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Reactivities of organic phase biosensors: 6. Square-wave and differential pulse studies of genetically engineered cytochrome $P450_{cam}$ (CYP101) bioelectrodes in selected solvents

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Abstract

Cytochrome $P450_{cam}$ (CYP101) bioelectrodes suitable for application in organic phases were prepared from genetically engineered CYP101 and vesicular dispersions of didodecyldimethylammonium bromide. The amperometric biosensor system was characterised under anaerobic conditions by cyclic and square-wave voltammetric methods. Cyclic- and square-wave-voltammetry studies showed that the biosensors exhibited direct reversible electron transfer between the haem iron atom and the glassy carbon electrode surface. The formal redox potential estimated for the electrode in acetonitrile was −380 mV/Ag–AgCl. The formal potential shifted anodically as the organic phase biosensor responded irreversibly to substrate (camphor) under anaerobic and aerobic conditions in acetonitrile. Differential pulse analysis of the reactivities of the CYP101 enzyme electrode confirmed the square-wave voltammetry result, which showed that the binding of substrate decreased the redox potential necessary for initiating the monooxygenation reaction of cytochrome $P450_{cam}$.

Keywords: Cytochrome $P450_{cam}$ (CYP101); Biosensor; Liquid crystal biosensor; Square-wave voltammetry; Differential pulse voltammetry; Organic phase biosensors

1. Introduction

A wide variety of both exogenous and endogenous compounds are known to undergo monooxygenation reactions catalyzed by a family of haem-thiolate enzyme systems, generally referred to as cytochrome P450 (CYP) (NC-IUB, 1991; Nebert and Gonzalez, 1987). The enzyme could be of bacterial, mitochondrial or microsomal origin. Type I cytochrome P450 systems (Degtyarenko, 1995) which are of mitochondrial and bacterial origins, usually consist of an iron–sulphur protein, NADPH or NADH-dependent reductase (an FAD-type flavoprotein) and P450 enzyme. On the other hand, microsomal P450 systems (Type II: Degtyarenko, 1995) are constituted by NADPH:P450 reductase (FAD- and FMN-containing flavoprotein), and P450 enzyme. Monooxygenation reaction of the CYP enzyme systems are useful in that they ensure the hydroxylation of a variety of compounds including steroids, fatty acids, drugs, food additives, alkanes and polyaromatic and polychlorinated hydrocarbons. Among the types of reactions associated with the P450 enzyme systems are hydroxylations, epoxidations, N-oxidations, N-dealkylations, O-dealkylations, and dehydrogenations. The unique monoxygenation capabilities of CYP mean that they can be applied as biochip sensing systems, and bioreactors for the biodegradation of environmental contaminants or biosynthesis of fine chemicals.

Camphor hydroxylating CYP101 monooxygenase (Nelson et al., 1993) has elicited a lot of interest among researchers in the field of biocatalysis and bioelectrochemistry (Reipa et al., 1997; Wong et al., 1995; Faulkner et al., 1995; Estabrook et al., 1996; Kazlauskaite et al., 1996; Zhang et al., 1997; Iwuoha et al., 1998,

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2000; Davis et al., 2000; Rusling et al., 2000; Mayhew et al., 2000; Sevrioukova et al., 2001; Shimada et al., 2001; Jones et al., 2001; Lee et al., 2001; Furukawa and Morishima, 2001; Woggon et al., 2001; Harris et al., 2001; Sjodin et al., 2001; Hata et al., 2001; Ivanov et al., 2001a,b; Davydov et al., 2001; Deng et al., 2001). The monooxygenase enzyme system is found in the bacterium *Pseudomonas putida* PpG786 when it is cultured on camphor. The enzyme catalyses camphor hydroxylation (5-*exo*-hydroxy-camphor) through a two electrons process, in which the 2Fe–2S protein, putidaredoxin, mediates electron transfer from NADH and the FAD-containing putidaredoxin reductase to cytochrome CYP101 (Sevrioukova et al., 2001). The enzyme is also known to show stereospecific hydroxylation of other substrates (Reipa et al., 1997).

