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Substrate radical intermediates in soluble methane monooxygenase ☆

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Abstract

EPR spin-trapping experiments were carried out using the three-component soluble methane monooxygenase (MMO). Spin-traps 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), α-4-pyridyl-1-oxide *N*-*tert*-butylnitrone (POBN), and nitrosobenzene (NOB) were used to investigate the possible formation of substrate radical intermediates during catalysis. In contrast to a previous report, the NADH-coupled oxidations of various substrates did not produce any trapped radical species when DMPO or POBN was present. However, radicals were detected by these traps when only the MMO reductase component and NADH were present. DMPO and POBN were found to be weak inhibitors of the MMO reaction. In contrast, NOB is a strong inhibitor for the MMO-catalyzed nitrobenzene oxidation reaction. When NOB was used as a spin-trap in the complete MMO system with or without substrate, EPR signals from an NOB radical were detected. We propose that a molecule of NOB acts simultaneously as a substrate and a spin-trap for MMO, yielding the long-lived radical and supporting a stepwise mechanism for MMO.

Keywords: Soluble methane monooxygenase; Spin-trapping; C–H oxidation; Oxygen activation; Radical intermediate; Nitrosobenzene; EPR

The soluble form of methane monooxygenase (MMO) catalyzes the NADH- and O_2-coupled conversion of methane to methanol in methanotrophic bacteria [1].

$$NAD(P)H + H^+ + CH_4 + O_2 \rightarrow NAD(P)^+ + CH_3OH + H_2O$$

The active site is located on the hydroxylase component (MMOH) and harbors a bis-μ-hydroxo-bridged diiron cluster known to catalyze both oxygen activation and hydrocarbon oxidation reactions [2,3]. This activity is strongly regulated by a cofactorless component termed "B" (MMOB) which binds strongly to the α subunit of MMOH [4,5]. A third component serves as a reductase (MMOR) and utilizes an Fe_2S_2 cluster and FAD to transfer the two electrons required by the reaction stoichiometry from NADH to MMOH [6,7]. Significant progress has been made during recent years in the study of MMO catalysis through the discovery of reaction cycle intermediates as well as the use of spectroscopic techniques and diagnostic chemical reactions [8,9]. The reaction clearly involves the conversion of the MMOH diiron cluster to a strongly oxidizing bis-μ-oxo-Fe(IV)$_2$ species termed compound **Q** (**Q**) that is capable of the fissure of stable C–H bonds in methane and many other hydrocarbons [3,10,11]. However, the chemical mechanism by which this occurs remains controversial [8,9]. One body of evidence supports a

☆ *Abbreviations:* MMO, soluble methane monooxygenase; MMOH, hydroxylase component of methane monooxygenase; MMOB, B component of methane monooxygenase; MMOR, reductase component of methane monooxygenase; **Q**, the key intermediate of the MMO catalytic cycle termed compound **Q**; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; POBN; α-4-pyridyl-1-oxide *N*-*tert*-butylnitrone; NOB; nitrosobenzene; EPR, electron paramagnetic resonance.

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stepwise mechanism in which **Q** first reacts with hydrocarbons by a hydrogen atom abstraction reaction to yield a substrate radical and what could be considered a hydroxyl radical bound to the diiron cluster [12–16]. Subsequent recombination of the radicals would yield the product. One alternative proposal based on ultrafast radical clock studies suggests a concerted C–H bond cleavage and oxygen insertion reaction [17–19]. Recent computational studies support a blend of these two extreme mechanisms in which a separate C–H bond cleavage step occurs, but the products do not depart from the bound hydroxyl radical, so that the rebound reaction occurs very rapidly [20,21]. Finally, mechanisms that propose formation of an intermediate Fe–substrate carbon bond also receive support from computational studies [22].

Attempts to resolve this controversy experimentally have centered on the detection of radical or cation intermediates [14,15,18,23–25]. Among the first of these attempts was to use water-soluble spin-trap molecules to capture radical species formed during the reaction [26,27]. A spin-trap, usually a nitrone or nitroso compound, can react with a transient radical and form a relatively long-lived spin adduct, typically a nitroxide, with a characteristic EPR spectrum [28,29]. Analysis of the EPR spectrum provides hyperfine splitting information about the nitroxide radical, thus allowing for the identification of the original radical species in favorable cases. Accordingly, Dalton and coworkers [26,27] reported that carbon centered radicals could be trapped using 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) or α-4-pyridyl-1-oxide *N-tert*-butylnitrone (POBN) as MMO turned over a wide variety of substrates. While most of these experiments were carried out using the soluble MMO from the type X methanotroph *Methylococcus capsulatus* (Bath), limited parallel experiments using the soluble MMO from the type II methanotroph *Methylosinus trichosporium* OB3b showed equivalent results.

