HYDROPEROXIDE CATALYZED LIVER MICROSOMAL AROMATIC HYDROXYLATION REACTIONS INVOLVING CYTOCHROME P-450

Anver D. Rahimtula and Peter J. O'Brien

Department of Biochemistry, Memorial University of Newfoundland, St. John's Newfoundland, CANADA, and
Department of Biochemistry, University of Texas Southwestern Medical School, 5323 Harry Hines Blvd., Dallas, Texas 75235.

Received July 25,1974

SUMMARY

Cumene hydroperoxide is capable of supporting the aromatic hydroxylation of a variety of compounds in the presence of hepatic microsomes. NADPH and molecular oxygen are not required. Cytochrome P-450 acts as the catalyst and could not be replaced by other hemoproteins. One mole of hydroperoxide is consumed for every mole of substrate hydroxylated. It is suggested that the oxenoid species of cytochrome P-450 involved in microsomal aromatic hydroxylation is present in a form equivalent to the ferryl from.

INTRODUCTION

Cytochrome P-450 is known to be a component of many monooxygenase reactions for the metabolism of steroids, fatty acids, drugs, alkanes and other xenobiotics in vertebrate tissues (1). In this reaction cytochrome P-450 is involved in the reduction and activation of molecular oxygen by a two electron transfer presumed not to yield H_2O_2. It is generally accepted that the mechanism involves the reduction of a cytochrome P-450 - substrate complex via a reduced flavoprotein involving a one electron transfer (2). The next step in the reaction sequence is considered to consist of a interaction with molecular oxygen of the complex of substrate with reduced cytochrome P-450 giving rise to a ternary reduced cytochrome P-450-substrate-oxygen complex (3,4). This complex subsequently accepts a second electron with the formation of a yet unidentified active oxygen-cytochrome P-450-substrate complex. After transfer within the complex of one oxygen atom and the uptake of a proton, the complex dissociates into oxidized cytochrome P-450, water, and hydroxylated product. The nature of the active oxygen which hydroxylates the sub-

strate in the ternary complex has been one of the most important as well as difficult questions to answer. In 1964, Hamilton (5) suggested that monooxygenases catalyze their reactions by an oxygen atom transfer or oxenoid mechanism because of their similarity to carbene and nitrene reactions. The fact that the entering hydroxyl group can displace a deuterium substituent to an adjacent position during aromatic hydroxylation also suggests that the active hydroxylating species is oxenoid (6).

Recently, Ziegler and coworkers (7) have shown that cytochrome P-450 can catalyze the organic hydroperoxide dependent C-oxidation of several amines in the absence of NADPH and molecular oxygen. They suggested that organic hydroperoxides may simply provide an alternate means of forming peroxy-heme which could function as an oxidant in certain cytochrome P-450 dependent oxidations. Also, Hrycay and O'Brien (8-10) have demonstrated that cytochrome P-450 can act as a peroxidase with various organic hydroperoxides. Using cumene hydroperoxide as a model organic hydroperoxide, we have now shown that it can also support the hydroxylation of benzpyrene, coumarin, biphenyl and aniline in the absence of NADPH and oxygen. It is suggested that the oxenoid species of cytochrome P-450 in microsomal aromatic hydroxylation is present as the ferryl ion.

MATERIALS AND METHODS

NADPH, $NADP^+$, catalase, myoglobin, horse radish peroxidase and hemin were purchased from the Sigma Chemical Company. Cumene hydroperoxide was obtained from Matheson, Coleman and Bell. All other chemicals and reagents were of the highest grade available. Cytochrome b_5 was a gift of Dr. Hrycay.

Rats or rabbits were pretreated with phenobarbital, 3-methylcholanthrene or corn oil and microsomes obtained from the livers by established methods (11). Coumarin 7-hydroxylase activity was measured by the method used by Ullrich and Weber (12) to assay 7-ethoxycoumarin O-deethylase except that the substrate coumarin was added in 50 µl of ethanol. The product in both cases is 7-hydroxycoumarin. Benzpyrene hydroxylase was measure according to Dowd

et al. (13). Aniline (14) and biphenyl (15) hydroxylase activities were measured by established methods. Benzpyrene hydroxylation was initiated by adding 75 μM cumene hydroperoxide; in all other cases 1.5 mM cumene hydroperoxide was used. All rates are initial rates.

