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# Substrate Specificity of Camphor-induced Cytochrome P-450 Immobilized on an Electrode

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## ABSTRACT

*The substrate specificity of a camphor-induced cytochrome P-450 (P-450$_{cam}$) was measured by using a new assay system: electrochemical control of P-450$_{cam}$ activity by protein immobilization on an electrode. Immobilized P-450$_{cam}$ showed the obvious substrate specificity for hydroxylation of the substrate, suggesting that the simple assay system is applicable for the study of the effect of the other components of the electron transfer system on activity. © 1998 John Wiley & Sons, Ltd.*

KEYWORDS: substrate specificity; cytochrome P-450$_{cam}$; immobilized enzyme; electrode; optical isomer; hydroxylation activity

## INTRODUCTION

Cytochrome P-450$_{cam}$ (P-450$_{cam}$), which is derived from *Pseudomonas putida*, catalyzes the hydroxylation of its physiological substrate, camphor [1]. Up to now, the substrate specificity of P-450$_{cam}$ has been mainly investigated by means of spectral change related to a spin state conversion of the heme iron [2–6]. However, the hydroxylation of substrate was not detected directly by the above method. We have first reported that P-450$_{cam}$ was immobilized on an indium tin oxide (ITO) electrode and its activity could be controlled artificially without other components of electron transfer system, such as NADPH, flavoprotein, putidaredoxin and putidaredoxin reductase [7]. Our assay system can directly detect the hydroxylation of substrate because oxygen in a solution decreases after the reaction. Therefore, in the present paper, the substrate specificity of P-450$_{cam}$ was examined by means of this system.

## EXPERIMENTAL

P-450$_{cam}$ was purified from camphor-induced *Pseudomonas putida PpG 786* by the method described previously [8, 9]. Immobilization of P-

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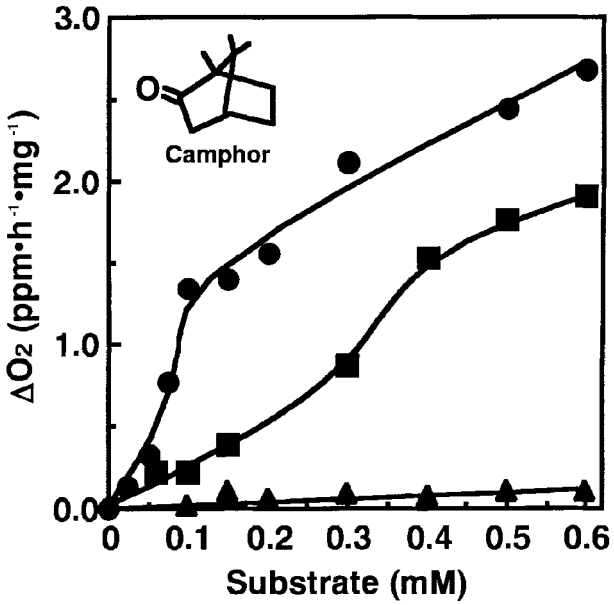


**FIGURE 1.** The hydroxylation activity of immobilized P-450$_{cam}$ for the camphor and its optical isomers as substrate: ● (+)-camphor; ▲ (−)-camphor; ■ (±)-camphor.

450$_{cam}$ on the ITO electrode and the measurement of its activity were performed according to the previous report [7]. P-450$_{cam}$ was immobilized on the electrode by covering with a polypyrrole chain, and the number of layers of the protein was at least 45. The activity of immobilized P-450cam was maintained for about one month after immobilization, and it was kept after repeated use of about 20 times. The electric potential was swept repeatedly between 0.4 and 0 V using a system of three kinds of electrodes, such as P-450$_{cam}$-immobilized ITO, Pt and SCE (saturated calomel electrode), and the amount of oxygen in the solution was measured with time. The specific activity of P-450$_{cam}$ was evaluated by the decrease of oxygen ($\Delta O_2$) per hour (ppm/hr) divided by the amount of P-450$_{cam}$ immobilized on the electrode (mg). The substrates used in this study were (+)-camphor, (−)-camphor, (±)-camphor, camphen, norcamphor and camphor-3-carboxylic acid.

## RESULTS AND DISCUSSION

Two optical isomers of camphor, (+)-camphor and (−)-camphor, and their racemic mixture, (±)-camphor, were added to the reaction solution, and the hydroxylation activity of immobilized P-450$_{cam}$ was measured. In the case of (+)-camphor the specific activity increased as its concentration increased in the solution, and the value of 2.6 ppm/h/mg was reached at the (+)-camphor concentration of 0.6 mM (Fig. 1). The activity was not observed for its enantiomer, (−)-camphor. Their racemic mixture, (±)-camphor, showed about a half-activity of (+)-camphor. The activity for (±)-camphor seems merely to reflect (−)-camphor in the mixture. In addition, the sigmoidal curves were observed in both (+)-camphor and (±)-camphor, probably because the substrate did not diffuse around immobilized P-450$_{cam}$ sufficiently in the first period.

