Oxidation of arylcyclopropanes by rabbit liver cytochrome *P*-450

Mechanistic implications of a multiple oxidation

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The arylcyclopropanes (cyclopropylarenes) cyclopropylbenzene and diphenylcyclopropane are oxidized by rabbit liver microsomal cytochrome *P*-450, both by the microsomal fraction and by the purified cytochrome in a reconstituted system. The products formed, principally benzoic acid, are due to an unusual triple oxidation of the substrate, which probably remains attached to the active site during the several steps of the oxidation. Both substrates were found to be inhibitors of the cytochrome *P*-450-dependent *O*-de-ethylation of 7-ethoxycoumarin. Model oxidation studies with cumene hydroperoxide as oxidizing agent and rabbit liver microsomal fraction as source of enzyme gave similar products to the microsomal and reconstituted systems. The significance of these results in the mechanism of oxidation catalysed by cytochrome *P*-450 is discussed.

INTRODUCTION

Although the mechanism of the hydroxylation reactions catalysed by cytochromes *P*-450 has been studied for many years, the detailed nature of the reactive intermediates formed during the hydroxylation step has remained obscure (White & Coon, 1980; Guengerich & Macdonald, 1984). One approach to this problem has been the use of substrates that contain functional groupings that are designed to react with these intermediates in a rational way (Guengerich & Macdonald, 1984). We have used cyclopropylbenzene as such a substrate in enzymic and in model non-enzymic oxidations (Suckling *et al.*, 1982). In these studies, partially purified rat liver cytochrome *P*-450 was found to oxidize this substrate principally to benzoic acid, and in addition a small quantity of 2-cyclopropylphenol was detected in the oxidation products. Benzoic acid was also a significant product in model chemical oxidations of cyclopropylbenzene. The formation of benzoic acid is unusual in that it requires a triple oxidation of the substrate in the presence of excess substrate. To rationalize this result, we hypothesized that the triple oxidation of cyclopropylbenzene might arise through the formation of a transient covalent intermediate (Scheme 1; Suckling *et al.*, 1982), in which case the product formed from the rest of the cyclopropylbenzene would be acetaldehyde. Alternatively, it is possible that a partially oxidized substrate molecule is more tightly bound to the enzyme, as is the case with the cholesterol-side-chain-cleavage cytochrome *P*-450 (Lambeth *et al.*, 1982). In the present paper we describe experiments with purified rabbit liver cytochrome *P*-450 and with related microsomal systems that characterize the interaction of cyclopropylbenzene with cytochrome *P*-450. We further show that 1,2-diphenylcyclopropane is also a substrate for oxidation and that its oxidation products are consistent with our proposed oxidation pathway.

Scheme 1. Proposed mechanism for the oxidation of cyclopropylbenzene by rat liver microsomes

MATERIALS AND METHODS

Cyclopropylbenzene was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K., and was redistilled before use. 1,2-Diphenylcyclopropane was prepared by the method of Applequist & Gdansk (1981) and was used as a mixture of the *cis* and *trans* isomers. Benzoic acid, acetophenone, phenylacetaldehyde and phenol were standard laboratory-reagent grade and were recrystallized or redistilled before use. The products of the oxidations were analysed by h.p.l.c. on octadecyl silylated silica with elution with methanol/0.05 M-sodium phosphate buffer, pH 3 (3:2, v/v), for acidic products and methanol/water (7:3, v/v) for phenolic products. Hepatic microsomal fractions (referred to below simply as microsomes) were prepared from male New Zealand-White rabbits (2–2.5 kg) that had been treated with phenobarbitone (1 mg/ml in drinking water) for 1 week before use. Microsomes were prepared by differential

centrifugation as described previously (Wolf *et al.*, 1980). Samples were either used fresh or stored at -80°C in 0.25 M-sucrose buffered with 10 mM-sodium phosphate, pH 7.4. Storage of the cytochrome at -80°C had no effect on the enzyme activity. Cytochrome *P*-450 (form 2) and cytochrome *P*-450 reductase were isolated from liver microsomes from phenobarbitone-treated rabbits as described previously (Wolf *et al.*, 1978, 1980). The purified cytochrome *P*-450 had a specific content of 17.6 nmol/mg of protein.

