Section V - Topics in Biology

Editor: Christopher T. Walsh, Massachusetts Institute of Technology
Cambridge, MA 02139

Chapter 22. Molecular Aspects of Drug Metabolism

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I. Introduction--The oxidation of medicinal chemicals by the cytochrome P450-containing monooxygenase system is an important initial step in determining the fate of both the chemical agent and the biological system. Metabolism can often lead to a reduction in duration of drug action through the formation of less active metabolites and the production of compounds more readily excreted into the urine by the kidneys. This process of metabolic inactivation followed by excretion is often the major pathway for terminating the action of a drug in biological systems.

Not all biotransformations catalyzed by this system are so benign, since this enzyme system can also play a central role in the formation of reactive metabolites which are either toxic, mutagenic or carcinogenic. Indeed, studies demonstrating the metabolic activation of reactive metabolites by this system have enjoyed tremendous growth in recent years[1-6]. It is now apparent that the chemistry of the metabolite(s) often determines the ultimate biological fate of an animal exposed to a chemical agent.

Although the hepatic, microsomal cytochrome P450 system metabolizes compounds at a relatively low rate (0.1 to 50 min^{-1}), it has several other features that make it uniquely suited to oxidizing nonpolar drugs. This enzyme system is able to oxidize and reduce a wide variety of structurally unrelated compounds. The membrane location and the hydrophobic nature of cytochrome P450 also favors the partitioning of nonpolar compounds from the plasma to the enzyme for metabolism[7-10]. Components of this system are located in high abundance in the liver--an organ to which about 25% of the total cardiac output is channeled. About 30% of this blood is from the hepatic artery while the remainder is supplied by the alimentary tract, pancreas and spleen via the portal vein. Thus, drugs absorbed from the gut pass directly to the liver and the cytochrome P450 enzyme system. The organism also appears to adapt to increased substrate levels through the synthesis or induction of higher amounts of these monooxygenases[11]. This feature along with adaptive increases in hepatic perfusion[12] contribute to decrease the biological half-life and duration of drug action.

This chapter is intended as an overview of the molecular aspects of drug metabolism catalyzed by this hepatic enzyme system. The recent progress in the isolation and characterization of the protein components constituting this enzyme system, the interaction between these components and the mechanism of oxygen activation are reviewed. The reader is directed to several excellent review articles which treat, in more detail, specific aspects of this system

ISBN 0-12-040513-X

such as the physical properties of its components[13-16], organization of enzyme components in the microsomal membrane[17,18], enzyme induction by chemical agents[11] and the purification and reconstitution of its components[19,20]. Three books have recently appeared treating the general properties of the microsomal cytochrome P450 drug metabolizing system and the variety of reactions catalyzed by this system[21-23]. In addition, the International Symposium on Microsomes and Drug Oxidations continues to publish its proceedings in book form[24-26].

II. Components of the Monooxygenase System--Cytochrome P450 and NADPH-cytochrome P450 reductase (or NADPH-cytochrome c reductase) are the two essential protein components of the hepatic microsomal monooxygenase system.[a] Cytochrome P450 is a b-type cytochrome with the protoporphyrin IX prosthetic group[28]. Cytochromes of the b-type are generally characterized by low-spin heme prosthetic groups octahedrally coordinated with the two z-axial positions coordinated to protein ligands[29] (Fig. 1). The two protein ligands prevent interaction of the heme iron with external ligands such as O_2, CO and CN^- and, therefore, these heme proteins are neither autoxidizable nor do they interact with the usual inhibitors of heme enzymes.

