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The Relationship Between the Redox Reaction of Camphor-induced Cytochrome P-450 and its Activity

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ABSTRACT

The relationship between the redox reaction of camphor-induced cytochrome P-450 (P-450$_{cam}$) and its activity was measured by using cyclic voltammetry. The redox potential of P-450$_{cam}$ solution shifted to the lower side of the potential by binding of substrate, and the change was proportional to the amount of the substrate binding to the protein. The substrate binding was inhibited at the low concentration of oxygen in the reaction solution. The reaction product, hydroxycamphor, was observed in the reaction mixture by gas chromatography/mass spectroscopy. However, hydroxycamphor was not observed at an oxygen concentration of about a tenth part of the saturated one. The shift of redox potential of P-450$_{cam}$ solution corresponded to the substrate specificity of the activity. These results suggest that the redox reaction of P-450$_{cam}$ related to the substrate-binding to the protein and its activity. Furthermore, the present system was very simple and speedy for the measurement of the activity. Copyright © 1999 John Wiley & Sons, Ltd.

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KEYWORDS: cytochrome P-450$_{cam}$; redox reaction; hydroxylation activity; cyclic voltammetry; substrate specificity

INTRODUCTION

Cytochrome P-450s (P-450s) are heme-containing monooxygenases [1] and are usually classified in two categories, mitochondrial [2–4] or microsomal [5, 6] types, depending on the component of the electron transport system. P-450s play important biochemical roles, such as drug metabolism [7–9], steroid hormone biosynthesis [10–12], and so on. Therefore, P-450s are very useful for sensing and reacting to important materials as related to the physiological function, but they are difficult to apply as such a biosensor and a bioreactor, because they are almost membrane-bound protein. A camphor-induced P-450 (P-450$_{cam}$) catalyzes the hydroxylation of its physiological substrate, camphor [13]. P-450$_{cam}$ is the only water-soluble P-450 species, and its electron transport system is the same as its mitochondrial type. We have already reported the immobilization of P-450$_{cam}$ by polypyrrole on the electrode and the electrochemical control of its activity [14]. It was also reported that substrate specificity of P-450$_{cam}$ did not change by

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immobilization [15]. At that time, the activity of P-450_{cam} was evaluated by the decrease of oxygen concentration in the solution, but at least 1 hr was needed for the measurement. Therefore, other experimental conditions, such as speed and ease of technique, are desirable.

It was reported that the redox potential of the heme-iron of P-450_{cam} was changed by the binding of the substrate [16–18]. So that, if the change of the potential relates the substrate specificity of P-450_{cam} activity, the new easy assay system of the activity will be established. However, the redox potentials are such that it is difficult to distinguish P-450_{cam} from polypyrrole because their potentials are near. Therefore, in the present study the relationship between the redox reaction of P-450_{cam} solution and its activity was examined without polypyrrole.

EXPERIMENTAL

Materials

P-450_{cam} was purified from camphor-induced *Pseudomonas putida PpG 786* by the method described previously [19, 20]. The purified P-450_{cam} was dialyzed against 50 mM potassium phosphate buffer (pH 7.4) containing 0.2 M KCl (Buffer A) immediately before use. Indium tin oxide (ITO) electrode was obtained from Nihon Ita Glass Co. Ltd. Various substrates, (+)-camphor and (±)-camphor were from Wako Pure Chem. Industries, and (−)-camphor, norcamphor and camphene were from Aldrich Chem. Co. Other reagents used were of high grade without purification.

Cyclic Voltammetry (CV) of P-450_{cam} Solution

The ITO electrode was cut into the size of $1 \times 10\,cm^2$, and washed with distilled water with sonication, and then further washed with organic solvents, such as acetone and ethanol. Part of the ITO ($1 \times 4\,cm^2$) was put into a semipermeable membrane that contained 7 ml of 0.07 mg/ml P-450_{cam} solution. Buffer A with the appropriate concentration of substrate was used as the outer solution of the membrane. The saturated calomel electrode (SCE) and Pt were used as reference and counter electrodes, and they were put into the outer solution, and then stirred slowly with a magnetic stirrer. The CVs were measured by sweeping repeatedly among +0.1 V and −0.5 V with the rate of 60 mV/sec at room temperature using Cyclic Polarograph P-900 (Yanako Co. Ltd).

