution (O_2 content 6.68 ppm/0.209 mM) were added to the cell (0.6 – 4.8 mM caffeine and 2.1 – 16.1 μM O_2 final concentrations, Fig. S5). Note that, we used caffeine concentrations above the reported K_M value in solution (240 – 280 μM range)^[23] to ensure the enzyme worked at maximum rate, thus facilitating detection of electrocatalytic activity. The cathodic peak intensity increased with caffeine concentration, however the current increase was identical to the one obtained by adding equivalent volumes of air-saturated water (Fig. S3). This suggests that the observed response was due to the catalytic reduction of O_2 and not caffeine turnover. In the control experiments, performed with argon-purged caffeine solutions, no catalytic activity was observed (Fig. S6), despite the presence of O_2 traces. Therefore, we conclude that the catalytically active state leading to substrate conversion was not formed under these experimental conditions. Uncoupling reactions occur frequently with mammalian CYPs; they yield O_2 reduction species but do not drive product formation.^[6,10d] Besides the peroxide shunt pathway and the production of H_2O_2 , the electrochemical set-up may also favor the occurrence of the oxidase shunt pathway. The potentials applied may have been sufficiently low to regenerate the resting state species (CYP-Fe(III)) without substrate conversion. In other words, the catalytic species CYP-(Fe(IV)=O)⁺⁺ would be rapidly reduced to CYP-Fe(III)/(II), increasing the flow through steps 10 and 1 and bypassing product formation (steps 6 and 7 in Fig. 4). In contrast to the on/off binding of CYP's redox partner CPR, the continuous electric fields generated at the electrode surface could explain the inability to form CYP-(Fe(IV)=O)⁺⁺. Also, the natural occurring intermittent attachment/detachment of CPR, with the involvement of CPR's

extensive open/close protein dynamics, could induce conformational changes in CYP, which might be of relevance for the reaction pathway in substrate conversion.^[24]

Overall, our results recommend caution when evaluating an increase of cathodic current in the presence of O₂ and organic substrates. This has been used as a measure for catalytic bioconversion in some studies reporting on the electrocatalytic activity of electrode immobilized CYPs towards organic substrates.^[11d,25] Care must be taken to guarantee that current increase is not mistaken by a higher rate of O₂ reduction, occurring when substrate is added to the cell, an issue that has not been suitably addressed so far. Electrochemically driven CYP biocatalysis studies must be looked at with caution and should include appropriate control experiments, to provide reliable results of protein activity.^[26] These include the control of O₂ concentration, the measurement of current upon solvent addition, as well as the detection of reaction products, a strategy previously used by several authors, but that is not always applicable.^[9,10c,14,17,27]

3.4. Direct electrochemistry of CYP1A2/CPR complex

As mentioned above, the hypothesis that an ET-coupled conformational change is required for O₂ reduction and regeneration of CYP-Fe(III)/(II) was evaluated in the presence of CPR. In the natural catalytic cycle of CYPs, the electron donor protein, CPR, forms a complex with CYP and is responsible for the electron transfer from NADPH to the enzyme.^[5]

The CYP1A2/CPR complex behaves similarly to the CYP1A2 electrodes in argon-purged supporting electrolyte and in the presence of the enzymatic O₂ scavenging system. In the first case, a cathodic peak, assigned to O₂ catalytic reduction, is detected at -0.320 V vs Ag/AgCl. Then, upon addition of GOx, Cat and glucose to the electrochemical cell, the catalytic

currents are eliminated and CYP's Fe(III)/Fe(II) reversible reaction is observed at a potential of -0.314 ± 8 V vs Ag/AgCl (Fig. S7). The low intensity peak pair, centered at -0.42 V vs Ag/AgCl was initially attributed to the components of the enzymatic O₂ scavenging system, as described in section 3.2 (cf. Fig. 2A). However, the control experiments performed with CPR alone, i.e. upon immobilization of CPR-containing membrane fragments in the sol-gel matrix (Fig. S8), showed that this signal was already present before the addition of the scavenging system (Fig. S8, solid black line). Therefore, the coinciding signal was here assigned to one of CPR's redox centers. By comparison with the electrochemical response of other CYPs, the signal observed at -0.42 V was attributed to the FAD center. Also note that the FMN cofactors typically have higher reduction potentials than the FAD and heme cofactors.^[10d,28]

