Oxidations of Cyclopropane, Methylcyclopropane, and Arenes with the Mono-oxygenase System from *Methylococcus capsulatus*

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Summary The mono-oxygenase system from *Methylococcus capsulatus* oxidises cyclopropane to cyclopropanol, methylcyclopropane to cyclopropylmethanol, and monosubstituted benzenes to *para*-substituted phenols (with accompanying NIH shift).

THE methane mono-oxygenases from *Methylococcus capsulatus* (Bath) and other sources have been reported to catalyse oxidations of a variety of substrates including alkanes, alkenes, and arenes.[1,2] To probe the mechanism and active site of the enzyme from *M. capsulatus* we have studied its behaviour towards cyclopropane, methylcyclopropane, and certain aromatic substrates (*cf.* Table).

Air and cyclopropane were incubated with a crude enzyme preparation[1] from *M. capsulatus* in phosphate buffer containing NADH. A single product of oxidation was identified as cyclopropanol by comparison (g.l.c.) with authentic cyclopropanol[3] (propanal and allyl alcohol were not found) and by its acid-catalysed conversion into propanal.[4] Thus, an aliquot of the reaction mixture did not produce a 2,4-dinitrophenylhydrazone when allowed to react with 0·4% (w/v) 2,4-dinitrophenylhydrazine in hydrochloric acid for 45 min/20 °C. However, after heating this mixture for 45 min at 60 °C, propanal 2,4-dinitrophenylhydrazone was obtained [identified by comparison (t.l.c.) with an authentic sample]. Ooyama and Foster[5] claimed that propanal is a product of oxidation of cyclopropane by *Mycobacterium vaccae* (strain JOB5) and dioxygen. However, they identified propanal after atmospheric distillation of the reaction mixture followed by addition of acidic 2,4-dinitrophenylhydrazine. It is likely that cyclopropanol was converted into propanal under these conditions.[4]

In a manner similar to that described for cyclopropane, methylcyclopropane gave cyclopropylmethanol which was identified by g.l.c. and by conversion into its α-naphthylurethane which was identified (comparison with an authentic sample) by t.l.c., ^{1}H n.m.r. spectroscopy, and electron impact (e.i.) mass spectrometry. According to the g.l.c. analyses, but-3-en-1-ol was *not* a product of the oxidation of methylcyclopropane by *M. capsulatus*.

Products from the oxidation of various aromatic substrates by the enzyme from *M. capsulatus* are shown in the Table. Note that the oxidation is regioselective for the *para*-position of the monosubstituted benzenes. Quantitative analysis by g.l.c. for the oxidation of both toluene and ethylbenzene showed $\leqslant 3\%$ *meta* and $\leqslant 5\%$ *ortho* isomers. The oxidation is susceptible to the size of the substituent. t-Butyl- and 1-methylcyclopropyl-benzene are not oxidised, indicating a certain spatial restriction at the active site. Whereas styrene gives similar amounts of styrene oxide and *p*-hydroxystyrene, α-methylstyrene gives *p*-hydroxy-α-methylstyrene almost exclusively. Naphthalene gives both α- and β-naphthol. The oxidation of ethylbenzene exhibits an 'NIH' shift.[6] *p*-Deuterioethylbenzene was prepared by quenching the Grignard reagent from *p*-bromoethylbenzene with deuterium oxide. Incubation of this substrate with the *M. capsulatus* enzyme

TABLE.[a]

Substrate	Product(s)[b,c]
Toluene	Benzyl alcohol(4), *p*-cresol(1)
Ethylbenzene	1-Phenylethanol(1), *p*-ethylphenol(1)
Styrene	Styrene oxide(1), *p*-hydroxystyrene(1)
α-Methylstyrene	*p*-Hydroxy-α-methylstyrene
Propylbenzene	*p*-Hydroxypropylbenzene
t-Butylbenzene	not oxidised
1-Methylcyclopropylbenzene	not oxidised
Naphthalene	α-(1·6) and β-Naphthol(1)