Measurement of Gas Chromatography/Mass Spectroscopy (GC-MS)

Camphor and oxidized analog were extracted by chloroform, and the molecular weight was analyzed with GC (Hewlett Packard 5890)-MS (EI) (Hitachi M-80B mass spectrometer) [21]. The column used was DB-1 (J & W fused silica megabore column). The temperature was over 110 °C for 1 min followed by elevating the temperature to 220 °C at 5 °C/min, the injection temperature was 220 °C, and the flow rate was 60 ml/min.

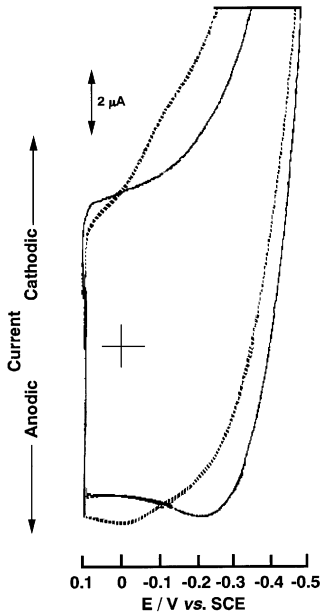

FIGURE 1. The CVs of P-450_{cam} solution in the absence (dotted line) and presence (solid line) of 0.1 mM camphor under saturated oxygen (1.10 ppm) in the reaction solution.

RESULTS

Figure 1 shows the CVs of P-450_{cam} solution in the absence and in the presence of substrate, camphor. Anodic and cathodic peaks were observed at 0.0 V and −0.1 V, respectively, in the absence of camphor. When 0.1 mM camphor was added to the solution, the anodic peak shifted from 0.0 V to −0.2 V, and the cathodic peak at −0.1 V disappeared. As shown in Fig. 1, the amounts of change of these peak currents were less than background currents, so the amounts of change were calculated as follows. At first, when the areas of two ranges, +0.1 V to −0.1 V (S_A) and −0.1 V to −0.3 V (S_B), institute as two anodic peaks that made the center each other, their minimum areas, S_{A0} and S_{B0}, correspond to the presence of excess camphor and the absence of camphor. When the areas, ΔS_A and ΔS_B, are calculated by subtracting each area, S_{A0} and S_{B0}, from the areas that are measured under various concentrations of camphor, the sum of ΔS_A and ΔS_B is approximately constant. Therefore, the amounts of change of the anodic currents, S_{AR} and S_{BR}, were obtained by the following equations:

$$S_{AR} = \frac{\Delta S_A}{\Delta S_A + \Delta S_B} \quad (1)$$

$$S_{BR} = \frac{\Delta S_B}{\Delta S_A + \Delta S_B} \quad (2)$$

These values, S_{AR} and S_{BR}, were plotted against the concentration of camphor, and the result showed that S_{AR} decreased with increasing camphor con-

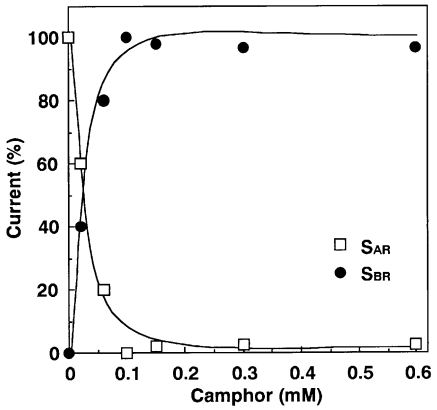

FIGURE 2. Plots of the ratios of the anodic peak currents, S_{AR} and S_{BR}, against the concentration of camphor under saturated oxygen (1.10 ppm) in the reaction solution.