RESULTS

Experiments described below show that cumene hydroperoxide can support the aromatic hydroxylation of a variety of substrates in the presence of hepatic microsomes (Table I). NADPH and molecular oxygen are not required for this reaction. Aniline hydroxylase activity was linear up to a protein concentration of 2 mg/ml while benzpyrene and coumarin hydroxylations were linear up to about 0.7 mg protein/ml. All the hydroxylation rates were linear with time for 1-2 minutes. The pH optimum for the hydroperoxide catalyzed coumarin hydroxylation was slightly higher (pH 9.0) than that for the NADPH catalyzed reaction (pH 8.4) (Fig. 1). The pH optimum for the aniline and benzpyrene hydroxylations using cumene hydroperoxide is 7.4 and 8.1 respectively. Heating the microsomes at 70°C for two minutes completely abolished the hydroxylase activity.

Effect of Varying the Cumene Hydroperoxide Concentration Fig. 2 shows the

TABLE I COMPARISON OF NADPH AND CUMENE HYDROPEROXIDE DEPENDENT AROMATIC HYDROXYLATION[a]

Species and Pretreatment		Substrate	NADPH and O_2 dependent	Hydroperoxide dependent
Rat	Corn oil	Biphenyl[b]	0.6	1.7
Rat	Corn oil	Benzpyrene	0.78	0.89
Rabbit	Phenobarbital	Coumarin	0.062	0.055
Rabbit	3-MC	Aniline	0.51	50

[a] These assays were carried out as described in Methods; rates are expressed as n.moles of product formed/min/mg protein.

[b] A.D. Rahimtula and M. D. Burke, unpublished observation.

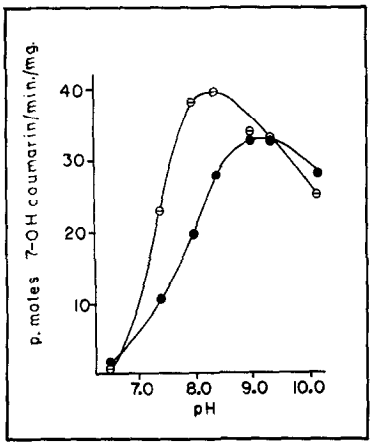

Fig. 1 pH profile of the NADPH (⊖—⊖—⊖) and cumene hydroperoxide (●—●—●) catalyzed coumarin 7-hydroxylation. Incubations were carried out as described in Methods. The buffer was 0.1 M tris adjusted to the pH with HCl at the incubation temperature (22°C).

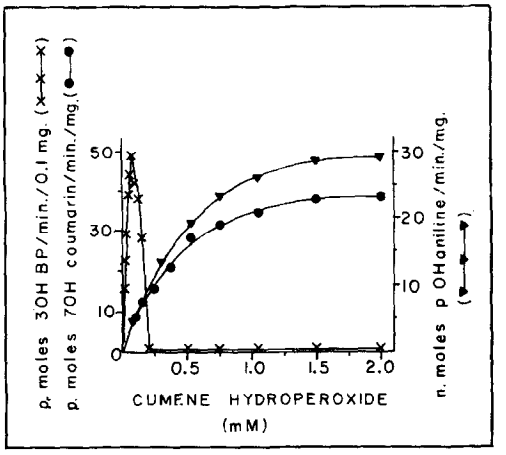

Fig. 2 Effect of increasing the cumene hydroperoxide concentration on the rate of hydroxylation of aniline (▼—▼—▼), benzpyrene (×—×—×), and coumarin (●—●—●). Incubations were carried out as described in Methods.

---

rate of hydroxylation with increasing cumene hydroperoxide concentrations. With aniline, coumarin and biphenyl (not shown in graph), maximal rates were obtained with a peroxide concentration of about 1.5 mM; the peroxide concentration required for half-maximal activity (apparent K_s) being roughly 0.5 mM. However, with benzpyrene hydroxylation the rate increased with cumene hydroperoxide concentration up to about 80 μM; further addition of

hydroperoxide caused a sharp decline in the reaction rate and at 0.2 mM cumene hydroperoxide there was no measureable activity. The reason for this critical effect on the hydroperoxide concentration used is not clear although similar results were obtained for the cumene hydroperoxide dependent oxidation of ethanol to acetaldehyde by liver microsomes*.

