The oxidation of cyclopropyl benzene by rat liver microsomal cytochrome *P*-450: an unusual triple oxidation of a substrate

Keith E. Suckling*, Candida G. Smellie*, I. El Sayeed Ibrahim+, Derek C. Nonhebel+ and Colin J. Suckling+

*Department of Biochemistry, University of Edinburgh, Scotland and +Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow, Scotland

Received 2 July 1982

Cytochrome P-*450* *Hydroxylation mechanism* *Cyclopropyl benzene*

1. INTRODUCTION

The mechanism of hydroxylation of both aliphatic and aromatic molecules by cytochromes *P*-450 and related chemical model systems has been studied in depth recently [1] and a wide range of approaches has been used. For example, Groves [2] has demonstrated the radical character of some model hydroxylating systems based upon tetraphenyl porphyrin by observing the ring opening of the cyclopropane ring in bicyclo(4,2,0)heptane and we have used a related approach to investigate the possibility of radical intermediates in hydrogen transfer reactions mediated by nicotinamide-dependent enzymes [3]. It was therefore of interest to attempt to extend the applicability of the cyclopropyl group as a probe for radical intermediates in aromatic substitution by examining the hydroxylation of cyclopropylbenzene by a cytochrome *P*-450 and related chemical systems. In this paper, we describe the reactions of cyclopropylbenzene with rat liver microsomal cytochrome *P*-450: related chemical reactions are discussed elsewhere [4].

2. MATERIALS AND METHODS

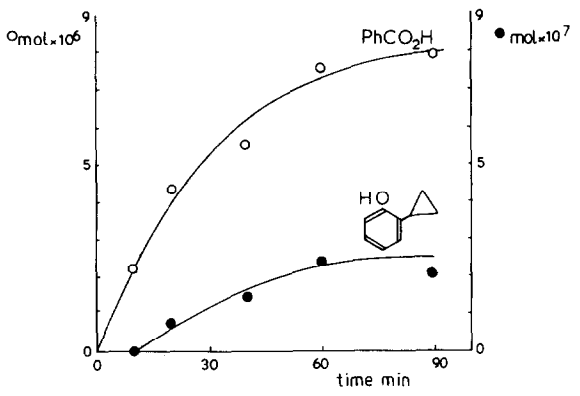
Cyclopropylbenzene was obtained from Aldrich Chemicals, Gillingham, Dorset, U.K., and was redistilled before use. Products of microsomal hydroxylation were assayed by HPLC on Partisil ODS (25 cm) eluting with 70% methanol:30% water in the reverse phase mode. Product peaks were characterised by comparison with synthetic reference compounds [4]. Quantitative results were obtained using 3-*t*-butylphenol as internal standard. Retention volumes (ml) at ambient temperature and a flow rate of between 0.6 and 0.7 ml · min^{-1} for significant compounds were as follows: NADP 1.7, benzoic acid 1.9, phenol 3.8, 2-cyclopropylphenol 5.1, 3-*t*-butylphenol 9.5, 4-cyclopropylphenol 10.4. It was essential to run reference samples prior to every analysis because some variation in retention volume was found with changing ambient temperature but more significantly between nominally identical columns. GLC analysis of the methyl esters of the products was carried out on 5% FFAP on Chromosorb G (2 m) at 170°C; methyl benzoate had a retention time of 3.4 min at a flow rate of 25 ml · min^{-1}. The microsomal preparation was obtained from the livers of rats treated with phenobarbitone (0.1% w/v in the drinking water [5]) and contained approximately 1 ± 0.4 nmol cytochrome *P*-450 per mg protein. Oxidation reactions were carried out in shaken tubes at 37°C and contained the following components for a 20 ml incubation in 0.1 M potassium phosphate buffer (pH 7.1): glucose 6-phosphate (30 mg), NADP (9 mg), microsomal protein (20 mg) and glucose 6-phosphate dehydrogenase (2.8 IU added last). These reagents were supplied by Boehringer. Cyclopropylbenzene (100 μl) was added in solution in acetone (10 μl) and was rapidly taken up by the microsomal suspension on shaking. After the appropriate time, a sample (4 ml) was removed and was quenched by the addition of methanol (8 ml) and the standard 3-*t*-butylphenol (0.15 mg) added. The protein was allowed to precipitate and was centrifuged off. Assays (HPLC) were carried out

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

on the supernatant methanolic solution. Control experiments were also carried out in the absence of the glucose 6-phosphate-NADP-glucose 6-phosphate reducing system.

