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Substrate radical intermediates in soluble methane monooxygenase ☆

Aimin Liu [a,c,1], Yi Jin [b,c,2], Jingyan Zhang [a,c], Brian J. Brazeau [a,c,3], John D. Lipscomb [a,c,*]

[a] Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Minneapolis, MN 55455, USA
[b] Department of Chemistry, University of Minnesota, Minneapolis, MN 55455, USA
[c] Center for Metals in Biocatalysis, University of Minnesota, Minneapolis, MN 55455, USA

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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.

* Corresponding author. Fax: +1 612 624 5121.
E-mail address: lipsc001@umn.edu (J.D. Lipscomb).
[1] Present address: Department of Biochemistry, University of Mississippi Medical Center, University of Mississippi, 2500 N. State St., Jackson, MS 39216, USA.
[2] Present address: Department of Pharmacology, University of Pennsylvania, Philadelphia, PA 19104, USA.
[3] Present address: Cargill Incorporated, Biotechnology Development Center, Minneapolis, MN 55440-5702, USA.

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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.

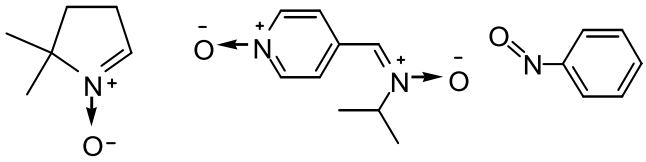
Spin-trapping experiments. Reactions were carried out in 50 mM Mops or sodium phosphate buffer, pH 7.6, containing one of the following, DMPO, POBN, or NOB. The reaction mixtures contained MMOH (0 or 50 μM), MMOR (0 or 100 μM), MMOB (0 or 100 μM), and substrate (0 to a concentration in millimolar range 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

temperature were monitored continuously over a 1 h period. Instrument parameters are reported in the figure legend for each individual scan. Spectra were simulated, and the simulation was optimized to a minimum sum of square residuals using a program developed by David Duling at NIEHS or the program SimFonia distributed by Bruker Inc.

Results

In this study, three spin-traps DMPO, POBN, and NOB, the structures of which are shown below, were used to investigate the possibility of substrate radical formation during the reactions catalyzed by MMOH from *M. trichosporium* OB3b.



Effects of spin-traps on MMO activity

Due to the broad substrate range of MMO, the effects of the spin-traps on activity were first determined. We found that DMPO and POBN have little effect on activity at low concentrations, but NOB strongly inhibits turnover of assay substrates. This is illustrated in Table 1, which shows the effects of the spin-traps on activity measured as either O_2 consumption in the presence or absence of the substrate furan (equivalent results were found by monitoring NADH consumption) or product formation using the substrate nitrobenzene. A mixture of NADH and MMOR slowly utilizes O_2 due to reduction and autooxidation of the redox cofactors present in this component. The low protein concentrations used here in assays do not cause O_2 to be utilized at a net rate that can be detected using an oxygen electrode. The spin-traps DMPO and POBN each accelerated this reaction slightly, while NOB had no effect. When all three components are present without a substrate, the O_2 utilization rate increases due to the formation of **Q** and its subsequent autodecay. High concentrations of DMPO inhibited this reaction slightly while POBN and NOB mildly accelerated the reaction. The complete system with furan as a substrate exhibited a high rate of oxygen utilization. DMPO and POBN inhibited this reaction slightly at very high concentrations. Interestingly, NOB accelerated this reaction, suggesting that it either increases the furan turnover rate or acts as a substrate itself. The results shown below support the latter possibility.

Product formation is conveniently monitored using nitrobenzene as a substrate because the product, predominantly *p*-NO_2 phenol, is chromophoric. DMPO and POBN slightly inhibited product formation from the complete system. In contrast, product formation was strongly inhibited, indeed completely eliminated, by low concentrations of NOB.

Spin-trapping experiments

Based on the results shown in Table 1, DMPO and POBN can be used as innocent spin-traps to intercept substrate radicals lost from the active site during abortive

Table 1
Effects of spin-traps on MMO activity

Spin-trap	Oxygen consumption			*p*-NO_2-phenol
	Rel. rate (N + R)[a]	Rel. rate (N + R + H + B)[b]	Rel. rate (N + R + H + B + S)[c]	Rel. rate (N + R + H + B + S)[d]
None	0	17	100	100
DMPO (mM)				
5	1	16	95	89
50	4	9	93	86
100	7	9	86	79
POBN (mM)				
10	4	23	86	95
50	10	30	81	92
100	12	35	62	84
NOB (μM)				
10	0	23	112	78
50	0	30	118	<10

[a] Relative rates of O_2 consumption by a mixture of NADH and MMOR in the absence and presence of spin-traps. Conditions are as described under Materials and methods. The error is ~10%.

