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(Authors' Review)

## Highly Purified Liver Microsomal Cytochrome P450: Properties and Catalytic Mechanism\*

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Recent studies in this laboratory on two forms of cytochrome P450 purified to homogeneity from rabbit liver microsomes are reviewed. The two forms, phenobarbital-inducible P450LM2 and 5,6-benzoflavone-inducible P450LM4, differ in subunit molecular weight, identity of the C-terminal amino acid, optical and EPR spectra, and other properties. As isolated, oxidized P450LM2 is in the low spin state, whereas P450LM4 is largely, but not entirely, in the high spin state. Mechanistic studies have shown the following: (a) P450LM2 may accept two electrons, calculated per heme, from dithionite or NADPH in the presence of catalytic amounts of the reductase, and may donate two electrons to various oxidizing agents, including molecular oxygen. (b) Hydrogen peroxide is formed in the reconstituted system in the presence of NADPH and oxygen, and the amount varies with the substrate added. (c) Hydrogen peroxide and other hydroperoxides apparently donate the oxygen atom inserted into substrate during hydroxylation in the absence of O<sub>2</sub> and an external donor. (d) Stopped flow spectrophotometry has provided evidence for two distinct oxygenated complexes of the reduced cytochrome. The reductase and cytochrome b<sub>5</sub> may play an effector role in increasing the rate of decomposition of the second complex during oxygen insertion into substrate. A scheme is proposed for the mechanism of action of purified P450LM2, based on these and other findings.

### INTRODUCTION

Cytochrome P450, the chief membrane-bound protein of hepatic cells, is an unusually versatile catalyst. This pigment brings about the hydroxylation of naturally occurring compounds such as fatty acids, prostaglandins, and steroids as well as a variety of reactions involving foreign compounds, including drugs, anesthetics, insecticides, petroleum products, and carcinogens. This laboratory resolved the mixed function oxidase system of liver microsomes into its components, including a solubilized form of P450LM\*\*\* which retained cata-

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\*\*\* The abbreviations used are: P450LM liver microsomal cytochrome P450; and P450CAM camphor-specific bacterial cytochrome P450.

lytic activity toward a variety of substrates<sup>1,2</sup>. Subsequent studies led to the purification and characterization of the cytochrome<sup>3,4</sup> as well as NADPH-cytochrome *P450* reductase<sup>5</sup> and a phospholipid<sup>6</sup> as summarized elsewhere<sup>7</sup>. Shortly following the isolation of electrophoretically homogeneous *P450*<sub>LM</sub> from phenobarbital-induced rabbit liver microsomes<sup>3,4</sup>, evidence for the occurrence of multiple forms of the cytochrome was obtained by enzyme fractionation<sup>8</sup>. The properties of the homogeneous phenobarbital- and 5,6-benzoflavone-inducible forms of the cytochrome, which are designated according to their relative electrophoretic mobilities on polyacrylamide gel as *P450*<sub>LM2</sub> and *P450*<sub>LM4</sub>, have recently been reported in detail<sup>9</sup>. Highly purified *P450*<sub>LM</sub> has also been obtained recently by two other laboratories. The isolation of the cytochrome from 3-methylcholanthrene-treated rats and rabbits and from phenobarbital-treated rats has been reported by investigators at Hoffmann-La Roche<sup>10,11</sup>, and from phenobarbital- and 3-methylcholanthrene-treated rabbits by investigators in Japan<sup>12,13</sup>.

The present paper briefly summarizes the spectral and other properties of the two purified forms of *P450*<sub>LM</sub> obtained in this laboratory and reviews our recent studies on the mechanism of action of *P450*<sub>LM2</sub>. Our present knowledge of electron uptake by the cytochrome, hydrogen peroxide generation during catalysis, peroxide utilization as an oxygen donor, and the formation of oxyferro intermediates as detected by rapid reaction techniques is presented and incorporated into a proposed mechanistic scheme.

#### PROPERTIES OF HOMOGENEOUS PHENOBARBITAL-INDUCIBLE AND 5,6-BENZOFLAVONE-INDUCIBLE FORMS OF LIVER MICROSOMAL CYTOCHROME *P450*

The best preparations of *P450*<sub>LM2</sub> from phenobarbital-induced rabbits and *P450*<sub>LM4</sub> from 5,6-benzoflavone-induced rabbits contain 20.1 and 18.1 nmol of the cytochrome per mg of protein, respectively, and are judged to be homogeneous on the basis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis following treatment with mercaptoethanol<sup>9</sup>, quantitative determination of the C-terminal amino acid residues<sup>9</sup>, and immunochemical methods, including Ouchterlony double-diffusion studies<sup>14</sup>. *P450*<sub>LM2</sub> has a subunit molecular weight of 48,700 and contains arginine as the C-terminal residue, whereas the subunit molecular weight of *P450*<sub>LM4</sub> is 55,300 and the C-terminal amino acid is lysine. The two enzyme preparations are similar, on the other hand, in containing one molecule of heme, three carbohydrate residues, and less than one molecule of phospholipid per polypeptide chain, and both are free of detectable amounts of NADPH-cytochrome *P450* reductase, cytochrome *b*<sub>5</sub>, NADH-cytochrome *b*<sub>5</sub> reductase, and epoxide hydrase.