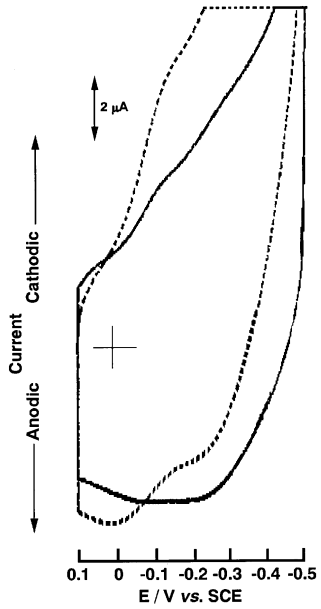

FIGURE 3. The CVs of P-450$_{cam}$ solution in the absence (dotted line) and presence (solid line) of 0.1 mM camphor at the low oxygen concentration (0.12 ppm).

centration, and then disappeared at a concentration of more than 0.1 mM (Fig. 2). Simultaneously, S_{BR} increased to correspond to that decrease. The oxygen in the reaction solution is generally needed for P-450 to catalyze the hydroxylation activity. Accordingly, the same experiment was performed by after removing the oxygen from the reaction solution as a reference. Here, oxygen was removed by bubbling the argon gas, and the concentration of oxygen in the solution was examined by using an oxygen electrode [22]. As the result, the oxygen concentration made 0.12 ppm from saturated state of 1.1 ppm by bubbling the argon gas. In the absence of camphor the shape of the CV of P-450$_{cam}$ at the low oxygen concentration was very similar to the case of saturated oxygen (Fig. 3). The anodic peak of 0.0 V shifted to −0.2 V by adding 0.1 mM camphor, but the peak of 0.0 V did not disappear, which has different from the case of saturated oxygen.

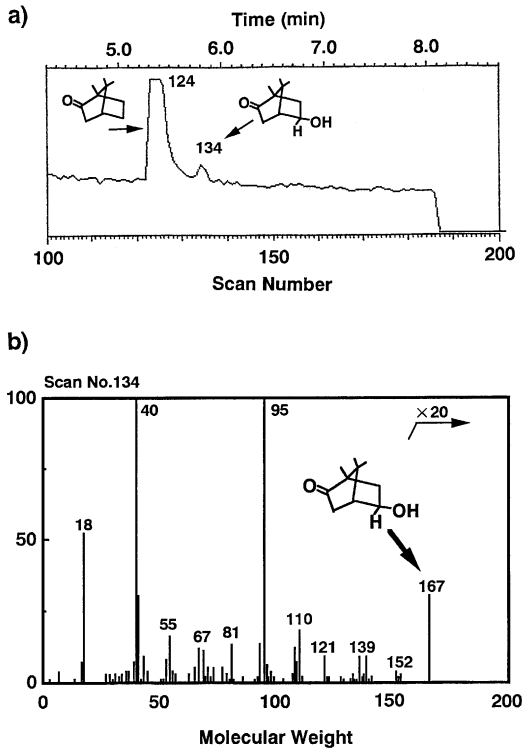

FIGURE 4. Gas chromatography and MS spectra of the extract reacted by P-450$_{cam}$ solution under saturated oxygen: (a) elution profile of gas chromatography; (b) MS spectra of no. 134. Experimental conditions were described in experimental section.

The extract by chloroform from the reaction mixture solution under saturated oxygen was applied to the GC analysis. Two peaks were observed in the elution profile (Fig. 4a). Their peaks were analyzed by MS, and it was found that the peak of no. 124 contained camphor and the peak of no. 134 contained the product of molecular weight of 167, which corresponds to hydroxycamphor (Fig. 4b). On the other hand, only one peak, which corresponds to camphor, was observed in the low concentration of oxygen (data not shown).

The semipermeable membrane separated the ITO electrode in P-450$_{cam}$ solution from the substrate solution in the present experimental system. The system was very useful because the P-450$_{cam}$ solution can be used repeatedly. However, it will be unsuitable if the membrane obstructs to approach the substrate to the P-450$_{cam}$ molecules near the electrode. So, the effect of diffusion of the substrate on the CV was measured by stirring the solution with a usual magnetic stirrer. The S_{AR} decreased from 100% to 0% with time, and S_{BR}, simultaneously, increased correspondingly (Fig. 5). Furthermore, their changes became constant at the time of more than 6–7 min.

Next, the relationship between the substrate specificity of P-450$_{cam}$ activity and its redox reaction was examined, and the result is shown in Table 1. In the case of enantiomer of (+)-camphor, (−)-camphor, the manner of changes of S_{AR} and S_{BR} were almost the same as in the absence of camphor.