[b] Relative rates of O_2 consumption by samples containing NADH, MMOR, MMOH, and MMOB in the absence and presence of spin-traps.

[c] Relative rates of O_2 consumption by samples containing NADH, MMOR, MMOH, MMOB, and 1.9 mM furan in the absence and presence of spin-traps.

[d] Relative rates of nitrophenol formation monitored by measuring the optical absorbance at 404 nm using samples containing NADH, MMOR, MMOH, MMOB, and 1.2 mM nitrobenzene in the absence and presence of spin-traps. Conditions are as described under Materials and methods. The error is less than 5%.

turnover cycles. Only the EPR spectra for the DMPO case are shown in Fig. 1, but those for the reactions with POBN present give equivalent results (data not shown). In a previous study, the complete system with methane or a variety of other substrates present was reported to yield a multiline EPR spectrum from a DMPO or POBN adduct radical with hyperfine coupling constants characteristic of the specific substrate radical produced [26,27]. Using our preparation of the *M. trichosporium* OB3b enzyme and the same experimental conditions as in the earlier study, no such DMPO or POBN adduct radical was detected (Fig. 1D). Samples made under different conditions, such as longer reaction times, different MMOH/MMOB ratios, different trap concentrations, or different buffer systems also failed to produce EPR radical signals.

A 10-line EPR spectrum from a DPMO adduct radical was observed when only MMOR and NADH were present with the spin-trap (Fig. 1B). Simulation of the spectrum yielded two species with coupling constants of $A_N = 14.5$ G, $A_H = 14.3$ G, and $A_N = 15.3$ G, $A_H = 22.0$ G, respectively. By comparison with the values of coupling constants reported in the literature [29], the first species is tentatively assigned to be the spin adduct of DMPO with hydroxyl radical (DMPO/·OH) and the second species a DMPO dimer radical (DMPO·DMPO) [38]. The presence of POBN under these conditions yields an EPR active species with coupling constants of $A_N = 15.09$ G and $A_H = 2.24$ G.

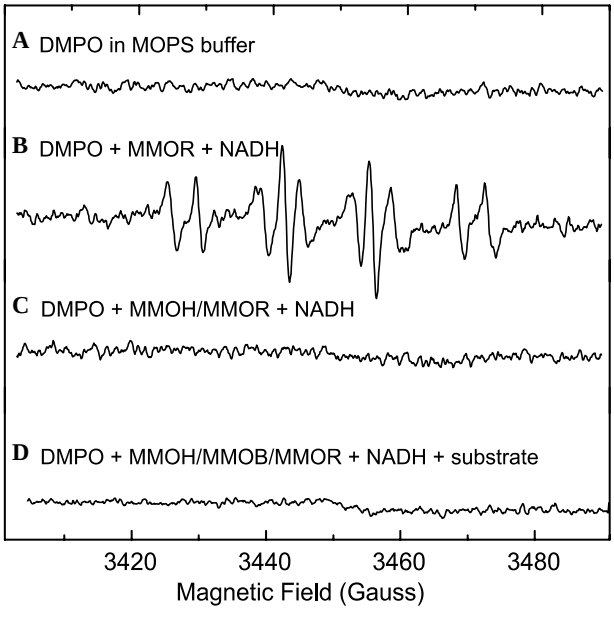


Fig. 1. EPR spectra from DMPO alone and MMO component reactions in the presence of DMPO. Reaction components are shown in the figure and the experimental conditions are described under Materials and methods. DMPO was present at approximately 10 mM. Instrument settings were: frequency, 9.770 GHz; modulation amplitude, 1 G; time constant, 0.128 s; number of scans, 5–10; power, 10 mW; ambient temperature.

No radical was observed from addition of DMPO or POBN to NADH or MMOR individually. Furthermore, addition of MMOH to the NADH and MMOR mixture, in the presence (data not shown) or absence (Fig. 1C) of MMOB, resulted in no observed signals. Thus, the presence of MMOH apparently abolishes the loss of radical species from autooxidation of MMOR.