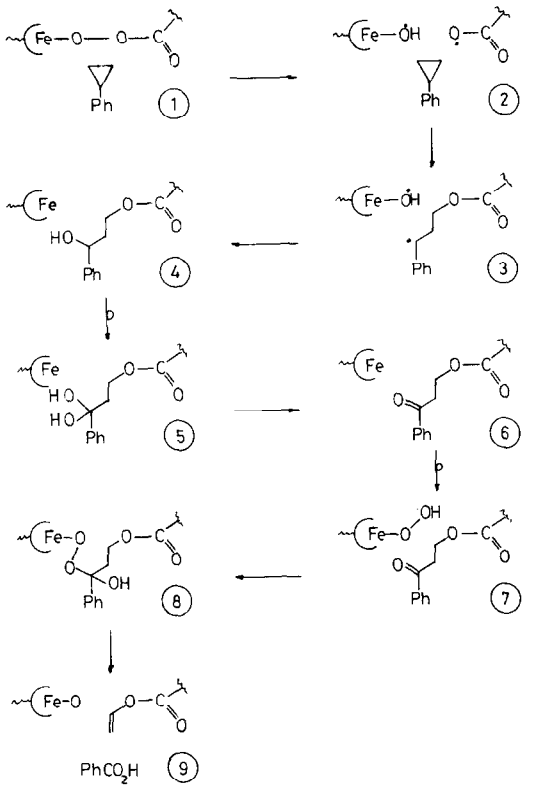
3. RESULTS AND DISCUSSION

The initial impetus to the investigation of the hydroxylation of cyclopropylbenzene by cytochrome *P*-450 was that addition of a radical oxidant to the aromatic ring might generate a radical with sufficient spin density at the carbon atom α- to the cyclopropane ring for the latter to open. Cyclopropylbenzene was found to bind to the microsomal cytochrome *P*-450 giving a type 1 binding spectrum (K_{diss} = 2.3 ± 0.5 (SE n = 3) mM). However analysis of the acidic products obtained showed that the only phenolic product was 2-cyclopropylphenol as detected by HPLC and high resolution mass spectrometry ($C_9H_{10}O$ requires m/e 134.0732 found 134.0711; M-1 C_9H_9O requires 133.0653 found 133.0638). The lack of ring opening of the cyclopropane by no means argues against radical intermediates in the microsomal hydroxylation because 2-substitution with an intact small ring was also observed using iron(II)/oxygen hydroxylating systems that have typical radical properties [4]: indeed 2-hydroxylation appears to be normal for this substrate. 2-Cyclopropylphenol was produced by the complete microsomal oxidation system in a time dependent manner (fig.1) and was absent in the control experiment lacking the NADPH generator. The absence of 4-hydroxylation was also observed in the chemical systems.



2-Cyclopropylphenol, however, accounted for only 5–10% of the microsomal hydroxylation products. The major product, surprisingly, was benzoic acid, detected by HPLC and GLC on the methyl esters, which was also produced in a time dependent manner (fig.1) and was absent in the control experiment. Benzoic acid was also obtained in the chemical model systems [4]. Although cyclopropylbenzene failed to be hydroxylated by the microsomal preparation along the expected pathway, the formation of benzoic acid is informative with regard to the mechanism of action of the enzyme. Evidence is accumulating that the activation of oxygen by cytochrome *P*-450 occurs by a homolytic process [1,6] and in particular, the role of an acyl peroxide or its equivalent has been emphasised [7].

The production of benzoic acid requires three oxidations to take place on the same substrate molecule. Although this is unusual in the hydroxylation of aromatic compounds by microsomal cytochromes *P*-450, it seems likely that a related enzyme is responsible for the removal of C-19 of the steroid skeleton during the biosynthesis of oestrones [8] and a triple oxidation also takes place during the side chain cleavage of cholesterol catalysed by mitochondrial cytochrome *P*-450 of steroid producing tissue. With these precedents in mind, it is attractive to rationalise the formation of benzoic acid as illustrated in fig.2. If the postulated acylperoxide intermediate (1) homolyses, addition of the resulting carboxy radical or its equivalent (2) to the cyclopropane ring instead of the usual hydrogen abstraction would lead to an intermediate (3) which can be readily hydroxylated to yield (4). Radical addition to the cyclopropane ring was observed in the model reactions and has been described for other radical reactions [9,10]. It is therefore reasonable that addition to the small ring should compete with attack on the benzene nucleus. Electrophilic acetylation and bromination, on the other hand, usually give rise to aromatic substitution [11]. The hydroxylation product at stage (4) would usually dissociate from the enzyme, but, because of the addition reaction (2)–(3), the substrate remains covalently bound. With a substrate molecule held at the active site, the enzyme can activate a second molecule of oxygen and catalyse benzylic hydroxylation which would be expected to lead to the ketone (6) possibly via



