# OXYGENATED CYTOCHROME P-450 AND ITS POSSIBLE ROLE IN ENZYMIC HYDROXYLATION

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SUMMARY: Upon addition of oxygen to the ferrous form of bacterial cytochrome P-450 ($P\text{-}450_{cam}$), a new spectral species of the enzyme was observed having absorption maxima at 555, 418 and 355 nm. This new species of $P\text{-}450_{cam}$ could be converted into carbon monoxy-$P\text{-}450_{cam}$ by the displacement of oxygen with carbon monoxide. Conversely, the carbon monoxide derivative could be transformed into the new species by extensive bubbling of the reaction mixture with oxygen. The EPR signal at g = 2.26, characteristic of low spin ferric $P\text{-}450_{cam}$, is absent in the new spectral species. Based on these findings, the new spectral species was interpreted to be an oxygenated form of $P\text{-}450_{cam}$, probably an intermediate in the overall hydroxylation reaction.

Cytochrome P-450 (P-450) has been shown to be a component of many monooxygenase reactions in the metabolism of steroids as well as various xenobiotics in vertebrate tissues (1,2). The detailed mechanism of its catalytic reaction, especially the mode of interaction with oxygen and carbon monoxide, has remained obscure. Recently, a soluble P-450 functional in methylene hydroxylation of D(+)-camphor from Pseudomonas putida was described by Katagiri et al. (3). The hydroxylation system consists of a reduced pyridine nucleotide dehydrogenase (flavoprotein), a non-heme iron protein (putidaredoxin) and the P-450 which was designated as $P\text{-}450_{cam}$ by the above authors. Subsequently, the physical, as well as chemical nature of the cytochrome has been extensively investigated (3-7).

## MATERIALS

$P\text{-}450_{cam}$ was extracted and purified 100-fold from cells of P. putida grown in a medium containing 6 mM camphor as sole carbon source. The cytochrome content of the preparation used in the present study was about 10 mμmoles per mg protein and contamination with other chromophores, such as hemoproteins, flavoproteins and non-heme iron, was negligible. The purification method, as well as

properties of our preparation, will be described elsewhere (7). Putidaredoxin was purified from the same bacterial extracts and the ratio of optical density at 455 nm to that at 278 nm of the preparation was 0.41, which is comparable to the value reported by others (8,9). A homogeneous sample of beef adrenodoxin was a kind gift of Mr. Wayne Taylor of this department.

## RESULTS

The P-450$_{cam}$ was isolated as the high spin ferric form having absorption maxima at 392, 540 and 645 nm (Figure 1A). Under anaerobic conditions it could be reduced to a ferrous state with absorption maxima at 409 and 545 nm, either by the addition of NADH in the presence of a catalytic amount of the flavoprotein dehydrogenase and putidaredoxin or by the addition of dithionite (Figure 1B). As reported by Katagiri et al. (3), the high spin form of P-450$_{cam}$ was changed into the low spin form by the removal of camphor. When

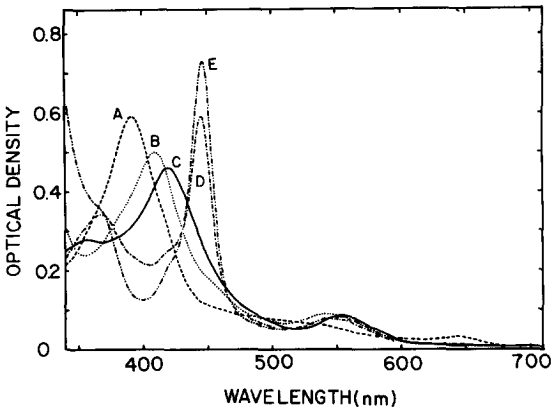


FIGURE 1 : Absorption Spectra of Various Forms of P-450$_{cam}$.

The reaction mixture contained 22 nmoles of P-450$_{cam}$, 0.35 mmoles of potassium phosphate buffer, pH 7.4, and 0.35 mmoles of potassium chloride in a final volume of 3.6 ml at 8°. The buffer was previously stirred for 15 minutes with an excess of solid D(+)-camphor at room temperature. Anaerobic conditions were obtained by gassing the reaction mixture with purified nitrogen and a small amount of antifoam B (Sigma) was used to prevent excess foaming. Spectra were recorded with a Cary 14R recording spectrophotometer equipped with a thermostated cell holder. A (-----), high spin ferric form; B (·····), ferrous form, obtained by the addition of sodium dithionite (0.3 μmoles to A under anaerobic conditions; C (———), the oxygenated form, obtained by bubbling oxygen at a rate of 40 ml per minute to B for 30 seconds; D (— · —), carbon monoxide form, obtained by bubbling CO for 30 seconds to C; E (— ·· —), carbon monoxide form, after addition of an excess of sodium dithionite to D and bubbling again with CO for 30 seconds.

oxygen was introduced into a reaction mixture containing ferro-P-450$_{cam}$ and camphor, a new spectral species appeared immediately, showing absorption maxima at 355, 418 and 555 nm and a shoulder around 580 nm (Figure 1C). Judged by the spectral characteristics of this compound, it is clearly distinguished from the other species of the enzyme.