Effect of Various Agents on Aromatic Hydroxylation Using coumarin as the substrate, it was found that both the NADPH and cumene hydroperoxide catalyzed reactions responded similarly to several cytochrome P-450 inhibitors (Table II). The lack of inhibition in the presence of EDTA or superoxide dismutase

TABLE II EFFECT OF VARIOUS INHIBITORS ON COUMARIN 7-HYDROXYLASE ACTIVITY[a]

Additions to the standard reaction mixture	NADPH & Oxygen dependent	Cumene hydroperoxide dependent
Control	100	100
+ EDTA (0.1 mM)	102	103
+ Mg^{2+} (5 mM)	50	46
+ TMPD* (0.01 mM)	69	54
+ TMPD* (0.1 mM)	0	0
+ KCN (0.1 mM)	59	82
+ KCN (1 mM)	13	36
+ SDS[x] (0.05%)	65	41
+ SDS[x] (0.1%)	0	0
+ Metyrapone (0.1 mM)	33	61
+ Androstenedione (0.1 mM)	33	74
+ SKF 525A (0.6 mM)	20	62
+ Superoxide dismutase (0.1 mg)	103	104
+ $NADP^+$ (1 mM)	39	100

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

* N,N,N',N'-Tetramethyl-p-phenylenediamine Dihydrochloride

[x] Sodium Dodecyl Sulfate

indicates that the peroxide dependent hydroxylation reaction is not mediated by metal ions or by the superoxide anion radical. $NADP^+$, which is known to inhibit only the flavoprotein NADPH-cytochrome P-450 reductase, does not have any effect on the peroxide dependent reaction.

*unpublished observation

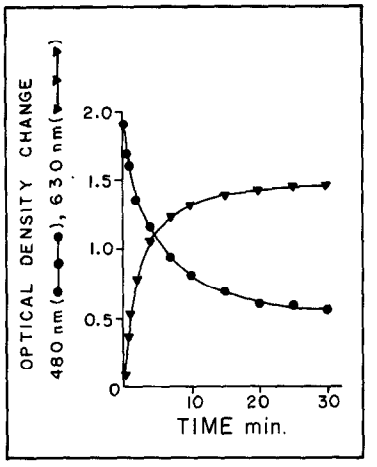

Fig. 3 Time course of aniline p-hydroxylation (▼—▼—▼) and cumene hydroperoxide decomposition (●—●—●). The slow endogenous rate of cumene hydroperoxide decomposition in the absence of aniline was subtracted.

Specificity of Cytochrome P-450 The requirement for cytochrome P-450 in the cumene hydroperoxide dependent aromatic hydroxylation reaction appears to be absolute. Using coumarin and aniline as substrates, it was found that microsomal cytochrome P-450 could not be replaced by hemin, catalase, horse radish peroxidase, myoglobin or cytochrome b_5 or by conversion of cytochrome P-450 to cytochrome P-420. In contrast, Ziegler and coworkers (7) found that catalase was a very effective catalyst for the cumene hydroperoxide dependent C-oxidation of dimethylaniline, aminopyrine and benzphetamine.

Stoichiometry of Hydroxylation Using aniline as the substrate it was found that the rate of p-hydroxyaniline formation closely followed the rate of cumene hydroperoxide decomposition (Fig. 3); one mole of hydroperoxide being consumed for every mole of aniline hydroxylated. Cumene hydroperoxide concentration was measured by the ferrithiocyanate method (16).

Addition of Cumene Hydroperoxide to Microsomes Addition of cumene hydroperoxide to rabbit liver microsomes gives rise to a unique spectral change (17) characterized in the difference spectrum by the appearance of absorption band peaks at 442 nm and 536 nm and troughs at 422 nm and 579 nm. This spectrum resembles the spectrum obtained by Yonetani and Schleyer (18) on the addition

of H_2O_2 to ferrimyoglobin and found by George and Irvine (19) to be ferrylmyoglobin.