In air-saturated supporting electrolyte (O₂ *approximately* 0.22 mM), CVs of CYP1A2/CPR/sol-gel PG electrodes revealed a sharp catalytic peak at a similar potential observed for CYP1A2/sol-gel system (-0.350 V and -0.400 V, respectively), Fig. 5. However, in the presence of CPR, the cathodic peak corresponding to O₂ catalytic reduction was maintained after an initial decrease. Upon stirring the solution, the O₂ concentration at the electrode interface was equilibrated with the bulk solution and the signal intensity was restored to values only slightly lower than the initial current. This indicates that unlike the CYP1A2/sol-gel electrodes (Fig. 3B), CYP1A2/CPR complex remains active towards O₂ reduction in the presence of high concentrations of this molecule. As already noted, in these conditions the conformational changes required to recycle CYP-Fe(III)/(II) may not occur, possibly because the electrode surface cannot mimic the interaction with CPR.

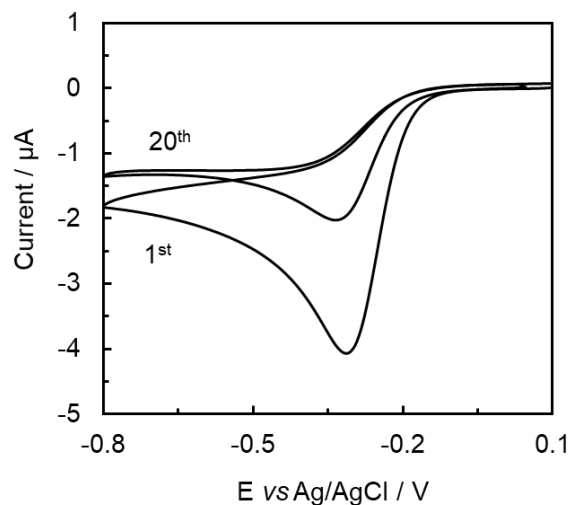


Fig. 5. Cyclic voltammetry response of CYP1A2/CPR/sol-gel electrodes in air-saturated supporting electrolyte. The first and twentieth CV cycles are represented. Measurements were performed in 0.05 M phosphate buffer, pH 7.0.

Nonetheless, no caffeine related catalytic currents could be detected with the CYP1A2/CPR/sol-gel electrodes in the presence of either argon-purged or air-saturated caffeine solutions (not shown). The current variations were identical to those obtained upon injection of equal volumes of solvent, suggesting a direct correlation with O₂ reduction by the immobilized CYP1A2, as observed with the CYP1A2/sol-gel electrodes. Importantly, we observed a consistent positive shift of the catalytic O₂ reduction peak between CYP1A2 and CYP1A2/CPR electrodes (Fig. 6). This higher reduction potential suggests a facilitated catalytic response of CYP1A2 in the presence of CPR.

Taken together, our results indicate that CPR has a critical role in the stabilization of CYP1A2 and in the maintenance of O₂ electrocatalysis. Nevertheless, the proposed sol-gel formulation does not ensure the preservation of the immobilized enzyme's activity towards caffeine. In order to understand the reasons behind the lack of activity, we investigated the

spectroscopic features of CYP1A2 in dried sol-gel films, which enable the identification of inactive CYP states, such as the P420 configuration. Unfortunately, all our attempts to characterize the enzyme/sol-gel films either by UV-Vis or resonance Raman spectroscopy, were thwarted by the low amount of immobilized sample and the complexity of microsome preparations (not shown).^[6-7]

Next, to rule out any possible influence from the sol-gel matrix on the CYP1A2/CPR activity, we checked the catalytic response, in the presence of the sol-gel, through a solution enzyme assay specific for CYP1A2.^[17] As shown in Fig. S9, the turnover rate obtained for the enzyme/sol-gel mixtures was only slightly lower ($k_{\text{cat}}^{\text{app}}$ 0.36 s⁻¹) than the value for the enzyme alone (k_{cat} 0.49 s⁻¹), which confirms that the matrix provides a friendly environment for the enzyme. Since the presence of sol-gel *per se* does not significantly impair the enzyme activity in solution, CYP1A2 immobilization on the electrode surface may result in steric hindrance, like an improper orientation on the electrode that blocks the substrate access to active site.