The optical spectra of *P450*<sub>LM2</sub> and *P450*<sub>LM4</sub><sup>9</sup> are presented in Figures 1 and 2, respectively. Oxidized *P450*<sub>LM2</sub> has a Soret band at 418 nm with distinct  $\alpha$  and  $\beta$  bands at 568 and 535 nm and no maxima in the range between 600 and 700 nm. Upon reduction of the protein, the Soret band moves to 413 nm, and exposure to CO then causes the Soret band to shift to 451 nm. In contrast, oxidized *P450*<sub>LM4</sub> has a Soret band at 394 nm, broad absorbance from 500 to 600 nm, and a weak but definite band at 645 nm which may be attributed to a charge transfer transition, characteristic of the high spin state. The Soret band of reduced *P450*<sub>LM4</sub> occurs at 411 nm and with CO as a ligand is observed at 448 nm. It may be noted that *P450*<sub>LM2</sub> is present in liver microsomes in significant amounts only after drug induction, whereas *P450*<sub>LM4</sub> is present

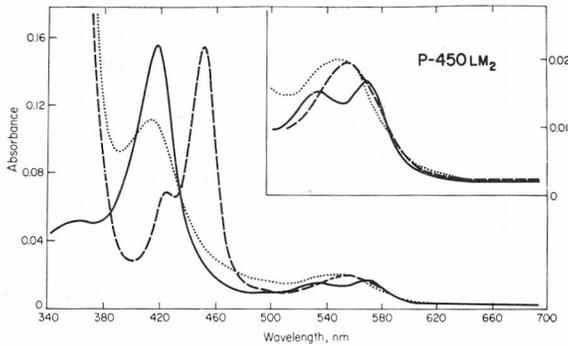


Figure 1. Spectrum of purified  $P450LM_2$  from phenobarbital-induced rabbit liver microsomes. The cytochrome concentration, based on heme analysis, was 1.40 nmol per ml of 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol. (Oxidized form, —; reduced form, .....; and reduced CO complex, - - - -).

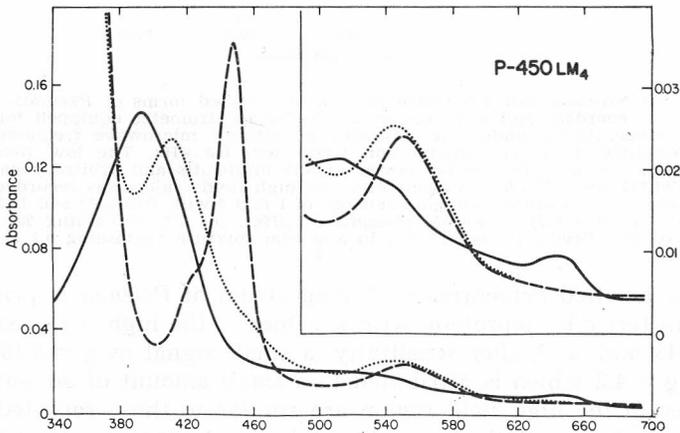


Figure 2. Spectrum of purified  $P450LM_4$  from 5,6-benzoflavone-induced rabbit liver microsomes. The cytochrome concentration, based on heme analysis, was 1.53 nmol per ml, and the buffer solution was the same as in Figure 1.

in microsomes from normal and drug-induced animals and is increased in amount upon induction with 5,6-benzoflavone.  $P450LM_4$  preparations isolated from normal, phenobarbital-induced, and 5,6-benzoflavone-induced rabbit liver microsomes exhibit identical optical spectra.

Octylamine difference spectra and EPR spectra have also been recorded with the purified cytochromes to determine the spin state<sup>9</sup>. Jefcoate *et al.*<sup>15</sup> have reported the use of octylamine for the quantitative determination of the high and low spin forms of cytochrome  $P450$  in rabbit liver microsomes. The spectra which we have obtained upon the addition of octylamine to  $P450LM_2$  and  $LM_4$  are highly similar to those which were reported by Jefcoate and Gaylor<sup>16</sup> with microsomal suspensions from animals treated with phenobarbital or 3-methylcholanthrene.  $P450LM_2$  gave a peak at 434 and a trough at 412 nm while  $LM_4$  gave a peak at 427 and a trough at 392 nm. The  $\Delta A_{410}/\Delta A_{392}$  ratios indicated that  $P450LM_2$  was at least 90% low spin, while  $LM_4$  was predominantly high spin. The EPR spectra shown in Figure 3 were determined on the oxidized