Reconstituted mono-oxygenase systems used to investigate the metabolism of cyclopropylbenzene contained cytochrome *P*-450 (5 nmol), cytochrome *P*-450 reductase (10000 units/nmol of cytochrome *P*-450, 1 unit being the amount of reductase required to reduce 1 nmol of cytochrome *c*/min at 37°C) and dilauryl phosphatidylcholine (500 μg /nmol of cytochrome *P*-450) in a maximum volume of 0.5 ml. This system is optimal for the mono-oxygenase activity of the purified cytochrome *P*-450 (Wolf *et al.*, 1978). The mixture was vortex-mixed and incubated for 5 min at room temperature followed by the addition of 5 ml of 0.1 M-Tris/HCl buffer, pH 7.4. Cyclopropylbenzene (1 mM) as a solution in ethanol (25 μl) was then added followed by NADPH (1 mM), and the sample was incubated for 1 h at 37°C . The products formed during the reaction were assayed by h.p.l.c. as described previously (Suckling *et al.*, 1982).

The effect of the cyclopropylbenzene on 7-ethoxycoumarin metabolism by purified cytochrome *P*-450 was determined by using the above reconstitution procedure with the exception that the reactants were used at one-tenth of the concentration. After the preincubation, 0.1 M-Tris/HCl buffer, pH 7.4 (2 ml), containing 7-ethoxycoumarin (1 mM) and various concentrations of cyclopropylbenzene were added, followed by the addition of NADPH (1 mM). The oxidation of 7-ethoxycoumarin was determined at 37°C by the direct fluorimetric method described by Ullrich & Weber (1972). This method was also used to determine the inhibition of 7-ethoxycoumarin *O*-de-ethylation by cyclopropylbenzene and diphenylcyclopropylbenzene in hepatic microsomal samples. Microsomal samples were also used to investigate the metabolism of cyclopropylbenzene and diphenylcyclopropylbenzene supported by NADPH or cumene hydroperoxide. Microsomes (50 mg of protein) in 0.1 M-Tris/HCl buffer (25 ml) were incubated with the above substrates (1 mM) and cofactors [glucose 6-phosphate (4 mM), glucose-6-phosphate dehydrogenase (0.5 unit/ml) and NADPH (1 mM)] for 1 h at 37°C or with cumene hydroperoxide (1 mM). Incubations with cumene hydroperoxide were gassed with N_2 for 5 min before the reaction was commenced and sealed during the course of the incubation.

Protein determinations were made by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Analysis of kinetic data used a linear-regression program (Curve Fit; Computace, Sutton, Surrey, U.K.) for the linear transformations plotted in the Figures. Kinetic parameters were calculated by numerical methods following those of Atkins & Nimmo (1980). The results shown in the Figures are typical experiments of at least two performed. The substrate concentrations used in the kinetic experiments were chosen on the basis of preliminary experiments to be those that gave a good range of reaction rate in the presence and in the absence of the inhibitors.

RESULTS

Our first experiments were planned to ensure that the products previously observed (Suckling *et al.*, 1982) were solely the result of cytochrome *P*-450-mediated reactions. We investigated the effect of the substrate 7-ethoxycoumarin on the oxidation of cyclopropylbenzene. When rabbit liver microsomes were incubated in the presence of cyclopropylbenzene (4.6 mM) and an NADPH-generating system in 0.1 M-Tris/HCl buffer, pH 7.7, the formation of both 2-cyclopropylphenol and benzoic acid was observed; however, when the same experiment was carried out in the presence of 7-ethoxycoumarin (0.46 mM), neither product was detected. Similar experiments with the purified rabbit cytochrome *P*-450 (form 2; 0.1 nmol) led to the production of benzoic acid but not of 2-cyclopropylphenol. Once again, 7-ethoxycoumarin inhibited the oxidation of cyclopropylbenzene.

The oxidation of cyclopropylbenzene by the purified cytochrome *P*-450 was also studied kinetically in competition with 7-ethoxycoumarin (Figs. 1 and 2). The inhibition of the oxidation of 7-ethoxycoumarin by cyclopropylbenzene approximated to a competitive pattern with a K_i of 0.057 mM. These results confirm that cyclopropylbenzene is a genuine substrate of the purified rabbit liver enzyme, that benzoic acid is an enzymic product and that no other enzymes are involved in the reaction. It is possible that another NADPH-dependent oxidase was responsible for the production of the 2-cyclopropylphenol.

