SODIUM PERIODATE, SODIUM CHLORITE, AND ORGANIC HYDROPEROXIDES AS HYDROXYLATING AGENTS IN HEPATIC MICROSOMAL STEROID HYDROXYLATION REACTIONS CATALYZED BY CYTOCHROME *P*-450

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1. Introduction

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 [1–5], the exact chemical structure of the species which actually hydroxylates the substrate has eluded definition. The initial findings of Hrycay and collaborators [5–7] that cyotchrome *P*-450 of liver microsomes can function as a peroxidase and recent discoveries [8–11] that certain organic hydroperoxides can support the hydroxylation of various drugs and fatty acids in the absence of NADPH and molecular oxygen have provided an impetus to the further study of the hydroxylation mechanism.

We now wish to report that $NaIO_4$ and $NaClO_2$ as well as various organic hydroperoxides can support the cytochrome *P*-450-catalyzed hydroxylation of steriod substrates in rat liver microsomes at reaction rates comparable to the NADPH-supported hydroxylations. It is proposed that these oxidizing agents promote steroid hydroxylation by forming a transient ferryl ion (compound I) of cytochrome *P*-450 which may be the common 'activated oxygen' species of cytochrome *P*-450-catalyzed hydroxylations. A preliminary report of this work has appeared in abstract form [12].

2. Materials and methods

Microsomes were prepared from livers of male Sprague-Dawley rats (220–250 g) as previously described [6]. Cytochrome *P*-450 and protein were determined by published methods [6,13].

Steroid hydroxylation was measured as follows: steroid substrates (^{14}C-labeled) were diluted with unlabeled steroid in acetone prior to use. A 25 μl solution (200 μg; 200 000 cpm) was added to a 4 ml final vol containing microsomal protein (6 mg/ml) and 0.1 M potassium phosphate (pH 7.5 or 8.0). The reaction was initiated by addition of the following final concentrations of hydroxylating agent: NADPH (1 mM), $NaIO_4$ (10 mM), $NaClO_2$ (4 mM), or cumene hydroperoxide (2 mM). Mixtures were incubated in air at 37°C for 1 to 10 min and reactions terminated with chloroform–methanol (2:1, v/v). Incubations containing NADPH were carried out at the pH optimum of 7.5 whereas all others were performed at pH 8.0. Steroid products were chromatographed, analyzed by autoradiography, and combined gas chromatography-mass spectrometry, and quantitated by measuring the radioactivity of the extracted samples as previously described [14,15]. Reaction rates were calculated from the linear portion of the time curve and expressed as nmoles hydroxylated product formed/min/mg protein.

4-[4-^{14}C] androstene-3,17-dione (androstenedione), 4-[4-^{14}C] pregnene-3,20-dione (progesterone), 17β-

hydroxy-4-[4-^{14}C] androsten-3-one (testosterone), and [4-^{14}C] estradiol were obtained from The Radiochemical Centre (Amersham, England) or from New England Nuclear Co. (Boston, Mass.). Cumene hydroperoxide was obtained form K and K Laboratories (Cleveland, Ohio) and NADPH from Sigma Chemical Co. (St. Louis, Mo.). Pregnenolone 17α-hydroperoxide was a gift of Dr J. E. van Lier and linoleic acid hydroperoxide was prepared as previously described [5].

3. Results

After incubation of 4-[4-^{14}C] androstene-3,17-dione with microsomes fortified with NADPH, $NaIO_4$, $NaClO_2$ or cumene hydroperoxide (cuOOH), total conversions of the substrate ranging from 20 to 45% were observed. No products were formed in the absence of microsomal fractions or hydroxylating agent, or with boiled microsomal fractions. Reaction rates with NADPH were linear with respect to microsomal protein concentration and incubation time. Velocities with $NaIO_4$, cuOOH, and $NaClO_2$ were linear with respect to microsomal protein concentration and linear with time only for 1, 3, and 5 min, respectively.

