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Drug metabolism biosensors: electrochemical reactivities of cytochrome $P450_{cam}$ immobilised in synthetic vesicular systems

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Abstract

Biosensors containing cytochrome $P450_{cam}$ in a didodecyldimethylammonium bromide vesicular system were prepared by cross-linking onto a glassy carbon electrode (GCE) with glutaraldehyde in the presence of bovine serum albumin. Cyclic voltammetric responses of the sensor in air-free buffer solution showed that the sensor exhibited reversible electrochemistry due to direct electron exchange between the haem $Fe^{3+/2+}$ redox system and the GCE surface. In air-saturated solution containing camphor, the biosensor gave an irreversible electrocatalytic current which is compatible with the monooxygenation of the substrate. Steady state amperometric experiments with camphor, adamantanone and fenchone were performed with a biosensor prepared by cross-linking $P450_{cam}$ with glutaraldehyde onto a Pt disc electrode. The sensor was characterised by fast amperometric responses, attaining steady-state in about 20 s in a cobalt sepulchrate mediated electrochemical system. The kinetic parameters of the biosensor were analysed using the electrochemical Michaelis–Menten equation. The estimated apparent Michaelis–Menten constant, K'_m, values for the biosensors were in the range of 1.41–3.9 mM.

Keywords: Amperometric biosensor; Cytochrome $P450_{cam}$; Electrochemistry; Enzyme electrode; Drug metabolism biosensor; Liquid crystal film; Amperometry; Cyclic voltammetry

1. Introduction

Biosensors which are intended to monitor drug metabolism, and based on the cytochrome P450 enzyme (P450), have been developed. Cytochrome P450s are haem-thiolate monooxygenase enzymes of microbial, mitochondrial or microsomal origin. Their catalytic reactivities result in the monooxygenation or reduction of a whole range of substances. The microsomal P450s catalyse the conversion of drugs and other xenobiotics to metabolites that are often more water soluble and easily excretable than the parent compound. Their actions result in steroid metabolism, drug deacti-

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vation, procarcinogen activation (e.g. benzo[*a*]pyrene and other polycyclic aromatic hydrocarbons, PAHs), xenobiotic detoxification and fatty acid metabolism. This enormous range of physiological processes involving these enzymes has generated widespread interest in them in medicine, pharmacology and biochemistry. In humans, P450s are the most important phase 1 drug metabolising enzymes. The methods that are now available for in vitro monitoring of drug metabolism are time consuming and expensive. Thus there is an urgent need to develop new analytical tools that would be easy to use, capable of identifying substrates and inhibitors of an enzyme, and allow quantification of substrate turnover. The use of appropriate biosensors would be a welcome alternative, because they are generally of small size, capable of continuous measurements, and can measure analytes faster and at a lower cost than traditional methods.

Cytochrome $P450_{cam}$ ($P450_{cam}$), a camphor hydroxylating bacterial P450, is water soluble, its 3-dimensional structure is known and its biochemistry well documented. However, the electrochemistry of this redox enzyme is not easily observed on unmodified electrode surfaces. In this study, model amperometric P450 biosensors were prepared by incorporating $P450_{cam}$ in vesicle dispersions of the synthetic lipid material didodecyldimethylammonium bromide (DAB). In aqueous solution DAB forms vesicles. Vesicular systems bear structural relationship with the phospholipid components of biologically important membranes. Other studies [1–3] have shown that the vesicular liquid-crystal films provide an excellent milieu for rapid electron exchange between redox proteins and electrode surfaces. A DAB liquid-crystal system was therefore used in this study to provide the biosensor with a membranous environment to facilitate rapid electron transfer between the enzyme's redox centre and the electrode surface.

2. Experimental

2.1. Reagents

Cytochrome $P450_{cam}$ (M_r 46 500) was prepared by genetic engineering [4,5] with subsequent purification as a 50 μM solution. The enzyme was stored in the freezer at −80°C when not in use. Didodecyldimethylammonium bromide (DAB) was obtained from Fluka, Germany and stored at <5°C. A 10 mM vesicle dispersion of DAB was prepared by sonicating 4.63 mg of the powdery compound in 1 ml of water. Camphor, adamantanone and fenchone were obtained from Aldrich, Gillingham, UK. Cobalt sepulchrate, which was the electron transfer mediator in the steady state experiments [6], ethylenediamine (EDA) and 25% glutaraldehyde were also purchased from Aldrich. 0.05 M phosphate buffer (pH 7.5) was prepared from AnalaR grade anhydrous disodium hydrogenorthophosphate and AnalaR grade sodium dihydrogenorthophosphate dihydrate from Merck (Poole, Dorset). The anhydrous salt was dried for 3 h at 110°C and cooled in a desiccator before it was used for buffer preparation.