In addition to methane, the reaction of many other substrates including ethane, propene, propane, isobutane, 2,2-dimethylpropane, chloromethane, dichloromethane, trichloromethane, 1,2-dimethylcyclopropanes, 1,1,2,2-tetramethylcyclopropane, 1,2-dimethylcyclohexane, and methylcyclopentane were also studied in the presence of DMPO and POBN spin-traps. No MMOH and substrate dependent spin adducts were detected from these experiments.

NOB as an active site probe

The failure to observed substrate radicals using DMPO or POBN as spin-traps may either mean that radicals do not form or that they do not escape the active site. DMPO and POBN are quite polar, so they are not expected to enter the hydrophobic MMOH active site, but this is not true of the nearly insoluble NOB. Accordingly, the results shown in Table 1 indicate that NOB strongly inhibits the nitrobenzene oxidation reaction. This suggests that it may compete with nitrobenzene, a relatively poor substrate, for the active site or the activated oxygen species. Fig. 2 shows that NOB behaves kinetically as a nearly pure noncompetitive inhibitor for the reaction with nitrobenzene as the substrate, exhibiting a K_i of approximately 33 μM. This result suggests that NOB can bind with high

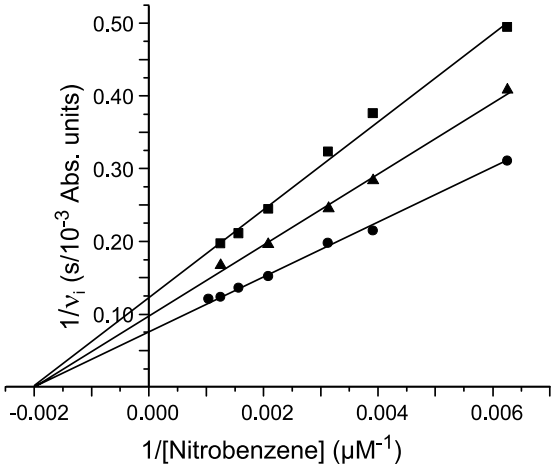


Fig. 2. Inhibition of the MMO-catalyzed oxidation of nitrobenzene by NOB. The MMO-catalyzed conversion of nitrobenzene to nitrophenol was monitored at 404 nm at pH 7.6 in 50 mM Mops buffer at 23 °C. The experiments for the $1/v_i$ vs 1/[nitrobenzene] plots shown included 0, 10, and 18.6 μM NOB, respectively, in plots of increasing slope. O_2 (250 μM) and NADH (200 μM) were supplied at saturating concentrations. The K_i value was estimated using the standard equation for noncompetitive inhibition.

affinity to the enzyme alone or together with this substrate to cause inhibition.

NOB spin-trapping

The EPR spectra from spin-trapping experiments using NOB are shown in Fig. 3. In this case, no radicals were detected from the NADH plus MMOR sample. Since it is likely that radical species were generated from this combination based on the results shown in Fig. 1, NOB is apparently less sensitive to hydroxide or superoxide radicals than the other spin-traps. The most significant difference when using NOB was the detection of a trapped radical from the complete MMO system as shown in Fig. 3D. This radical has an unusually long half-life of ca. 1 h. The coupling constants for this radical ($A_N = 10.43$ G and $A_H^{NH} = 13.15$ G, $A_H^{o,p} = 3.26,\ 3.60$ G, and $A_H^{m} = 1.0$ G) identify it as the neutral radical of NOB with a strongly coupled hydrogen bounded to the nitrogen [39]. Surprisingly, the radical was observed with or without a known substrate added.

