Substrate Radical Intermediates are Involved in the Soluble Methane Monooxygenase Catalysed Oxidations of Methane, Methanol and Acetonitrile

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Carbon-centred radical intermediates have been identified by spin-trapping in the soluble methane monooxygenase catalysed hydroxylation of alkyl substrates.

Methane monooxygenase (MMO) from *Methylococcus capsulatus* (Bath) catalyses the oxidation of methane to methanol, [eqn. (1)]. In addition it catalyses the hydroxylation or epoxidation of a broad range of substrates.[1,2] The soluble enzyme has been purified and consists of three components. The hydroxylase[3] (M_r = 250 000) contains a bridged diiron site similar to those in hemerythrin,[4] rubrerythrin,[5] ribonucleotide reductase B2 protein,[6] purple acid phosphatases[7] and the initial stages of iron accumulation in apoferritin.[8] The reductase[9,10] (M_r = 38 500) contains flavin adenine dinucleo-

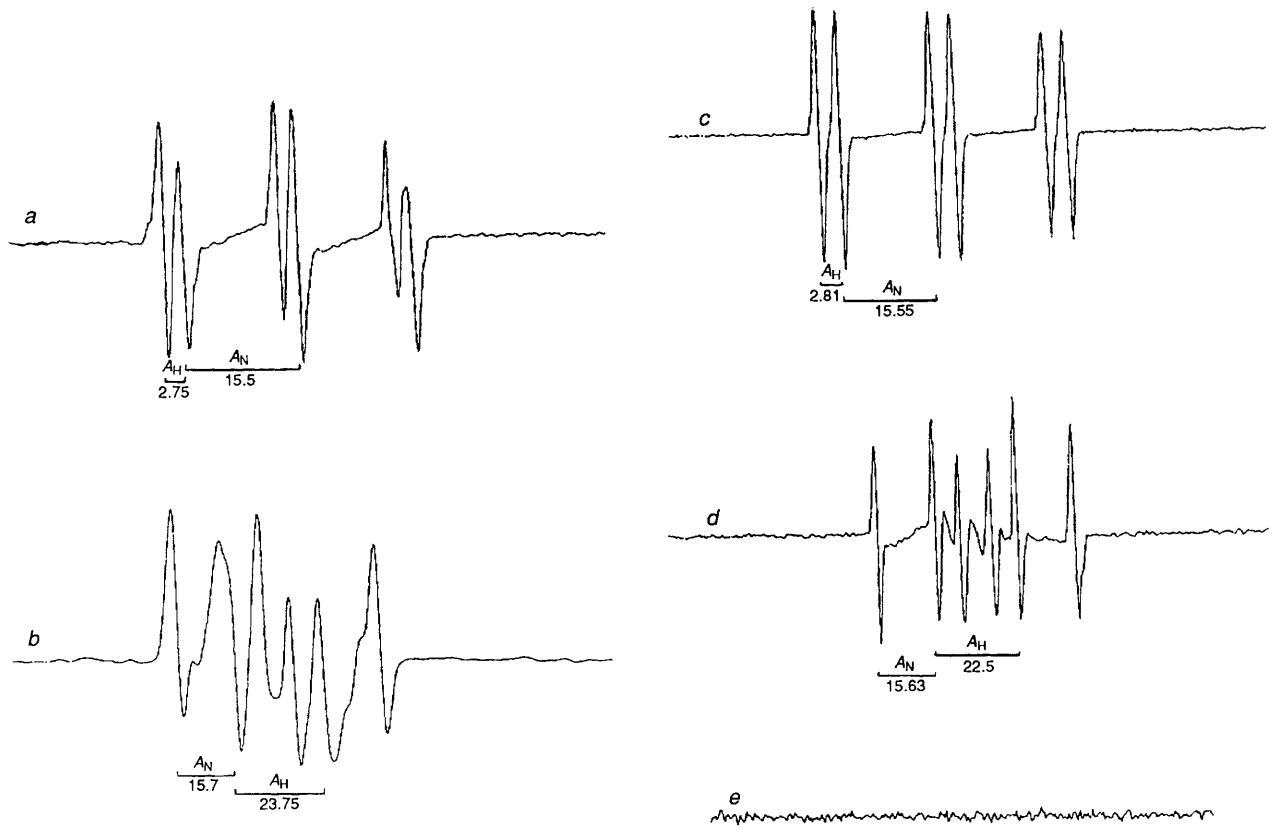

Fig. 1 X-Band ESR spectra of the: (*a*) POBN–Me adduct; (*b*) DMPO–Me adduct; (*c*) POBN–CH_2OH adduct; (*d*) DMPO–CH_2OH adduct and (*e*) enzyme, POBN, MeOH solution without added NADH. Instrument settings were: (*a*) field centre, 3365 G; modulation amplitude, 1 G; frequency, 9.4603; (*b*) 3365 G, 1 G, 9.4627; (*c*) 3358 G, 0.32 G, 9.4396; (*d*) 3336 G, 1 G, 9.4627; (*e*) 3358 G, 0.8 G, 9.463. Range, 100 G; time constant, 0.06 s; scan time, 7.8 min; power, 1 mW; approximately 25 °C.

tide (FAD) and Fe_2S_2 centres and the third component (referred to as B) is a small protein (M_r = 16 000) which contains no metal ions or cofactors.[11] All three components are required for substrate turnover. The hydroxylase is believed to be the site of catalytic activity.[12] The reductase transfers electrons, in stages, from reduced nicotinamide adenine dinucleotide (NADH) to the hydroxylase and component B has a regulatory function. Soluble MMO's from three other sources, *Methylosinus trichosporium* OB3b,[12] *Methylobacterium* CRL-26[13,14] and *Methylosinus sporium*[15] have also been purified and characterised. All are very similar to the *M. capsulatus* enzyme.