Several approaches have been adopted to, on one hand, eliminate the need for co-factors and electron-mediating proteins in CYP101 monooxygenation reactions (Reipa et al., 1997), and on the other hand, improve substrate specificity of the enzyme (Walsh et al., 2000). Over the last 6 years, cathodically polarised CYP101 bioelectrodes have been employed by various laboratories (Reipa et al., 1997; Wong et al., 1995; Faulkner et al., 1995; Estabrook et al., 1996; Kazlauskaite et al., 1996; Zhang et al., 1997; Iwuoha et al., 1998; Davis et al., 2000; Rusling et al., 2000; Mayhew et al., 2000; Ivanov et al., 2001a) to drive the biocatalytic process, as a replacement for the physiological co-factor and mediator proteins.

One method through which the CYP101 enzyme system has been modified for use in electrode-driven biocatalysis, is by active-site mutation (Mayhew et al., 2000). Parameters such as, active-site polarity, volume and hydrophobicity have been genetically manipulated (Walsh et al., 2000) to probe their effect on the reaction of CYP101 with hydrocarbons. Wong et al. (Jones et al., 2001) have investigated the oxidation of polychlorinated benzenes by mutants of the CYP101 enzyme. Increasing the active-site hydrophobicity with the Y96F mutation increased the activity up to 100-fold, and both pentachlorobenzene and hexachlorobenzene were oxidized slowly to pentachlorophenol.

An area of focus in CYP101 bioelectrode research has been the development of electrode designs that allow direct electron transfer between electrode surface and the enzyme haem redox centre, which is protected within the protein interior. Rusling et al. showed direct electron transfer from lipid-modified (Zhang et al., 1997) and polyion-modified (Rusling et al., 2000) pyrolytic graphite electrodes to CYP101). Hill et al. (Kazlauskaite et al., 1996) reported reversible electrochemical response from glassy carbon electrodes. Vilker's research group demonstrated (Reipa et al., 1997) the achievement of direct electron transfer from a semiconductor electrode to CYP101 mediated by putidaredoxin, a natural component of the CYP101 monooxygenation reaction. Unmediated electrochemical responses have also been reported for glassy carbon electrodes modified with CYP101 immobilised in synthetic lipid material (Iwuoha et al., 1998) or methyltriethoxysilane sol–gel (Iwuoha et al., 2000). In the present study, vesicular dispersions of the synthetic lipid material didodecyldimethylammonium bromide (DDAB) will be employed in the fabrication of the CYP101 biochips for application in non-aqueous media. DDAB forms vesicular systems that bear structural relationship with the phospholipid components of biologically important membranes (Zhang et al., 1997; Iwuoha et al., 1998, 2000). At ambient temperature, DDAB forms stable lamella liquid–crystal films (Blandamer et al., 1995). In this study, the DDAB liquid–crystal system was used to provide the biosensor with a membranous environment that facilitated rapid electron transfer between the enzyme's redox centre and the electrode surface. The effects of organic solvent on the reactivities of the liquid–crystal CYP101 enzyme electrode were studied by Osteryoung-type square voltammetry, differential pulse voltammetry (DPV) and cyclic voltammetry.

2. Experimental

2.1. Reagents

A stock 50 μM solution of cytochrome $P450_{cam}$ (CYP101; EC 1.14.15.1, Mr 46 500) was prepared by genetic engineering followed by subsequent purification, as described by Fisher et al. (1996). The enzyme was stored in the freezer at −80 °C when not in use. DDAB which was a product of Fluka (Germany) and stored in the refrigerator set at 5 °C. Vesicle dispersion of 10 mM DDAB was prepared by sonicating for 8 h 4.63 mg sample of the compound in 1 ml of water. The solvents for organic phase experiments were chloroform and HPLC grade acetonitrile supplied by Lab Scan (Dublin, Ireland). Camphor, adamantane and glutaraldehyde were products of Aldrich (Gillingham, UK) The electrolyte used for organic phase studies was 1 mM tetraethylammonium *p*-toulenesulphonate (Aldrich, Milwaukee, USA). Analytical grade argon (Air-Products Plc., UK) was used to degas all samples. Buehler (IL, USA) alumina micropolish and polishing pads were used for electrode polishing. Distilled water that was deionised with Milli-Q-water purification system was used for the preparation of all solutions used in this study. Phosphate buffer solutions (0.05 M, pH 7.5) were prepared with AnalaR grade compounds supplied by Merck (Poole, Dorset): anhydrous disodium hydrogenorthophosphate and potassium dihydrogenphosphate dihydrate. The anhydrous salt was dried for 3 h at 110 °C and cooled in a desiccator before it was used for