the hydrate (5). Once again, the enzyme could then react with a third molecule of oxygen to give the hydroperoxide (7). It is well established that hydroperoxides add readily to aralkyl ketones to form hemiacetal derivatives (8) without catalysis [12] and radical decomposition of such intermediates to give esters has precedent [13]. The formation of benzoic acid (9) could take place by an analogous process. Alternatively, the intermediate (8) could fragment in a manner similar to that proposed by Akhtar [14] to account for the fate of isotopically labelled oxygen in the biosynthesis of oestrone.

Thus the unexpected oxidation of cyclopropylbenzene to benzoic acid can be seen to be consistent with both chemical precedent and with the current mechanistic understanding of cytochrome *P*-450. The mechanistic hypotheses outlined above pose a number of interesting questions one of which is whether the possible alkylation of cytochrome *P*-450 by cyclopropylbenzene causes partial inhibition of the enzyme as has been observed for cyclopropylamine derivatives [15,16]. We intend to investigate this and other facets of the reaction using purified enzyme preparations.

ACKNOWLEDGEMENTS

This work was supported in part by MRC grant G801067SB, by the award of the Royal Society Smith and Nephew Senior Research Fellowship to C.J.S. and by an SERC postdoctoral research assistantship to I.E.I..

REFERENCES

[1] White, R.E. and Coon, M.J. (1980) Ann. Rev. Biochem. 49, 315–356.
[2] Groves, J.T., Kruger, W.J. jr and Haushalter, R.C. (1980) J. Am. Chem. Soc. 102, 6375–6377.
[3] MacInnes, I., Nonhebel, D.C., Orszulik, S.T. and Suckling, C.J. (1982) J. Chem. Soc., Chem. Commun. 121–122.
[4] Ibrahim, I.E., Nonhebel, D.C. and Suckling C.J., in preparation.
[5] Brown, M.J.G. and Boyd, G.S. (1974) Eur. J. Biochem. 44, 37–47.
[6] White, R.E., Sligar, S.G. and Coon, M.J. (1980) in: Biochemistry, Biophysics and Regulation of Cytochrome *P*-450 (Gustaffson, J.-A. et al. eds) pp. 307–314, Elsevier Biomedical, Amsterdam, New York.
[7] Sligar, S.G., Besman, M., Gelb, M., Gould, P., Heimbrook, D. and Pearson, D. (1980) in: Biochemistry, Biophysics and Regulation of Cytochrome *P*-450 (Gustaffson, J.-A. et al. eds) pp. 379–382, Elsevier Biomedical, Amsterdam, New York.
[8] Holland, H.R. (1982) Chem. Soc. Rev. 10, 435–453.
[9] Applequist, D.E. and Mackenzie, L.F. (1976) J. Org. Chem. 41, 2262–2266.
[10] Young, L.B. (1968) Tetrahedron Lett. 5105–5108.
[11] Levina, R.Y. and Gembitskii, P.A. (1961) J. Gen. Chem. USSR 31, 3242–3243.
[12] Sosnowsky, G. and Rawlinson, D.J. (1971) in: Organic Peroxides (Swern, D. ed) Vol. 2, p. 183, Wiley Interscience, New York.
[13] Sharp, D.B. and Patrick, T.M. (1961) J. Org. Chem. 26, 1389–1394.
[14] Akhtar, M., Calder, M.R., Corina, D.L. and Wright, J.N. (1981) J. Chem. Soc., Chem. Commun. 129–130.
[15] Hanzlik, R.P. and Tullman, R.H. (1982) J. Am. Chem. Soc. 104, 2048–2050.
[16] MacDonald, T.L., Zirvi, K., Burka, L.T., Peyman, P. and Guengerich, F.P. (1982) J. Am. Chem. Soc. 104, 2050–2052.