$$CH_4 + NADH + H^+ + O_2 \rightarrow MeOH + H_2O + NAD^+ \quad (1)$$

Oxidation of methane to methanol requires activation of dioxygen and the extremely stable C–H bond in CH_4. A mechanism similar to that for cytochrome P-450 has been proposed for s-MMO which takes these difficult activations into account.[2,16,17] Irrespective of whether homolytic or heterolytic cleavage of the O–O bond occurs, both pathways postulate the existence of a high valent iron intermediate and H atom abstraction to produce the methyl radical. In the heterolytic pathway a substrate radical is produced *via* the ferryl (Fe^{IV}=O) species whereas in the homolytic pathway a hydroxyl radical is generated. To date there has been no hard evidence to indicate which pathway is operative. We now present evidence that it is the substrate radical and not a hydroxyl radical which is involved in the catalytic cycle. Three different substrate radicals have been trapped using the spin-traps 5,5-dimethylpyrroline 1-oxide (DMPO) and α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone [POBN; more systematically *N*-(4-pyridylmethylene)-*tert*-butylamine *N*,*N*′-dioxide].

Table 1 Hyperfine splitting constants for substrate radical spin-trap adducts[a]

Substrate	Spin-trap[b]	A_N/G	A_H/G
CH_4	POBN	15.5	2.75
MeOH	POBN	15.6	2.8
MeCN	POBN	15.4	2.4
$HOCMe_3$	POBN	15.5	2.6
CH_4	DMPO	15.7	23.8
MeOH	DMPO	15.6	22.5
·OH[c]	DMPO	14.9	14.9

[a] Measured at ambient temperature, 0.025 mol dm^{-3} MOPS, pH 7 (1 G = 10^{-4} T). Reaction time was five minutes at 45 °C. [b] [Trap] = 0.1 mol dm^{-3}. [c] ·OH produced with H_2O_2 and UV light.