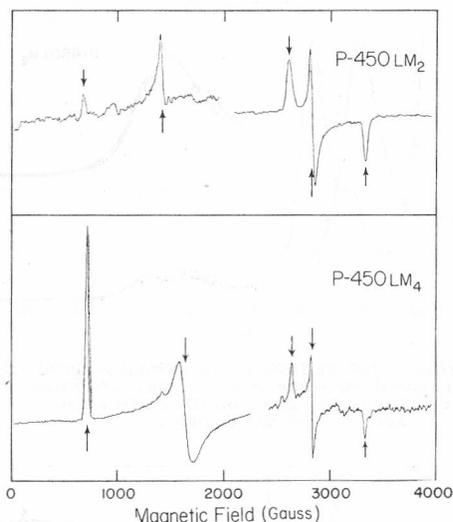


Figure 3. Electron paramagnetic resonance spectra of oxidized forms of  $P450_{LM2}$  and  $P450_{LM4}$ . The spectra were recorded with a Varian model V-4502 spectrometer equipped for operation at liquid helium temperatures, under the following conditions: microwave frequency, 9.242 GHz; modulation amplitude, 9 G; and modulation frequency, 100 kHz. The low field region was recorded at about 7 K at a microwave power of 6.15 milliwatts and arbitrary gain settings of 4 and 1 for  $P450_{LM2}$  and  $P450_{LM4}$ , respectively. The high field region was recorded at 20 K at a microwave power of 0.2 milliwatt and gain settings of 1 and 2 with  $P450_{LM2}$  and  $P450_{LM4}$ , respectively.  $P450_{LM2}$  was 0.15 mM in 0.03 M phosphate buffer, pH 7.4, containing 20% glycerol and 1.0 mM EDTA, and  $P450_{LM4}$  was 0.13 mM in a similar mixture containing 0.1 M phosphate.

forms of the purified cytochromes. The spectrum of  $P450_{LM2}$  is primarily that of a low spin ferric hemeprotein, with  $g$  values in the high field region of 1.93, 2.25, and 2.43 and, at higher sensitivity, a small signal at  $g = 8.46$  as well as a signal at  $g = 4.3$  which is attributed to a small amount of adventitious iron. The  $g$  values in the high field region are similar to those reported earlier by this laboratory for a relatively crude solubilized preparation<sup>17</sup> and by Mason *et al.*<sup>18</sup> for a microsomal suspension. On the other hand, the EPR spectrum of  $P450_{LM4}$  purified from 5,6-benzoflavone-induced microsomes is at least 75% high spin and only partly low spin. The  $g$  values are 3.84 and 8.36 in the low field region and, at higher sensitivity, 1.93, 2.26, and 2.42 in the high field region. EPR spectral evidence for both high and low spin forms of cytochrome  $P450$  in liver microsomes has been reported by several laboratories<sup>16,19-21</sup>.

Data presented elsewhere<sup>9</sup> show that the addition of Renex 690 (the nonionic detergent used by this laboratory in the purification of membrane-bound enzymes) to  $P450_{LM4}$  causes a pronounced change in the optical spectrum. The Soret band shifts from 394 to 418 nm to give a spectrum closely resembling that of  $P450_{LM2}$ . The detergent may cause this spectral change by displacing bound substrate<sup>9</sup>. Hashimoto and Imai<sup>13</sup> have recently reported the isolation from 3-methylcholanthrene-treated rabbit liver microsomes of cytochrome  $P450$  complexed with this inducer. In their experiments the addition of Emulgen, a nonionic detergent, caused partial conversion to a low spin form, which was attributed to partial release of methylcholanthrene from the substrate-binding site. On the other hand, Witmer *et al.*<sup>22</sup> have treated rabbits with <sup>14</sup>C-3-methylcholanthrene and shown that the carcinogen was present at only low levels

in the isolated hepatic microsomes. From such experiments they concluded that the high spin state of the cytochrome is not the result of formation of an enzyme-substrate complex.

#### MECHANISM OF ACTION OF PURIFIED LIVER MICROSOMAL CYTOCHROME P450

The hydroxylation reactions catalyzed by the mixed function oxidase system of liver microsomes require the transfer of two electrons from NADPH to a molecule of molecular oxygen, resulting in the reduction of one of the oxygen atoms to water and insertion of the other into the substrate. Much remains to be learned, however, about the details of this process, in which it is believed that oxygen activation occurs with generation of a species considerably more reactive than the parent molecular oxygen. The studies summarized below were aimed at elucidation of the details of this overall process with the use of highly purified P450<sub>LM2</sub> and reductase by providing information on such questions as whether the cytochrome accepts one or more electrons, whether oxygen-containing products are formed other than water and the hydroxylated substrate, whether other oxygen donors may be substituted for O<sub>2</sub>, and whether oxygen-containing intermediates bound to P450<sub>LM</sub> may be detected by rapid reaction techniques. The possibility may also be considered that P450<sub>LM</sub> catalyzes substrate activation, as by proton or hydride ion abstraction. This seems somewhat unlikely in view of the poor chemical reactivity of many of the substrates, such as alkanes, but cannot be ruled out at this time.

