SODIUM PERIODATE, SODIUM CHLORITE, ORGANIC HYDROPEROXIDES, AND H_2O_2 AS HYDROXYLATING AGENTS IN STEROID HYDROXYLATION REACTIONS CATALYZED BY PARTIALLY PURIFIED CYTOCHROME P-450

Eugene G. HRYCAY, Jan-Åke GUSTAFSSON, Magnus INGELMAN-SUNDBERG, and Lars ERNSTER

Department of Biochemistry, Arrhenius Laboratory, University of Stockholm and Department of Chemistry, Karolinska Institutet, Stockholm, Sweden

Received June 12,1975

SUMMARY: The mechanism of androstenedione hydroxylation has been examined by employing NADPH, $NaIO_4$, $NaClO_2$, H_2O_2, and organic hydroperoxides as hydroxylating agents and partially purified cytochrome P-450, prepared from phenobarbital-induced rat liver microsomes, as the catalyst. The NADPH-supported hydroxylation also required NADPH-cytochrome P-450 reductase. Androstenedione was hydroxylated in the 6β-, 7α-, and 16α-positions to varying degrees depending on the hydroxylating agent. $NaIO_4$ was the most effective agent followed by cumene hydroperoxide, NADPH, linoleic acid hydroperoxide, $NaClO_2$, t-butyl hydroperoxide, and H_2O_2. It was suggested that the ferryl ion (compound I) of cytochrome P-450 is the common "activated oxygen" species in these hydroxylations.

The terminal "oxygen-activating" enzyme of liver microsomes, cytochrome P-450, catalyzes the hydroxylation of a wide variety of substrates in the presence of NADPH and molecular oxygen (1). Although several "activated oxygen" species have been proposed to function as hydroxylating intermediates (1-6), the true identity of the species which actually hydroxylates the substrate has eluded definition. The initial findings of Hrycay and collaborators (7-9) that cytochrome P-450 can function as a peroxidase and recent discoveries (6,10-12) that certain organic hydroperoxides can replace NADPH and molecular oxygen in supporting the hydroxylation of various drugs and fatty acids have led us to examine the nature of the "activated oxygen" species more closely.

Following an earlier report (13) in which higher oxidation states of cytochrome P-450 were proposed to function in the hydroxylation mechanism, we have recently shown that $NaIO_4$ and $NaClO_2$ as well as various organic hydroperoxides can support the cytochrome P-450-dependent hydroxylation of a

number of steroid substrates in rat liver microsomes at reaction rates comparable to the NADPH-dependent hydroxylation (14-16).

This study reports that $NaIO_4$ $NaClO_2$, H_2O_2, and various organic hydroperoxides can support the hydroxylation of androstenedione in the presence of partially purified cytochrome P-450 prepared from phenobarbital-induced rat liver microsomes. A mechanism for substrate hydroxylation is proposed in which the ferryl ion (i.e. compound I) of cytochrome P-450 functions as the common "activated oxygen" species.

Methods

Microsomes were prepared from livers of male Sprague-Dawley rats (220-250 g) that had been pretreated with sodium phenobarbital (PB) once daily for 3 days at a dose of 100 mg/kg body weight as described previously (8). Partially purified cytochrome P-450 and NADPH-cytochrome c reductase were prepared from PB-induced rat liver microsomes essentially by the method of Lu et al. (17) as described (18). Total phosphorus in these preparations was measured as described by Bartlett (19). Cytochrome P-450 and NADPH-cytochrome c reductase were determined by established methods (8,20). Protein was estimated by the procedure described by Lowry et al. (21) using bovine serum albumin as a standard.

