CYTOCHROME P450 AS AN OXENE TRANSFERASE

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SUMMARY

In the presence of iodosobenzene, liver microsomes catalyze the O-dealkylation of 7-ethoxycoumarin. The reaction proceeds in the absence of NADPH and O_2 and is dependent on cytochrome P450. The results indicate that cytochrome P450 acts as an oxene transferase probably involving $[FeO]^{3+}$ as the transient intermediate of active oxygen.

INTRODUCTION

Cytochrome P450 from various cells and tissues has been identified as the dioxygen-activating component of a series of monooxygenases. The nature of the active oxygen species, however, has remained a matter of speculation. From the similarity between monooxygenation and carbene reactions an oxenoid structure has been suggested for the hydroxylating species (1). Such a species could possess either the structure of a ferric peroxide or of a ferryl ion depending on whether water has been released before or after the monooxygenation process (1,2).

One approach to elucidating the structure of the oxenoid intermediate is the use of oxygen donors other than molecular oxygen. It has recently been reported that organic peroxides, especially cumene hydroperoxide, can support hydroxylations by microsomes in the absence of dioxygen and NADPH (3,4). Even sodium periodate, sodium chlorite and hydrogen peroxide have been found active in the hydroxylation of some

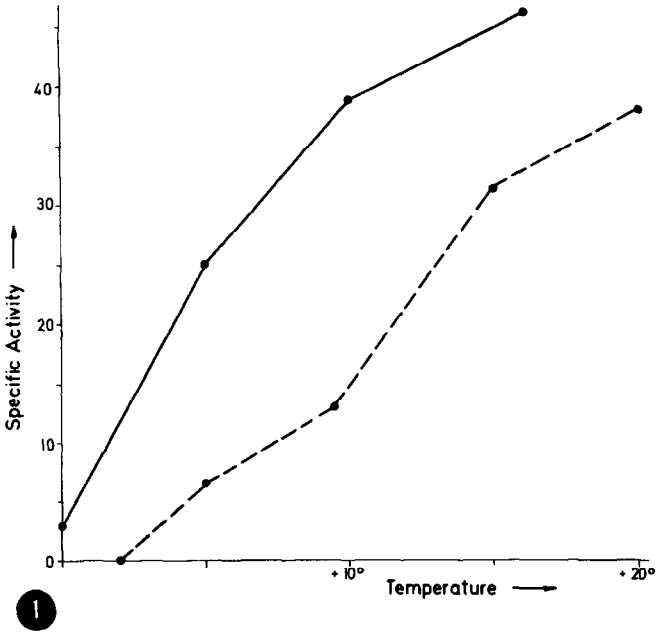
steroid substrates when liver microsomal cytochrome P450 was present as a catalyst (5). From these results it was postulated that the ferryl ion, $Fe^{IV}O^{-}$, could be the hydroxylating species. Unfortunately, in our hands these compounds proved to be inactive in the microsomal monooxygenation of typical drug substrates. Therefore, in order to provide evidence for an active oxygen species containing a single oxygen atom bound to the ferric cytochrome, we looked for lipophilic organic molecules which could act as oxygen atom donors for cytochrome P450. From all compounds tested only iodosobenzene fulfilled these requirements and investigations on its mechanism of action were carried out.

MATERIALS AND METHODS

Male Sprague-Dawley rats (80-110 g body weight) were used in all experiments. Phenobarbital was administered intraperitoneally at a dose of 80 mg/kg b.w. over three days. In other experiments 3,4-benzo(a)pyrene (20 mg/kg b.w.) was injected i.p. over two days. The animals were then starved overnight and killed by decapitation. The microsomal fraction was isolated as described previously (6). NADPH (grade I) was purchased from Boehringer Mannheim GmbH, GFR, and metyrapone was a gift from Ciba-Geigy Pharmaceutical Company, Basel, Switzerland. Iodosobenzene was prepared as described by Willgerodt (7). The O-dealkylation of 7-ethoxycoumarin (8) was measured in an Eppendorf fluorimeter using a 366 nm filter for excitation and a secondary filter cutting off light below 400 nm. At the end of each experiment 1 nmol umbelliferone was added for calibration purposes.