2.2. Apparatus

All electrochemical experiments were carried out using a BAS 100W integrated, automated electrochemical workstation from BioAnalytical Systems (BAS), Stockport, UK. Amperograms and voltammograms were recorded with a computer interfaced to the BAS 100W electrochemical workstation. A 20 ml electrochemical cell thermostated at 25°C, was used in a three electrode configuration, consisting of a Pt or glassy carbon (GCE) disc working electrode (0.071 cm^2, Metrohm, Herisau, Switzerland), Pt wire mesh auxiliary electrode and Ag/AgCl reference electrode (BAS Technicol). The Pt and glassy carbon electrodes were polished with aqueous slurries of 1.0, 0.1 and 0.05 micron alumina (Buehler, Coventry, UK), respectively, before use.

2.3. Preparation of biosensor for voltammetric measurements

The biosensors for voltammetric experiments were prepared by immobilising $P450_{cam}$ contained in a didodecyldimethylammonium bromide vesicle dispersion on a glassy carbon electrode. In this method [7], 50 μl of 10 mM aqueous vesicle

dispersion of DAB was mixed with 2 mg BSA to form solution **A**. 50 μl of 38 μg ml^{-1} $P450_{cam}$ was added to solution **A**, which produced a vesicle–enzyme complex, solution **B**. 3 μl of solution **B** was mixed with 2 μl of 2.5% glutaraldehyde on a 0.071 cm^2 GCE. The sensor solution was allowed to dry at room temperature for 24 h to form a thin $P450_{cam}$-DAB liquid-crystal film on the electrode surface. The final composition of the biosensor on the electrode was 0.08 mg cm^{-2} $P450_{cam}$, 15 nmol DAB, 0.05 mg BSA and 1.25% glutaraldehyde.

2.4. Preparation of biosensor for steady-state amperometry

Two methods were used in this preparation. In the first method a 5 μl solution containing 0.5 mg cm^{-2} $P450_{cam}$, 0.1 mg BSA and 1.25% glutaraldehyde was placed on the surface of a 0.071 cm^2 Pt disc electrode. The electrode was allowed to dry in open air for 12 h. The resulting Pt/$P450_{cam}$/BSA/Glutarldehyde electrode was stored in the refrigerator when not in use. The Pt disc electrode was polished with alumina before enzyme film deposition.

The second type of biosensor was prepared by placing a 5 μl solution containing 0.5 mg cm^{-2} $P450_{cam}$, 3.75% ethylenediamine and 1.25% glutaraldehyde on the surface of a 0.071 cm^2 Pt disc electrode. The Pt/$P450_{cam}$/EDA/Glutaraldehyde electrode was allowed to dry in open air for 12 h and stored in the refrigerator when not in use. The Pt disc electrode was polished with alumina and treated with Trixton X-100 before enzyme film deposition.

2.5. Procedures

All electrochemical measurements were carried out at 25°C in either degassed or undegassed 0.05 M phosphate buffer (pH 7.5) solution containing 0.1 M KCl. Anaerobic conditions were achieved by passing argon gas through the electrolyte for 20 min and maintaining an argon blanket on the top of the solution. Voltammograms were recorded in the presence and absence of 2 mM camphor (ethanolic solution) at a potential sweep rate of 500 mV vs SCE, from an initial potential, $E_i = 0$ mV to a switch potential, $E_\lambda = -800$ mV.

Unlike the biosensors for the potentiodynamic measurements, the sensors used for the potentiostatic determinations did not contain the synthetic lipid membrane, DAB. This was because in its present form, the DAB-containing electrode was not stable when rotated or placed in a stirred solution for a long time. In the steady-state amperometric determinations, $P450_{cam}$ biosensors immobilised in glutaraldehyde matrix in the presence or absence of ethylenediamine (EDA) were used. In this procedure, the enzyme electrode was polarised at an electrode potential of −800 mV/Ag–AgCl in an electrochemical cell thermostated at 25°C. The electrolyte was 10 ml of 0.05 M phosphate buffer (pH 7.5) containing 0.01 M KCl solution, and 25 μM cobalt sepulchrate $\{Co(Sep)^{3+}\}$ as the electron transfer mediator. The electrolyte was degassed as described above. The background current was allowed to decay to a steady state, then aliquots of 0.1 M camphor were added at regular intervals to the cell solution, which was constantly stirred at 500 rpm. Steady-state amperograms were recorded for experiments with camphor, adamantanone and fenchone.