#### A. Evidence that P450<sub>LM2</sub> May Contain a Second Electron Acceptor

The reduction of P450<sub>LM2</sub> requires two electrons, as shown by titration with standardized dithionite in the presence of carbon monoxide under highly anaerobic conditions<sup>23</sup>. This unexpected finding was confirmed in a series of experiments carried out under a variety of conditions<sup>24</sup>. Some typical results are shown in Table I. Two electrons, calculated per heme, are accepted from dithionite in the presence of cyanide, imidazole, CO, or with no added ligand, or from NADPH in the presence of NADPH-cytochrome P450 reductase as a catalyst and with CO as a ligand. Two electrons are also donated by the reduced P450<sub>LM2</sub> to molecular oxygen, dichlorophenolindophenol, or cytochrome c. Furthermore, photochemical reduction of P450<sub>LM2</sub> also involves the uptake of two electrons, as shown by back titration with dichlorophenolindophenol.

The significance of such experiments is not yet clear, particularly since the rapid autoxidizability of P450<sub>LM</sub> prevents experiments being carried out on the magnitude of electron uptake under aerobic conditions. Some obvious possible interpretations are that the second electron acceptor is artifactual in the sense of not occurring in the membrane-bound cytochrome, that this acceptor remains reduced rather than transferring electrons during substrate hydroxylation, and that it is an essential donor of an electron at some stage of the catalytic mechanism. The identity of the acceptor, tentatively called Factor C<sup>24</sup>, is not yet known, but it appears not to be a metal or other group detectable by EPR in the reduced or oxidized state of the enzyme. Attempts are in progress to characterize the acceptor and to determine its role, if any, in hydroxylation reactions. It may be noted that, under our experimental con-

TABLE I

*Quantitative determination of electron uptake and donation by P450LM*

Reductant or oxidant added	Ligand added	Electrons transferred per molecule of cytochrome*
Dithionite	None	+2.0
"	Cyanide	+1.9
"	Imidazole	+2.1
"	CO	+2.1
NADPH	CO	+1.9
O <sub>2</sub>		-2.1
Dichlorophenolindophenol		-2.0
Cytochrome <u>c</u>		-2.2

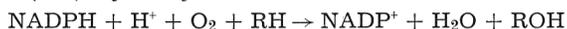
\* Electron uptake by the cytochrome is indicated by a positive sign and electron donation by a negative sign.

ditions, P450CAM is a one electron acceptor<sup>23</sup>, as already reported by others<sup>25,26</sup>, and therefore may be different in this respect from P450LM<sub>2</sub>. More recent experiments in collaboration with Dr. J. A. Peterson indicate that some P450LM<sub>2</sub> preparations require only one electron for full reduction and are functional in substrate hydroxylation.

### B. Hydrogen Peroxide Formation Accompanying Substrate Hydroxylation

In a recent study of the stoichiometry of hydroxylation reactions catalyzed by P450LM<sub>2</sub>, hydrogen peroxide was identified as a side product and shown to vary in amount depending on the substrate added<sup>27</sup>. The NADPH-dependent production of hydrogen peroxide by liver microsomes was first reported by Gillette *et al.*<sup>28</sup> and has also been described by others in recent studies<sup>29-32</sup>. The results of our experiments are shown in Table II. NADPH oxidation and O<sub>2</sub> consumption were found to be equimolar, whereas the product formed (formaldehyde, in the case of benzphetamine) was less than half as great in amount. However, when hydrogen peroxide formation was taken into account, the electron uptake and O<sub>2</sub> consumption were well accounted for, as would be predicted by the occurrence of the two competing reactions<sup>27</sup>:

(a) Substrate (RH) hydroxylation:



(b) Oxidase activity:

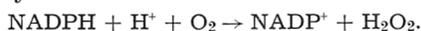


TABLE II

*Stoichiometry of P450LM<sub>2</sub>-catalyzed reactions in reconstituted enzyme system*

Compound added	Change in components (nmol/min/nmol P450)			
	-NADPH	-O <sub>2</sub>	+CH <sub>2</sub> O	+H <sub>2</sub> O <sub>2</sub>
Benzphetamine	77	75	33	42
Desmethylbenzphetamine	45	44	0	42
None	21	22	0	21

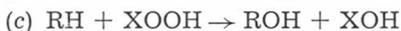
Desmethylbenzphetamine (benzylamphetamine) acts as a pseudosubstrate in that it stimulates peroxide formation to the same extent as the parent compound (benzphetamine) but does not undergo hydroxylation. Accordingly, benzylamphetamine may be used in control experiments to correct for the NADPH and O<sub>2</sub> consumption not associated with benzphetamine hydroxylation. In the experiment shown, subtraction of the rates observed with desmethylbenzphetamine gives the following corrected rates for substrate hydroxylation, expressed as turnover numbers: NADPH disappearance, 32; oxygen uptake, 31; and formaldehyde formation, 33. Within experimental error, such values agree nicely with the 1 : 1 : 1 stoichiometry predicted for these three components of the reaction. It should be noted that the level of NADPH oxidase activity of the system, which is apparently largely due to the autoxidizability of P450LM<sub>2</sub>, is lower when neither the substrate nor the pseudosubstrate is added.