RESULTS AND DISCUSSION

For all experiments iodosobenzene was dissolved in methanol at a concentration of 10^{-1}M. When 10 ul of this solution at room temperature was added to 1 ml of a microsomal suspension containing 1 mg of protein and 10^{-3}M 7-ethoxycoumarin a rapid increase of umbelliferone fluo-



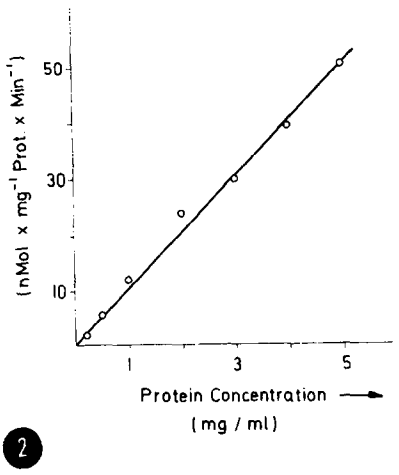


Fig. 1 Temperature dependence of the iodosobenzene-supported O-dealkylation. Microsomes from phenobarbital-pretreated rats: dashed line. Microsomes after benzpyrene pretreatment: solid line.

Fig. 2 Dependence on microsomal protein concentration of the iodosobenzene-supported O-dealkylation. The specific activity corresponds to umbelliferone formed.

rescence was recorded which ceased after about 20 sec. The initial velocities of this reaction were determined down to $0^{\circ}C$ and are presented in Figure 1.

At $8^{\circ}C$ the hydroxylation reaction was linear for about one minute and, therefore, further measurements were carried out at this temperature. A pH-optimum of 7.4 was obtained in microsomes from phenobarbital - as well as from benzpyrene-pretreated rats.

In order to prove that the iodosobenzene-dependent O-dealkylation of 7-ethoxycoumarin is truly a microsomal reaction, the dependence of the rate on the microsomal

Table 1. Effect of Treatment of Rat Liver Microsomes on the Iodosobenzene-Supported O-Dealkylation of 7-Ethoxycoumarin

Treatment of Rat Liver Microsomes	Spec. Activity[a] of O-Dealkylation			
	Pb-Pretreated		Bp-Pretreated	
	NADPH	ϕ-J=O	NADPH	ϕ-J=O
None (Control)	3.6	5.8	3.5	25.3
Heat Denatured	0	0	0	0
pH 3 $\rightarrow$ pH 7.4[b] (P420)	0	0.1	0	2.6
Anaerobic Incubation	0	5.6	0	24.1

a nMol umbelliferone x mg prot.$^{-1}$ x min^{-1}. Average of 5 experiments.
b titration with .5 M HCl to pH 3 and readjusting to pH 7.4 with .5 M NaOH

protein concentration was studied. From Fig. 2 a linear relationship is seen which indicates that indeed catalysis by microsomal proteins is involved.

Table 1 supports the suggestion that cytochrome P450 is the most likely catalyst for the O-dealkylation reaction. For comparison the specific activities measured with NADPH are also listed. Heat denaturation or conversion of cytochrome P450 to cytochrome P420 by decreasing the pH from pH 7.4 to 4.0 for 30 sec completely abolished the activity in microsomes from phenobarbital- and benzpyrene-pretreated rats. Dioxygen was not required for the iodosobenzene-dependent O-dealkylation. It is remarkable that microsomes from benzpyrene-pretreated rats have a much higher specific activity in this reaction compared with that for the NADPH and dioxygen dependent reaction (Table 1).

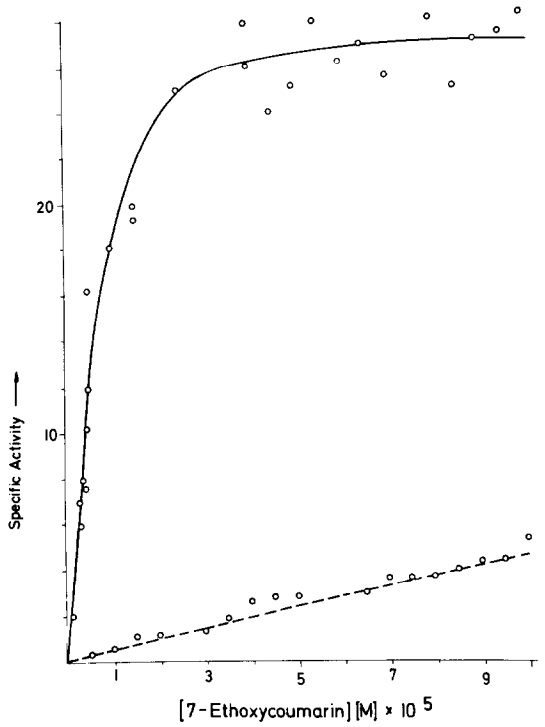


Fig. 3 Dependence on substrate concentration of the iodosobenzene-supported O-dealkylation. Microsomes from phenobarbital-pretreated rats: dashed line. Microsomes after benzpyrene pretreatment: solid line.