The steady state responses, I_{ss}, of the sensor were analysed using the electrochemical Michaelis–Menten equation given by [8,9]:

$$I_{ss} = \frac{nFALk_{cat}[P450_{cam}]}{1 + K_M/K_S[S]} \quad (1)$$

where k_{cat}, K_M and K_S are the biosensor turnover rate constant, Michaelis–Menten constant and the partition coefficient of the substrate, respectively. n is the number of electrons transferred in the reaction, F is Faraday constant (96500 C mol^{-1} K^{-1}), A is the surface area of the biosensor (0.071 cm^2) and L is the thickness of the biosensor film.

3. Results

3.1. Cyclic voltammetry of GCE/P450$_{cam}$-DAB biosensor

The cyclic voltammogram (CV) of GCE/P450$_{cam}$-DAB biosensor in substrate-free anaerobic solution is shown in Fig. 1(a), for an experiment carried out at a potential sweep rate of 500 mV s^{-1}. The voltammogram consists of both oxidation and reduction waves, with reduction (cathodic, $E_{p,c}$) and oxidation (anodic $E_{p,a}$) peak potential values of -278 and -242 mV, respectively. The peak separation, $\Delta E_p (= E_{p,a} - E_{p,c})$ value was 36 mV. A mid point potential, $E_m \{= 1/2(E_{p,a} + E_{p,c})\}$ value of -260 ± 10 mV vs SCE was estimated from the cyclic voltammetric data. The ratio of the anodic peak current to the cathodic peak current ($I_{p,a}/I_{p,c}$) was estimated to be 1.02.

Fig. 1(b) shows the cyclic voltammetric responses of the biosensor under anaerobic or aerobic conditions, in the presence and absence of camphor. The voltammograms showed that as the potential was scanned from $+100$ to -650 mV, the reduction (cathodic) current steadily increased to a maximum value, after which the current decreased until the switch potential was reached. The reverse voltammetric waves did not have anodic currents. The first cyclic voltammogram from top showed that in aerobic solution containing 3 mM camphor, the biosensor gave only cathodic current with a peak potential, E_p^{cam} (aerobic) of -350 mV and peak current, I_p^{cam} (aerobic) of 50 μA. The second CV from the top represents the biosensor's response in substrate-free aerobic solution. The biosensor gave a CV similar to the case where 3 mM camphor was present. The peak parameter values were; $E_p^{cam\text{-}free}$ (aerobic) $= -375$ mV and $I_p^{cam\text{-}free}$ (aerobic) $= 44$ μA. The biosensor response obtained in anaerobic solution containing 3 mM camphor, is shown by the third CV from top. The peak cathodic potentials and the corresponding peak current values calculated from Fig. 1(b) were: E_p^{cam} (anaerobic) $= -430$ mV; I_p^{cam} (anaerobic) $= 14$ μA. The lowest CV in Fig. 1(b) represents the biosensor response in anaerobic substrate-free solution (i.e. the same as the CV shown in Fig. 1(a)).

3.2. Cyclic voltammetry of Cobalt sepulchrate

The cyclic voltammogram of 40 μM cobalt sepulchrate {Co(Sep)$^{3+}$} on a plain GCE working electrode performed at 5 mV s^{-1} in an argon-degassed phosphate buffer solution is shown in Fig. 2(a). The cyclic voltammogram had a cathodic peak ($E_{p,c} = -700$ mV/SCE, $I_{p,c} = 540$ nA) and an anodic peak ($E_{p,a} = -640$ mV/SCE, $I_{p,a} = 495$ nA). The peak separation (ΔE_p) value was 60 ± 7 mV and the $I_{p,a}/I_{p,c}$ value was 0.92 ± 0.09. Fig. 2(b) contains the 5 mV s^{-1} scan rate CVs of P450$_{cam}$(Fe$^{3+/2+}$) and Co(Sep)$^{3+/2+}$ redox species. Cytochrome P450$_{cam}$(Fe$^{3+/2+}$) was surface-bound (contained in the biosensor), while Co(Sep)$^{3+/2+}$ was in solution. The cathodic wave of the voltammogram, in the absence of camphor, consisted of two peaks corresponding to $E_{p,c}$ values of 279 and 708 mV. The peaks of the oxidation wave were not as distinct as those of the cathodic wave. However, the small anodic peaks were observed at $E_{p,a}$ values of -640 and -242 mV. When 3 mM camphor was added to the solution, the resultant cyclic voltammogram (top CV of Fig. 2(b)) showed an increase in the cathodic current, with the I_p^{cam} (279 mV) value showing a three-fold increase in value. The reverse voltammetric wave showed a decrease in the anodic current particularly in the region where the electrochemistry of cobalt sepulchrate is observed.