In order to characterize further the multiple oxidation of the cyclopropane ring, the oxidation of 1,2-diphenylcyclopropane by the microsomal preparations was studied. This molecule was selected as a probe

Fig. 1. Lineweaver-Burk plot of the oxidation of 7-ethoxycoumarin by purified rabbit liver cytochrome *P*-450

●, No additions; ○, 2 mM-cyclopropylbenzene added. The assays were carried out as described in the Materials and methods section. The specific content of the cytochrome *P*-450 was 17.6 nmol/mg of protein. The limiting velocity was calculated to be 39.3 nmol of product formed/min per nmol of cytochrome *P*-450 in the control experiment and was 25.5 nmol/min per nmol of cytochrome *P*-450 in the presence of the cyclopropylbenzene. K_m in the absence of cyclopropylbenzene was 0.35 mM, and was 1.2 mM in the presence of the inhibitor.

Fig. 2. Dixon plot of the inhibition by cyclopropylbenzene of the oxidation of 7-ethoxycoumarin by rabbit liver cytochrome *P*-450

The concentrations of 7-ethoxycoumarin were 0.1 mM (○) and 0.05 mM (●). K_i was calculated to be 0.057 mM.

X = H, Fe(haem), COEnz

Scheme 2. Possible oxidation products of diphenylcyclopropane by cytochrome *P*-450

because, if the mechanism of Scheme 1 is followed, benzoic acid as well as either phenylacetaldehyde or acetophenone or both should be detected as products (Scheme 2). H.p.l.c. analysis of the products of oxidation after removal of highly polar material by percolation through silica and fractionation into neutral and acidic components showed the presence of all three predicted

Fig. 3. Lineweaver-Burk plot of the oxidation of 7-ethoxycoumarin by rabbit liver microsomal cytochrome *P*-450

●, No additions; ○, 0.05 mM-diphenylcyclopropane added. The specific content of the cytochrome *P*-450 in the microsomal preparations used was 1.54 nmol/mg of protein. The limiting velocity was 6.8 nmol of product formed/min per nmol of cytochrome *P*-450 in the control experiment and was 1.3 nmol/min per nmol of cytochrome *P*-450 in the presence of the inhibitor. K_m for the control was 0.053 mM, and was 0.064 mM in the presence of the inhibitor.

products. The major neutral product was phenylacetaldehyde, suggesting that the initial attack by the microsomal enzyme takes place at that less-hindered carbon atom of 1,2-diphenylcyclopropane. These results are consistent with a complex radical oxidation mechanism and require that the enzyme-mediated cleavage of 1,2-diphenylcyclopropane generates C_7 and C_8 fragments. As with cyclopropylbenzene, non-enzymic model oxidation experiments afforded similar products (C. J. Suckling, D. C. Nonhebel & L. Brown, unpublished work), but also further oxidation products of phenylacetaldehyde (benzyl alcohol and benzaldehyde) were significant. Small amounts of these two products were also detected in the neutral fraction of the microsomal oxidation products.

Kinetic experiments on the oxidation of 1,2-diphenylcyclopropane and 7-ethoxycoumarin were also carried out (Fig. 3). The data clearly show that 1,2-diphenylcyclopropane inhibits oxidation of 7-ethoxycoumarin by the microsomal cytochrome *P*-450. Kinetically, non-competitive inhibition was observed with an apparent inhibition constant of 11 μ M. In neither set of kinetic experiments with cyclopropylbenzene or 1,2-diphenylcyclopropane was time-dependent or irreversible inhibition detected. No destruction of cytochrome *P*-450 could be found by measurement of the CO-absorption spectrum after incubation with the arylcyclopropanes (results not shown).

As a further demonstration that the oxidation of diphenylcyclopropane and cyclopropylbenzene was catalysed by cytochrome *P*-450 in a manner consistent with that of other substrates (White & Coon, 1980), we investigated the oxidation of these compounds by rabbit liver microsomes in the presence of cumene hydroperoxide,

a compound that can act as an oxygen donor for hydroxylation reactions carried out anaerobically. When cyclopropylbenzene or 1,2-diphenylcyclopropane was incubated alone with cumene hydroperoxide, no detectable products were formed. If the incubation was carried out in the presence of microsomes, acetophenone was a major product, together with many acidic further oxidation products. In a control incubation in which the substrate was omitted, only traces of phenyl-containing products were detected, showing that the cumene hydroperoxide was itself negligibly transformed under these conditions.