4-Androstene,3,17-dione (androstenedione) hydroxylation was measured as follows: 4-[4-^{14}C]androstene-3,17-dione was diluted with unlabeled androstenedione in acetone prior to use. A 25 µl solution (200 µg; 200,000 cpm) was added to a 1 ml final vol containing partially purified cytochrome P-450 (2.6 nmoles) and 0.1 M potassium phosphate (pH 7.5). The hydroxylation reaction was initiated by addition of the following final concentrations of hydroxylating agent: NADPH (1 mM), $NaIO_4$ (7.5 mM), cumene hydroperoxide (1 mM), $NaClO_2$ (4 mM), linoleic acid hydroperoxide (0.4 mM), t-butyl hydroperoxide (3 mM), or H_2O_2 (10 mM). In incubations containing NADPH, an excess of partially purified NADPH-cytochrome c reductase (300 units) was included. One unit of reductase catalyzed the reduction of one nmole cytochrome c/min. Mixtures were incubated in air at 37° for 1 to 10 min and reactions terminated with 20 vol of chloroform-methanol (2:1, v/v). The precipitate was filtered off and 0.2 vol of 0.9 % NaCl solution was added. The chloroform phase was collected, the solvent evaporated, the residue dissolved in 0.5 ml methanol and applied onto precoated silica gel plates (250 µm thick). The tlc plates were developed once in chloroform-ethyl acetate (4:1, v/v) and autoradiographed. The radioactive zones were determined exactly from the X-ray film and measured for radioactivity using Instagel as scintillator liquid. Steroid products were analyzed by combined gas chromatography-mass spectrometry as previously described (15,16,22).

Hydroxylation rates were calculated from the linear portion of the time curve and expressed as nmoles hydroxylated product formed/min incubation time. Activities were not proportional to cytochrome P-450 concentration at low levels (below 0.05 nmoles/ml) or at high levels (above 0.4 nmoles/ml). It was necessary to use high concentrations of cytochrome P-450 (2.6 nmoles/ml) since certain hydroxylations could not be detected at lower levels and certain hydroxylating agents caused a rapid destruction of cytochrome P-450 at low concentrations. Because of these conditions, activities could not be expressed per nmole of cytochrome P-450.

Results

After incubation of 4-[4-^{14}C]androstene-3,17-dione with partially purified cytochrome P-450 preparations fortified with optimal levels of hydroxylating agent, total conversions of the steroid substrate ranging from 1 to 25 % were observed. No steroid products were formed in the absence of cytochrome P-450 or in the absence of hydroxylating agent. Reaction rates with NADPH were linear with respect to incubation time (up to 10 min at 37°) whereas velocities with other hydroxylating agents were linear with respect to incubation time only for about 1 min.

Figs. 1A and 1B show the effect of increasing concentrations of cumene hydroperoxide (cuOOH) and $NaIO_4$, respectively, on androstenedione hydroxylation catalyzed by cytochrome P-450. Androstenedione was converted to its respective 6β-, 7α-, and 16α-hydroxy derivatives. In the cuOOH-fortified reaction (Fig. 1A), the 6β-hydroxylase exhibited the highest affinity for cuOOH. At optimal cuOOH levels, the 6β-hydroxylase was approximately 2.2- and 3.2-times more active, respectively, than the 7α- and 16α-hydroxylase. In the $NaIO_4$-supported hydroxylation (Fig. 1B), the 7α-hydroxylase was slightly more active than the 6β-hydroxylase but approximately 10-times more effective than the 16α-hydroxylase. The optimal concentrations for cuOOH and $NaIO_4$ were 1 mM and 7.5 mM, respectively. When concentrations of cuOOH above 1 mM were used, the velocities for 6β- and 16α-hydroxylation began to decline below the optimal levels due to destruction of the cytochrome P-450 heme moiety (16).