buffer preparation. Buffer solutions used in the experiment contained 0.01 M potassium chloride, Fluka. Bovine serum albumin (BSA) was obtained from Sigma (MO, USA).

2.2. Instrumentation

BioAnalytical Systems (Stockport, UK (BAS)) electrochemical workstation BAS 100B was used for all the electrochemical measurements. The electrochemical cell consisted of a 10 ml cell containing a glassy carbon (GCE) working electrode (0.071 cm^2 Metrohm, Herisau, Switzerland), platinum wire-mesh counter electrode and Ag/AgCl (NaCl type) as the reference electrode (BAS Technicol, Stockport, UK). The temperature of the electrochemical cell was thermostatically controlled at 25 °C with a Heto (Denmark) water-bath. The glassy carbon electrode was polished with 1.0, 0.3 and 0.05 μm grade alumina, rinsed thoroughly with deionised water and allowed to dry before surface modification with DDAB and CYP101.

2.3. Preparation of biosensor

The cytochrome $P450_{cam}$ amperometric electrode was prepared as described below. Fifty microlitre of aqueous vesicle dispersion of 10 mM DDAB was mixed with 2 mg BSA. Then 50 μl of 38 μg/ml CYP101 was added to the DDAB/BSA solution to form the enzyme-synthetic membrane complex. Then 3 μl of the complex was mixed with 2 μl of 2.5% glutaraldehyde solution on a 0.071 cm^2 GCE. The electrode coated with the enzyme-synthetic membrane (DDAB) film was spun at with a home-made spin coater at 400 rpm for of 1 h under an air dryer. The enzyme-coated electrode was stored to cure for 12 h before use. The final composition of the biosensor on the electrode was 0.8 μg/cm^2 CYP101, 15 nmol DDAB, 0.05 mg BSA and 1.25% glutaraldehyde.

2.4. Procedure

2.4.1. Cyclic voltammetry

A 10 ml acetonitrile, 0.1 M in TEATS, was placed in a water-jacketed electrochemical cell thermostatically controlled at a temperature of 25 °C for all measurement. The biosensor (GC/CYP101-DDAB), the platinum wire-mesh auxiliary and the Ag/AgCl reference electrodes were placed in the cell and connected to BAS 100B electrochemical workstation. For determinations under anaerobic conditions, the cell solution was degassed for 30 min with argon and maintained under an atmosphere of argon throughout the duration of each voltammetric measurement. Cyclic voltammetric responses of the biosensor were recorded from an initial potential, $E_i = +100$ mV, to a switch potential, $E_\lambda = -650$ mV; at a suitable scan rate, usually 500 mV/s, although measurements were also made at 20 mV/s when chloroform was used as solvent. Voltammogrammes were recorded in the presence and absence of 3 mM ethanolic solution of camphor. Determinations under aerobic conditions were performed with freshly prepared electrodes in undegassed acetonitrile (0.1 M TEATS) media that contained no camphor or 3 mM camphor (ethanolic solution).

Experiments for the determination of adamantane were performed in chloroform (0.1 M TEATS) that was either air-saturated or argon-degassed for 30 min and kept under argon atmosphere. The stock 0.1 M adamantane solution used is this study was prepared in chloroform. Cyclic voltammetric responses were recorded in the presence and absence of 0.3 mM adamantane at a scan 20 mV/s.