DISCUSSION

Cyclopropane derivatives bearing heteroatom substituents have been found to act as irreversible inhibitors of cytochromes *P*-450 (Hanzlik & Tullman, 1982; Macdonald *et al.*, 1982; Guengenrich & Macdonald, 1984). It has been argued that the mechanism of inhibition involves alkylation of the haem by a radical formed by one-electron transfer from oxygen-activated cytochrome *P*-450 (Scheme 3). This scheme depends upon the well-known ring opening of cyclopropyl alcohols and their derivatives (Griller & Ingold, 1980). We have shown that arylcyclopropanes are typical substrates for phenobarbitone-induced cytochromes *P*-450 from rat and rabbit liver, and that with these cyclopropane derivatives inhibition does not occur. Consequently it is probable that a variant of the mechanism of action of cytochrome *P*-450 involving initial addition of a radical to the cyclopropane ring is operating. Such addition reactions are of course normal in the epoxidation of alkenes catalysed by cytochrome *P*-450 and in the oxidation of arenes to phenols. The unusual feature of the reactions of the arylcyclopropanes is that in each case a multiple oxidation of the substrate occurs.

Since the oxidation reactions were carried out in the presence of a vast excess of substrate, it is probable that the multiple oxidation took place sequentially with the substrate remaining at the active site. A mechanism for the reduction of polyhalogenated methanes to carbenes by cytochrome *P*-450 has been proposed in which an unstable intermediate generated by a one-electron reduction is stabilized by the haem iron of the cytochrome before the addition of a second electron (Wolf *et al.*, 1977). We hypothesized that a fragmentation reaction might lead to benzoic acid, together with acetaldehyde, which was not detected (Suckling *et al.*, 1982). The finding that acetophenone and phenylacetaldehyde accompany benzoic acid is consistent with the fragmentation reaction proposed, and requires that each fragment of 1,2-diphenylcyclopropane becomes bonded to oxygen and is oxidized to the oxidation state of carbonyl and carboxylic acid. Such a result arises naturally from the sequence

Scheme 3. Mechanism of irreversible inhibition of cytochrome *P*-450 by cyclopropane derivatives

of reactions outlined in Schemes 1 and 2; chemical precedents exist for each step, and we have also reproduced the same pattern of reactivity in non-enzymic model radical oxidations, for example an $\text{Fe}^{\text{II}}/\text{EDTA}/\text{O}_2$ system (C. J. Suckling, D. C. Nonhebel & L. Brown unpublished work).

However, in the case of the enzyme-catalysed reactions we have no evidence as to the nature of X in Schemes 1 and 2. A recent chemical model shows that acyl groups may perform this function (Khenkin & Shteinman, 1984). Since addition to the cyclopropane ring is the likely first step, it is possible that both fragments of the homolysed activated oxygen add in a rapid stepwise manner. It is attractive to suggest that one of these radicals is a carboxy radical, as proposed by Sligar *et al.* (1980), because such covalent-bond formation would hold the substrate at the active site and promote further oxidation, albeit by a different mechanism. Product release could then occur by ester hydrolysis. Alternatively, intermediate dihydroxyaralkanes (Schemes 1 and 2, X = OH) may be formed and remain tightly bound to the enzyme. In none of our experiments was irreversible inhibition of the enzyme observed, in contrast with observations with alkynes, activated alkenes and heteroatom-substituted cyclopropanones (Guengenrich & Macdonald, 1984). There are several possible explanations for this distinction. Firstly, the presumed radical intermediates in the oxidation of arylcyclopropanes may not be accessible to the haem. Secondly, the stability of these radicals, which are benzylic, may mitigate against reaction with the haem in competition with combination with an available oxygen radical. Recently a correlation has been made between the single-electron oxidation potential of a series of heteroatom-substituted cyclopropanes that are inhibitors of cytochrome *P*-450 and the rate of the inhibition reaction (Guengenrich *et al.*, 1984). Our substrates would have a very low tendency to undergo one-electron oxidation to yield substituted cyclopropylmethyl radicals from which homoalkyl radicals that could alkylate the haem could arise. Thus our results are not inconsistent with the observations of others concerning inhibition of cytochrome *P*-450. With arylcyclopropanes as substrates, cytochrome *P*-450 selects addition to the cyclopropane as the preferred reaction course from the many possibilities.

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