The trapped radicals are $\cdot CH_3$, $\cdot CH_2OH$ and $\cdot CH_2CN$ when methane, methanol and acetonitrile are the substrates used. Methanol and acetonitrile have been shown to be good substrates for s-MMO.[1] Experiments were performed with three different protein samples. The spin adducts were identified by the characteristic hyperfine splitting constants in their ESR spectra caused by the spin-trap nitrogen, A_N and β proton, A_H.[18] The ESR spectra of the POBN- and DMPO-trapped $\cdot CH_3$ and $\cdot CH_2OH$ radicals are shown in Fig. 1. The hyperfine splitting constants for each of the spin adducts are collected in Table 1. All are within the range of expected values.[18] The spin-trapping experiments were carried out with the usual assay conditions[2] (*i.e.* appropriate amounts of the component proteins, substrate and NADH in a 45 °C shaker bath) except that POBN or DMPO was added. Parallel

gas-chromatographic assays were performed to assess enzyme inhibition by the spin-traps. Both POBN and DMPO inhibit s-MMO activity, but there was no quantitative correlation between inhibition and ability to trap substrate radical. Control experiments were carried out with all the usual assay components with the exception of NADH, but including POBN or DMPO and also with solutions containing only substrate, spin-trap and NADH or enzyme, spin-trap and NADH. No spin-adduct was detected in any of these controls. Each control solution was prepared in 0.025 mol dm^{-3} 3-morpholinopropanesulphonic acid (MOPS) pH 7, precluding any artefact signals due to the buffer. The ESR spectrum of the enzyme–methanol–POBN control shows no detectable features (*e* in Fig. 1). After the ESR spectrum (*e*) was recorded, NADH was added to the solution and spectrum (*c*) was generated.

In none of the experiments was an oxygen-based radical trapped. If an ·OH radical were produced *via* O–O bond homolysis it would be trapped as efficiently as is an alkyl radical. The hydroxyl radical POBN or DMPO adducts would be easily identified by their A_N and A_H values. The values we obtained for the DMPO–·OH adduct generated by a Fenton reaction are listed in Table 1. This, we believe, rules out the homolytic pathway in the proposed mechanism.

Our spin-trapping experiments are strong support for a substrate radical mechanism in the soluble methane monooxygenase catalysed hydroxylation of alkanes. We have no direct evidence for a high valent iron species, but we now favour formation of the alkyl substrate radical by the ferryl species in the heterolytic pathway.

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References

1 J. Colby, D. I. Stirling and H. Dalton, *Biochem. J.*, 1977, **165**, 395.
2 J. Green and H. Dalton, *J. Biol. Chem.*, 1989, **264**, 17 698.
3 M. P. Woodland and H. Dalton, *J. Biol. Chem.*, 1984, **259**, 53.
4 P. C. Wilkins and R. G. Wilkins, *Coord. Chem. Revs.*, 1987, **79**, 195.
5 J. LeGall, B. C. Prickril, I. Moura, A. V. Xavier, J. Moura and B.-H. Huynh, *Biochem.*, 1988, **27**, 1636.
6 J. Stubbe, *J. Biol. Chem.*, 1990, **265**, 5329.
7 K. Doi, B. C. Antanaitis and P. Aisen, *Struct. Bonding (Berlin)*, 1988, **70**, 1.
8 P. M. Hanna, Y. Chen and N. D. Chasteen, *J. Biol. Chem.*, 1991, **266**, 886.
9 J. Colby and H. Dalton, *Biochem. J.*, 1978, **171**, 461.
10 J. Colby and H. Dalton, *Biochem. J.*, 1979, **177**, 903.
11 J. Green and H. Dalton, *J. Biol. Chem.*, 1985, **260**, 15 795.
12 B. G. Fox, W. A. Froland, W. A. Dege and J. D. Lipscomb, *J. Biol. Chem.*, 1989, **264**, 10 023.
13 R. N. Patel and J. C. Savas, *J. Bacteriol.*, 1987, **169**, 2313.
14 R. N. Patel, *Arch. Biochem. Biophys.*, 1987, **252**, 229.
15 S. J. Pilkington and H. Dalton, *FEMS Microbiol. Lett.*, 1991, **78**, 103.
16 H. Dalton, D. D. S. Smith and S. J. Pilkington, *FEMS Microbiol. Revs.*, 1990, **87**, 201.
17 B. G. Fox, J. G. Borneman, L. P. Wackett and J. D. Lipscomb, *Biochem.*, 1990, **29**, 6419.
18 G. R. Buettner, *Free Rad. Biol. Med.*, 1987, vol. 3, 259.