3.3. Steady-state amperometry

When polarised at -800 mV s^{-1} in presence of 25 μM Co(Sep)$^{3+}$, the P450$_{cam}$-based biosensors gave background currents which stabilised to ~1 μA within 250 s. The biosensor exhibited fast amperometric responses to the substrates, attaining steady state in 20 s. The calibration curves for the electrode systems, plotted with the steady state currents less the background currents, are given in Fig. 3. The

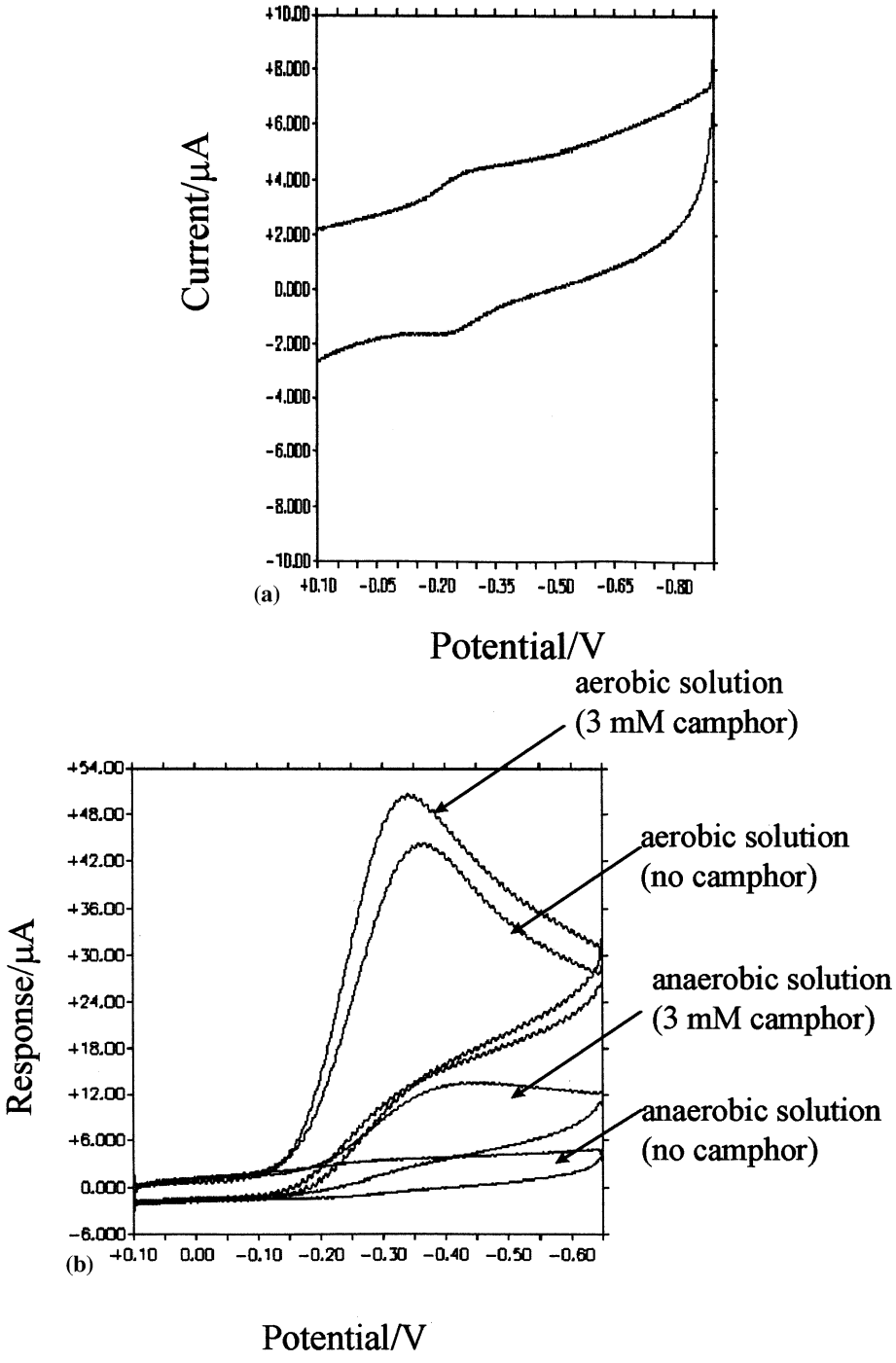

Fig. 1. (a) Cyclic voltammogram of Cytochrome $P450_{cam}$ biosensor at 500 mV s^{-1} versus SCE in argon degassed 0.05 M potassium phosphate buffer (pH 7.5, 0.1 M KCl). The biosensor contained 0.8 mg cm^{-2} *Cyt* $P450_{cam}$ in 15 nmol didodecyldimethylammonium bromide vesicle system, 0.05 mg BSA and 1.25% glutaraldehyde on a 0.071 cm^2 GCE. Anodic and cathodic waves are indicated by negative and positive currents, respectively. (b) Cyclic voltammetric responses of *Cyt* $P450_{cam}$ biosensor at 500 mV s^{-1} versus SCE. Experimental conditions are as described in (a).

