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Spectroelectrochemistry of cytochrome P450cam

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Abstract

The spectroelectrochemistry of camphor-bound cytochrome P450cam (P450cam) using gold electrodes is described. The electrodes were modified with either 4,4′-dithiodipyridin or sodium dithionite. Electrolysis of P450cam was carried out when the enzyme was in solution, while at the same time UV–visible absorption spectra were recorded. Reversible oxidation and reduction could be observed with both 4,4′-dithiodipyridin and dithionite modified electrodes. A formal potential ($E^{0\prime}$) of -373 mV vs Ag/AgCl 1 M KCl was determined. The spectra of P450cam complexed with either carbon monoxide or metyrapone, both being inhibitors of P450 catalysis, clearly indicated that the protein retained its native state in the electrochemical cell during electrolysis.
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There are many compounds in the human's environment which are harmful. Many of them are metabolized by the monooxygenase systems cytochrome P450. This enzyme reaction may also be used to detect such compounds using appropriately designed biosensors. The main problem, however, in doing so is the complexity of the monooxygenase systems which require flavin and iron–sulfur proteins and NAD(P)H as electron supplying components. Electrochemical delivery of the required electrons may therefore be an interesting alternative to facilitate biosensor development. One important but still not solved question is whether cytochrome P450 remains native when interacting with electrodes. Spectroelectrochemistry allows to get an insight into the structural changes accompanied by the electrochemical redox cycling [1]. In this work we describe the first spectroelectrochemical study on mediator-free wild type P450cam reduction using thiol modified Au electrodes.

Cytochrome P450cam, also known as (1R)-camphor 5-exo-monooxygenase (EC 1.14.15.1), is an oxidoreductase isolated from the bacterium *Pseudomonas putida*. Its crystal structure was first elucidated by Poulos [2]. It consists of 414 amino acids with a molecular weight of 46,600 Da. Its active site is deeply buried within the protein and consists of an iron protoporphyrin IX complex with a deprotonated cysteine as proximal iron ligand (heme). P450cam catalyzes the regio- and stereo-specific hydroxylation of (1R)-camphor to 5-exo-hydroxycamphor according to the reaction 1.

$$+ \; NADH \; + \; O_2 \; + \; H^+$$

$$\xrightarrow{2e^-} \cdots OH \; + \; NAD^+ \; + \; H_2O \qquad (1)$$
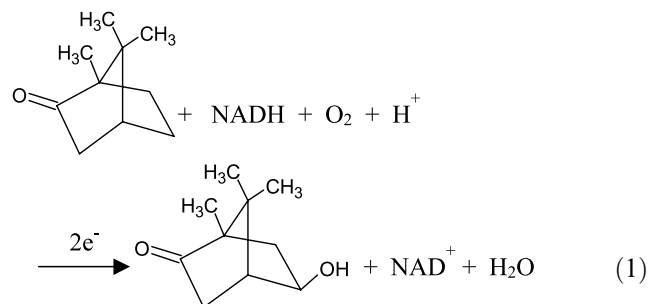
Camphor binds to the active site just above the heme distal surface and is held in place by a hydrogen bond between its carbonyl and the side chain hydroxyl group of Tyr96 and by hydrophobic contacts of its methyl

groups C-8, C-9 to Val-295, and Asp-297 in the β_3-sheet, and of the methyl group C-10 to Val-247 in the I-helix and Thr-185 in the F-helix [2]. The reaction occurs in several steps including two reduction reactions followed by oxygen activation. The electrons are derived from NADH and transferred first to putidaredoxin reductase. They are then transferred to the heme of P450cam through an iron–sulfur protein putidaredoxin (Pdx), which is the direct redox partner of P450cam and forms a complex with it. The first reduction step is considerably slower in the absence of camphor than in its presence [3]. The ferric heme center in the absence of camphor is six-coordinate and in the iron low-spin state (~95%), with a cluster of six water molecules complexed to the iron at the distal position. Substrate binding excludes water from the active site causing a change to the high-spin state accompanied by a decrease in the polarity of the heme environment. This causes a positive shift of the formal potential ($E^{0\prime}$) from $-303\,mV$ vs NHE to $-170\,mV$ vs NHE (corresponding to the shift from -540 to $-407\,mV$ vs Ag/AgCl 1 M KCl) making the first reduction step thermodynamically favourable [4,5].