DISCUSSION

The data shown above indicate that organic hydroperoxides can support aromatic hydroxylations in the presence of liver microsomes. Cytochrome P-450 is implicated as the catalyst for this reaction. The nature of the oxygenating species in the enzymatic hydroxylation of aromatic substrates is still a subject of much speculation. In 1964, Hamilton (5) proposed the oxygen atom transfer or oxenoid mechanism for aromatic hydroxylation reactions. Support for such a mechanism came from the work of Jerina, Daly and coworkers (6) who showed that enzymic aromatic hydroxylations displayed the 'NIH Shift'. Recently, Hamilton et al (20) suggested that the most likely structure for such an oxenoid reagent would be similar to that suggested for Compound I in the catalase reaction. Also, Hrycay and O'Brien (8-10) have shown that cytochrome P-450 can act as a peroxidase. The readiness with which an organic hydroperoxide can catalyze aromatic hydroxylations by liver microsomes at rates at least as rapid as those obtained in the presence of NADPH and oxygen strongly suggests that a peroxidase mechanism is operating. The oxenoid species involved is therefore very likely a ferryl ion and similar to compound I or compound II in catalase or peroxidase reactions. The ferryl species has been considered to be only capable of hydrogen abstraction but the N-dealkylation of sec.- and tert. amines by microsomes in the presence of sec. and tert. hydroperoxides (7) again suggests a ferryl involvement in aromatic hydroxylation reactions.

ACKNOWLEDGEMENTS

Supported by the National Research Council of Canada (Grant No. A4918). We wish to express our appreciation to Professor Ronald W. Estabrook for his interest and encouragement and for partial financial support (Grant No: 2 ROl GM 1648806).

REFERENCES

1. Estabrook, R. W., Schenkman, J. B., Cammer, W., Remmer, H., Cooper, D. Y., Narashimhulu, S., and Rosenthal, O. (1966) in 'Biological and Chemical Aspects of Oxygenase' p. 153 (K. Bloch and O. Hayaishi, editors) Mazuzen Co. Ltd Tokyo.
2. Ullrich, V., Cohen, B., Cooper, D. Y., and Estabrook, R. W. (1968) in 'Structure and Function of Cytochromes', p. 649, (K. Okunuki, M. D. Kamen, and I. Sekuzu, editors) Univ. Park Press, Baltimore, Md.
3. Gunsalus, I. C. (1970) Reported at the Werner-Gren Symposium on Structure and Function of Oxidation-Reduction Enzymes, Stockholm.
4. Ishimura, I., Ullrich, V., and Peterson, J. A. (1971) Biochem. Biophys. Res. Comm. 42, 140.
5. Hamilton, G. A. (1964) J. Amer. Chem. Soc. 86, 3391.
6. Jerina, D. A. (1973) Chem. Technol. 4, 120.
7. Kadlubar, F. F., Morton, K. C., and Ziegler, D. M. (1973) Biochem. Biophys. Res. Comm. 54, 1255.
8. Hrycay, E. G., and O'Brien, P. J. (1972) Arch. Biochem. Biophys. 153, 480.
9. Hrycay, E. G., and O'Brien, P. J. (1973) Arch. Biochem. Biophys. 157, 7.
10. Hrycay, E. G., and O'Brien, P. J. (1974) Arch. Biochem. Biophys. 160, 230.
11. Remmer, H., Greim, H., Schenkman, J., Estabrook, R.W., (1967) in "Methods in Enzymology" Vol. X p. 703-708 (R. W. Estabrook & M. Pullman, editors) Academic Press, London, New York.
12. Ullrich, V., and Weber, Peter (1972) Hoppe Seyler's Z. Physiol. Chem. 353, 1171.
13. Dowd, D., Burke, M. D., Prough, R. A., and Estabrook, R. W. to be published.
14. Fujita, T., and Mannering, G. J. (1973) J. Biol. Chem. 248, 8150.
15. Creaven, P. J., Parke, D. V., and Williams, R. T. (1965) Biochem. J. 96, 879.
16. Thurman, L. G., Ley, Heinz Georg and Stotz Roland (1972) European J. Biochem. 25, 420.
17. Rahimtula, A. D., O'Brien, P. J., Peterson, J. A., and Estabrook, R. W. manuscript in preparation.
18. Yonetani, T., and Schleyer, H. (1967) J. Biol. Chem. 242, 1974.
19. George, P., and Irvine, D. H. (1952) Biochem. J. 52, 511.
20. Hamilton, G. A., Giacin, J. R., Hellman, T. M., Snook, M. E., and Weller, J. W., (1972) Ann. N.Y. Acad. Sc. page 4.