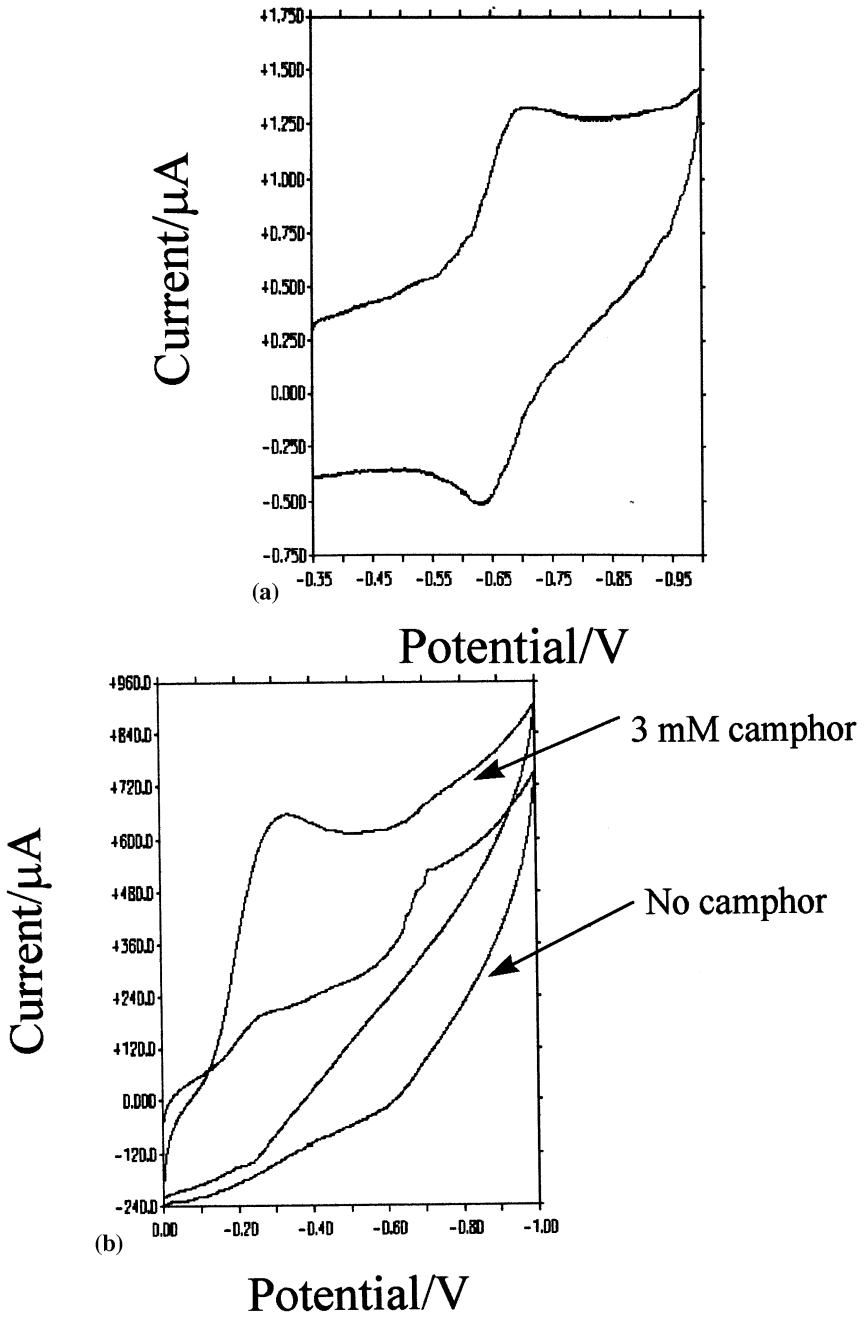

Fig. 2. (a) The cyclic voltammogram of 40 μM $Co(Sep)^{3+}$ on a 0.071 cm^2 plain GCE in argon degassed 0.05 M phosphate buffer solution (pH 7.5, 0.1 M KCl). Scan rate was 5 mV s^{-1} vs. SCE at 25°C. $E_{p,c} = -700$ mV and $E_{p,a} = -640$ mV. (b) *Cyt* $P450_{cam}$ $Fe^{3+/2+}$ and $Co(Sep)^{3+/2+}$ cyclic voltammograms recorded with *Cyt* $P450_{cam}$-DAB-based biosensor at 5 mV s^{-1} versus SCE at 25°C. The electrolyte was argon-degassed 0.05 M potassium phosphate buffer (pH 7.5, 0.1 M KCl) containing 40 mM $Co(Sep)^{3+}$. The electrode composition is as described in Fig. 1.

