HYDROPEROXIDE DEPENDENT O-DEALKYLATION REACTIONS CATALYZED BY LIVER MICROSOMAL CYTOCHROME P450

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Summary: The organic hydroperoxide, cumene hydroperoxide was capable of supporting the O-dealkylation of a variety of methoxy and ethoxy compounds in the presence of hepatic microsomes - NADPH and molecular oxygen were not required for this reaction. Cytochrome P450 was implicated as the catalyst. Hemin and other hemoproteins were ineffective. The K_m for the substrate was much higher with the cumene hydroperoxide dependent reaction than for the NADPH dependent reaction. The significance of the cumene hydroperoxide dependent reaction in elucidating the mechanism of cytochrome P450 reactions is discussed.

Introduction: The hepatic mixed function oxidase system metabolizes a variety of drugs, steroids, fatty acids, alkanes and other foreign compounds (1,2). In rat and rabbit liver microsomes this system has been resolved into three fractions consisting of cytochrome P450, NADPH-cytochrome c reductase, and a lipid component (3). Cytochrome P450 has been identified as the terminal oxidase of this multi component system (4). Recently, Hrycay and O'Brien (5,6) have shown that cytochrome P450 can also act as a peroxidase by demonstrating its ability to catalyze the oxidation of a variety of electron donors such as NADH, NADPH and TMPD in the presence of organic hydroperoxides. Furthermore, Kadlubar et al. (7) have described the ability of similar hydroperoxides to support the C-oxidation of several amine substrates in a reaction that does not require molecular oxygen or NADPH. More recently, we have shown that cumene hydroperoxide (CHP)[1] can sustain the aromatic hydroxylation of a variety of substrates such as coumarin, benzpyrene, biphenyl and aniline also in the absence of NADPH and molecular oxygen (8). One mole of CHP was shown to be consumed for every mole of substrate hydroxylated. 2-phenyl-2-propanol (cumenol) was identified as the major reduction product of CHP.

[1]Abbreviations: CHP=cumene hydroperoxide; TMPD=tetramethyl-p-phenylenediamine

In this communication, we show that cumene hydroperoxide is also capable of supporting the O-dealkylation of a variety of methoxy and ethoxy substrates. NADPH and molecular oxygen are not required. Hemin or other hemoproteins could not replace cytochrome P450 in this reaction.

Materials and Methods: $NADP^+$, NADPH, horse radish peroxidase, catalase, myoglobin and hemin were purchased from the Sigma Chemical Company. Cumene hydroperoxide was obtained from Matheson, Glemen and Bell. All other reagents and chemicals were of the highest purity commercially available.

Rats or rabbits were pretreated with phenobarbital (PB) or 3-methylcholanthrene (3-MC) and the microsomes obtained from the livers by established methods (9). The dealkylation of 7-ethoxycou marin was assayed fluorometrically by the method of Ullrich and Weber (10) except that the substrate 7-ethoxycoumarin was added to the assay mixture in 50 µl of ethanol. p-Nitroanisole demethylase was assayed by the method of Netter and Seidel (11). In all cases, O-demethylation was assayed by measuring the formaldehyde formed (12) and demethylation measured by the amount of acetaldehyde formed (13). All the CHP dependent dealkylations were initiated by the addition of 0.5 mM CHP (final concentration) to the reaction mixture. All rates are initial rates.

Results: Experiments described in this section indicate that CHP is capable of sustaining the O-dealkylation of a variety of substrates in the presence of liver microsomes (Table I). NADPH, the flavoprotein NADPH-cytochrome c reductase and molecular oxygen were not required for this reaction. p-Nitroanisole demethylation was linear up to a protein concentration of at least 3.0 mg/ml and the dealkylation up to at least 1.0 mg/ml. The CHP dependent O-deethylation of 7-ethoxycoumarin had a slightly higher pH optimum (9.0) than the NADPH dependent reaction (8.4). In other cases the pH optimum was in the region of pH 7.5 - 8.0. Under our assay conditions, all the dealkylation rates were linear with time for 1-2 minutes. Heating the microsomes at 70° C for two minutes completely abolished the dealkylation activity.