Other compounds than camphor, such as compounds of environmental and industrial interest, have also been used as substrates for P450cam. Oxidation of styrene, for example, has been achieved using both wild type and the Y96F mutated form of the enzyme [6]. Naphthalene, pyrene, butane, and propane have also been reported as substrates for P450cam [7,8]. Furthermore, Castro et al. and Walsh et al. [9,10] have described the dehalogenation reactions of hexachloroethane, carbon tetrachloride, and other polyhalomethanes by P450cam and some of its mutants.

All these reactions have been monitored and investigated in the presence of P450cam biological redox partners. Several reports have already been published where the biological electron delivery is replaced by electrochemical means and P450cam is either immobilized or in solution [3,11–14]. The enzyme has been immobilized on carbon electrodes such as glassy carbon and pyrolytic graphite modified with sodium montmorillonite [11], synthetic membrane film (didodecyldimethylammonium bromide) [12], and dimyristoyl-L-α-phosphatidylcholine film [13]. Zu et al. [14] have used P450cam in thin protein-polyanion film on mercaptopropane sulfonate coated Au electrodes.

Recently, a spectroelectrochemical study of P450cam and its active site mutant Y96F has been reported using nano-crystalline antimony-doped tin oxide electrodes [15] where phenosafranine as soluble mediator transferred the redox equivalents between the electrode and the proteins. In contrast to this study, however, we were able to monitor spectroscopically the mediator-free reduction of wild type P450cam using thiol modified Au electrodes and present proofs for the native state of the electrochemically reduced P450cam.

Experimental

Cytochrome P450cam from *Pseudomonas putida* expressed in *Escherichia coli* TB1 was isolated and purified as described [16]. The absorbance ratio $\varepsilon_{392\,nm}/\varepsilon_{280\,nm}$ of the purified protein was 1.4. The purified protein was finally dialyzed against 100 mM potassium phosphate buffer, pH 7, 500 μM (1R)-camphor (Sigma, Deisenhofen, Germany).

Spectroelectrochemical measurements were carried out at a thiol-modified gold (Au) capillary electrode, 1 cm long and 350 μm in diameter, which was machined from a 3 mm diameter Au rod. The electrode was placed between two PEEK crossings and came into contact with the optical fibers FCB-UV 400/050-2 and FC-UV 200 (TOP Sensor systems, Eerbreek, Netherlands) (Fig. 1B). The optical fibers were connected to a light source DH-2000 (TOP Sensor systems, Eerbreek, Netherlands) on one side and a fiber optic spectrometer SD 2000 (Ocean Optics, Dunedin, Florida, USA) on the other side (Fig. 1A). The spectrometer was connected to a personal computer and the spectra were handled by the computer software Spectra Win version 4.2 (TOP Sensor systems, Eerbreek, Netherlands). A platinum wire and an Ag/AgCl 1 M KCl electrode were used as counter- and reference electrodes, respectively. The electrodes were connected to a LC-3E potentiostat (BAS, West Lafayette, USA).

Prior to modification the electrode was cleaned with Piranhas solution (concentrated H_2SO_4: 30% H_2O_2 1:4) overnight. It was then immersed into either saturated 4,4′-dithiodipyridin (Aldrich, Taufkirchen, Germany) or sodium dithionite (Aldrich, Taufkirchen, Germany) solution for 1 h. Excess 4,4′-dithiodipyridin or sodium dithionite was removed by injecting through the capillary several milliliters of distilled H_2O. The electrode was then placed between the two PEEK crossings and 30 μl of 100 mM K-phosphate buffer/50 ml KCl, pH 7.4, was injected. The light source was activated and allowed for 1 h to reach a stable performance. A blank spectrum was recorded only in the presence of the 100 mM K-phosphate buffer/50 ml KCl, pH 7.4. Thirty microliters of 12.5 μM of oxidized P450cam was then injected into the capillary while at the same time the previously injected buffer exited the capillary from an oulet. A potential was applied on the electrode and the protein was left for 10 min equilibration before spectra were measured. Spectra were recorded while the electrode potential was changed from +100 to −700 mV in steps of 100 mV.