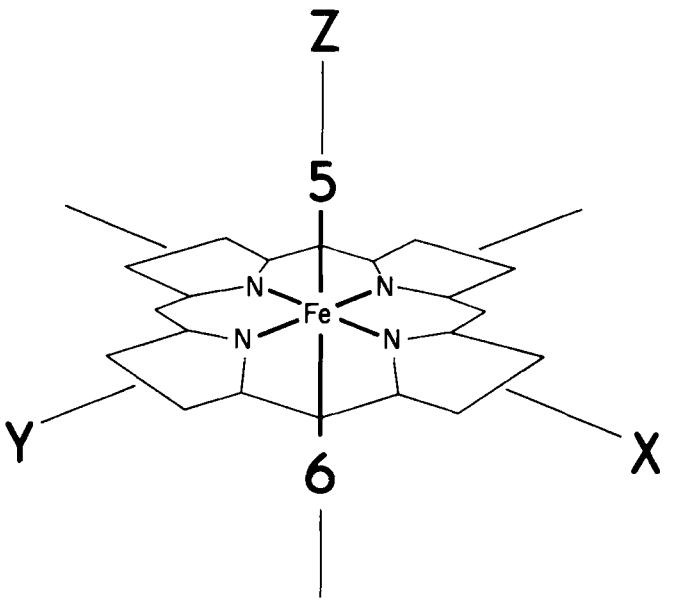


Figure 1

[a]Although cytochrome b_5 and NADH-cytochrome b_5 reductase have also been implicated in NADPH-dependent drug metabolism, these components have never been shown to be essential in reconstituting catalytic activity with purified enzyme components. A discussion of these components has therefore been omitted from the present chapter although the reader is referred to a review article on this subject[27].

Cytochrome P450 is an exceptional b-type cytochrome since it has either no protein residue[30] or an easily displaced protein residue coordinated in the sixth z-axial position[31,32]. As a result, it is readily autoxidizable[33] and combines with external ligands such as CN^-[34], CO[35], ethylisocyanide[36]. O_2[37,38] and many drug and steroid substrates[39-41] to give complexes that can be observed by visible spectroscopy. Cytochrome P450 therefore possesses the iron and heme properties of cytochromes necessary for electron transport. Moreover, it can also bind O_2 and substrate molecules--properties that make it uniquely suited as a terminal oxidase.

The solubilization and purification of this protein from mammalian sources has permitted extensive biochemical characterization. The cytochrome exists in multiple forms with a minimum molecular weight ranging between 45,000 and 60,000 (Table 1). Multiple forms may exist in the same species of animal[42-47] and may be differentially induced by exposure of the animal to various chemical agents[11,45-48]. These different species of cytochrome P450 demonstrate different affinities or absorption maxima for ligands such as CN^-, CO and ethylisocyanide[34,47,48]. Different, but overlapping substrate specificities have also been demonstrated for these forms[45,47,49,50]. The amino acid composition has been reported for two forms of cytochrome P450 from mammalian sources [51] and, more recently, the initial amino-terminal sequence of two forms of this protein from rabbit liver microsomes has been reported[52].

The fifth z-axial position of the oxidized cytochrome is believed to be occupied by a cysteine mercaptide anion. This hypothesis, first suggested by Mason[33] is supported by several investigations. Model studies have demonstrated that a sulfhydryl ligand coordinated to the heme iron satisfactorialy explains the unusual absorption maximum at 450 nm of the reduced cytochrome-CO complex[54-58] and the characteristically low-spin EPR spectrum . Of particular interest is the recent study by Kawalek, et al[59] suggesting that the role of the sulfhydryl group in catalysis may be dependent on the specific form of cytochrome P450. More structural and functional studies on the purified cytochrome P450 are obviously needed to definitely establish the role of the sulfhydryl group in catalysis. Chevion, et al[32] have recently suggested that the sixth ligand of the oxidized cytochrome P450 may be a histidine residue.

During the course of cytochrome P450-catalyzed oxidation reducing equivalents are transferred from NADPH to the oxidized cytochrome-substrate complex by NADPH-cytochrome P450 reductase. This enzyme has been shown by various criteria to be identical with NADPH-cytochrome c reductase[53,60-62]. The enzyme contains 1 mole of FAD and 1 mole of FMN per mole of enzyme and has a molecular weight of about 79,000[61,63,64]. The circular dichroism spectrum and amino acid composition have also been reported[65]. There is some evidence[20] for at least two forms of this enzyme in mammalian liver samples although immunological evidence suggests that only a single form exists from these sources[66,67].