Table I shows a comparison of the effectiveness of various hydroxylating agents in supporting androstenedione hydroxylation with partially purified cytochrome P-450. When total velocities were compared using optimal levels of hydroxylating agent, $NaIO_4$ was by far the most effective hydroxylating agent followed by cuOOH, NADPH, linoleic acid hydroperoxide, $NaClO_2$, t-butyl hydroperoxide, and H_2O_2. Examining the activities individually, 6β-hydroxylation of androstenedione generally was supported effectively by all hydroxyl-

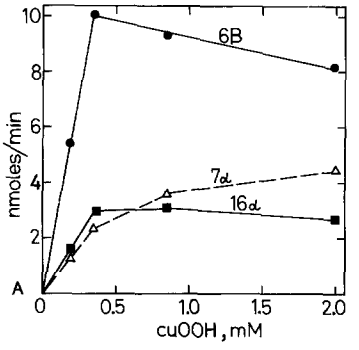

Fig. 1A: The effect of increasing cuOOH concentration on androstenedione hydroxylation catalyzed by partially purified cytochrome P-450.

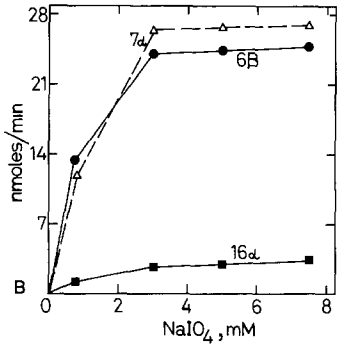

Fig. 1B: The effect of increasing $NaIO_4$ concentration on androstenedione hydroxylation catalyzed by partially purified cytochrome P-450. See Methods for details.

---

ating agents. H_2O_2 could only support 6β-hydroxylation under these assay conditions. With $NaIO_4$ and t-butyl hydroperoxide, 7α-hydroxylation proceeded at a slightly more rapid rate than did 6β-hydroxylation. The formation of 17β-hydroxy-4-androstene-3,16-dione occurred only in the NADPH-sustained incubations. This product was formed presumably by rearrangement of either a 16α- or 16β-hydroxy derivative of androstenedione (16). Other oxidizing agents such as $K_2Cr_2O_7$, CrO_3, $KClO_4$, and $NaIO_3$ could not support androstenedione hydroxylation with partially purified cytochrome P-450.

Table I

Effectiveness of Various Hydroxylating Agents in Supporting Androstenedione Hydroxylation with Partially Purified Cytochrome P-450

Hydroxylating agent	Reaction rates (nmoles/min)				
	6β-OH	7α-OH	16α-OH	16-keto, 17β-OH [a]	Total
$NaIO_4$ (7.5 mM)	24.70	26.73	3.46	0	54.89
Cumene hydroperoxide (1 mM)	9.25	3.90	3.09	0	16.24
NADPH (1 mM) [b]	2.73	1.14	1.53	1.64	7.04
Linoleic acid hydroperoxide (0.4 mM)	1.07	0	1.98	0	3.05
$NaClO_2$ (4 mM)	1.35	0	0.96	0	2.31
t-Butyl hydroperoxide (3 mM)	0.55	0.68	0.78	0	2.01
H_2O_2 (10 mM)	1.09	0	0	0	1.09

Partially purified cytochrome P-450 (2.6 nmoles/ml) was incubated with androstenedione and indicated amounts of hydroxylating agent as described in Methods. Reaction rates are expressed as nmoles hydroxylated product formed/min.

[a] 16-keto,17β-OH = 17β-hydroxy-4-androstene-3,16-dione.

[b] In incubations with NADPH, partially purified NADPH-cyt. c reductase (300 units) was included. One unit catalyzed the reduction of 1 nmole cyt. c/min.

Exogenous phosphatidylcholine was not required in these incubations because the cytochrome P-450 preparation still contained phospholipid. Omission of the reductase fraction from the NADPH incubations containing cytochrome P-450 resulted in no significant hydroxylation rates. Addition of the reductase fraction to incubations containing cytochrome P-450 and hydroxylating agents other than NADPH did not produce any significant alteration in the initial activities.

Other hemoprotein peroxidases such as horseradish peroxidase, catalase, and metmyoglobin (up to 10 μM) could not catalyze androstenedione hydroxylation with any of the hydroxylating agents.

