BIOCHEMICAL STUDIES ON CYTOCHROME P-450 SOLUBILIZED FROM LIVER MICROSOMES: PARTIAL PURIFICATION AND MECHANISM OF CATALYSIS*

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The cytochrome P-450 of the hepatic endoplasmic reticulum is of particular interest to biochemists and pharmacologists, because of its inducibility by many agents and its remarkably broad substrate specificity. Indeed, it is probably no exaggeration to state that it is the most versatile biological catalyst known. Not only physiological substrates, such as steroids and fatty acids, but also many foreign compounds, including drugs, anesthetics, petroleum products, insecticides, and so on, are attacked by molecular oxygen in the presence of NADPH as an electron donor and cytochrome P-450 as the catalyst. The hydroxylation of foreign substances is generally viewed as an important initial step in their detoxication, but in some instances the compounds so modified may become more toxic or even carcinogenic. Although much has been learned about the inducibility, specificity, and other properties of this enzyme system through work with liver microsomal suspensions, it seems likely that some of the more puzzling questions will not be answered without purification and characterization of the individual components. Two of the more difficult questions are: whether one or more forms of cytochrome P-450 account for the broad specificity, and whether oxygen undergoes activation in the presence of cytochrome P-450 to produce some species more reactive toward these substrates, most of which are lipophilic in nature and are often attacked at positions that do not appear to be highly chemically reactive. Hopefully, the conclusions reached will be applicable to the membranous system of microsomes and will prove useful to our understanding of the disposition of drugs and other foreign substances in the intact organism.

Several years ago, this laboratory reported the resolution of the liver microsomal enzyme system by treatment with deoxycholate in the presence of stabilizing agents and ion-exchange chromatography of the resulting extract.[1,2] The three components that were found to be necessary for the hydroxylation of a variety of substrates are cytochrome P-450, NADPH-cytochrome P-450 reductase, and a heat-stable, chloroform-soluble fraction which contains phosphatidylcholine as the active component.[1,3,4] The reductase, which has NADPH-cytochrome *c* reducing activity, has been partially purified, and the lipid factor may be replaced by synthetically prepared phosphatidylcholines.

This paper is concerned with the properties of the cytochrome P-450 fraction, which has recently been partially purified but is not yet homogeneous, and with the possible role of superoxide in substrate hydroxylation in this reconstituted enzyme system. As has been described elsewhere, the cytochrome P-450 is "solu-

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ble" as judged by several criteria that are usually applied to enzymes derived from membranes, and the addition of the phospholipid does not cause it to form large aggregates or membrane-like structures.[5-7] The evidence so far obtained suggests that cytochrome P-450, the reductase, and phosphatidylcholine form a catalytically active complex which is readily dissociable.

Resolution and Partial Purification of Liver Microsomal Cytochrome P-450

Cytochrome P-450 was solubilized in several different ways for use in the studies to be described. Preparations solubilized with deoxycholate and then submitted to column chromatography on DEAE-cellulose[1,3] will be referred to as column P-450. They contain varying amounts of cytochrome P-420; sometimes none is present, and sometimes as much cytochrome P-420 as P-450 is present. Such preparations are particularly useful in studies on mechanism, because of their ability to couple with nonphysiological electron donors. More recently, cytochrome P-450 was solubilized by sonic oscillation and treatment with sodium cholate.[6,8] The extract was then treated with ammonium sulfate (AS), and the fraction that precipitated between 40 and 50% saturation was found to contain the cytochrome P-450 at a concentration of 1.5–3.0 nmol/mg protein. Such AS P-450 preparations not only have a higher cytochrome P-450 content than the column P-450 preparations but are also more consistently free of cytochrome P-420.

To obtain a partially purified preparation, another procedure was used which is a modification of the method of MacLennan and colleagues,[9] in which microsomes are extracted with *t*-amyl alcohol. This was followed by solubilization with cholate and fractionation with AS. The fraction that precipitated between 40 and 50% saturation contained cytochrome P-450 at two to three times the concentration in typical AS P-450 preparations. The best preparations so far obtained had 6.7 nmol cytochrome P-450/mg protein.