In studies with the purified enzyme components and microsomal preparations, the NADPH-dependent enzyme reduces cytochrome P450 in two single electron transfer steps[68,69]. In the presence of substrate, O_2 and lipid this results in a reconstituted system capable of hydroxylating substrates without the requirement for other protein components such as cytochrome b_5 and adrenodoxin[20,70].

III. Multiple Forms of Cytochrome P450--The existence of isozymes of cytochrome P450 in liver microsomes has been suspected for many years [11,71-74]. The purification of many of these cytochrome P450 species in recent years represents a significant achievement in the area of drug metabolism. From studies with the purified enzymes, it is now clear that the species of cytochrome P450 confers the substrate specificity to the monooxygenase system [43,45,46,48,49].

Exposure of an animal to chemical agents such as phenobarbital or 3-methylcholanthrene results in the induction of cytochrome P450 species that differ in terms of the absorption maxima for the reduced cytochrome P450-carbon monoxide complex, molecular weight (Table 1) and immunological properties [76-78]. Table 1 also summarizes the metabolism of a precarcinogen, benzo[a]pyrene, and a drug substrate, benzphetamine, by various forms of cytochrome P450 in reconstituted systems containing fixed quantities of NADPH-cytochrome P450 reductase and lipid. Although all the species of cytochrome P450 catalyze the metabolism of benzo[a]pyrene and benzphetamine, the activity is markedly dependent on the particular form of cytochrome P450 examined. The activity varies over a hundred-fold range for benzo[a] pyrene hydroxylation and over a forty-fold range for benzphetamine N-demethylation. Since the isozymes of cytochrome P450 also have overlapping substrate specificities, the complex kinetics observed for substrate metabolism, patterns of inhibition [71-75] and the species, strain and sex differences, can now be explained in terms of these multiple forms in microsomal preparations.

In addition to differences in substrate specificity (Table 1), these hemeproteins also demonstrate differences in regiospecificity (different sites of oxidation on the same molecule) as has been demonstrated for testosterone [14,43,48] and benzo[a]pyrene [79,80]. These differences in catalytic properties would therefore influence the formation of potentially benign, cytotoxic or carcinogenic metabolites from drugs, polycyclic aromatic hydrocarbons and other foreign compounds.

IV. Component Organization and Interaction within the Microsomal Membrane-Cytochrome P450 is a major protein in microsomal preparations constituting up to 10% of the total, membrane-bound proteins [14]. There is a 20 to 30-fold mole excess of this protein relative to NADPH-cytochrome P450 reductase in these microsomal preparations. The spatial organization and mode(s) of interaction between these two proteins within the microsomal membrane are subjects of continued controversy. This has been comprehensively reviewed by Depierre and Dallner [17].

The surface localization of these proteins on the microsomal membrane has been investigated mainly through the use of macromolecule agents which do not penetrate the microsomal membrane. For example, the reductase flavoprotein is readily solubilized from the microsomal membrane by protease digestion [17,81] indicating that much of this protein is localized on the outer or cytoplasmic surface of the membrane. In contrast, protease treatment does not solubilize significant quantities of cytochrome P450 [17,81]. Furthermore, antibodies prepared against partially purified cytochrome P420 (a degradation product of cytochrome P450 resulting from protease treatment) do not inhibit