2.4.2. Square-wave voltammetry

Osteryoung-type square-wave voltammetry was performed with biosensors in acetonitrile (0.1 M TEATS) under anaerobic conditions (degassed for 30 min and kept under argon atmosphere). The experimental conditions were 75 mV square-wave amplitude, 4 mV potential step and potential range of 0 to −500 mV. The net square-wave responses (differential square-wave voltammogrammes) were plotted for cathodic (0 to −500 mV) and anodic (−500 to 0 mV) scans at frequencies of 100, 140 and 200 Hz. Furthermore, square-wave experiments were performed with the biosensors in the presence and absence of 3 mM camphor under anaerobic conditions at 200 Hz, 75 mV square-wave amplitude and 4 mV potential step.

2.4.3. Differential pulse voltammetry

Differential pulse experiments were performed on the GC/CYP101/DDAB biosensors in acetonitrile (0.1 M TEATS) containing 3 mM ethanolic solution of camphor. The experimental conditions were: scan rate 20 mV/s; pulse amplitude 50 mV; sample width of 17 ms; pulse width of 50 ms; and pulse period 200 ms. The potential was scanned cathodically from an initial to a final potential of 0 and −650 mV, respectively. Voltammogrammes were recorded under anaerobic (argon-degassed for 30 min and argon-capped) and aerobic (undegassed) conditions.

3. Results and discussion

3.1. Cyclic voltammetry of GC/PYP101/DDAB

Fig. 1 shows the cyclic voltammogrammes of GC/PYP101/DDAB biosensors in acetonitrile substrate-free argon-degassed acetonitrile and buffer solutions. The voltammogrammes were obtained at 500 mV/s. The anodic peak potential (E_{pa}) and cathodic peak potential (E_{pc}) values for acetonitrile were −380 and −400 mV,

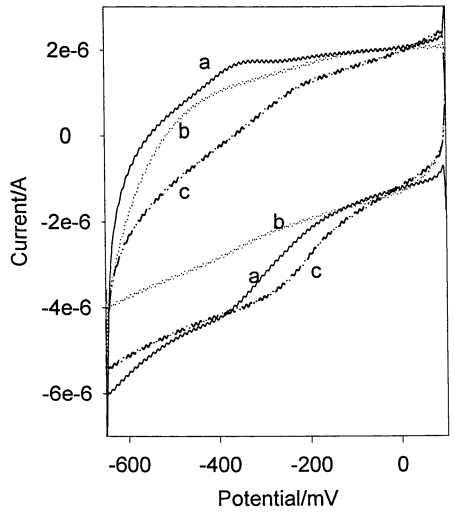

Fig. 1. Cyclic voltammogrammes for $Fe^{3+/2+}$ of GC/CYP101/DDAB biosensors in (a) acetonitrile (0.1 M TEATS), (b) chloroform (0.1 M TEATS) and (c) phosphate buffer (0.1 M, pH 7.2, 0.1 M KCl). Experiments were performed in argon-degassed solution at 500 mV/s vs. Ag/AgCl at 25 °C. Initial scan direction is cathodic. Cathodic currents: negative direction; anodic currents: positive direction. Biosensors composition: 0.8 μg/cm^2 CYP101, 15 nmol DDAB, 0.05 mg BSA and 1.25% glutaraldehyde.

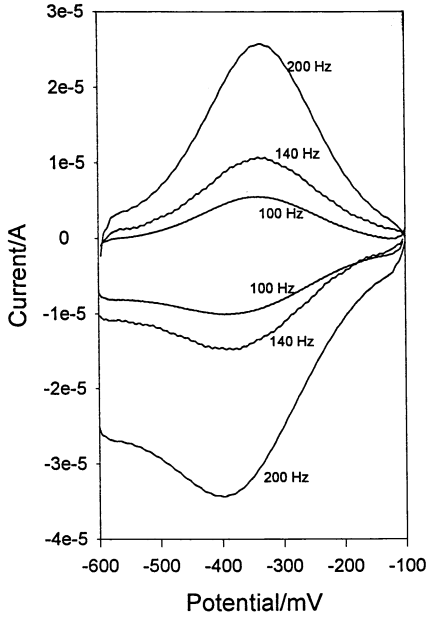

Fig. 2. Square-wave voltammogrammes of the biosensor's haem $Fe^{3+/2+}$ system in argon-degassed acetonitrile (0.1 M TEATS) performed at 100, 140 and 200 Hz. Anodic scans (−500 to 0 mV): positive waves; and cathodic scans (0 to −500 mV): negative waves. Experimental conditions are: square-wave amplitude 75 mV; potential step 4 mV. Biosensors composition is as in Fig. 1. (Plots show net currents of the forward and reverse SW currents.)