For the spectra of the carbon monoxide (CO) complex, P450cam was purged with CO gas for 5 min and then injected into the dithionite modified Au capillary. First a potential of +100 mV was applied and the spectrum was recorded. A potential of −700 mV was then applied and the spectrum was also recorded.

For the metyrapone spectra, 3.67 mM of metyrapone was mixed with 12.5 μM of oxidized P450cam and 1 mM (1R)-camphor. The mixture was then injected into the dithionite modified Au capillary and spectra were taken first at +100 mV and then at −700 mV.

Results and discussion

It is already known that the electrochemistry of proteins such as P450cam is extremely difficult to be seen in solution with an unmodified metal electrode because the heme is buried deeply in the protein [2]. Modified electrodes such as glassy carbon (GC), pyrolytic graphite (PG), carbon cloth, tin oxide, and Au have therefore been widely used to investigate the electrochemical characteristics of cytochrome P450cam. In particular, Lei et al. [11] and Iwuoha et al. [12,17] have used GC electrodes modified with sodium montmorillonite and didodecyldimethylammonium bromide,
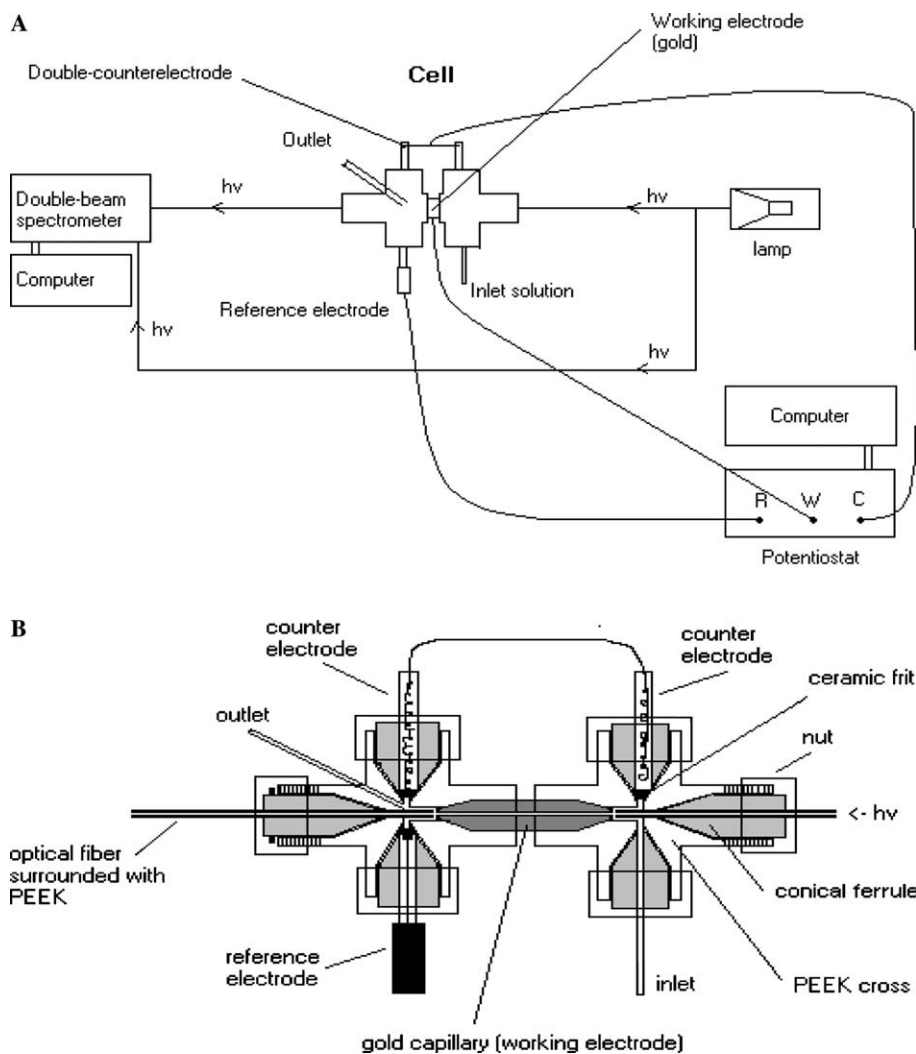
Fig. 1. (A) Scheme of the spectroelectrochemical setup. Light is emitted from the light source and then split to two beams. One beam passes through the cell before arriving to the double beam spectrometer and the other goes directly to the spectrometer and is used as reference. (B) Scheme of the spectroelectrochemical cell. The Au capillary electrode in the center is sandwiched between two PEEK crossings, where it comes into contact with the optical fiber. A double counter electrode and a Ag/AgCl-reference electrode are also placed close to the Au electrode.