Discussion

The results presented here have shown that in addition to NADPH, various organic hydroperoxides, $NaIO_4$, $NaClO_2$, and H_2O_2 were capable of supporting androstenedione hydroxylation in the presence of partially purified cyto-

chrome P-450 preparations, with $NaIO_4$ being by far the most effective hydroxylating agent. These hydroxylation reactions appear to be specific for cytochrome P-450 since other hemoprotein peroxidases such as horseradish peroxidase, catalase, and metmyoglobin are non-functional.

Rat liver microsomes contain multiple forms of cytochrome P-450 (17,18, 23,24) and several of these forms have been partially purified from PB-induced rat liver microsomes (23,24). Our present data have shown that partially purified cytochrome P-450 from PB-induced rat liver microsomes catalyzes the hydroxylation of androstenedione in the 6β-, 7α-, and 16α-positions to varying extents depending on the hydroxylating agent used. A plausible explanation for this phenomenon is that our cytochrome P-450 preparation contains multiple forms of cytochrome P-450 which exhibit different affinities for the various hydroxylating agents.

Our finding that $NaIO_4$, $NaClO_2$, H_2O_2, and various organic hydroperoxides can support androstenedione hydroxylation with partially purified cytochrome P-450 may be a key observation with regard to defining the chemical nature of the "activated oxygen" species since these oxidizing agents also react with other hemoprotein peroxidases (e.g. horseradish peroxidase, cytochrome c peroxidase, chloroperoxidase, catalase, metmyoglobin) to form the respective compound I derivatives which are believed to contain heme iron in a quadrivalent state bound to one atom of oxygen (i.e. the ferryl structure) (25-28).

We propose the following mechanism for cytochrome P-450-catalyzed hydroxylation (Fig. 2). In the NADPH supported reaction, ferric cytochrome P-450 binds substrate (AH) to form a complex which accepts one electron from NADPH to give a ferrous enzyme complex. This species binds molecular oxygen and accepts a second electron from NADPH to yield a ferrous enzyme-superoxide intermediate which is a resonance form of the ferric enzyme-hydroperoxo complex. This species is unstable and decomposes to give water and a ferric enzyme-monooxygen species which is a resonance form of the ferryl ion complex ($Fe^{4+}O^{-}$) and is therefore equivalent to compound I. This ferryl ion species

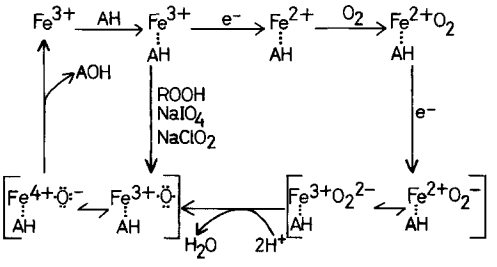

Fig. 2: A proposed mechanism for substrate hydroxylation catalyzed by cytochrome P-450. Fe signifies the heme iron of cytochrome P-450; AH, substrate; ROOH, hydroperoxides.

---

decomposes with the liberation of hydroxylated product (AOH) and regenerated ferric enzyme. Hydroxylating agents such as $NaIO_4$, $NaClO_2$, H_2O_2, and organic hydroperoxides (ROOH) by-pass the reduction steps of the NADPH pathway and form the ferryl ion by donating one oxygen atom to the ferric enzyme (Fig. 2).

From the results reported in this study, the ferryl ion of cytochrome P-450 appears to be the most likely candidate for the "activated oxygen" species of substrate hydroxylation reactions.

Acknowledgments: We thank Mrs. G. Elfström, Miss G. Björk, and Miss I. Johansson for skillful technical assistance and thank the Canadian Medical Research Council, the Swedish Medical Research Council (grant no. 03X-2819), and NIH (grant no. NO1 CP33363) for support.