lines running through the data points are the Michaelis–Menten fits of the experimental data based on Eq. (1) with $n = 2$. The apparent Michaelis–Menten constant ($K'_m = K_m/K_s$) and the maximum current obtainable ($I_{max} = nFALk_{cat}[P450_{cam}]$) for the biosensors are shown in Table 1.

4. Discussion

4.1. Electrochemical behaviour of Cyt $P450_{cam}$-DAB biosensor

The oxidation and reduction cyclic voltammetric curves described in Fig. 1(a) showed that the GCE/$P450_{cam}$-DAB biosensor contained only one redox species. This is due to the observation of only one anodic and one cathodic peaks in the CV. In a similar study, Zhang et al. [7] performed CV experiments with a $P450_{cam}$-DAB electrode at 100 mV vs SCE on pyrolytic graphite. The authors obtained anodic and cathodic peaks characterised by an E_m value of -290 ± 35 mV (which is in agreement with the E_m value of -260 ± 10 obtained in this study). Considering that oxygen-free reaction medium was used, the anodic and cathodic peaks of the CV were attributed [5] to the haem $Fe^{3+/2+}$ redox species contained in $P450_{cam}$. Since the E_m value is independent of potential sweep rate [10], the redox couple in the present study would be the haem $Fe^{3+/2+}$ of cytochrome $P450_{cam}$. Thus when the potential was scanned in the cathodic mode, the Fe^{3+} species of $P450_{cam}$ was reduced to Fe^{2+} giving a maximum reduction current at a potential of -278 mV. Re-oxidation of Fe^{2+} occurred during the reverse (anodic) scan. Maximum oxidation of the $P450_{cam}$ (Fe^{2+}) occurred at a potential of -242 mV. The ΔE_p and $I_{p,a}/I_{p,c}$ values of a redox species give information about the reversibility (Nernstian behaviour) of the electrochemical process [10–13]. An $I_{p,a}/I_{p,c}$ value of 1.0 shows that the electron transfer reaction at the electrode is reversible [10,11]. Also, a ΔE_p value lower than 65 mV is indicative of a surface-bound electroactive substance undergoing fast reversible electron transfer reaction at the electrode [14,15]. The $I_{p,a}/I_{p,c}$ and ΔE_p values of 1.02 and 36 mV, respectively, were estimated in this study. The values of these parameters suggest that the haem $Fe^{3+} \leftrightarrow Fe^{2+}$ transition in the $P450_{cam}$-DAB-based biosensor is rapid and reversible.

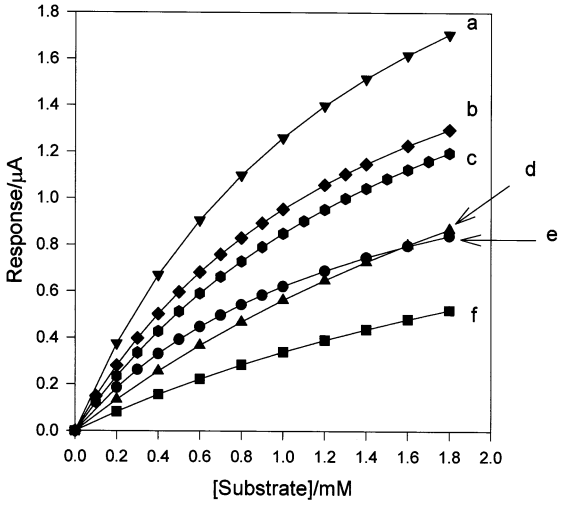

Fig. 3. The calibration plots of *Cyt* $P450_{cam}$ amperometric biosensor for camphor (a, b), adamantanone (c, d) and fenchone (e, f). Biosensor contained 2 μM *Cyt* $P450_{cam}$/1.25% glutaraldehyde immobilised on 0.071 cm^2 Pt disc electrode in the absence (curves b, c, e) or presence (a, d, f) of 1 mM ethylenediamine.