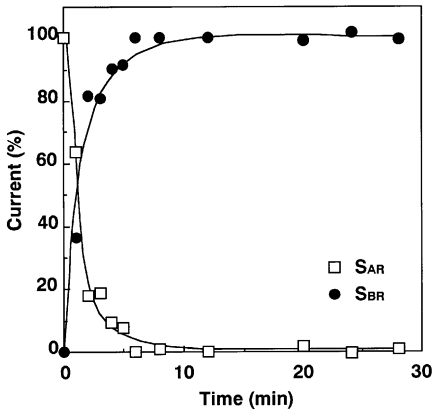

FIGURE 5. Time-dependent changes of the anodic peak currents, S_{AR} and S_{BR}.

TABLE 1. The Ratio of the Anodic Peak Currents, S_{AR} and S_{BR}, for Various Substrates. The Concentrations of All Substrates Were 0.1 mM

Substrate	Current (%)	
	S_{AR}	S_{BR}
–	100	0
(+)-Camphor	0	100
(−)-Camphor	98	2
(±)-Camphor	51	49
Norcamphor	99	1
Camphene	99	1

While in the case of their racemic mixture, (±)-camphor, S_{AR} and S_{BR} showed about a half value each other. In addition, the anodic peak did not shift for either norcamphor or camphene, as in the absence of camphor.

DISCUSSION

The mechanism of substrate hydroxylation cycle by P-450$_{cam}$ has been well studied [13]. Then it was known that the binding of substrate to P-450$_{cam}$ makes the redox potential of the heme-iron decrease [16–18]. Therefore, the present result of the shift of the anodic peak from 0.0 V to −0.2 V seems to be caused by the substrate binding. Although the sum of $\Delta S_A + \Delta S_B$ was approximately constant under low oxygen concentration as well as under saturated oxygen, the values of the sums were different from each other. Therefore, the amount of change of their anodic peak currents could not simply be compared. However, the shape of CV under low concentration was similar to that of about a half percentage of the maximum amount of change under saturated oxygen. The result suggests that about a half of P-450$_{cam}$ reacted to the ITO electrode does not bind to the substrate. The reason is not known, but the amount of substrate binding might decrease because the dissociation of substrate from P-450$_{cam}$ seems to be suppressed by the coordination of oxygen to the heme-iron. As shown in Fig. 1, the cathodic peak that corresponds to the anodic peak at −0.2 V was not observed. If the electrons transferred from the heme-iron of P-450$_{cam}$ to the substrate hydroxylation cycle, the anodic peak current would be reduced. However, the result did not show the reduction of the current, probably because the amounts of electrons were very few. Furthermore, the reaction product, hydroxycamphor, was observed only in the saturated state of oxygen in the reaction solution. Therefore, at least some electrons could undoubtedly be transferred to the cycle. However, it seems to need the detailed quantitative analysis of the reaction product to clear the reaction mechanism of the present experimental system.

The substrates were sufficiently diffused to the inner membrane solution for about 6–7 min even if the electrode separated from the substrate solution. The result suggests that the times of measurement of P-450$_{cam}$ activity could be reduced substantially in comparison with our previous reports [14, 15]. Furthermore, when the present system is applied to other P-450s in the future, such a partition by a semipermeable membrane would inhibit the many inclusions in blood, such as proteins, from obstructing the electrode reaction of P-450s.

The change of anodic peak current in the CV was clearly different during optical isomers of camphor, and the substrate specificity of P-450$_{cam}$ activity was almost the same as the native one. However, the CV of norcamphor did not change in spite of previous reports that norcamphor was the substrate for P-450$_{cam}$ [15]. The reason for this is twofold: by one is low affinity of norcamphor for P-450$_{cam}$ [23] and the other is the detection limit for the present CV measurement.

Above all, it was found that the redox reaction of P-450$_{cam}$ could be related to its enzymatic activity. The present experimental system was very simple and speedy, and will be useful to examine the interaction of P-450s and other components of electron transport system, such as ferredoxin, ferredoxin reductase and flavoprotein.

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