[a] Oxidations were done with *e.g.* 10 μl of liquid substrate, a crude enzyme preparation (0·2 cm^3 containing *ca.* 4 mg of protein), NADH (0·05 cm^3 of a 0·1 M solution in phosphate buffer) and 0·4 cm^3 of a 20 mM phosphate buffer (pH 7·0) at 45 °C for 15 min—1 h. For larger scale reactions quantities of components were proportionally increased. The reaction mixture was contained in a stoppered (Subaseal) flask and agitated in a reciprocating water-bath. [b] Relative amounts of products are given; conversions of substrate into products are up to *ca.* 2%. [c] Products were identified by t.l.c. and/or g.l.c. and by ^1H n.m.r. spectroscopy after chromatographic separation.

gave *p*-ethylphenol, isolated by solvent extraction and purified by preparative layer chromatography. The 61·4 MHz ^2H n.m.r. spectrum of this product shows a single resonance corresponding in chemical shift to 2-H (δ 6·6 in CCl_4) of *p*-ethylphenol (*n.b.* 3-H at δ 6·9). The ^1H n.m.r. spectrum and the e.i. mass spectrum of its methyl ether indicate a deuterium retention of *ca.* 60%.

The results presented suggest that the *M. capsulatus* system operates *via* a mechanism in which dioxygen is converted into a presumably metal-bound oxygen species that is capable of insertion into a C–H bond and addition to a C=C bond.[7] The regioselective formation of *p*-substituted phenols from monosubstituted benzenes (*cf.* Table) and the accompanying NIH shift require the intermediacy of either an arene 3,4-oxide or *p*-substituted cationic σ-complex. Both electronic and steric factors could favour these intermediates over their corresponding isomers. The non-formation of allyl alcohol from cyclopropane and but-3-en-1-ol from methylcyclopropane argues against a reaction pathway featuring an intermediate that is either a charged species (*e.g.* cyclopropyl carbocation) or a radical (*i.e.* cyclopropylmethyl radical).[8]

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[1] J. Colby, D. I. Stirling, and H. Dalton, *Biochem. J.*, 1977, **165**, 395.
[2] J. Colby and H. Dalton, *Biochem. J.*, 1978, **171**, 461.
[3] C. H. Depuy, G. M. Dappen, K. L. Eilers, and R. A. Klein, *J. Org. Chem.*, 1964, **29**, 2813.
[4] C. H. Depuy, *Acc. Chem. Res.*, 1968, **1**, 33.
[5] J. Ooyama and J. W. Foster, *Antonie van Leeuwenhoek*; *J. Microbiol. Serol.*, 1965, **31**, 45.
[6] J. W. Daly, D. M. Jerina, and B. Witkop, *Experientia*, 1972, **28**, 1129.
[7] For recent mechanistic discussions of oxygenases see J. T. Groves, G. A. McClusky, R. E. White, and M. J. Coon, *Biochem. Biophys. Res. Commun.*, 1978, **81**, 154; J. T. Groves, *Adv. Inorg. Biochem.*, 1979, **1**, 119; S. W. May, S. L. Gordon, and M. S. Steltenkamp, *J. Am. Chem. Soc.*, 1977, **99**, 2017; A. T. Pudzianowski and G. H. Loew, *ibid.*, 1980, **102**, 5443.
[8] The ease of ring-opening of cyclopropyl to allyl is carbocation ≫ carbanion ~ radical (P. Merlet, S. D. Peyerimhoff, R. J. Buenker, and S. Shih, *J. Am. Chem. Soc.*, 1974, **96**, 959). The rate constant for the conversion cyclopropylmethyl radical into but-3-enyl radical is $1·3 \times 10^8\ s^{-1}$ at 25 °C (B. Maillard, D. Forrest, and K. U. Ingold, *ibid.*, 1976, **98**, 7024). Groves *et al.*, have very recently suggested a mechanism involving a caged radical intermediate for the oxidation of 1,2-dideuteriocyclohexene effected by cumene hydroperoxide–cytochrome P450, which gives dideuteriocyclohex-2-en-1-ols indicative of partial allylic rearrangement: J. T. Groves, O. F. Akinbote, and G. E. Avaria, Microsomes Drug Oxid., Chem. Carcinog. (Fourth Int. Symp. Microsomes Drug Oxid.), 1979, ed. M. J. Coon, A. H. Conney, and R. W. Estabrook, Academic Press, New York, 1980, vol. 1, p. 253; see also D. W. Potter and D. J. Reed, *ibid.*, p. 371.