### C. Peroxide-Dependent Substrate Hydroxylation by Purified P450LM<sub>2</sub>

The ability of liver microsomal suspensions to utilize organic hydroperoxides for the hydroxylation of various substrates and the possible role of cytochrome P450 in these reactions have been reported by several laboratories<sup>33-38</sup>. We have recently shown that highly purified P450LM<sub>2</sub> catalyzes such reactions<sup>39</sup>; the enzyme preparations used were known to be free of the other known microsomal electron carriers, that is, the NADH- and NADPH-dependent flavoproteins and cytochrome b<sub>5</sub>. Substrates which underwent hydroxylation in the presence of cumene hydroperoxide included *N,N*-dimethylaniline, *N*-methylaniline, cyclohexane, *N*-methylbenzylamine, methyl cumyl ether, benzphetamine, *N*-methyl-*n*-butylamine, aminopyrine, and methyl octanoate, and with radioactive benzphetamine as substrate the oxygenating agents which were effective included cumene hydroperoxide, *p*-nitroperbenzoic acid, *m*-chloroperbenzoic acid, *p*-menthyl hydroperoxide, hydrogen peroxide, sodium chlorite, and alkyl hydroperoxides such as *t*-butyl, *n*-hexyl, and ethyl. Because of P450LM heme destruction by peroxides, it was necessary to carry out quantitative studies under specified conditions, including short time intervals. Benzphetamine demethylation showed a complete dependence on hydrogen peroxide as well as on P450LM<sub>2</sub>, and the latter could not be replaced by cytochrome P420, hemin chloride, or ferric chloride at the same concentration. As with

other reactions involving purified  $P450_{LM}$ , phospholipid is necessary for full activity. Of particular interest, NADPH and the reductase are not required in the reaction mixture when peroxides are present, and hydroxylation occurs as well under anaerobic conditions as with  $O_2$  present. CO is not inhibitory, which strongly suggests that the ferrous form of  $P450_{LM2}$  is not involved. Inhibition by cyanide and by ethyl isocyanide demonstrates the importance of the ferric state in the catalytic cycle, while the inhibition in the presence of SKF-525A indicates that the reaction occurs at the usual substrate-binding site.

These reactions may be written as follows, where RH represents a substrate and XOOH a peroxide:



The stoichiometry of the reaction was determined using  $N,N$ -dimethylaniline as substrate, and it was shown that equimolar amounts of  $N$ -methylaniline, formaldehyde, and cumyl alcohol were formed. Such results are in accord with the equation written above. Evidence that the oxygen atom in the hydroxylated product comes from the added peroxide was provided by an experiment carried out in  $H_2^{18}O$ , with cyclohexane as substrate and with cumene hydroperoxide present. The resulting cyclohexanol was found to have derived less than 10% of its oxygen from water. Since, as already mentioned, peroxide-dependent hydroxylations do not require molecular oxygen, it appears that over 90% of the oxygen in the cyclohexanol must have been derived from cumene hydroperoxide. The low incorporation of  $^{18}O$  from water may be due to a slow exchange reaction with some oxygen-containing intermediate in the reaction, but this has not been studied further.

#### D. Detection of Intermediates by Rapid Reaction Studies

We have recently used stopped flow spectrophotometry in an attempt to detect intermediates in the reaction of oxygen with the reduced form of highly purified  $P450_{LM2}$ <sup>40</sup>. The reaction showed biphasic kinetics, which when studied in detail as a function of wavelength provided evidence for two distinct intermediates, as indicated by the difference spectra in Figure 4. In these experiments, one syringe of the stopped flow apparatus contained photochemically reduced  $P450_{LM2}$  in the presence of substrate (benzphetamine), and the other contained an aerobic solution of the substrate. The reaction was initiated by

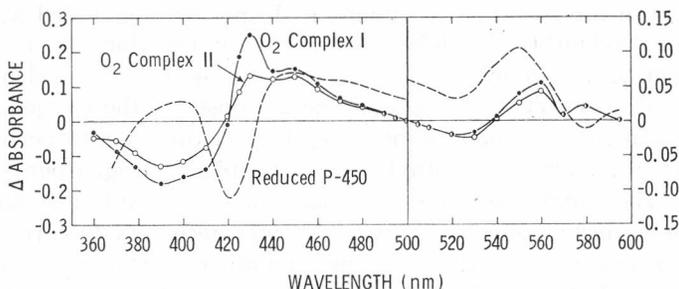


Figure 4. Difference spectra of complexes resulting from reaction of  $P450_{LM2}^{red}$  (reduced  $P450_{LM2}$ ) with oxygen as determined by stopped flow techniques. This figure is taken from an earlier publication<sup>38</sup>.