Some analytical data on the partially purified cytochrome P-450 and AS P-450 preparations are given in TABLE 1. The results show that both preparations contained more heme than could be accounted for by cytochromes P-450 and b_5. Non-heme iron was also present in these fractions, but the amount appeared to decrease during purification of the cytochrome P-450. The nature and possible

TABLE 1
ANALYSIS OF CYTOCHROME P-450 PREPARATIONS*

Preparation	Components				
	Cytochrome P-450	Cytochrome b_5	Total Heme	Total Iron	Non-Heme Iron
	nmol/mg protein				
Rabbit liver AS P-450	2.5	0.1	3.5	16.7	13.2
Partially purified P-450 from rabbit liver	5.7	0.3	7.2	9.5	2.3

* Cytochrome P-450 was determined from the reduced CO difference spectrum, and cytochrome b_5 and total heme were determined according to the method of Omura and Sato.[31] Non-heme iron was calculated as the difference between total iron[32] and total heme. The values as given represent an average of determinations on at least three preparations.

function of the excess heme and the non-heme iron components are not known at this time. It may be noted that no acid-labile sulfide was found in either of the cytochrome P-450 preparations, nor could ferritin be detected by immunological methods. Preliminary experiments have shown that, upon titration of the resolved AS P-450 preparation by dithionite under anaerobic conditions, up to three times as many electrons were consumed as would be predicted from the cytochrome P-450 content.[10]

During disc electrophoresis of partially purified rabbit liver cytochrome P-450, in polyacrylamide gel that contained mercaptoethanol and sodium dodecyl sulfate (FIGURE 1), most of the protein migrated as a double band in the 55,000 molecular weight region. These bands appeared to increase in relative intensity with purification of the cytochrome P-450. Since the apparent molecular weight of the resolved cytochrome P-450 is about 350,000 (as judged by sedimentation velocity measurements, sucrose density gradient centrifugation, and gel exclusion chromatography),[6] it may be concluded that sodium dodecyl sulfate and mercaptoethanol disrupt the protein complex into individual polypeptide chains.

Mechanism of Catalysis by Cytochrome P-450: Studies on the Role of Superoxide

We have recently presented evidence that superoxide ($O_2 \cdot^-$) may play an important role in cytochrome P-450-catalyzed hydroxylation reactions.[5,11,12] Use was made of enzymes that catalyze superoxide generation or dismutation, in order to determine the effect of alterations in the concentration of this free radical

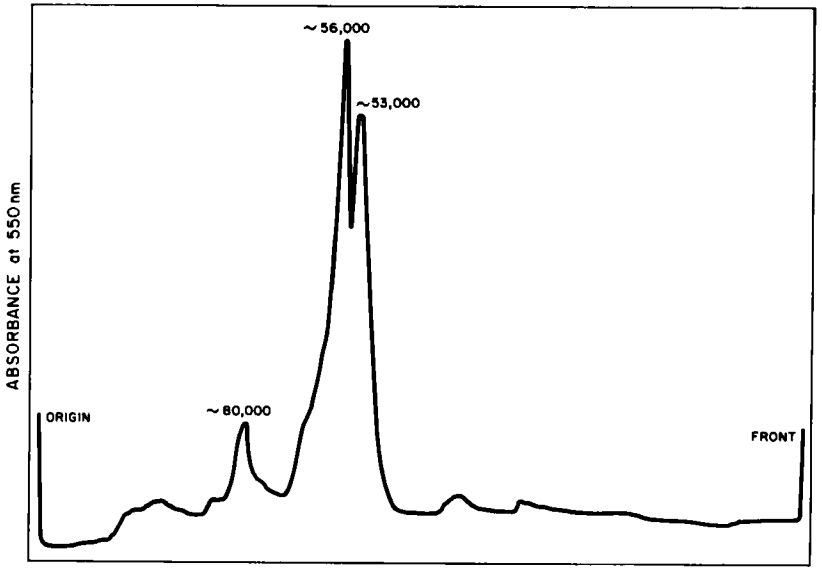

FIGURE 1. Gel electrophoresis of partially purified rabbit liver cytochrome P-450. Rabbit liver cytochrome P-450 (6.4 nmol/mg protein) was treated with sodium dodecyl sulfate and mercaptoethanol and subjected to electrophoresis on 7.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate at pH 7.0, as described by Weber and Osborn.[34] The gels were stained with Coomassie® Blue[35] and scanned at 550 nm in a Gilford spectrophotometer.