Table 1
The kinetic parameters of the *Cyt* $P450_{cam}$ enzyme electrodes in 0.05 M phosphate buffer, pH 7.5 containing 0.10 M KCl and 25 μM cobalt sepulchrate

Substrate	I_{max} (μA)	K'_m (mM)	Sensor matrix[a]
Fenchone	1.5	1.41	Glutaraldehyde/ BSA
Adamantanone	2.45	1.90	Glutaraldehyde/ BSA
Camphor	2.36	1.47	Glutaraldehyde/ BSA
Fenchone	1.6	3.6	Glutaraldehyde/ EDA
Adamantanone	2.73	3.9	Glutaraldehyde/ EDA
Camphor	3.1	1.41	Glutaraldehyde/ EDA

The working potential was −800 mV/Ag–AgCl at 25°C. Other conditions are as in Fig. 3.
[a] BSA, bovine serum albumin; EDA, ethylenediamine.

4.2. Electrocatalytic responses of Cyt $P450_{cam}$-DAB

The two top cyclic voltammograms in Fig. 1(b) have clearly defined cathodic peaks with no corresponding anodic peaks, which depicts the coupling of fast electron transfer reaction at the electrode surface to a fast chemical process in which the reduced electroactive species have been used up [12,13]. In this case, the haem Fe^{2+} of $P450_{cam}$ produced during the cathodic scan, was used up in a fast follow-up chemical reaction, thus making it unavailable for re-oxidation during the anodic scan. As can be seen in Fig. 1(b), irreversible cyclic voltammetric wave-forms were recorded in air-saturated solutions both in the absence and presence of camphor. This suggests that what is actually observed is the binding of oxygen to the haem redox centre. However, the presence of camphor increases the rate of this process as shown by an increase in the voltammetric current. Oxygen binding to Fe^{2+} of haem proteins is known to be fast [16,17]. Thus the two top CVs in Fig. 1(b) presumably refer to the coupling of the fast $Fe^{3+} \leftrightarrow Fe^{2+}$ electron transfer reaction and the rapid electrocatalytic reactions involved in the binding of oxygen to the haem Fe^{2+} of reduced $P450_{cam}$. These electro-catalytic processes involving the $P450_{cam}$ biosensor were so fast that they required a monitoring scan rate as high as 500 mV s^{-1}. The CV obtained in the presence of 3 mM camphor in anaerobic medium gave a typical electrocatalytic voltammogram in which the fast reversible electrochemistry of the haem $Fe^{3+/2+}$ redox species was coupled to subsequent processes, such as the hydroxylation of camphor or H_2O_2 production by uncoupled turn-over. It is noteworthy that because the ethanolic camphor solution used was not degassed, there exists the possibility of having enough oxygen for the hydroxylation process to occur. Comparisons of the voltammograms in the presence and absence of camphor (both in aerobic and anaerobic media) suggest that camphor increases the rate of oxygenation of $P450_{cam}$. The implication is that the enzyme electrode can be used as a camphor biosensor by varying the camphor concentration and constructing a calibration curve for camphor. A suggested reaction scheme for the oxygenation of $P450_{cam}$ enzyme electrode in the presence of substrate is summarised in Fig. 4.

4.3. Cobalt sepulchrate as soluble mediator for $P450_{cam}$ biosensor

Many redox proteins do not give faradaic currents either with a carbon electrode or metal electrodes (Au, Ag, Pt), possibly due to electrode fouling, slow interfacial electron transfer, and the location and orientation of the redox centre in the enzyme [18,19]. Electroactive mediators can be used to facilitate electrical communication between the redox centres of the enzymes and the electrode surface. The main requirement is that the electron transfer mediator compound exhibits fast reversible electrochemistry [20]. In principle, ΔE_p and $I_{p,a}/I_{p,c}$ values for a reversible one electron cyclic voltammetric process at moderate scan rate (~100 mV s^{-1}) would be 59 mV and 1.0, respectively, at 25°C [10]. Cobalt sepulchrate was used as a soluble electron transfer mediator for the $P450_{cam}$ enzyme electrode. The ΔE_p value of 60 ± 7 mV and the $I_{p,a}/I_{p,c}$ value of 0.92 ± 0.09 computed from the cyclic voltammogram in Fig. 2(a), showed that $Co(Sep)^{3+}$ exhibited reversible electrochemistry. Within the limits of experimental error, the two electrochemical parameters for reversibility have values that confirm fast reversible behaviour of the mediator.