mixing equal volumes of the two reaction mixtures at 12 °C. Data were obtained from the kinetic traces at various wavelengths at the time intervals indicated and are presented as the reduced minus oxidized and the reduced, oxygenated minus oxidized difference spectra. The first oxygenated complex, with maxima at 430 and 450 nm, was formed in the dead time of the instrument and was largely unchanged at 10 ms. At longer times, however, a second complex was formed with a broad maximum at about 440 nm. The spectrum of Complex II is similar to that seen in steady state experiments with the reconstituted system containing P450LM<sub>2</sub><sup>40</sup> as well as with liver microsomal suspensions<sup>41</sup> and with purified P450CAM from *Pseudomonas putida*<sup>42,43</sup>.

Complex I may be a species containing dioxygen coordinated to ferrous iron, that is, (Fe<sup>2+</sup>)(O<sub>2</sub>), although the possible presence of a second electron acceptor makes the interpretation difficult. Neither Complex I nor II resembles horseradish peroxidase Compound I or catalase Compound I, both of which show broad absorbance centered at 400 to 410 nm<sup>44</sup> and are thought to contain (Fe—O)<sup>3+</sup>.<sup>45</sup> Furthermore, the indications that the decomposition of Complex II in the absence of substrate results in formation of hydrogen peroxide<sup>27,40</sup> suggest that Complex II retains two oxygen atoms. The absolute spectrum of Complex I, with a maximum at 423 nm, resembles that of cytochrome c peroxidase Compound II, which has a Soret band at 425 nm<sup>46</sup>. One of the P450LM complexes may involve an iron-coordinated peroxide, (Fe<sup>3+</sup>)(O<sub>2</sub><sup>2-</sup>), for which no spectral models are currently available.

The apparent first order rate constants were determined for the reaction sequence, P450 red.  $\xrightarrow[k_1]{O_2}$  Complex I  $\xrightarrow{k_2}$  Complex II  $\xrightarrow{k_3}$  P450 ox., in which  $k_1$  represents the formation of the first oxygenated complex,  $k_2$  the second oxygenated complex, and  $k_3$  the decay of the latter with regeneration of the oxidized cytochrome. The formation of Complex I was found to be complete in the dead time of the instrument with  $k_1$  equal to or greater than 60,000 min<sup>-1</sup>. The conversion of Complex I to II had a rate constant of 270 min<sup>-1</sup>, and the regeneration of P450 ox. had a rate constant of 3 min<sup>-1</sup>. For comparison, the turnover number of the enzyme in the complete reconstituted system (expressed as mol benzphetamine hydroxylated per mol cytochrome per min) was determined at the same temperature (12 °C) and found to be 24 min<sup>-1</sup>. Clearly, therefore,  $k_3$  was too small under these experimental conditions to account for the known catalytic activity of the enzyme. However,  $k_3$  appeared to be increased to 90 min<sup>-1</sup> when cytochrome b<sub>5</sub> was added at the same concentration as P450LM<sub>2</sub> prior to photoreduction; the cytochrome b<sub>5</sub> was not oxidized significantly in the time required for Complex II to form oxidized P450LM<sub>2</sub>. Similar experiments with NADPH-cytochrome P450 reductase added in place of cytochrome b<sub>5</sub> were more difficult to interpret but provided indirect evidence that  $k_3$  was increased to 23 min<sup>-1</sup>. Accordingly, it is possible that cytochrome b<sub>5</sub> and NADPH-cytochrome P450 reductase play an effector role when added to P450LM in such single turnover experiments. The studies of Lipscomb *et al.*<sup>47</sup> describing an effector role for putidaredoxin in a bacterial cytochrome P450-containing hydroxylation system provide a precedent for an electron carrier (putidaredoxin) with such a dual role.

As described in the paper by Debey, Begard, Balny, and Douzou presented at this Conference, two intermediates in the reaction of cytochrome P450 with

oxygen have been detected spectrally in intact microsomes at sub-zero temperatures. Of particular interest, the intermediates they have detected at low temperatures appear to have spectral properties very similar to those we have observed by rapid reaction techniques<sup>40</sup>.