From recent studies on the differences of microsomal cytochrome P450 from phenobarbital- and benzpyrene-pretreated rats we have concluded that the O-dealkylation of 7-ethoxycoumarin in microsomes from benzpyrene-pre treated animals proceeds with a much higher affinity for the substrate (9). The same was found with iodosobenzene. The apparent K_m in microsomes from benzpyrene-induced rats was about $5 \cdot 10^{-4}$M whereas after phenobarbital pretreatment a K_m of about 10^{-2}M was calculated from a Lineweaver-Burk plot since saturation could not be obtained (Fig. 3).

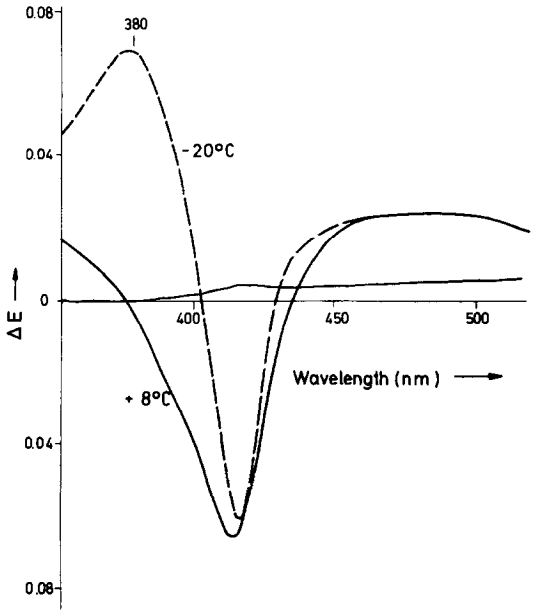


Fig. 4 Difference spectra of iodosobenzene in microsomes from phenobarbital-pretreated rats at -20 and +8°C. Each cuvette contained 3 mg of microsomal protein/ml. 10 µl of a 0.1 M solution of iodosobenzene in methanol was added to the sample cuvette and 10 µl of methanol to the reference cuvette.

The same differences in affinities were observed with NADPH and oxygen after pretreatment of rats with phenobarbital and benzpyrene (9), although the K_m-values were about two magnitudes lower. This, however, is reasonable since iodosobenzene competes with substrates for the binding site as shown in Fig. 4.

At -20°C iodosobenzene forms an enzyme-substrate complex as indicated by the peak at 390 nm (Fig. 4). This peak disappears at about 0°C and a trough is formed at around 416 nm. This was shown to be a consequence of the destruction of cytochrome P450 similar to the reaction observed with iodine (10).

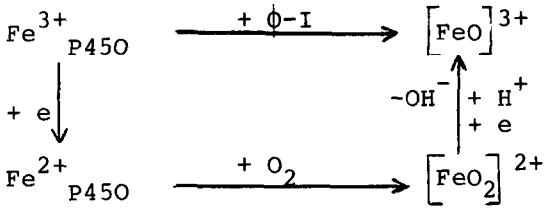
In microsomes prepared from phenobarbital-pretreated

rats, the simultaneous addition of 10^{-5}M metyrapone inhibited the reaction by 48 %. Microsomes from benzpyrene-pretreated rats showed a 40 % inhibition under the same conditions.

We conclude from our experiments that cytochrome P450, but not cytochrome P420, can catalyze the following reaction:

$$RH + \phi\text{-}I = O \xrightarrow{Fe^{3+}\ P450} ROH + \phi\text{-}I$$

Since the catalytically active intermediate has properties very similar to that obtained with NADPH and dioxygen in microsomes the following mechanism of oxygen activation can be assumed:



The special coordination sphere of cytochrome P450 with sulphur as the trans-ligand could be responsible for the stabilization of the electrophilic oxygen atom species.

ACKNOWLEDGEMENTS

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