respectively. Synthetic membrane films have also been used to modify PG electrodes [13], Au and carbon cloth electrodes [13]. Direct electron transfer has also been observed using multilayer modified Au and PG electrodes based on the use of alternating P450cam/polycation layers [33]. Mediated spectroelectrochemistry based on the use of antimony-doped tin oxide electrodes has been used to determine the influence of mutations on the redox potentials [15]. Using the newly developed spectroelectrochemical system we have now been able to observe for the first time mediator-free direct electron transfer between P450cam with the 4,4′-dithiodipyridin and dithionite modified Au electrode. Although the spectrum of the oxidized protein in the absence of gold modifier (4,4′-dithiodipyridin and dithionite) was clearly visible at 100 mV (spectra not shown), upon application of a potential of −700 mV the spectrum of the reduced protein was not detected. Instead, the Soret peak re-

mained at 393 nm but the absorbance decreased. After 50 min the Soret peak shifted to 420 nm and could not undergo further oxidation or reduction (data not shown). This is because the inactive form of cytochrome P450cam, cytochrome P420, was formed. Thus, no electron transfer could be observed in the absence of thiols but protein denaturation. These results indicate that an important function of the thiols is to prevent direct adsorption of P450cam on the electrode, which may cause protein denaturation. Taniguchi et al. [18] were the first to use thiols to investigate the electrochemistry of heme proteins by immobilizing sulfur bridged dipyridines to gold electrodes. In addition to preventing protein adsorption on the electrode, thiols may also orientate the active site of the enzyme toward the surface of the electrode, so decreasing the distance between the protein and the electrode and enhancing electron transfer. This has previously been shown to

occur with the heme enzyme horseradish peroxidase [19]. Furthermore, the large surface–volume ratio of the Au capillary electrode together with the 10-min incubation period used in this study may also explain the ability to observe electron transfer between P450cam in solution and the electrode.

At a 4,4′-dithiodipyridin modified electrode reversible oxidation and reduction was evident (Fig. 2). The spectra of the oxidized and reduced state obtained were characteristic for P450. Direct electron transfer has also been shown with other P450 species such as P450 1A1 [20], P450 1A2 [21,22], P450 3A4 [23], P450 4A1 [24], P450 2B4 [22,36], P450 BM3 [25], P450cin [26], and P450scc [22], but no information about whether the P450 protein was indeed in the native state could be provided. On the other hand, spectroelectrochemistry has been achieved with other heme proteins such as cytochrome *c* [1], cellobiose dehydrogenase [27], hemoglobin [28], myoglobin [29,30], and cytochrome *c* oxidase [31] to be helpful in characterizing structural properties.