### E. Proposed Scheme for Mechanism of Action of Liver Microsomal Cytochrome P450

In Figure 5, a scheme is proposed to account for the mechanism of substrate hydroxylation catalyzed by  $P450_{LM_2}$ . The oxidized form of the cytochrome, arbitrarily shown with Factor C in an uncharged state, binds substrate (RH) before rapid electron transfer can occur. The uptake of two electrons (from NADPH, catalyzed by the reductase) gives the ferrous heme protein, with a negative charge arbitrarily shown on Factor C. Alternatively, only one electron

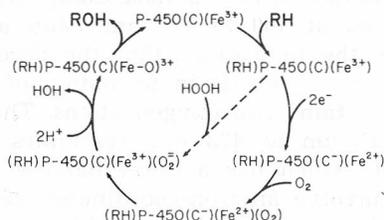


Figure 5. Proposed mechanism of action of liver microsomal cytochrome P450.

may be taken up in this step. Next, oxygen combines with the protein and undergoes a two-electron reduction to yield  $O_2^{2-}$ , the dianion of hydrogen peroxide. Superoxide may be a transient intermediate in this step (cf. ref. 48). The peroxide anion is then pictured as undergoing protonation and elimination of water with formation of an Fe-O complex having an overall charge of  $3^+$  and a formal oxidation state of  $5^+$ . The activated oxygen may be an oxenoid species, which we have written as a ferric-bound oxygen atom, although other resonance structures involving higher oxidation states of the iron or the porphyrin may also contribute<sup>44</sup>. Next, insertion of the oxygen atom into a favorably positioned carbon-hydrogen bond of the bound substrate would yield the hydroxylated product, ROH, and oxidized  $P450_{LM}$  would be regenerated.

The scheme shown is in accord with the known stoichiometry of substrate hydroxylation. It also indicates the manner in which added hydrogen peroxide may support hydroxylation in the absence of molecular oxygen and an external electron donor. Considerable evidence has been accumulated from studies of model compounds that the fifth ligand to the heme iron in cytochrome P450 is a thiolate anion, probably contributed by a cysteine residue<sup>49-55</sup>. Recently, it has been suggested that the presence of a coordinated thiolate anion is necessary for oxygen activation, perhaps by providing sufficient charge repulsion to facilitate the crucial dioxygen bond cleavage yielding the  $(Fe-O)^{3+}$  intermediate<sup>56</sup>. Justification for the proposal of  $(Fe-O)^{3+}$  as the activated oxygen moiety is provided by studies of hydroxylation of unactivated hydrocarbons with mixtures of iron ions and peroxides<sup>57</sup> or with mixtures of various transition metal ions, oxygen, and a reductant<sup>58,59</sup>, each of which apparently involves an oxygenated metal ion as the active intermediate. Added peroxides

and other oxygenating agents may produce the same species of activated oxygen as generated from molecular oxygen and NADPH in the complete reconstituted system, but this remains to be established.

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## DISCUSSION

**H. Schleyer:**

(a) In the EPR spectrum of LM2 some  $g \sim 4.26$   $\text{Fe}^{3+}$  resonance was present. How much, approximately? Is this related to the partial loss of heme from the preparation?

(b) ROOH-supported hydroxylation parallels roughly the »normal« activity (reconstituted system + NADPH) in terms of apparent turnover numbers, comparing a series of substrates. If so — we have other results in the case of rat liver microsomes and find under comparable experimental conditions (PB-infusion, by David Y. Cooper) that, for instance,  $\text{CH}_2\text{O}$  is formed with benzphetamine as substrate, but not with codeine, while turnover numbers with the normal system are about 13 and  $10 \text{ min}^{-1}$ , respectively.

**M. J. Coon:**

(a) The signal at about  $g = 4.26$  in this preparation represents only a few percent of the total iron. We don't know whether this small amount of adventitious iron resulted from heme loss. As judged by atomic absorption, the iron content of LM2 preparations is usually fully accounted for by the heme content.

(b) Yes, the substrate specificity in rabbit liver is roughly the same in the complete reconstituted system and in the peroxide-dependent system. We haven't tested codeine, however.

**K. Ruckpaul:**

How many molecules of oxygen are consumed by the simultaneous generation of  $\text{H}_2\text{O}_2$  per molecule of product formed?

**M. J. Coon:**

The amount of hydrogen peroxide formed varies with the substrate used. For example, the molar ratio of  $\text{O}_2$  used for  $\text{H}_2\text{O}_2$  formation relative to  $\text{O}_2$  used for substrate hydroxylation is about 1.3 with benzphetamine and 0.3 with cyclohexane.

**H. Rein:**

(a) How much LM4 is in a microsomal preparation from phenobarbital induced rabbits? (b) How do you dissolve the hydrophobic substrates in relatively high concentration?

**M. J. Coon:**

(a) We have no way of knowing the LM4 concentration in the membrane accurately, but I would estimate that it amounts to about 15% of the total P450 in such drug-induced microsomes or in microsomes from animals to which no inducer was administered.

(b) Alkanes and other hydrophobic substrates are added in 1 to 10  $\mu\text{l}$ iters of methanol or acetone to reaction mixtures containing reductase, P450LM, and phosphatidylcholine, final volume 1.0 ml.

**I. Blanck:**

Have you any idea about the two oxygen species in the stopped flow experiment? Are both »on the way to become activated  $\text{O}_2$ -species«, or is the first an activated complex and the second one a secondary product?