Figs.1A and 1B show the effect of increasing concentrations of cuOOH and $NaIO_4$, respectively, on androstenedione hydroxylation in liver microsomes. The major products formed were the 6β, 7α, and 16α-hydroxy derivatives. In the cuOOH-fortified reaction, the 6β-hydroxylase exhibited the highest affinity for cuOOH. At saturating cuOOH levels, the 6β-hydroxylase was as effective as the 7α-hydroxylase but 1.8 times more active than the 16α-hydroxylase. In the $NaIO_4$-supported reaction, the 7α-hydroxylase was about 10 times more active than the 16α-hydroxylase and twice as active as the 6β-hydroxylase.

Table 1 shows the steroid specificity of microsomal hydroxylases. In terms of total velocities, androstenedione and testosterone were the most effective substrates. Examining the hydroxylases individually, the 6β-hydroxylase was the most active in reactions supported by NADPH and cuOOH whereas the 7α-hydroxylase was the most efficient in $NaIO_4$-dependent hydroxylations of androstenedione and testosterone. The formation of the minor product, 17β-hydroxy-4-androstene-3,16-dione, occurred probably by rearrangement of either a 16α- or 16β-hydroxy derivative of androstenedione.

Table 2 shows a comparison of various hydroxylating agents in supporting androstenedione hydroxylation. When total velocities were compared using saturating levels of hydroxylating agent, $NaIO_4$ was by far the most effective hydroxylating agent followed by cuOOH, NADPH, $NaClO_2$, pregnenolone 17α-hydroperoxide. Other oxidizing agents such as H_2O_2, $K_2Cr_2O_7$, CrO_3, K_2IrCl_6, $KClO_4$, $KBrO_3$, and $NaIO_3$ could not support androstenedione hydroxylation in liver microsomes.

Androstenedione hydroxylation supported by NADPH, cuOOH, and $NaIO_4$ was inhibited by various modifiers of cytochrome *P*-450. The type II ligands such as aniline (5 mM) and metyrapone (0.5 mM) were particularly effective (range of inhibition, 50–100%) as were reagents that convert cytochrome *P*-450

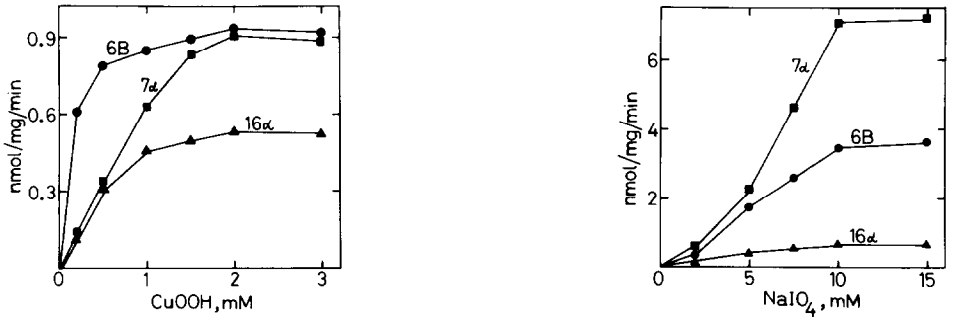

Fig.1(A) The effect of increasing cuOOH concentration on androstenedione hydroxylation. (B) The effect of increasing $NaIO_4$ concentration on androstenedione hydroxylation. See text for details.