The new form of P-450$_{cam}$ is rather stable at low temperatures (4-8°) in the presence of excess oxygen, but it is slowly converted into the high spin ferric state upon storage (Figure 2). The half-time for the decay of a 6 μM solution to the high spin form was about five minutes and forty minutes at room temperature and 6°, respectively. The half-time for the decomposition of the new species at 6° to the high spin ferric form was less than one minute in the presence of an amount of putidaredoxin stoichiometric with respect to the P-450$_{cam}$. Neither the same amount of putidaredoxin, which had been previously bleached by heating for fifteen minutes in a boiling water bath nor native oxidized adrenodoxin had an effect on the rate of decomposition of the new

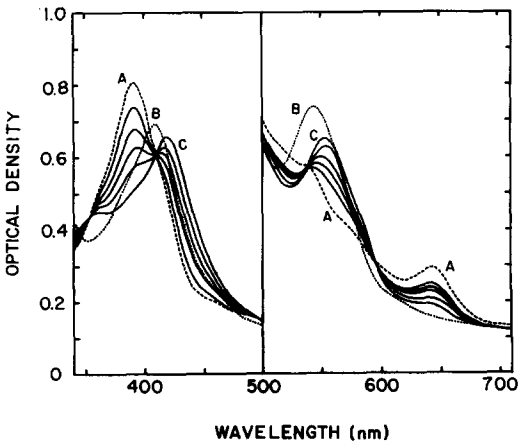


FIGURE 2 : Autodecomposition of Oxyferro P-450$_{cam}$ at 5°.

The oxygenated form of P-450$_{cam}$ was prepared by essentially the same procedure as that described in Figure 1. Left hand figure: P-450$_{cam}$ concentration was 8 μM in a buffer containing 20 mM potassium phosphate, pH 7.4, 20 mM potassium chloride; A (-----), ferric form; B (·····), ferrous form; C (———), the oxygenated form, at 2 minutes after addition of oxygen to B. The spectra were then recorded at 2, 28, 44 and 60 minutes after C, respectively. Right hand figure: P-450$_{cam}$ concentration was 48 μM in the same buffer as the left hand figure; A (-----), ferric form; B (·····), ferrous form; C (———), the oxygenated form at 2 minutes after addition of oxygen to B. The spectra were then recorded at 3, 11, 24 and 43 minutes after C, respectively.

species.

The ferrous enzyme, obtained either by chemical reduction with dithionite or enzymatic reduction by NADH, is capable of forming the new species only in the presence of oxygen. The new species could not be detected, either upon addition of oxygen or hydrogen peroxide to the ferric enzyme, or upon addition of an oxidant, such as hydrogen peroxide or ferricyanide, to the ferrous enzyme. The addition of ferricyanide to the new spectral species caused an instantaneous conversion to the ferric form. The formation of the new species required an excess of oxygen with respect to the ferrous enzyme. Addition of a small amount of oxygen to the ferrous enzyme caused its slow oxidation to the ferric state without forming the new species in a detectable quantity. Similar observations have been reported in the oxygenation of ferrous horseradish peroxidase and interpreted to be due to the fast interaction of oxyperoxidase with ferro-peroxidase (10,11).

When carbon monoxide was introduced into the solution containing the new

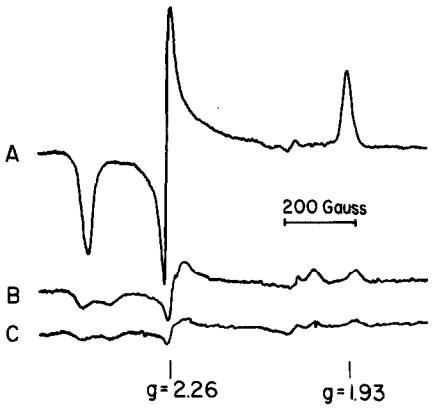


FIGURE 3 : EPR Spectra of Various Forms of P-450$_{cam}$.