Effect of Various Agents on the O-dealkylation Activity: Both the NADPH- and CHP-dependent dealkylation of 7-ethoxycoumarin (Table II) and p-nitroanisole responded similarly to several cytochrome P450 inhibitors. TMPD inhibited the dealkylation by competing with the substrate for oxidation. TMPD was previously shown to be an effective hydrogen donor in the peroxidase activity of cytochrome P450 (5). Carbon monoxide, which is known to combine with the reduced form of cytochrome P450 (Fe^{2+}) inhibited

Table I

Comparison of NADPH and CHP Dependent O-Dealkylation

Species	Pretreatment	Substrate	NADPH and O_2 Dependent	CHP Dependent
Rabbit	PB	7-ethoxycoumarin	1.5	3.6
Rabbit	PB	p-nitroanisole	2.3	12.0
Rabbit	PB	Norcodeine	6.2	6.2
Rabbit	PB	P. anisic acid	5.1	6.1
Rabbit	PB	Phenacetin	2.3	2.7

These assays were carried out at 22° - 25° C as described in Methods. Rates are expressed as nmoles of product formed/min/mg/protein

only the NADPH dependent reaction. The CHP dependent reaction does not proceed via ferrous cytochrome P450 (see Discussion). $NADP^+$, which is known to inhibit only the flavoprotein NADPH-cytochrome c reductase, affected only the NADPH dependent reaction. The lack of inhibition in the presence of EDTA or superoxide dismutase indicates that the CHP-dependent demethylation reaction is not mediated by metal ions or by the superoxide anion radical. Conversion of cytochrome P450 into P420 with sodium dodecyl sulfate inhibited the dealkylation reaction.

Effect of Varying the CHP Concentration: The rate of dealkylation of 7-ethoxycoumarin and p-nitroanisole increased with increasing CHP concentrations. With 4.5 mM p-nitroanisole the maximum dealkylation rate was obtained with a hydroperoxide concentration of about 0.5 mM. A Lineweaver-Burke plot gave an apparent K_m for hydroperoxide of 0.25 mM.

Effect of Varying the Substrate Concentration: Using PB-induced rabbit liver microsomes it was found that the K_m for the same substrate is different in the NADPH and CHP catalyzed reactions. In the case of p-nitroanisole demethylation, the K_m for p-nitroanisole for the NADPH dependent reaction was 39 μM while for the CHP dependent system it was about 4.0 mM for a hydroperoxide concentration of 1.0 mM. Similar results were observed in the O-deethylation of 7-ethoxycoumarin and the hydroxylation of aniline. These differences were maintained in a reconstituted system using highly purified rabbit PB-induced cyt. P450 and either CHP or NADPH+NADPH-cyt. P450 reductase.

Table II

Effect of Various Inhibitors on the Dealkylation of 7-ethoxycoumarin[a,b]

Additions to the Standard Reaction Mixture	NADPH & O_2 Dependent	CHP Dependent
Control	100	100
+ EDTA (0.1 mM)	101	100
+ CO	43	100
+ Mn^{2+} (1.0 mM)	114	98
+ Mg^{2+} (5.0 mM)	126	100
+ KCN (0.1 mM)	63	80
+ KCN (1.0 mM)	13	32
+ SDS[c] (0.05%)	65	56
+ SDS[c] (0.1%)	5	8
+ TMPD[d] (75 μM)	64	37
+ 1P1[e] (15 μM)	0	0
+ Androstenedione (0.1 mM)	63	48
+ SKS 525A (0.6 mM)	22	26
+ Metyrapone (0.1 mM)	28	18
+ Superoxide dismutase (0.1 mg/ml)	92	91
+ $NADP^+$ (1.0 mM)	7	101

[a] These assays were carried out with phenobarbital pretreated rabbit liver microsomes as described in Methods; rates are expressed relative to control.

[b] Qualitatively similar results were obtained with p-nitroanisole demethylation.

[c] Sodium dodecyl sulfate.

[d] N, N, N', N' - Tetramethyl-p-phenylenediamine Dihydrochloride.

[e] 1-(2-Isopropyl-phenyl)-Imidazole.

The reason for this difference in K_m may reflect a different rate limiting step in the NADPH dependent system from the hydroperoxide catalyzed reaction. The reduction of cytochrome P450 is believed to be the rate limiting step in the NADPH dependent system. Alternatively, or in addition, the

bulk of the cumene hydroperoxide molecule sterically hinders access of the substrate to the active site.

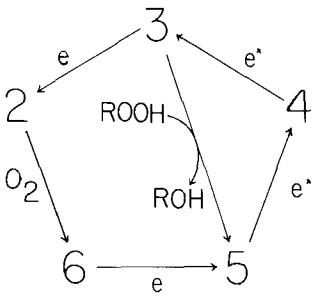
Specificity of Cytochrome P450: The requirement for cytochrome P450 in the CHP dependent O-dealkylation reaction appears to be essential. Using 7-ethoxycoumarin and p-nitroanisole as substrates, it was found that microsomal cytochrome P450 could not be replaced by hemin, catalase, horse radish peroxidase or myoglobin. Furthermore, conversion of cytochrome P450 into P420 resulted in inactivation. In contrast, Kadlubar et al. (7) found that catalase could catalyse the CHP dependent C-oxidation of diethyl aniline, aminopyrine and benzphetamine.