In our studies with the 4,4′-dithiodipyridin modified electrode the Soret band for the oxidized P450cam at +100 mV appeared as a broad band with the main maximum at 393 nm indicating that the high-spin state is mainly populated. The shoulder at about 420 nm may originate from P450cam in the low-spin state although a 100% high-spin state population should be expected for the camphor-bound protein. The origin of this high-spin/low-spin state mixture in this sample is not clear. However, the small band at 646 nm of the oxidized form is consistent with the 393 nm Soret band and confirmed the high-spin state of the enzyme. When a potential of −700 mV is applied the Soret band shifts to 417 nm.
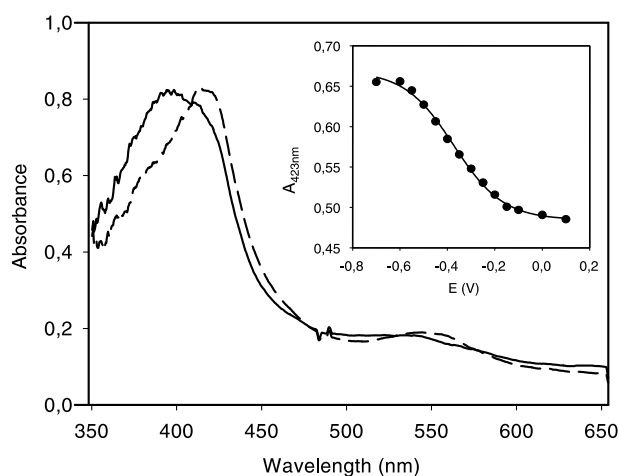
Although this Soret band position is slightly red-shifted compared to the Soret band of the protein chemically reduced with dithionite (at 408–412 nm) the visible band at 550 nm clearly indicates the reduced state. This reduced state is formed with increasing negative potential applied. The formal potential ($E^{0\prime}$) was found from the graph of the absorbance at 423 nm against the applied potential (Fig. 2). The points were fitted to the Eq. (2), which was derived from the Nernst equation and Lambert–Beer law

$$A = \varepsilon_{\mathrm{red}} \cdot l \cdot C_{\mathrm{t}} + l(\varepsilon_{\mathrm{ox}} - \varepsilon_{\mathrm{red}})$$
$$\times \frac{C_{\mathrm{t}} \cdot \exp\left[(E - E^{0\prime})\,\mathrm{n}F/RT\right]}{1 + \exp\left[(E - E^{0\prime})\,\mathrm{n}F/RT\right]}. \tag{2}$$

An $E^{0\prime}$ of −373 mV was then found from the intercept at the mid-point of the linear part of the curve. This value is in agreement with previously reported values for immobilized P450cam such as −361 mV [11] and −380 mV [12] vs Ag/AgCl 1 M KCl but slightly more positive than the reported theoretical value of $E^{\circ\prime} = -407$ mV vs Ag/AgCl 1 M KCl for the protein in solution [4]. On the other hand, the use of graphite and Au electrodes gave a variety of $E^{0\prime}$ [13,14,33]. In particular, using graphite electrode Rusling and co-workers [13] found a distribution of $E^{0\prime}$ in films containing P450cam; values of $E^{0\prime}$ ranging from −231 to −350 mV vs Ag/AgCl 1 M KCl were obtained. Similar values were also obtained with Au electrodes [14,33].

Spectra obtained with the dithionite modified electrode also indicated reversible oxidation and reduction of P450cam. The spectrum of the oxidized protein has the Soret band at 393 nm and the small band at 646 nm indicating the high-spin state of the heme iron. Some contribution of low-spin state population is seen as shoulder at 420 nm (Fig. 3). The Soret peak for the reduced form appeared at 417 nm with a shoulder at about 442 nm. This shoulder together with a broad band at ∼360 nm is sometimes observed for the reduced P450cam and may reflect the coordination of a nitrogen base as sixth iron ligand [39]. Whether it results from the agent which is attached on the electrode after modifying is not yet clarified. Nevertheless, the protein is not denatured as the spectrum for the CO complex clearly shows (Fig. 4). A solution of oxidized P450cam was exposed to CO and then reduced by the dithionite modified Au capillary electrode. The spectrum in Fig. 4 shows the typical P450cam–CO Soret band at 446 nm and the UV band at ∼370 nm [16,35], which is normally not seen when reduced with dithionite itself because of the overlay with the long-wavelength wing of the strong dithionite absorption band at 312 nm. The Soret band at 446 nm arises only when the structure of P450cam is in its native state as a result of a mixing of a thiolate sulfur/iron orbital charge transfer transition with the porphyrin π–π* transitions [34,35]. Protonation or displacement