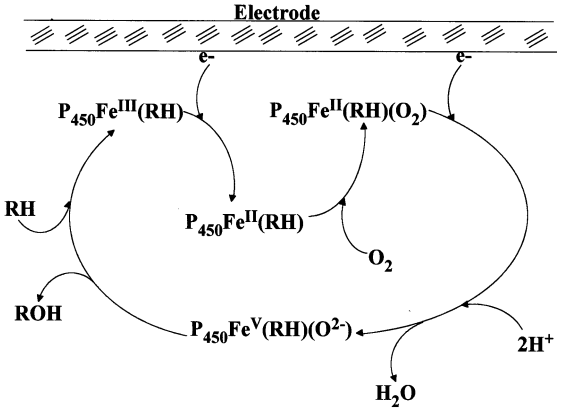

Fig. 4. Suggested scheme for the electrocatalytic oxygenation reaction of substrate-bound $P450_{cam}$.

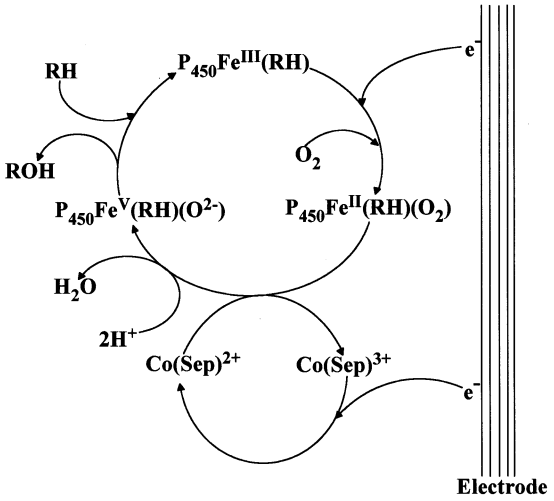

Fig. 5. Suggested scheme for the cobalt sepulchrate-mediated electrocatalytic hydroxylation reaction of *Cyt* $P450_{cam}$.

4.4. Steady-state amperometry

Amperometric determinations were not carried out with the $P450_{cam}$-DAB electrode because the electrode in its present form was not stable under hydrodynamic conditions. However, electrodes containing the enzyme in glutaraldehyde matrix were stable when used in the rotating disk electrode mode. The reaction scheme for the $Co(Sep)^{3+/2+}$-mediated amperometric determinations of camphor with a $Pt/P450_{cam}$ electrode is given in Fig. 5. Because I_{max} for a biosensor is related to the turnover rate constant of the sensor, it can be used as an index of sensor sensitivity. From the data in Table 1, it is clear that the biosensor containing ethylenediamine is more sensitive to substrates than the sensor that contains bovine serum albumin. The enhanced sensitivity is probably due to the improvement in electron transport in the biosensing layer. The substrate concentration range where the sensor is applicable may be indicated by the apparent Michaelis–Menten constant (K'_m) value of the biosensor. The K'_m value for a substrate/sensor system gives an indication of substrate concentration above which the biosensor is saturated. The K'_m values depict the upper limit of the linear range of the calibration curve attainable by the biosensor [21]. From the results in Table 1, changing from an enzyme electrode containing bovine serum albumin to that containing ethylenediamine, increases the K'_m value by factors of 2.55 and 2.05 for fenchone and adamantanone, respectively. Change of electrode material did not seem to affect the binding characteristics of camphor. The implication is that the range of linear response of the biosensor for substrates such as fenchone and adamantanone can be increased by introducing ethylenediamine in the sensor matrix.

5. Conclusion

The electrochemical reactivity of $P450_{cam}$ can be enhanced by immobilising the electrode in the membrane-like DAB vesicle dispersion which dries on the electrode surface to form $P450_{cam}$-DAB liquid crystal films. These films permit fast reversible electrochemistry of the haem $Fe^{3+/2+}$ redox system in $P450_{cam}$. Cyclic voltammetric and amperometric experiments show that the observed current can be related to the amount of substrate present in the solution. Thus the $P450_{cam}$-DAB electrode can be used as a biosensor for the various substrates. The $P450_{cam}$-DAB system was found to be unstable under hydrodynamic conditions. Studies are now in progress to stabilise the biosensor by encapsulation with a methyltriethoxysilane (MTEOS) sol-gel. Figs. 4 and 5 represent suggested reaction schemes rather than detailed mechanism for the proposed electrocatalytic hydroxylation of camphor using $P450_{cam}$ biosensor. It is quite possible that, as for most haem-containing proteins, the haem Fe passes through a ferryl oxo intermediate and there is an involvement of H_2O_2 in the electro-hydroxylation reaction of $P450_{cam}$. Further experiments need to be carried out to ascertain the role of these species in the electrocatalytic reaction between camphor and the cytochrome $P450_{cam}$ bioelectrodes.

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