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Differences between the hydroperoxide-dependent and NADPH-dependent microsomal aryl hydrocarbon hydroxylase activities

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The liver microsomal mono-oxygenase system catalyzes the NADPH-dependent oxygenation and hydroxylation of various hydrocarbons. This activity is also responsible for the metabolic activation of polycyclic carcinogens and is usually assayed as aryl hydrocarbon hydroxylase (AHH) activity with benzo[a]pyrene (BP) as a substrate [1]. Pretreatment of rats with 3-methylcholanthrene induces the synthesis of a form of cytochrome P-450 known as cytochrome P-448 or P_1-450 which is more efficient in catalyzing the hydroxylation of BP than the enzyme of phenobarbital-pretreated rats (PB cytochrome P-450) or control rats (control cytochrome P-450) [2–4]. The difference in catalytic activity between these two forms of cytochrome P-450 is not well understood at the molecular level. Recently it has been shown that cumene hydroperoxide is capable of supporting the cytochrome P-450-dependent dealkylation and hydroxylation of various substrates [5–9]. Although the hydroperoxide-dependent mono-oxygenase reactions are generally comparable to the NADPH-dependent ones [5, 7, 9], it is not known whether the mechanisms of oxygenation in these two systems are the same. In the present study, we have compared these two systems with regard to the relative catalytic activities of cytochrome P-450- and cytochrome P-448-containing microsomes as well as the actions of specific inhibitors of the mono-oxygenase system.

Sources of chemicals are as follows: NADPH, BP and butylated hydroxyanisole (BHA) [2,(3)-*tert*-butyl-4-hydroxyanisole, mixed isomers] from Sigma Chemical Co., St. Louis, MO; cumene hydroperoxide from Matheson, Coleman & Bell, Norwood, OH; 3-methylcholanthrene from Mann Research Laboratories, New York, N.Y.; 7,8-benzoflavone from Eastman Organic Chemicals, Rochester, N.Y.; and phenobarbital from Merck & Co., Rahway, N.J. Liver microsomes were prepared according to methods described previously [10] from male Long–Evans rats (body weight, 75–125 g) which had been pretreated with 3-methylcholanthrene (injected i.p. daily at 25 mg/kg body weight for 4 days) or phenobarbital (1 mg/ml in drinking water for 5–7 days). These will be referred to as MC microsomes and PB microsomes respectively. The cytochrome P-450 contents of MC, PB and control microsomes were usually 1.5 to 2.0, 2.2 to 2.8 and 0.7 to 1.1 nmoles/mg of protein respectively. The methods for these determinations have been described previously [10]. The NADPH-dependent AHH was assayed according to the procedure of Nebert and Gelboin [11] with slight modifications [10]. The reaction mixture contained 100 μmoles potassium phosphate (pH 7.4), 5 μmoles $MgCl_2$, 0.1 μmole EDTA, 0.4 μmole NADPH, and microsomes in a final volume of 1.0 ml. After a preincubation of 2 min at 37°, the reaction was initiated by the addition of 80 nmoles BP in 25 μl acetone, and the mixture was incubated for 5 min at 37°. The activity was expressed in turnover numbers, i.e. nmoles of phenolic products [11] formed/min/nmole of cytochrome P-450. Similar conditions were used for assaying the cumene hydroperoxide-dependent AHH. The reaction mixture contained 100 μmoles potassium phosphate (pH 8.0), 5 μmoles $MgCl_2$, 0.1 μmole EDTA, 80 nmoles BP, and microsomes in a final volume of 1 ml. After preincubating at 37° for 2 min, the reaction was initiated by the addition of 75 nmoles cumene hydroperoxide in 10 μl acetone and allowed to proceed for 1 min. Both the NADPH- and cumene hydroperoxide-dependent oxygenation reactions were also studied by a direct fluorescence method [12]. The decrease of the BP fluorescence (excitation, 387 nm; emission, 407 nm) was measured with a Perkin Elmer double beam recording spectrofluorometer (model 512).