respectively. This gave a formal potential, $\{E^{0\prime} = (E_{pa} + E_{pc})/2\}$ of −380 mV. The peak separation ΔE value for of the biosensor *n* acetonitrile was 40 mV. Analysis of the voltammogramme from experiments performed in phosphate buffer (0.1 M, pH 7.2) gave $E_{pa} = -230$ mV, $E_{pc} = -242$ mV, $\{E^{0\prime} = -260$ mV, and $\Delta E = 36$ mV$\}$. The peak separation values obtained under anaerobic conditions in this substrate-free media were lowers than 65 mV, which is characteristic of surface bound fast one electron transfer process (Iwuoha et al., 1998), in this case involving the haem Fe of CYP101 (i.e. $Fe^{3+/2+}$ redox couple). The electron transfer process was so fast that a scan rate of 500 mV/s was necessary to observe the electron transfer voltammetric peaks. Experiments performed at lower scan rates did not show clearly discernible reversible peaks. The sensitivity of the biosensors calculated from the faradaic peak currents, was 1.5±0.3 ng $Fe^{3+/2+}$/μA for both organic and aqueous phases. It is important to mention here that, the formal potential of the biosensor system was shifted cathodically by 120 mV as the reaction medium was changed from the aqueous phase to organic phase. This effect of solvent may be related to the stabilisation of the ferri-haem rest state of CYP101 by acetonitrile which affects the reduction potential of the ferri-haem.

3.2. Square-wave voltammetry of GC/PYP101/DDAB

Fig. 2 depicts the organic phase square-wave voltammetric (SWV) responses of the CYP101 bioelectrode as a function of square-wave frequency. The voltammogrammes were recorded under anaerobic conditions and in the absence of substrate. The voltammogrammes with peak maxima are the anodic wave, while those with peak minima are cathodic waves. The voltammogrammes represent the net (difference between forward and reverse currents) SWV responses when the electrode was scanned oxidatively (anodic wave) or reductively (cathodic wave). The peak cathodic potential was −380±20 mV/Ag–AgCl. The corresponding peak for the anodic scan was −340±25 mV/Ag–AgCl. The formal redox potential calculated from this data was −360±23 mV/Ag–AgCl. These results agree with what was calculated using cyclic voltammetry. Thus the square-wave voltammogrammes obtained represent the CYP101 haem $Fe^{3+/2+}$ redox couple undergoing direct electron transfer at the electrode. It has to be mentioned here that the oxidative waves are more symmetrical than the reductive wave. However, the faradaic currents of the reduction wave are higher than those of the corresponding oxidation wave. This suggests that CYP101 bioelectrode has its haem iron atom in the Fe^{3+} form in the CYP101 rest state. On the other hand during oxidative scan, the amount of Fe^{2+} produced at the initial potential of −600 mV are oxidized as the potential is scanned anodically. The peak current will depend on the amount of Fe^{2+} produced at the initial potential. The peak potentials were found to be independent of the square-wave frequency as expected for simple reversible electrode process of the form: Fe^{3+} +

$e^{-}\Delta Fe^{2+}$. In this type of system, the peak current is proportional to the square-wave frequency (Osteryoung and O'Dea, 1986) as obtained in this study.