TABLE 1

BENZO[a]PYRENE AND BENZPHETAMINE METABOLISM BY RECONSTITUTED SYSTEMS CONTAINING VARIOUS SPECIES OF CYTOCHROME P-450

Cytochrome P-450 Species[a]	Absorption maximum of reduced CO-complex (nm)	Subunit M.W.[b]	Benzo[a]-pyrene nmol products/ min/nmol P-450	Benz-phetamine nmol products/ min/nmol P-450	Reference
Rat (PB)	450	(44,000 (46,000 (47,000	0.2	52	15
Rat (3-MC)	447	53,000	3.9	2	15
Mouse A_2(PB)	451	50,000	0.3	5	43
Mouse C_2 (PB)	450	56,000	0.1	8	43
Rabbit LM_1	448.6	47,000	1.5	3	45
LM_2(PB)	450.8	49,000	0.6	80	
LM_{4a}(β-NF)	446.6	53,000	1.3	2	
LM_{4b}(β-NF)	447.1	.53,000	0.2	5	
LM_7	449.3	60,000	1.2	2	
LM_x(PB)	448.4	53,000	0.8	2	
Rabbit form a (TCDD)	450.5	48,000	0.4	12	50
form b (TCDD)	448	60,000	4.1	4	
form c (TCDD)	447	54,500	0.03	2	

[a]Various forms of cytochrome P-450 were purified from liver microsomes of animals treated with different inducers indicated in parentheses. PB, phenobarbital; 3-MC, 3-methylcholanthrene; β-NF, β-naphthoflavone; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

[b]Molecular weight of subunit determined by gel electrophoresis in the presence of sodium dodecyl sulfate.

microsomal benzphetamine N-demethylation[82]. These data minimally suggest that the prosthetic group of cytochrome P450 is imbedded in the microsomal membrane and is not sensitive to either proteolytic enzymes or antibodies.

On the other hand, Welton et al[83] have demonstrated the ^{125}I-radiolabelling of this enzyme by lactoperoxidase while Thomas, et al[84] have shown the inhibition of various microsomal monooxygenase reactions by antibodies prepared against highly purified, detergent solubilized, cytochrome P450 preparations. These results are consistent with the surface localization of a significant portion of these hemeproteins on the microsomal membrane.

Several studies have examined the interaction between cytochrome P450 and reductase proteins within the microsomal membrane. This has been recently reviewed by Yang[18]. Two models have been proposed for the arrangement of reductase and cytochrome P450 molecules in the membrane. A "rigid" model in which a single reductase molecule is surrounded by a cluster or sphere of the more numerous cytochrome P450 molecules[85]. Ordered regions or clusters of these enzymes form discrete electron transport complexes. Thus, translational motion of the cytochrome is not required for the electron transport between these proteins necessary for substrate oxidation.

Alternatively, a "nonrigid" model has been proposed[86] in which both the reductase and cytochrome P450 molecules are randomly distributed within the matrix of the membrane. Lateral diffusion mobility is required between reductase and cytochrome P450 for efficient electron coupling between these proteins.

V. Mechanism of the Cytochrome P450 Monooxygenase Reaction--Investigations into the mechanism of substrate oxidation by the cytochrome P450 system were initiated soon after the discovery of microsomal cytochrome P450[35,87] and the class of enzymes collectively known as oxygenases[88,89]. The monooxygenase nature of cytochrome P450-catalyzed reactions, in which one atom of molecular oxygen is transferred to the substrate, was demonstrated by Posner, et al[90] with $^{18}O_2$ experiments.

$$NADPH + SH + {}^{18}O_2 + H^+ \longrightarrow NADP^+ + S^{18}OH + H_2{}^{18}O \qquad (1)$$

Unfortunately, the microsomal enzyme system contains several enzymes such as catalase and pyrophosphatases that impeded early attempts to quantitatively elucidate the stoichiometry and mechanism of cytochrome P450-catalyzed reactions. Nevertheless, considerable knowledge about the sequence of events leading to substrate oxidation by cytochrome P450 has been obtained. This has been recently reviewed by Estabrook and Werringloer[91] and is summarized in Figure 2. Briefly, the substrate (SH) binds reversibly to the oxidized cytochrome to form a binary complex. NADPH-cytochrome P450 reductase catalyzes a one electron transfer to form a ferrous cytochrome P450-substrate complex. The reduced cytochrome can bind either CO to give a λ_{max} 450 nm absorbing species, characteristic of this protein, or to O_2 forming the ternary SH-cytochrome P450$(Fe)^{2+}$-O_2 complex. The latter species has been observed,