Examination of the assay conditions of the cumene hydroperoxide-dependent AHH indicated that a cumene hydroperoxide concentration of 75–100 mM, a microsomal concentration corresponding to 0.3 to 0.5 nmole cytochrome P-450/ml, and an incubation period of 1–2 min were optimal. The results are consistent with those observed by Rahimtula and O'Brien [6]. The effect of pH on the cumene hydroperoxide AHH is shown in Fig. 1. A pH optimum of 8.0 was observed with both control and MC microsomes, although the latter appeared to be less responsive to the pH change. The hydroperoxide-dependent hydroxylase activities of control, PB and MC microsomes are shown in Table 1. Most of the assays were carried out simultaneously with the NADPH-dependent reactions which are also shown in the same table. The rates of the hydroperoxide-dependent reactions were lower than the NADPH-dependent ones. Moreover, the ratio of the AHH activities of control, PB and MC microsomes was drastically different from that of the NADPH-dependent system. In contrast to NADPH-dependent reactions, the AHH activity of the MC microsomes was much lower than that of the control microsomes in the hydroperoxide-dependent system. This difference in activity was also

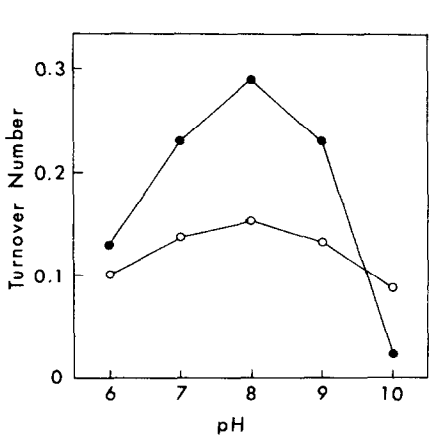

Fig. 1. Effect of pH on the cumene hydroperoxide-dependent hydroxylation of benzo[a]pyrene. The reaction mixtures contained control microsomes (●) or MC microsomes (○) corresponding to 0.52 and 0.79 nmole cytochrome P-450 respectively.

Table 1. NADPH-dependent and hydroperoxide-dependent microsomal aryl hydrocarbon hydroxylase activities*

Microsomes	NADPH-dependent	Hydroperoxide-dependent
Control	0.82 ± 0.06 (5)	0.44 ± 0.04 (5)
PB	0.48 ± 0.03 (4)	0.12 ± 0.03 (4)
MC	2.29 ± 0.10 (5)	0.18 ± 0.04 (5)

* Results are expressed as turnover numbers in the form of mean ± S.E.; the number of determinations is given in parentheses. For the NADPH-dependent reaction, the reaction mixture contained control or PB microsomes corresponding to 0.13 nmole cytochrome P-450, or MC microsomes corresponding to 0.06 nmole cytochrome P-450. For the hydroperoxide-dependent reaction, the reaction mixture contained 0.51, 0.43 or 0.22 to 0.42 nmole cytochrome P-450 from control, PB or MC microsomes respectively.

observed (1) with methanol as the solvent for BP, (2) when the incubation time, the amount of microsomes, the order of substrate addition or the concentration of cumene hydroperoxide was varied, or (3) when cumene hydroperoxide was added continuously during the incubation at 1-min intervals for 5 min. Denaturation of the microsomal enzymes by heating resulted in the total loss of hydroxylase activity.

The lack of parallelism between the NADPH- and hydroperoxide-dependent reactions was also observed with a direct fluorescence assay of BP oxygenation (Fig. 2). A low BP concentration (1.4 μM) was used in the assay, and a more dramatic difference between the control and