**M. J. Coon:**

That is an important question. Since we do not yet know the identity of these species, other than that they are oxygenated complexes of the reduced form of P450LM, it is not possible to state whether one of these is the »activated oxygen« required for insertion into substrate. On the other hand, the kinetics indicate that both Complex I and Complex II are formed rapidly enough that they could serve this function.

**T. G. Traylor:**

What was the concentration of oxygen used in your kinetic studies?

**M. J. Coon:**

In the experiment shown the oxygen was in large excess, at about half the concentration at maximum solubility in the buffer used. In other experiments the  $O_2$  concentration was varied from 25 to 500  $\mu M$ ; the lack of dependence on the oxygen concentration over this range indicated that the oxygenation reaction was complete in the dead time of the stopped flow instrument.

**P. Debey:**

Did you find any difference spectrum upon addition of peroxides to the purified preparation?

**M. J. Coon:**

I assume you are referring to the stopped flow studies. We have carried out a few such experiments and so far have no clear spectral evidence for the same intermediate as obtained by the reaction of molecular oxygen with reduced cytochrome P450.

**K. Ruckpaul:**

What can you say about the stability of your system after having incubated P450 with ROOH? In our group, experiments have been done with cathodically generated  $H_2O_2$  and enzymatic activity was maintained for up to 2 hours.

**M. J. Coon:**

The stability depends upon the peroxide concentration; I assume that you are producing low steady state concentrations under your conditions. We usually work with peroxide concentrations which provide less than the maximal rate but give linearity for up to 5 minutes. At longer times or higher peroxide concentrations, a significant amount of heme loss occurs.

**F. Jung:**

(a) Did you have difficulties using chlorite as oxidant? It is very reactive and should destroy your preparation. (b) In your system with benzphetamine you should also use chlorate as oxidant.

**M. J. Coon:**

(a) Chlorite supported benzphetamine hydroxylation, but the result is only a qualitative observation. We have worked out optimal conditions only with hydrogen peroxide and cumene hydroperoxide. (b) I am interested in your suggestion about chlorate; we have not tested it.

**D. L. Williams-Smith:**

Have you attempted to measure the number of electrons transferred to P450LM in a dithionite reduction by EPR, in the absence of CO?

**M. J. Coon:**

In a preparation with half of the P450LM reduced, about half of the EPR signal had disappeared, and in a preparation with almost all of the P450LM reduced, the signal was largely absent. This experiment was in accord with the results expected. We have not carried out a thorough reductive titration using EPR, however.

**H. Rein:**

Is there a change of the redox-potential in the presence of substrate?

**M. J. Coon:**

Neither substrate nor phosphatidylcholine has a significant effect on the redox potential of P450LM2, which is about  $-330$  mvolts.

**T. G. Traylor:**

Can you change the ratio of hydroxylation to hydrogen peroxide production by changing things other than substrate structure, e. g. more substrate, pH change, etc.?

**M. J. Coon:**

We have not determined whether such variables have an effect on the relative amount of hydrogen peroxide formed. The various substrates were tested at about the minimal concentrations necessary to give maximal hydroxylation rates. Desmethylbenzphetamine, the product of benzphetamine demethylation, acts as a pseudo-substrate and causes the same rate of  $H_2O_2$  formation as does the parent compound.

**SAŽETAK****Mikrosomski citokrom P450 visoke čistoće: svojstva i katalitički mehanizam**

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Dan je pregled najnovijih istraživanja iz ovog laboratorija o citokromu P450 pročišćenom do homogenosti. Dva oblika, P450LM2 induciran fenobarbitalom, i P450LM4 induciran 5,6-benzoflavonom, razlikuju se po molekulskoj težini podjedinice, C-terminalnoj aminokiselini, optičkim i EPR-spektrima, i po drugim svojstvima. Izolirani i oksidirani P450LM2 u niskospinskom je stanju, dok je P450LM4 najvećma, iako ne sasvim, u visokospinskom stanju. Proučavanje mehanizma pokazalo je: (a) P450LM2 prima dva elektrona računano po hemu, od ditionita ili NADPH u prisustvu katalitskih količina reduktaze, a predaje dva elektrona različnim oksidativnim agensima, uključujući i molekulski kisik. (b) Vodikov se peroksid stvara u rekonstituiranom sistemu u prisustvu NADPH i kisika, a količina mu zavisi o dodanom substratu. (c) Vodikov peroksid i drugi hidroperoksidi, čini se, predaju kisikov atom za ugradnju u supstrat za vrijeme hidroksilacije u odsustvu  $O_2$  i vanjskog donora. (d) Spektrofotometrija brze kinetike pokazuje dva različna oksigenirana kompleksa reduciranog citokroma. Reduktaza i citokrom  $b_5$  vjerojatno igraju ulogu efektora povećavajući brzinu razlaganja drugog kompleksa prilikom ugradnje kisika u supstrat. Na temelju ovih i drugih podataka predložena je shema enzimskog mehanizma za pročišćeni P450LM2.

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