under steady state conditions, in both microsomal[37] and purified enzyme[38] systems. A second electron equivalent is transferred from NADPH via the reductase to the ternary complex. The actual charge distribution between the cytochrome iron and O_2 are unknown in this complex and therefore a formal charge of plus one is indicated outside the brackets containing the entire complex. One possible distribution of charge within the complex giving an "active oxygen" species is also illustrated. In this case, O_2 has taken on the formal oxidation state of H_2O_2. This ternary complex, containing the "activated oxygen" then breaks down to give the hydroxylated product (SOH), water and oxidized cytochrome P450.

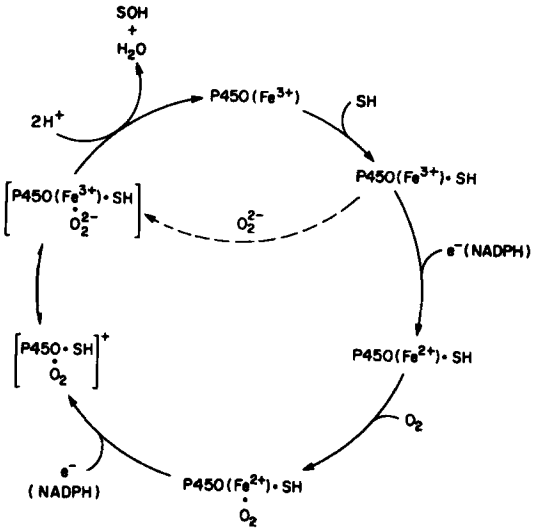


Figure 2

In recent years, the combined efforts of biochemists and organic chemists have resulted in the successful purification of various components of this multienzyme system and the elucidation of several chemical models for cytochrome P450-catalyzed reactions. Although no definitive mechanism has emerged, the arena of possible mechanisms has been considerably narrowed.

The purified and reconstituted cytochrome P450 system has been particularly useful in mechanism studies. For example, Nordblom and Coon[92] have demonstrated the 1:1:1 stoichiometry between NADPH oxidation, O_2 utilization and product(s) formation required by Equation 1 which had eluded earlier attempts with the microsomal system. In addition to the substrate hydroxylating capacity of cytochrome P450, NADPH is also oxidized by O_2 to form $NADP^+$ and H_2O_2 (Equation 2). Whether this reaction results from the autoxidation of cytochrome P450 following the second electron transfer (Equation 3) or from a

reduced flavin-catalyzed formation of H_2O_2 from O_2, via free radical intermediates, by NADPH-cytochrome P450 reductase (Equation 4) or a combination of both pathways is unknown.

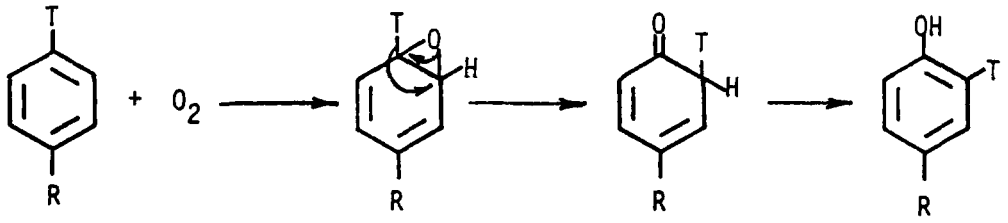
$$NADPH + O_2 + H^+ \longrightarrow NADP^+ + H_2O_2 \quad (2)$$

$$P450(Fe^{++})O_2 + e^- + 2H^+ \longrightarrow P450(Fe^{+++}) + H_2O_2 \quad (3)$$

$$FADH_2 + O_2 \longrightarrow FADH\cdot + HO_2\cdot \longrightarrow FAD + H_2O_2 \quad (4)$$

Both reaction 1 leading to substrate hydroxylation and reaction 2 leading to H_2O_2 formation occur in microsomal[93-95] and reconstituted systems[92]. The sum of reactions 1 and 2 account for the total quantities of substrate and cofactor oxidized versus hydroxylated product(s) and H_2O_2 formed[92].