3.3. Camphor determination with CYP101 biosensor

Fig. 3 shows the responses of the CYP101 bioelectrode to camphor in acetonitrile (0.1 M TEATS). The voltammetric wave labelled 'a' represents the reactivity of the biosensors under substrate-free anaerobic environment. The response of the biosensor to camphor under aerobic condition is shown by curve 'b'. As has been explained in previous communication (Iwuoha et al., 1998, 2000), the reaction is initiated by the reduction of Fe^{3+} to Fe^{2+}. The ferro-haem then binds oxygen. The rate of this binding of oxygen is increased in the presence of substrate. Thus it would be possible to observe catalytic voltammetric wave due to the coupling of the $Fe^{3+} + e^{-}\Delta Fe^{2+}$ electrode process to the binding of oxygen. As shown by curve 'c', the inclusion of camphor in the reaction process enhances the reduction current. The cyclic voltammetric response is related to the amount of camphor present (Iwuoha et al., 1998).

3.4. Effect of substrate on the reactivity of the bioelectrode

We have shown during the analysis of the square-wave voltammogrammes of the GC/CYP/DDAB biosensors, that differences in the nature of the electroactive species can be determined by the magnitude of the cathodic or anodic SWV currents. However, for a reaction where electron transfer process is coupled to a catalytic process, both the magnitude of the currents

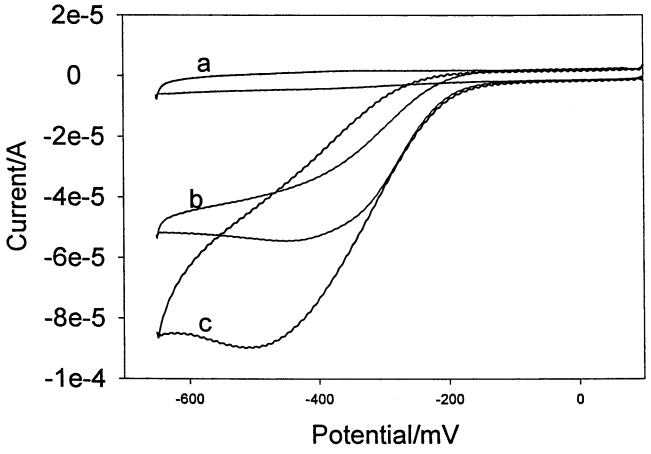

Fig. 3. Cyclic voltammogrammes of the biosensor's $Fe^{3+/2+}$ in (a) argon-degassed acetonitrile (0.1 M TEATS), (b) undegassed acetonitrile (0.1 M TEATS), and (c) undegassed acetonitrile (0.1 M TEATS) containing 3 mM camphor. Experiments were performed at 500 mV/s vs. Ag/AgCl at 25 °C. Initial scan is cathodic. Biosensors composition: 0.8 μg/cm^2 CYP101, 15 nmol DDAB, 0.05 mg BSA and 1.25% glutaraldehyde.

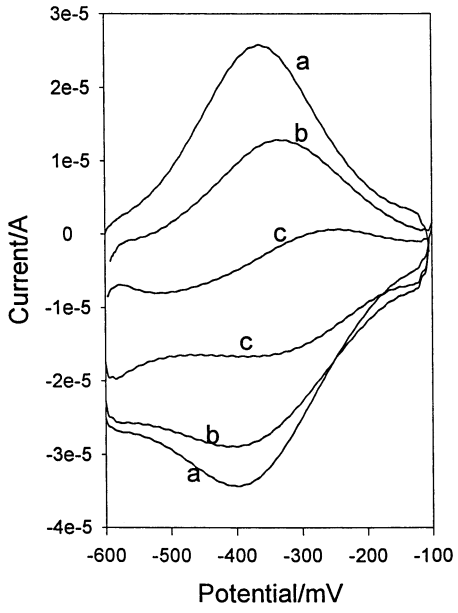

Fig. 4. Square-wave voltammetry responses of the CYP101 bioelectrode at 200 Hz: (a) argon-degassed acetonitrile, (b) 3 mM camphor in argon-degassed acetonitrile, and (c) 3 mM camphor in un-degassed acetonitrile. All the solvent systems contained 0.1 M TEATS. Anodic scans (−600 to −100 mV) positive waves; and cathodic scans (−100 to −600 mV): negative waves. Experimental conditions are: square-wave amplitude 75 mV; potential step 4 mV. Biosensor composition is as in Fig. 1. (Plots show net currents of the forward and reverse SW currents.)