Next, the activity of immobilized P-450$_{cam}$ was measured in the presence of several kinds of camphor analogs, such as camphor-3-carboxylic acid, norcamphor and camphen. In the case of camphor-3-carboxylic acid the activity increased with increasing its concentration similar to that of (+)-camphor to 0.1 mM (Fig. 2). However, the activity gradually decreased at a concentration of more than 0.1 mM. In the case of norcamphor a

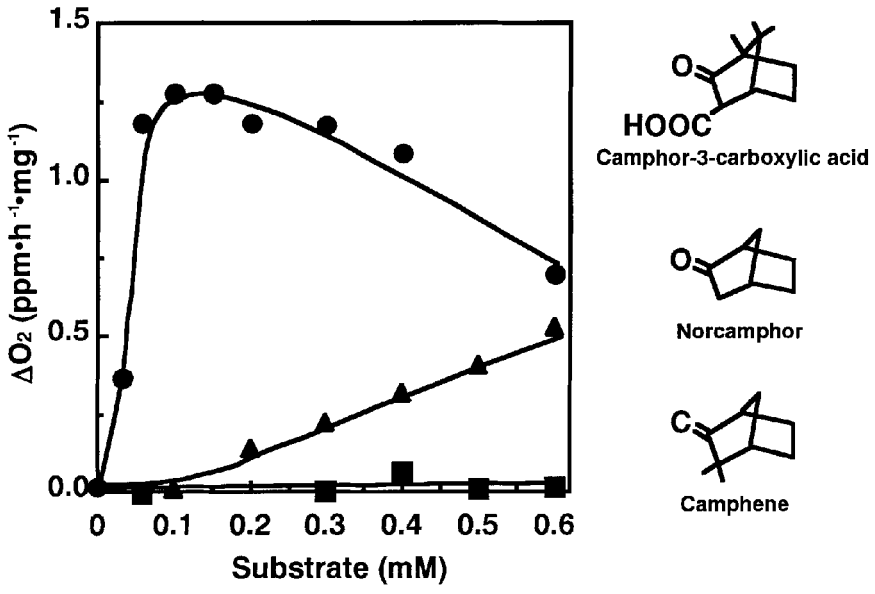


**FIGURE 2.** The hydroxylation activity of immobilized P-450$_{cam}$ for the camphor analogues as substrate: ● camphor-3-carboxylic acid; ▲ norcamphor; ■ camphen.

slight increase in activity was observed, and no activity for camphen was observed. It is known that for the hydroxylation of (+)-camphor by P-450$_{cam}$ both the coordination of carbonyl oxygen to heme iron and the binding of methyl groups to the hydrophobic region of the protein are necessary [1]. Therefore, the reason norcamphor and camphen were not hydroxylated seems to be that they have no methyl groups at 1- and 7-carbons of the camphor structure, and besides camphen has no carbonyl group. These results corresponded relatively well to the previous reports of binding affinity; the $K_d$ values of (+)-camphor and norcamphor for P-450$_{cam}$ were 1.6 μM and 150 μM, respectively [4, 6]. On the other hand, the carboxyl group of camphor-3-carboxylic acid does not seem to prevent the binding to P-450$_{cam}$. This consideration was supported by the fact that the $K_d$ value of 3-endo-bromocamphor for P-450$_{cam}$ was 48 μM [6]. It seems possible that the diffusion velocity of the substrate in polypyrrole chain changes by hydroxylation. Therefore, the decrease in activity observed in camphor-3-carboxylic acid might be caused by the decrease of turnover number of the enzyme.

It was reported that the activity was increased as the scan rate increased, so drawing a sigmoidal curve, in the case of (+)-camphor [7]. The same results were observed in (±)-camphor, norcamphor and camphor-3-carboxylic acid. The sigmoidal curve seemed to be caused by the balance between the rate of electron transfer in the substrate hydroxylation cycle and the rate of electron transpot from electrode to the protein.

Above all, it was found that the immobilized P-450$_{cam}$ showed the obvious substrate specificity with a hydroxylation of the substrate. Until now, other components of electron transfer system have been necessary for the measurement of P-450$_{cam}$ activity, so that it must take the interaction of the substrate and the other components into account for its activity, but the present system was very simple without such a consideration. Therefore, this system seems to be applicable for the study of the effect of the components on the activity.

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