species on the extent of drug hydroxylation in the reconstituted liver microsomal enzyme system. Superoxide dismutase[13] (erythrocuprein) was shown to inhibit benzphetamine hydroxylation in the presence of NADPH, molecular oxygen, and the three microsomal components. The dismutase causes complete inhibition, however, only in the presence of a concentration of sodium chloride which is itself partly inhibitory. The salt may have a chaotropic effect on the system and thereby enable the dismutase to reach the active site more readily, but the effect of ionic strength on the physical properties of the enzyme system is not yet known. Tiron®, which is known to decompose superoxide,[14-16] was also shown to inhibit drug hydroxylation.

In other experiments it was found that xanthine and xanthine oxidase, which serve as a superoxide-generating system under aerobic conditions,[17] couple with the cytochrome P-450 fraction to support the hydroxylation of benzphetamine and ethylmorphine in the absence of NADPH and the reductase fraction.[11] This coupling is dependent upon the presence of phosphatidylcholine and is abolished by the addition of Tiron or of the dismutase. We have also employed a nonenzymatic photochemical system to generate the superoxide; in such studies, a mixture of riboflavin and methionine exposed to light was shown to couple with the cytochrome P-450 fraction to effect drug demethylation in the absence of the reductase fraction and NADPH.[18] This coupling is phosphatidylcholine-dependent and does not occur when Tiron or superoxide dismutase is added to the reaction mixture.

The scheme in FIGURE 2 summarizes our present understanding of the mechanism of action of cytochrome P-450.[19] The binding of the substrate by the oxidized form of the cytochrome is shown in Step a. This reaction is rapid as judged from the appearance of the substrate difference spectrum, but, as is shown by stopped-flow measurements, need not necessarily precede electron transfer from NADPH.[4] Next, in Step b, the iron atom is reduced to the ferrous state by

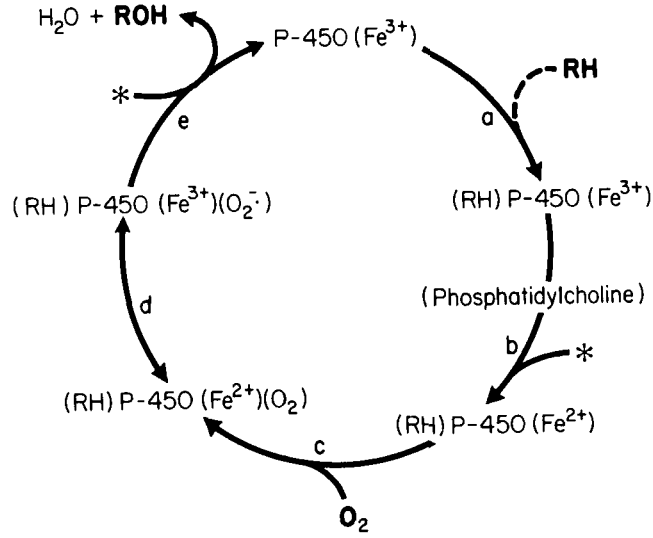

FIGURE 2. Proposed mechanism of reactions catalyzed by cytochrome P-450.

NADPH in the phosphatidylcholine-dependent reaction catalyzed by the reductase,[20,21] Molecular oxygen is then believed to combine with the reduced hemoprotein to give a ternary complex (Step c). Evidence for the formation of such a complex has been obtained for cytochrome P-450 from *Pseudomonas putida*,[22,23] as well as from liver microsomes.[24] In Step d, an electron transfer within the active complex is proposed, with generation of the superoxide radical bound to the oxidized form of cytochrome P-450. This radical species is written as an anion rather than as $HO_2\cdot$ (perhydroxyl radical), because the pK is about 4.8,[25] and deprotonation would therefore be largely complete at neutral pH. It is assumed that oxygen and superoxide are coordinated to the iron atom of cytochrome P-450 and that the resulting $Fe^{2+}O_2$ and $Fe^{3+}O_2^-$ species represent resonance forms. The uptake of a second electron in Step e, accompanied by the attack of superoxide on the substrate, would yield water and the expected product containing a hydroxyl group. Whether electron transfer precedes oxygen insertion, thereby yielding an intermediate complex that contains reduced cytochrome P-450 and superoxide [(RH)P-450(Fe^{2+}) ($O_2\cdot^-$)], remains to be established. This reaction sequence is in accord with the overall stoichiometry of the hydroxylation reaction,[26] as follows:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