and the peak potentials would be affected (Osteryoung and O'Dea, 1986). Fig. 4 represent the square-wave voltammogrammes of the biosensors measured at 200 Hz. Curves 'a' show the response of the electrode, in oxygen-free, camphor-free medium. What we observe here is strictly the electrochemistry of the haem iron atom in acetonitrile as previously explained. Curves 'b' are the responses when 3 mM camphor is present under anaerobic conditions. Under this condition of coupling the electrode process to oxygen binding, the system becomes more irreversible, which results in the decrease of the square-wave current. As the system becomes more irreversible with the addition of camphor under aerobic conditions, the magnitude of the observed current drops even further from the value for the reversible $Fe^{3+}\Delta Fe^{2+}$ process. This agrees with theoretical predictions as shown by Osteryoung and O'Dea (1986).

It is important to note that the peak potentials also change as the reaction species change. The oxidative and reductive waves become more anodic as the system becomes more irreversible, i.e. as the reaction changes from being a simple reversible electron transfer reaction to catalytic reaction. The beauty of this technique is that the *peak potentials*, and hence, the formal potentials of the system, can still be measured even for irreversible systems. The anodic peak potential are −380, −350 and −250 mV for anaerobic/substrate-free, anaerobic/3mM

camphor and aerobic/3mM camphor systems, respectively. The corresponding cathodic peak potentials are −400, −370, −340 mV. The estimated formal redox potentials are −390, −360, −295 mV/Ag–AgCl for anaerobic/substrate-free, anaerobic/3mM camphor and aerobic/3mM camphor systems, respectively. This result show how square-wave voltammetry may be used for speciation of electroactive systems. The decrease in the reduction potential with the binding of camphor is characteristic of the monooxygenation reaction of CYP.

3.5. Differential pulse voltammetry of CYP101 bioelectrode

This study was used to correlate the observed SWV trend in the redox potential of CYP101 bioelectrode under various conditions of oxygen and camphor binding. The differential pulse data in Fig. 5 show the (cathodic) responses of the biosensors to: (i) 3 mM camphor under anaerobic conditions ('a': $E_{peak} = -260$ mV); (ii) no camphor under aerobic condition ('b': $E_{peak} = -340$ mV); and 3 mM camphor under aerobic conditions ('c': $E_{peak} = -290$ mV). The inference from this DPV studies is that oxygen is required for the monooxygenation reaction to proceed, however, the binding of oxygen occurs at lower potentials when camphor is bound to CYP101.

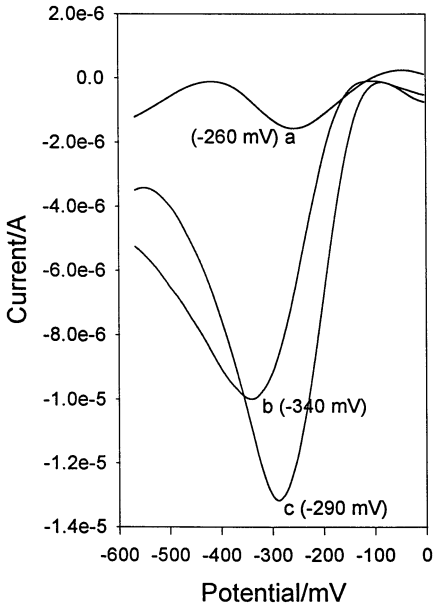

Fig. 5. DPV responses of the CYP101-DDAB-based amperometric sensor in (a) 3 mM camphor in argon-degassed acetonitrile; (b) 0 mM camphor in air-saturated acetonitrile (c) 3 mM camphor in air-saturated acetonitrile. All solutions contained 0.1 M TEATS. DPV conditions: scan rate 20 mV/s; pulse amplitude 50 mV; sample width of 17 ms; pulse width of 50 ms; and pulse period 200 ms. Biosensors composition is as described in Fig. 1. Scan direction is cathodic.