Discussion: The mechanism of oxygen activation required for substrate hydroxylation in cytochrome P450 catalyzed reactions has eluded definition. A cyclic reaction sequence describing the interaction of various substrates, electrons and oxygen with cytochrome P450 has been proposed (14-18) and the details for some of these individual steps elucidated. However, the chemical nature of the oxygenated form of reduced cytochrome P450, as well as the subsequent steps associated with the insertion of one oxygen atom of molecular oxygen into the substrate molecule, remain unknown.

The data presented in this communication and in others (7,8) indicate that CHP is capable of supporting the N- and O-dealkylation as well as aromatic hydroxylation of a variety of substrates in a reaction that does not require NADPH, the flavoprotein NADPH: cytochrome c reductase or molecular oxygen. Also, the peroxidative function of cytochrome P450 has been well documented (5,6). Presumably, the substrates undergo hydroxylation by acting as an electron donor in a peroxidase mechanism.

In addition, we have recently shown (19,20) that cytochrome P450 can function as a dioxygenase in the peroxidation of polyunsaturated lipids in the presence of hydroperoxides. In this instance, the mechanism is believed to involve first the peroxidase action of cytochrome P450 with polyunsaturated lipid as donor, followed by an oxygen dependent chain reaction. In view of this, it is conceivable that the monooxygenase property of cytochrome P450 may consist of a dioxygenase activity leading to the formation of a substrate hydroperoxide followed by a peroxidase activity causing the formed hydroperoxide intermediate to be reduced to a hydroxylated product and water. Indeed, such hydroperoxide intermediates have been proposed for a number of reactions (21,22).

On the other hand, Schonbaum and Lo (23) have shown that the conversion of HRP to compound I by the addition of a stoichiometric amount of



e* from NADPH, NADH, TMPD or a suitable substrate

Fig. 1. Proposed scheme for the cyclic reduction and oxidation transitions of cytochrome P450 during hydroxylation reactions. The site of interactions of organic hydroperoxides and the high valence states of heme iron are indicated. 2 and 3 are ferrous and ferric cytochrome P450.4, 5 and 6 are peroxidase Compounds II, I and III, respectively.

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ethyl hydrogen peroxide is attended by a release of ethanol but without concomitant prototropic changes. This establishes that HRP compound I is not an enzyme peroxide complex but a derivative in which the active site is oxidized. If this analogy is extended to cytochrome P450 reactions it means that the active complex consists of only one oxygen atom attached to the heme iron. In this case, cumenol (CHP catalyzed reactions) or H_2O (TPNH and O_2 catalyzed reactions) would be eliminated first.

Addition of CHP (but not H_2O_2) to rabbit liver microsomes results in the appearance of a transient spectral species characterized by an intense absorbance change at 441 nm in the difference spectrum concomitant with a loss of absorbance at about 420 nm (24). George and Irvine (25) observed a similar spectral change when H_2O_2 was added to methyoglobin and they attributed it to the ferryl state of the heme (4+). When samples of microsomes were examined by EPR spectroscopy, the presence of CHP (but not (H_2O_2) results in a decrease in the magnitude of the low spin form of ferric cytochrome P450 and the appearance of a unique EPR signal at g=2.01 and g= 2.04 (24). A similar EPR signal, typical for a spin one-half species with axial symmetry, was observed when H_2O_2 was added to cytochrome c peroxidase (26). In this case, the signal was thought to be due to a peroxide derived oxygen atom near the heme-associated iron system and found in the cytochrome c peroxidase hydrogen peroxide product (26,27), i.e., the peroxidase had undergone a two-electron equivalent oxidation. The mechanism

described in Fig. 1 using the pentagonal diagram of the five oxidation states of peroxidase (from Yamazaki et al., 28) suggests that with cytochrome P450 the substrate to be hydroxylated or other sources of reducing equivalents derived from NADH, NADPH or TMPD[1] can be utilized to discharge this proposed higher valence intermediate of cytochrome P450 in a manner analogous to that proposed for peroxidase catalyzed reactions. The ease with which CHP carries out N- and O-dealkylation as well as aromatic hydroxylations in the presence of hepatic microsomes suggest that such a peroxidase type mechanism may be operating.

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