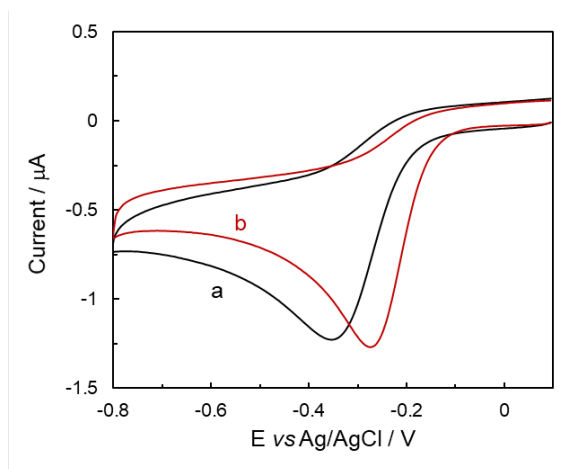


Fig. 6. Cyclic voltammetry response of (a) CYP1A2/sol-gel and (b) CYP1A2/CPR/sol-gel electrodes to the addition of air-saturated water (O_2 7.34 ppm/0.229 mM). Concentration of added O_2 was $9.0 \mu\text{M}$. Measurements were performed in argon-purged 0.05 M phosphate buffer, pH 7.0.

4. Conclusions

Herein we report on the DET of a hybrid CYP1A2/sol-gel film on pyrolytic graphite electrodes. Our results showed that the synergic combination of sodium silicate and PEG 400 provided a friendly environment for the enzyme immobilization in an electrochemically active form. Also, we demonstrated that a GOx/Cat-based O_2 scavenging system was able to efficiently remove O_2 from the sol-gel film, contrary to the commonly used argon purging method. A non-turnover response from the immobilized CYP1A2, without O_2 interference, could only be attained in the presence of this O_2 scavenging system.

Furthermore, this work proves that the measurement of CYP electrochemical activities in the presence of O_2 is not straightforward. Control assays concerning the regulation of the co-substrate's concentration are mandatory in studies aiming to correlate CYP catalysis with

current variations, as they are essential to correctly assign catalytic currents. In particular, the addition of O₂-containing substrate solutions to the electrochemical cell should be carefully monitored.

Despite the absence of monooxygenase activity, the immobilization of CYP1A2 in the presence of its physiological electron transfer partner CPR (i.e. the CYP1A2/CPR complex) was shown to be fundamental for the maintenance of O₂ electrocatalysis. Reduction of O₂ in air-saturated solutions could be measured with the CYP1A2/CPR complex/sol-gel electrode but not with the CYP1A2/sol-gel system.

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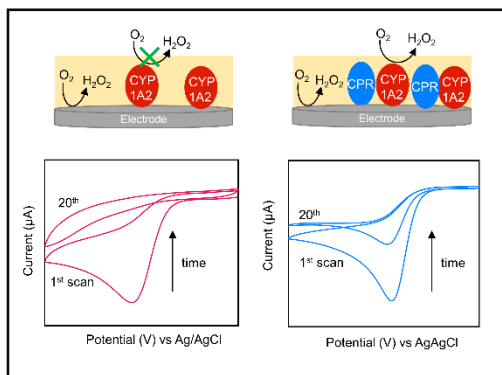
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Entry for table of contents:



Cytochrome P450 1A2 (CYP1A2) direct electrochemistry was obtained in a biocompatible sol-gel film.

Control of O_2 levels is critical to get reliable results on the enzyme's activity.

The electrocatalytic activity towards O_2 depends on interaction with the electron donor CYP oxidoreductase (CPR).