Fig. 2. Spectra of 12.5 μM P450cam in 100 mM K-phosphate buffer containing 50 mM KCl and 1 mM (1R)-camphor, taken with a 4,4′-dithiodipyridin modified Au electrode. Solid line, oxidized P450cam at +100 mV. Dashed line, reduced P450cam at −700 mV. Inset: relationship of absorbance at 423 nm with applied potential *E*. $E^{0\prime}$ was calculated as described in the text.
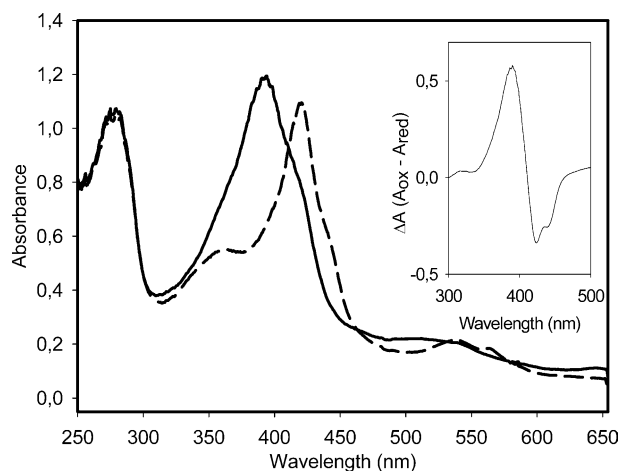
Fig. 3. Spectra of 12.5 μM P450cam in 100 mM K-phosphate buffer containing 50 mM KCl and 1 mM (1R)-camphor taken with a dithionite modified Au electrode. Solid line, oxidized P450cam at +100 mV. Dashed line: reduced P450cam at −700 mV. Inset: difference spectrum of the absorbance of oxidized minus reduced P450cam.
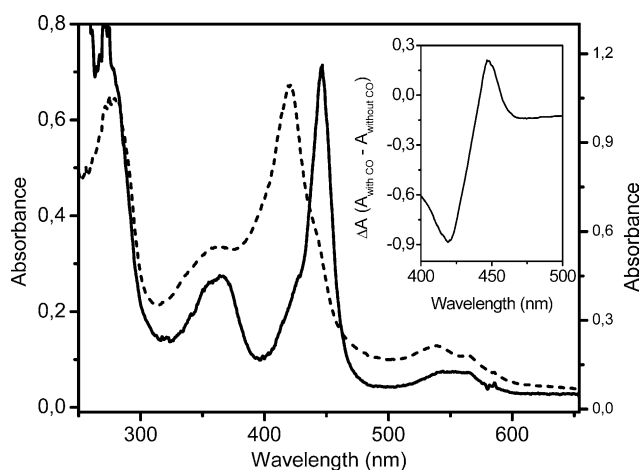

Fig. 4. Effect of CO. Spectra of electrochemically reduced P450cam in the presence and absence of CO. Dashed line, reduced P450cam at −700 mV without CO. Solid line, reduced P450cam at −700 mV with CO. Inset: difference spectra of the absorbance of reduced P450cam with CO minus reduced P450cam without CO. A dithionite modified Au-capillary electrode was filled with a solution containing 12.5 μM P450cam in 100 mM K-phosphate buffer, 50 mM KCl, and 1 mM (1R)-camphor. CO was introduced prior to introduction to the capillary. The protein was reduced by applying a potential of −700 mV and CO binding was observed (solid line).

of the thiolate or even lengthening of the Fe–S(thiolate) bond would give rise to the inactive P420 species [34,35]. The Soret absorption maximum for the CO–P420 complex lies at 420 nm. In our case, a peak at 420 nm was not observed and also no shoulder at 420 nm in the CO difference spectrum (inset of Fig. 4) was seen indicating that P450cam was native. This is the first spectroscopic proof that the P450cam remains in the native state during electrolysis although several reports on the

direct electron transfer already exist [6,12,14,15,32,33]. Only in one report [13], the native P450 structure was demonstrated afterwards by taking the absorption spectrum of the P450cam–CO complex where the P450 was obtained after the electrolysis by redispersing oxidized P450cam from the lipid layers, reducing it with sodium dithionite and exposing to CO.