Table 1
Steroid specificity of microsomal hydroxylases

Steroid substrate	Major hydroxylated products	Reaction rates (nmoles/mg/min)		
		NADPH	$NaIO_4$	cuOOH
Androstenedione	6β-OH	0.58	3.97	0.85
	7α-OH	0.13	7.13	0.65
	15-OH	0.13	0.90	0.12
	16α-OH	0.27	0.58	0.43
	17β-hydroxy-4-androstene-3,16-dione	0.12	0.70	0.10
	Total	1.23	13.28	2.15
Testosterone	6β-OH	0.74	2.30	1.22
	7α-OH	0.34	3.98	0.25
	16α-OH	0.16	0.71	0.17
	Total	1.24	6.99	1.64
Progesterone	2α-OH	0.23	0.62	0.12
	6β-OH	0.47	1.20	0.48
	7α-OH	0	0.44	0
	15α-OH	0	0.22	0
	15β-OH	0	0.26	0.14
	16α-OH	0.41	0.44	0.26
	Total	1.11	3.18	1.00
17β-Estradiol	6α-OH	0.10	1.59	0.39
	6β-OH	0.14	1.60	0.48
	16α-OH	0.14	1.58	0.15
	Total	0.38	4.77	1.02

Liver microsomes were incubated with steroid substrate and NADPH, $NaIO_4$, or cuOOH as described in Methods.

Table 2
Effectiveness of various agents in supporting androstenedione hydroxylation

Hydroxylating agent	Reaction rates (nmoles hydroxylated product formed/mg protein/min)					
	6β-OH	7α-OH	15-OH	16α-OH	16-keto, 17β-OH[a]	Total
$NaIO_4$ (10 mM)	3.33	7.10	1.12	0.67	0.62	12.84
Cumene hydroperoxide (2 mM)	0.87	0.75	0.19	0.48	0.11	2.40
NADPH (1 mM)	0.52	0.14	0.11	0.41	0.13	1.31
$NaClO_2$ (4 mM)	0.28	0.16	0.09	0.25	0.10	0.88
Pregnenolone 17α-hydroperoxide (1.4 mM)	0.48	0	0	0.05	0.01	0.54
t-Butyl hydroperoxide (3 mM)	0.14	0.10	0.07	0.12	0.09	0.52
Linoleic acid hydroperoxide (0.8 mM)	0.06	0	0	0	0.04	0.10

Incubations were carried out using indicated amounts of hydroxylating agent and products were determined as described in Methods.

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

to cytochrome *P*-420 such as acetone (10%, v/v) and deoxycholate (0.5%, w/v). The type I modifiers such as progesterone (0.2 mM) and testosterone (0.2 mM) produced varied inhibitions.

4. Discussion

The results presented here have shown that, in addition to NADPH, various organic hydroperoxides, $NaIO_4$, and $NaClO_2$ were capable of supporting steroid hydroxylation in liver microsomes, with $NaIO_4$ being by far the most effective hydroxylating agent. These hydroxylation reactions are dependent on cytochrome *P*-450 since various modifiers of cytochrome *P*-450 act as good inhibitors and partially purified cytochrome *P*-450 preparations are effective in catalyzing steroid hydroxylation supported by these oxidizing agents [16]. Liver microsomes contain multiple forms of cytochrome *P*-450 [17,18] and the different ratio of steroid products observed with these various hydroxylating agents may reflect different affinities of the various cytochrome *P*-450's for hydroxylating agent.

Our finding that $NaIO_4$, $NaClO_2$, and various organic hydroperoxides can support steroid hydroxylation may be a key observation in terms of defining the nature of the 'activated oxygen' species since these oxidizing agents also interact 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) [19–21].

In view of our findings, 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 complex which is a resonance form of the ferric enzyme–hydroperoxo complex. Because of its instability, this complex 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

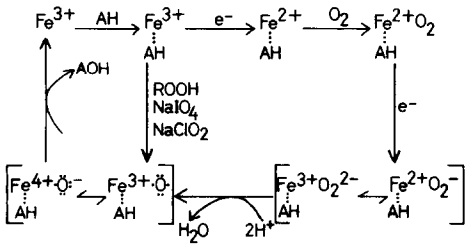

Fig.2. A proposed mechanism for substrate hydroxylation. Fe signifies the heme iron of cytochrome *P*-450; AH, substrate; ROOH, organic hydroperoxides.

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

From the results obtained 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.

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