3.6. Responses of GC/CYP101/DDAB in chloroform

The behaviour of the biosensor in an organic phase other than acetonitrile was demonstrated with the application to adamantane detection in chloroform (0.1 M TEATS). The alkane is insoluble in acetonitrile or ethanol, but soluble in chloroform. Fig. 6 shows the cyclic voltammetric responses of the CYP101 biosensor to 3 mM adamantane in chloroform. Voltammogrammes are displayed for 'a': biosensor electrochemistry in substrate-free argon-degassed chloroform at 20 mV/s; 'b': biosensor response to substrate-free air-saturated chloroform; and 'c': biosensors response to 3 mM adamantane in aerobic chloroform medium. These initial studies show that stereospecific substrates of CYP101 that are soluble in chloroform can be detected using the CYP101 bioelectrode.

4. Conclusion

Cytochrome P450 bioelectrode was prepared in liquid crystal membranous system consisting of vesicular dispersions of the synthetic lipid material DDAB. The bioelectrode exhibited direct electron transfer with the glassy carbon electrode in acetonitrile organic phase, as shown by both cyclic voltammetry and square-wave voltammetry in de-aerated solvent media. The estimated formal potential of the biosensor in acetonitrile ($E^{0\prime} = -380$ mV/Ag–AgCl) is 100 mV more than that determined in aqueous buffer medium ($E^{0\prime} = -260$ mV/Ag–AgCl). The stabilisation of the ferri-haem rest state of the CYP101 enzyme in acetonitrile may be responsible for the requirement for higher reduction

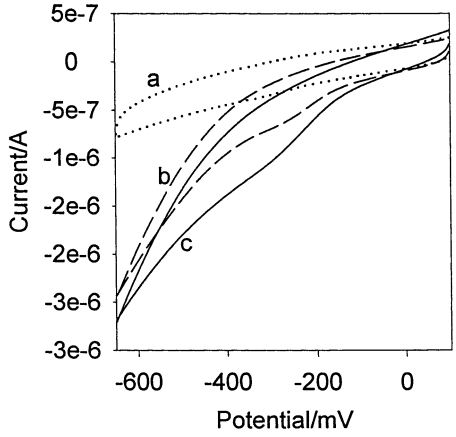

Fig. 6. Cyclic voltammetric responses of the GC/CYP101/DDAB biosensor's $Fe^{3+/2+}$ at 20 mV/s vs. Ag/AgCl and 25 °C. (a) 0 mM adamantane in argon-degassed chloroform; (b) 0 mM adamantane in air-chloroform; and (c) 3 mM adamantane in air-chloroform. All the solvent systems contained 0.1 M TEATS. Initial scan is cathodic. Biosensors composition: 0.8 μg/cm^2 CYP101, 15 nmol DDAB, 0.05 mg BSA and 1.25% glutaraldehyde.

potential in acetonitrile. From square-wave studies, it was found that the formal potential of the $Fe^{3+/2+}$ redox couple in the biosensor shifted anodically as the biosensor responded irreversibly to the presence of substrate (camphor) under anaerobic and aerobic conditions in acetonitrile organic phase. Differential pulse analysis of the reactivities of the CYP101 enzyme electrode confirmed the square-wave voltammetry result, which showed that the presence of substrate reduced the redox potential necessary for initiating the monooxygenation reaction of cytochrome $P450_{cam}$. The square-wave responses of the biosensors offer a method for studying the reversibility of the biosensor reactivities under various conditions. In particular, it would be possible to use the GC/CYP101/DDAB bioelectrode to determine the presence or not of oxygen in a system, and the amount of substrate present in a given reaction media, by analysing both the potential shift and the change in observed current. Organic phase electrochemical behaviour of the CYP101 electrode was demonstrated with camphor detection in acetonitrile and adamantane in chloroform. This means that environmentally relevant polyaromatic hydrocarbons may be studied with biosensors based on CYP101/DDAB liquid crystal membrane system. Other areas where the technique developed in this study can be applied, is in the development of biosensors for monitoring drug metabolism.

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