The observation of trapped substrate radicals is a very important result, but it is inconsistent in some ways with more recent advances in both mechanistic theory and structural studies of the MMO components. Most importantly, it is clear that the reaction occurs in an active site pocket which is completely occluded from bulk solution by the protein structure [30–32]. Moreover, all experimental approaches suggest that the lifetime of the putative substrate radical is very short, perhaps only a few hundred femtoseconds [14,19,21,23,25]. Thus, in order to trap substrate radicals, either the water-soluble spin-traps would have to access the hydrophobic active site or substrate radical would have to escape the site before the oxygen rebound could occur. Both possibilities seem unlikely, especially in view of recent studies of the effects of MMOB on substrate access to and product egress from the active site, which show that the processes are relatively slow [33,34].

Here we re-examine the spin-trap approach using the MMO system isolated *M. trichosporium* OB3b by procedures that give a highly active enzyme in which NADH utilization and product formation are tightly coupled. Our trapping experiments produced significantly different results than those previously reported, despite the fact that they were conducted under the same experimental conditions and with the same substrates and spin-traps. The current study suggests that substrate radicals do not escape the active site. However, observations made during the study led to a new approach in the use of spin-traps by combining the trap and the substrate in the same molecule. This approach supports the formation of substrate radicals in the reaction cycle of soluble MMO.

Materials and methods

Materials. MMO protein components were purified from *M. trichosporium* OB3b as previously described [35,36]. The specific activity of MMOH preparations used for the experiments was in the range of 600–1000 nmol/min/mg, assayed at 23 °C. Spin-traps and other chemicals were of the highest purity available from Aldrich Chemical, Milwaukee, WI. DMPO was purified by distillation to a colorless liquid from yellow starting material, whereas POBN and NOB were used without further purification.

Polarographic activity assays. MMO activity was assayed at 23 °C by monitoring O_2 depletion, as previously described [35]. In all cases, the buffer solution was air saturated with 50 mM Mops buffer, pH 7.6. A typical concentration of MMOH ($(\alpha\beta\gamma)_2$ quaternary structure) used was 0.7 μM. MMOB and MMOR were used in stoichiometric amounts relative to MMOH active sites, i.e., 1.4 μM. The reaction was initiated by addition of ethanol-free NADH (final concentration of 200 μM) and repeated at least three times for each different condition. Furan was used as the standard assay substrate (final concentration of 1.9 mM). For DMPO and POBN, calculated amounts of high concentration stock solution were added to the reaction chamber to obtain the desired final concentration. NOB stock solutions were made by stirring an excess of NOB in buffer under argon at 4 °C for 2 h in the dark to generate a saturated solution. The actual NOB concentration was confirmed just before use based on the absorbance at 309 nm ($\varepsilon = 10{,}000\ M^{-1}\ cm^{-1}$) [37].

Absorption spectroscopic activity assays. All assays were performed at 23 °C using nitrobenzene as the substrate. The formation of its hydroxylation product, 4-nitrophenol, was monitored optically at 404 nm ($\varepsilon = 15\ mM^{-1}\ cm^{-1}$ at pH 7.6) using a Hewlett–Packard 8451A diode array spectrophotometer. The reaction was carried out in a cuvette by the addition of enzyme components (final concentration of MMOH 1 μM; MMOR and MMOR in stoichiometric amount relative to MMOH active sites) and ethanol-free NADH (final concentration 200 μM) to 50 mM Mops buffer, pH 7.6, containing nitrobenzene (final concentration 1.2 mM) and monitored at 404 nm over time. Spin-traps were added as indicated in the figure legends.

Spin-trapping experiments. Reactions were carried out in 50 mM Mops or sodium phosphate buffer, pH 7.6, containing one of the following, DMPO, POBN, or NOB. The reaction mixtures contained MMOH (0 or 50 μM), MMOR (0 or 100 μM), MMOB (0 or 100 μM), and substrate (0 to a concentration in millimolar range depending upon the substrate). Addition of ethanol-free NADH (final concentration 3 mM) initiated the reaction. Incubations were undertaken in a shaking water bath maintained at 30 °C for 5, 10, or 20 min. After the incubation, each solution was immediately frozen in liquid nitrogen and maintained at that temperature until immediately before its EPR spectrum was recorded. In the case of NOB, some experiments were carried out directly in an EPR flat cell so that there is no freezing and thawing step and the time course of the reaction could be more accurately monitored.

EPR analysis. X-band EPR spectra were recorded on a Bruker Elexsys E-500 spectrometer. Frozen samples were thawed and transferred to capillary tubes and the spectra were measured at ambient temperature. In the case of NOB, the experiments carried out in EPR flat cells at ambient