The native structure of P450cam in the electrolysis cell is indicated also by binding of metyrapone, a common inhibitor of P450cam preventing hydroxylation of cam-
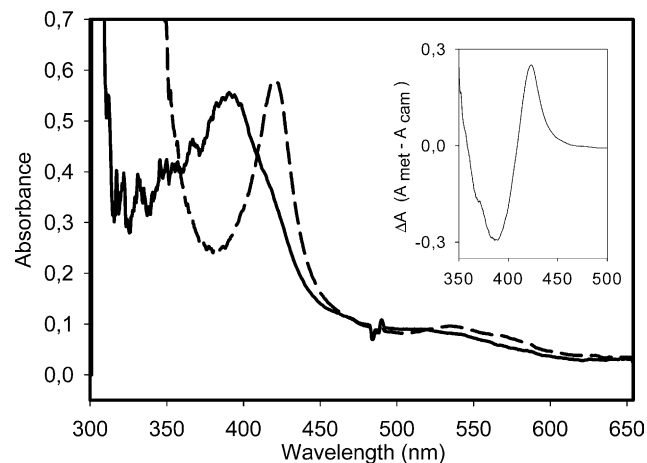

Fig. 5. Spectra of camphor bound P450cam in the presence of 3.67 mM metyrapone (met) and without metyrapone. P450cam-solution was as in Fig. 4. Spectra were recorded with a dithionite modified Au electrode at a potential of +100 mV. Solid line, oxidized P450cam in the presence of camphor (cam). Dashed line, oxidized P450cam in the presence of camphor and metyrapone. Inset: difference spectra of the absorbance of oxidized P450cam with metyrapone minus oxidized P450cam with camphor.
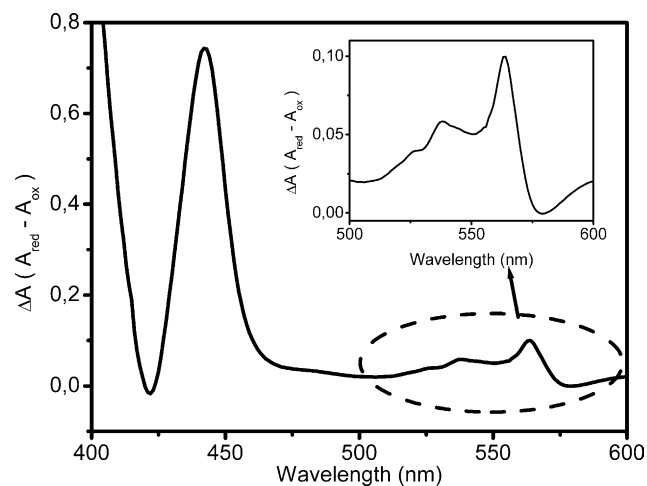

Fig. 6. Difference spectrum of the absorbance of electrochemically reduced P450cam at −700 mV with metyrapone minus electrochemically oxidized P450cam at +100 mV with metyrapone. Spectra were taken with a dithionite modified Au electrode. 12.5 μM P450cam, in 100 mM K-phosphate buffer containing 50 mM KCl, were mixed with 1 mM (1R)-camphor and 3.67 mM metyrapone. Inset: magnification of the region from 500 to 600 nm.

phor [37–39]. As shown in Fig. 5, addition of metyrapone to the oxidized enzyme caused a shift of the Soret band from 393 to 420 nm. Electrochemical reduction led to the appearance of two peaks at 441 and 564 nm (Fig. 6). These peaks as well as the pattern of peaks observed in the 500–600 nm region (Fig. 6, inset) are characteristic of the binding of metyrapone to the heme center of the native enzyme [39]. This is in agreement with previously reported works, where P450cam was reduced chemically with sodium dithionite and metyrapone was bound [39].

Conclusions

The spectroelectrochemistry of cytochrome P450cam using a 4,4′-dithiodipyridin and dithionite modified gold capillary electrode is described. Reversible oxidation and reduction has been seen with both modified electrodes. An $E^{0\prime}$ of -373 mV vs Ag/AgCl 1 M KCl for camphor-bound P450cam in the absence of inhibitors could be calculated. Finally, spectra obtained in the presence of either CO or metyrapone during electrolysis were indicative that P450cam remains in the native state during electrolysis This is the prerequisite for a further development of a P450cam biosensor based on 4,4′-dithiodipyridin and dithionite modified gold capillary electrodes for monitoring compounds of environmental interest.

Acknowledgments

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