It should be apparent from the scheme presented that upon the addition of the dismutase, Step e might be blocked, because of the loss of superoxide from the active complex, and that when a superoxide-generating system (such as xanthine and xanthine oxidase) is substituted for NADPH and the reductase, the oxidized P-450-substrate complex [(RH)P-450(Fe^{3+})] might be transformed directly into the superoxide-containing complex [(RH)P-450(Fe^{3+}) ($O_2\cdot^-$)], thereby bypassing Steps b, c, and d.

Several points should be emphasized concerning this scheme, which is presented as a working hypothesis.

1. Phosphatidylcholine must be present for rapid electron flow to occur from NADPH to cytochrome P-450 in the presence of the reductase. The mechanism by which the phospholipid exerts this effect is not yet known, but evidence has been obtained that its function is neither to cause the formation of a membrane-like complex or aggregate from the various components nor to assist in the binding of substrates to cytochrome P-450.[6,7] The studies so far carried out indicate that a dissociable complex containing cytochrome P-450, reductase, and phosphatidylcholine may function in catalysis. It should be noted that the phospholipid is required not only for electron transfer to cytochrome P-450, but also for hydroxylation in the presence of NADPH and the reductase or of superoxide-generating systems. It may be an essential component for all reactions catalyzed by liver microsomal cytochrome P-450.

2. The experiments thus far carried out on the inhibition of drug hydroxylation by the addition of superoxide dismutase or Tiron to the reconstituted liver microsomal enzyme system do not distinguish among three possible roles of superoxide. These are: an electron donor in Step b, the activated oxygen in the ternary complex, or the electron donor in Step e. Further comment on the role of superoxide as a possible diffusible electron carrier is given below.

3. As we have stated elsewhere,[11] biochemical studies on enzymatically or chemically generated superoxide must be interpreted cautiously, since some other species generated from or in equilibrium with superoxide could serve as the

actual activated oxygen. In this connection it may be noted that singlet molecular oxygen is produced from superoxide under certain conditions,[27] and that xanthine oxidase produces not only superoxide and hydrogen peroxide as the immediate products of oxygen reduction, but also hydroxyl radicals as a result of the interaction of these products.[28] Furthermore, recent evidence indicates that superoxide dismutase may act as a singlet oxygen quencher.[29] Much remains to be learned about the fate of superoxide in biological systems, but the scheme presented should serve as a useful working hypothesis.

Possible Role of a Diffusible Electron Carrier in the Membrane-Bound Hydroxylation System

The ability of the solubilized cytochrome P-450 (column P-450) to couple with various superoxide-generating systems is shown in TABLE 2. When xanthine and xanthine oxidase were substituted for NADPH and the reductase, drug demethylation occurred at about half the usual rate. Both the photochemical system that contained riboflavin and methionine[18] and a mixture of NADPH and the bromelin-solubilized NADPH-cytochrome *c* reductase were effective in enabling the cytochrome P-450 to effect benzphetamine demethylation. The results presented also indicate that superoxide dismutase readily inhibits the coupling of these nonphysiological reducing systems with cytochrome P-450. When the deoxycholate-solubilized reductase is present, however, a high salt concentration is required in order for the dismutase to be inhibitory. Presumably the salt has a chaotropic effect and is required only when the electron-transferring protein is firmly bound to the cytochrome P-450.