A, ferric P-450$_{cam}$ in 20 mM Tris chloride buffer, pH 7.4, in the absence of camphor; B, ferric P-450$_{cam}$ in 20 mM potassium phosphate buffer, pH 7.4, with 20 mM potassium chloride and camphor; C, an aliquot of a reaction mixture which corresponds to spectrum C, Figure 2, with 70-90% oxygenated form. The aliquot was taken about 4 minutes after the addition of oxygen to the ferrous form. The P-450$_{cam}$ concentration in each of these experiments was 48 μM. The EPR spectra were recorded with a Varian E4 EPR spectrometer equipped with a variable temperature attachment. The various instrument parameters for this experiment were: modulation amplitude, 12.5 gauss; gain, 1.25 x $10^3$; temperature, -172°; scan rate, 250 gauss per min; time constant, 0.3 sec.

species, the major portion of the enzyme was found to be converted into carbon monoxy-P-450$_{cam}$ (Figure 1D). In addition, when oxygen was bubbled into the solution of carbon monoxy-P-450$_{cam}$, the spectrum reverted gradually to that of the new species, although a small portion of the enzyme was converted to the ferric enzyme.

The new species exhibited no significant EPR signal between 500 and 4,000 gauss at -172° using the experimental conditions described in the legend to Figure 3. On the other hand, EPR spectra of both high spin and low spin ferri-P-450$_{cam}$ were essentially the same as the recent reports of Tsai et al. (4) and Peterson (7), who have shown that the high spin form contained about 20% of the low spin form. The high spin EPR signal at g = 7.9 could not be detected at this temperature, as has been reported previously (4).

## DISCUSSION

The new spectral species described in this paper is considered to be an oxygenated compound of P-450$_{cam}$ for the following reasons: (a) dependency of its formation on the presence of both oxygen and the ferrous enzyme, and (b) its reversible transformation into carbon monoxy-P-450$_{cam}$ simply by changing the ratio between carbon monoxide and oxygen concentrations in the reaction mixture. These results fit well with current concepts of oxygenated hemoproteins. The new species is probably not a hydrogen peroxide complex since addition of hydrogen peroxide to ferrous P-450$_{cam}$ failed to demonstrate the formation of the new species, and it could be formed even in the presence of a large amount of catalase. Either dithionite-reduced or enzymatically-reduced P-450$_{cam}$ was capable of forming the oxygenated compound. The lack of an EPR signal attributable to the low spin form of oxy-P-450$_{cam}$ lends further support to our interpretation.

The role of oxy-P-450$_{cam}$ in the overall catalytic reaction is not clear at this moment because it could be either an intermediate or a side product of the reaction. However, the immediate decomposition of the oxygenated compound observed in the presence of putidaredoxin may indicate that the new species

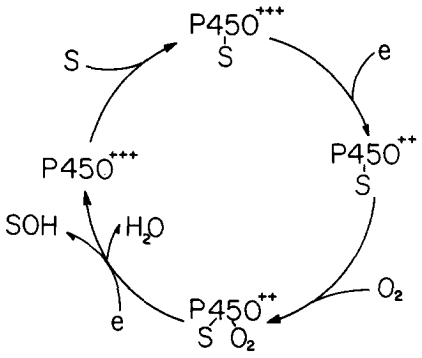


FIGURE 4 : Proposed Reaction Mechanism of Methylene Hydroxylation of D(+)-Camphor by P-$450_{cam}$.

S, D(+)-camphor as substrate; SOH, the hydroxylated reaction product; e, an electron from putidaredoxin.

undergoes a rapid turnover in the presence of the other components of the coupled hydroxylation system. The rapid decay in the presence of putidaredoxin may be a reductive or an oxidative process, but reductive decomposition seems more likely, since our preliminary experiments showed that reduced putidaredoxin was more effective than oxidized putidaredoxin in accelerating the rate of decomposition of the new species. Hydrogen peroxide which could oxidize oxyperoxidase (10) did not accelerate the decay of the oxygenated compound of P-$450_{cam}$, but it oxidized the ferrous enzyme to ferric enzyme.

The following reaction mechanism which was originally postulated by Estabrook et al. (1) is therefore compatible with our findings (Figure 4). First, organic substrate, D(+)-camphor in this case, combines with ferric P-$450_{cam}$ facilitating the reduction of heme through putidaredoxin. A significant change in the oxidation-reduction potentials of P-$450_{cam}$ has recently been noticed by Peterson et al. (12). The reduced heme reacts then with oxygen to form a ternary complex of oxygen, camphor and the enzyme, which subsequently would be reduced by receiving an additional electron from putidaredoxin, and finally converted into reaction products and ferri-P-$450_{cam}$.

The accompanying paper (13) demonstrates the presence of a spectral species similar to the oxygenated form of P-$450_{cam}$ during the steady state

of hydroxylation of various compounds catalyzed by rat liver microsomes. Further investigations on the formation and decomposition of the oxygenated form, as well as the exact role of putidaredoxin in the catalysis, are now in progress.

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