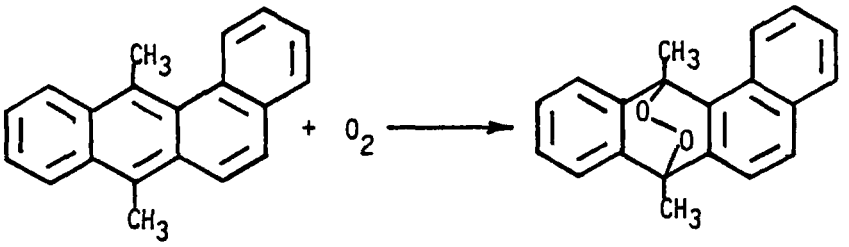
Perhaps the most plausible description of the "active oxygen" species involved in cytochrome P450-catalyzed oxidations was proposed by Hamilton[92]. By analogy to carbene and nitrene chemistry, Hamilton postulated a transition state for an electrophilic "active oxygen" species with electronic properties similar to a singlet oxygen atom. The oxygen insertion reaction catalyzed by the cytochrome P450 system of unactivated alkanes to give alcohols and of aromatic compounds to form phenols and arene oxides, finds analogy in the carbene insertion reaction between C-H bonds of alkanes to give alkylbenzenes and of aromatic compounds to form norcaradienes. This oxenoid or oxygen atom transfer mechanism is also supported by the "NIH shift" reaction[97] observed with the microsomal cytochrome P450 system. The product, 4, in which tritium



1 2 3 4

migration is observed is best explained by the epoxide intermediate, 2, formed by the electrophilic attack of an oxenoid reagent. The "NIH shift" mechanism has been recently confirmed[98] with a reconstituted system employing partially purified enzyme components.

It is generally agreed that oxidation of the substrate occurs after the second electron reduction of the ternary complex by NADPH-cytochrome P450 reductase to form $[SH\text{-}P450\text{-}O_2]^{+91}$. Thus, there are three possible oxenoid reagents; (1) SH-P450(Fe^{+})-O_2 complex, (2) SH-P450(Fe^{3+})-O_2^{2-} or (3) SH-P450(Fe^{5+})-O complex. Of the three, the second complex appears to be the most probable since H_2O_2[99], electrochemically generated HO_2^-[100], and organic hydroperoxides[101,102] can, in many cases, support substrate hydroxylation (Figure 2) in systems containing cytochrome P450 but devoid of O_2, NADPH and NADPH-cytochrome P450 reductase. The cytochrome P450-dependent oxygen insertion reaction supported by cumene hydroperoxide results in the incorporation of oxygen primarily from the peroxide[99]. Furthermore, ferrous cytochrome P450 is not involved since CO does not inhibit this reaction. Thus, the role of both inorganic and organic peroxides may be in the formation of an "active oxygen" species similar to the enzyme-peracid or enzyme-peramide intermediates postulated by Hamilton[103] for the cytochrome P450 monooxygenases.



5 6

Molecular singlet oxygen has largely been ruled out for energetic reasons as an oxenoid reagent in cytochrome P450-catalyzed reactions[103]. One possible exception, however, is the addition of O_2 to 9,10-dimethyl-1,2-benzanthracene, 5, to form the transannular 1,4-peroxide, 6, reported by Chen and Tu[104]. The transannular product, 6, is the expected product from a singlet oxygen reaction with 5. Singlet O_2 originating from the Haber-Weiss[105,106] reaction has been documented and may be a possible explanation for this singlet oxygen reaction in microsomal incubations.

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