As stated above, our experiments suggest that superoxide plays a role in

TABLE 2

DRUG DEMETHYLATION CATALYZED BY SOLUBILIZED LIVER MICROSOMAL CYTOCHROME P-450 COUPLED WITH VARIOUS SUPEROXIDE-GENERATING SYSTEMS*

Reducing System	Drug Demethylation	
	Dismutase Absent	Dismutase Present
	% maximal rate	
NADPH + deoxycholate-solubilized NADPH-cytochrome P-450 reductase	100	70
	70†	27†
	45‡	0‡
Xanthine + xanthine oxidase	50	0
Riboflavin + methionine (reaction mixture light-irradiated)	70	0
NADPH + bromelin-solubilized NADPH-cytochrome *c* reductase	70	18

* The experiments were carried out under the usual assay conditions[26] with the column P-450 fraction[1, 3] as the rate-limiting component, and with the phospholipid and the reducing system (as indicated) at saturating concentrations. The rates were determined by the liberation of formaldehyde from benzphetamine as the substrate. The detergent-solubilized cytochrome P-450 reductase was prepared as described elsewhere.[3] A preparation of liver microsomal NADPH-cytochrome *c* reductase solubilized with bromelin and purified to homogeneity[33] was kindly provided by Mr. T. C. Pederson and Dr. S. D. Aust. Where indicated, superoxide dismutase (60 μg Pentex® preparation) was added to the reaction mixture.

† 0.4 M NaCl was present.

‡ 0.6 M NaCl was present.

hydroxylation reactions catalyzed by cytochrome P-450, but with such a complex system it is not yet possible to conclude whether this free radical species is an essential electron donor or plays a central role in the stage at which molecular oxygen is inserted into the various substrates. As was shown in other experiments not presented in the table, nonphysiological superoxide-generating systems appear to couple more readily with column P-450 preparations than with AS P-450 preparations. The question of whether an additional required component of the system is limiting in some of the AS P-450 preparations is under investigation. Presumably further purification of the system will indicate whether other electron carriers, such as non-heme iron proteins, are involved in liver microsomal hydroxylation reactions.

The possibility should also be considered that a diffusible electron carrier, such as superoxide, may serve to transfer electrons from the reductase to cytochrome P-450 in the intact membrane. It has been estimated that flavoprotein and cytochrome P-450 are present in molar ratios approaching 1:50 in microsomal membranes.[30] Presumably either the membrane must have a sufficiently mobile structure to permit the reductase to come into contact with the various cytochrome P-450 molecules, or a diffusible electron carrier may provide for efficient electron transfer. Superoxide would be a likely candidate for this role, assuming that it is sufficiently stable when in the hydrophobic environment of the membrane.

Summary

The mechanism of liver microsomal hydroxylation reactions has been investigated in a reconstituted enzyme system that contains solubilized cytochrome P-450, solubilized NADPH-cytochrome P-450 reductase, and phosphatidylcholine. The ability of superoxide-generating systems to couple with cytochrome P-450 to support the hydroxylation of benzphetamine and the ability of superoxide-generating agents to inhibit the reaction indicate that the superoxide radical anion ($O_2 \cdot^-$) or some closely related species is involved in the overall reaction mechanism. A scheme has been presented to indicate that superoxide may function as the donor of one or both of the electrons that enter the system, or as the source of the oxygen atom in the hydroxylated product.

Partially purified liver microsomal cytochrome P-450 contains additional heme and non-heme iron. Whether electron-transferring proteins other than the reductase and cytochrome P-450 are involved in this enzyme system remains to be established. Disc gel electrophoresis of the partially purified cytochrome P-450 showed the presence of proteins which migrate as a double band in the 55,000 molecular weight region.

The proposal has been made that the occurrence of a diffusible electron carrier, such as superoxide, may account for the ability of the reductase to catalyze the reduction of cytochrome P-450, which is present in relative excess in the endoplasmic reticulum.

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Discussion of the Paper

DR. SCHENKMAN (*Yale University School of Medicine, New Haven, Connecticut*): Dr. Coon, do I understand you to say that you think the phosphatidylethanolamine is functioning as a superoxide generator?

DR. COON: No. One must have phosphatidylcholine present for hydroxylation to occur, whereas phosphatidylethanolamine is inactive. So far as we know, the lipid has no effect on superoxide generation, but it must be there for electrons to flow to cytochrome P-450.

DR. HARVEY COHEN (*National Institute of Environmental Health Sciences*): Have you checked the effect of catalase on this system?

DR. COON: Yes, and catalase has absolutely no effect.