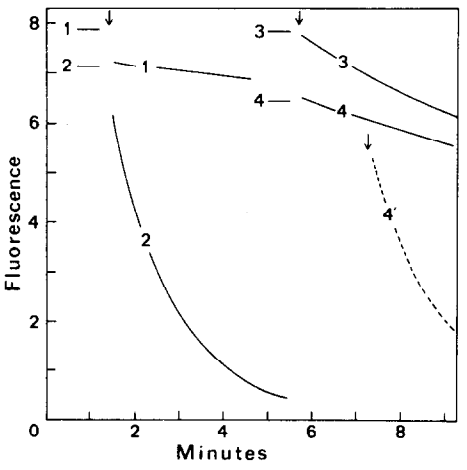

Fig. 2. NADPH- and hydroperoxide-dependent oxidations of benzo[a]pyrene assayed by a direct fluorometric method. Microsomes (0.6 mg protein) and 2.8 nmoles BP were preincubated in 2 ml buffer at 30°. The reactions were initiated, as indicated by the arrows, by either 0.4 μmole NADPH (traces 1, 2 and 4') or 0.15 μmole cumene hydroperoxide (traces 3 and 4). In trace 4', the NADPH was added 1.5 min after the addition of hydroperoxide. Traces 1 and 3 contained control microsomes (0.52 nmole cytochrome P-450). Traces 2 and 4 contained MC microsomes (0.96 nmole cytochrome P-450). The NADPH- and hydroperoxide-dependent reactions were assayed at pH 7.4 and 8.0, respectively, in a 0.1 M phosphate buffer containing 5 mM $MgCl_2$ and 0.1 mM EDTA.

Table 2. Specificity of the inhibitors of the aryl hydrocarbon hydroxylase systems*

	NADPH-dependent		Hydroperoxide-dependent	
Microsomes	7,8-Benzoflavone	BHA	7,8-Benzoflavone	BHA
Control	15	74	54	40
PB	5	79	36	53
MC	83	25	54	60

* Per cent inhibition caused by 0.1 mM 7,8-benzoflavone or BHA, which was added in 10 μl acetone. The assay conditions were similar to those in Table 1.

MC microsomes was observed in the NADPH-dependent reaction: turnover numbers were 0.07 and 1.89 respectively. In contrast, the turnover numbers of the hydroperoxide-dependent reactions were 0.37 and 0.11, respectively, for control and MC microsomes. A turnover number of 1.40 was observed when NADPH was added to a reaction mixture in which the MC microsomes had already been incubated with BP and cumene hydroperoxide for 1.5 min. This rate is similar to that observed in the absence of cumene hydroperoxide under comparable conditions. The results suggest that the lower rate observed with MC microsomes and cumene hydroperoxide is not due to the inactivation of cytochrome P-450 or inhibition of the reaction by the hydroperoxide. It can be concluded that there is no parallelism between the NADPH- and hydroperoxide-dependent systems with regard to the AHH activities of the different types of microsomes. A difference between NADPH- and hydroperoxide-dependent reactions was also observed with *N*-demethylase activities. For example, the NADPH-dependent aminopyrine demethylase activities of PB microsomes and MC microsomes, assayed as formaldehyde produced [10], were 4.8 and 1.6 nmoles/min/nmole of cytochrome P-450, respectively, whereas the hydroperoxide-dependent activity was about 7 nmoles/min/nmole of cytochrome P-450 for both types of microsomes (unpublished results).

Another major difference between these two types of reactions is demonstrated by the actions of specific inhibitors, as shown in Table 2. With the NADPH-dependent reaction, 7,8-benzoflavone is a potent inhibitor of the AHH activity of MC microsomes but not of PB or control microsomes [13]. On the other hand, BHA is more effective in inhibiting the hydroxylase of the latter two types of microsomes but is rather ineffective toward MC microsomes [14]. The specificity of inhibitors was not observed with the hydroperoxide-dependent reactions. At 0.1 mM, both inhibitors caused 36–60 per cent inhibition of the AHH activity of all three microsomal preparations.

Although the above results cannot be fully interpreted at the molecular level, the differences observed between the NADPH- and hydroperoxide-dependent AHH activities may be useful in elucidating the mechanism of the hydroxylation of aryl hydrocarbons. It has been suggested that, during the hydroperoxide-dependent hydroxylation reaction, the oxygen atoms of the hydroperoxide can bind to the heme iron of cytochrome P-450 to form an oxygenated complex cytochrome P-450 ($Fe^{3+}O_2^{2-} \equiv Fe^{5+}O_2^{4-}$) [15] or $(Fe\text{-}O)^{3+}$ [16]. If such an oxygenated complex is also an intermediate of the NADPH-dependent mono-oxygenase reactions [15, 16], the difference in catalytic activity between cytochrome P-450 and cytochrome P-448 is probably due to steps prior to this intermediate in the catalytic cycle.

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