References

1. Estabrook, R.W., Baron, J., Peterson, J., and Ishimura, Y. (1972) Biological Hydroxylation Mechanisms, pp. 159-185 (Boyd, G.S. and Smellie, R.M.S., eds), Academic Press, New York.
2. Ullrich, V., and Staudinger, H.J. (1966) Biological and Chemical Aspects of Oxygenases, pp. 235-249 (Bloch, K. and Hayaishi, O., eds), Maruzen, Tokyo.
3. Sih, C.J. (1969) Science 163, 1297-1300.
4. Hamilton, G.A. (1974) Molecular Mechanisms of Oxygen Activation, pp. 405-451 (Hayaishi, O., ed), Academic Press, New York.
5. Coon, M.J., Strobel, H.W., Autor, A.P., Heidema, J., and Duppel, W. (1972) Biological Hydroxylation Mechanisms, pp. 45-54 (Boyd, G.S. and Smellie, R.M.S., eds), Academic Press, New York.
6. Rahimtula, A.D., and O'Brien, P.J. (1975) Biochem. Biophys. Res. Commun. 62, 268-275.
7. Hrycay, E.G., and O'Brien, P.J. (1972) Arch. Biochem. Biophys. 153, 480-494.
8. Hrycay, E.G., and Prough, R.A. (1974) Arch. Biochem. Biophys. 165, 331-339.

9. Hrycay, E.G., Jonen, H.G., Lu, A.Y.H., and Levin, W. (1975) Arch. Biochem. Biophys. 166, 145-151.
10. Rahimtula, A.D., and O'Brien, P.J. (1974) Biochem. Biophys. Res. Commun. 60, 440-447.
11. Kadlubar, F.F., Morton, K.C., and Ziegler, D.M. (1973) Biochem. Biophys. Res. Commun. 54, 1255-1261.
12. Ellin, A., and Orrenius, S. (1975) FEBS Lett. 50, 378-381.
13. Rahimtula, A.D., O'Brien, P.J., Hrycay, E.G., Peterson, J.A., and Estabrook, R.W. (1974) Biochem. Biophys. Res. Commun. 60, 695-702.
14. Hrycay, E.G., Gustafsson, J.-A., Ingelman-Sundberg, M., and Ernster, L. (1975) Abstr. 6th Int. Congr. Pharmacol., in press.
15. Hrycay, E.G., Gustafsson, J.-A., Ingelman-Sundberg, M., and Ernster, L. (1975) FEBS Lett., in press.
16. Hrycay, E.G., Gustafsson, J.-A., Ingelman-Sundberg, M., and Ernster, L. (1975) Eur. J. Biochem., in press.
17. Lu, A.Y.H., Kuntzman, R., West, S., Jacobson, M., and Conney, A.H. (1972) J. Biol. Chem. 247, 1727-1734.
18. Gustafsson, J.-A., and Ingelman-Sundberg, M. (1975) Biochemistry, in press.
19. Bartlett, G.R. (1959) J. Biol. Chem. 234, 466-468.
20. Masters, B.S.S., Williams, C.H., Jr., and Kamin, H. (1967) Meth. Enzymol. 10, 565-573.
21. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
22. Einarsson, K., Gustafsson, J.-A., and Stenberg, A. (1973) J. Biol. Chem. 248, 4987-4997.
23. Comai, K., and Gaylor, J.L. (1973) J. Biol. Chem. 248, 4947-4955.
24. Ryan, D., Lu, A.Y.H., West, S., and Levin, W. (1975) J. Biol. Chem. 250, 2157-2163.
25. George, P. (1953) J. Biol. Chem. 201, 413-426.
26. Schonbaum, G.R., and Lo, S. (1972) J. Biol. Chem. 247, 3353-3360.
27. Yamazaki, I. (1974) Molecular Mechanisms of Oxygen Activation, pp. 535-558 (Hayaishi, O., ed), Academic Press, New York.
28. Hollenberg, P.F., Rand-Meir, T., and Hager, L.P. (1974) J. Biol. Chem. 249, 5816-5825.