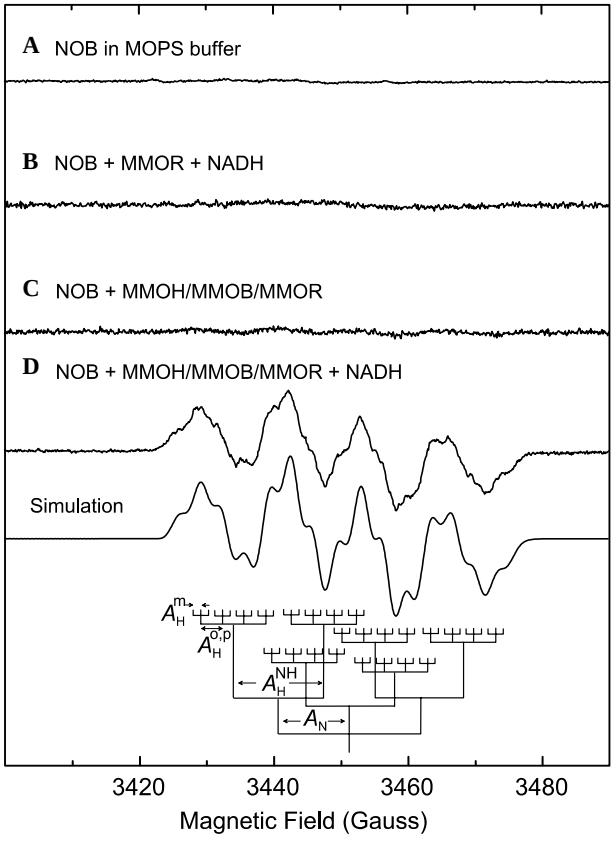


Fig. 3. EPR spectra from NOB alone and MMO component reactions in the presence of NOB. Reaction components are shown on the figure and the experimental conditions are described under Materials and methods. NOB was present at approximately 700 μM. The bottom spectrum is a simulation for the neutral NOB radical based on the hyperfine parameters listed in the text. The origin of the splitting seen in the spectrum is illustrated in the diagram. The splitting is not fully resolved in the spectrum. Instrument settings were: frequency, 9.771 GHz; modulation amplitude, 3 G; time constant, 0.40 s; number of scans, 5–10; power, 0.5 mW; ambient temperature.

Discussion

It is shown here that the reaction of the complete MMO system from *M. trichosporium* OB3b with substrates is unlikely to result in the generation of substrate radicals that diffuse out of the active site. The only diffusible radicals detected result from the known autooxidation reaction of reduced MMOR. However, the detection of a characteristic multi-line EPR spectrum when using the hydrophobic spin-trap NOB in the complete MMO system shows that radicals are generated at some point in the catalytic cycle and suggests a new strategy for their characterization. The implications of these observations in comparison to results of analogous previous studies are discussed in the following sections.

Uncoupling reaction(s) of MMOR

The nitrone-based spin-traps, DMPO and POBN, are known to react with activated oxygen species that might be produced directly or indirectly during the autooxidation reaction of MMOR, such as superoxide and hydroxyl radical, to yield EPR active species [38]. These species might be spin-trap adducts of the reactive oxygen species or simple oxidation of the radical trap. As stated above, the EPR spectrum observed here is consistent with the presence of a hydroxyl radical adduct as well as a DMPO dimer that might result from further reaction of an oxidized monomer. The scavenging of the reactive oxygen species would prevent dismutation reactions that release O_2, accounting for the apparent increase in oxygen utilization rate observed in the presence of the probes.

The suppression of radical release by the NADH and MMOR mixture in the presence of MMOH is consistent with the high affinity MMOH–MMOR complex and the rapid electron transfer and between these components [5,7]. Apparently, the transfer of the electrons to MMOH eliminates the MMOR autooxidation reaction thereby assuring tight coupling and control of potentially damaging reactive oxygen species by the intact system. Since MMOB has no effect on this reaction, its role in coupling is likely to be at the level of intermediates in the MMOH reaction cycle rather than in altering the nature of the electron transfer reaction between MMOR and MMOH, as previously hypothesized [4,40]. Substrate also has no effect on this reaction, which is in full agreement with previous findings that the electron transfer of the MMO enzyme system is not regulated by the substrate as it is in the P450 systems [41,42].

Comparison with previous results

The results presented here differ fundamentally from those reported in an earlier study [26,27]. We believe that it is unlikely that the enzyme used for most of the experiments in that study, MMO from *M. capsulatus* (Bath), differs mechanistically from that of *M. trichosporium* OB3b

used here. All other probes of the mechanism show these two enzymes to be remarkably similar despite substantial differences in the host bacteria [8,9]. While it is impossible to determine why these two studies differ, three related possibilities are: (i) The MMO used in the earlier study exhibits a much lower specific activity than the *M. trichosporium* OB3b enzyme used here, so the probability is higher for the loss of reactive oxygen species that may react with the spin-traps. (ii) In the earlier study, the formation of a substrate radical adduct of the spin-trap was established by comparison with published *A* values. These are known to differ substantially with solvent and other conditions, making such identifications difficult [38]. (iii) Substrates may act as intermediate scavengers for hydroxyl and similar radicals generated by uncoupling reactions and then react later with the spin-trap. The substrate ethanol, for example, reacts much faster with hydroxyl radical than the spin-traps employed [38,43].

NOB as a novel MMO probe

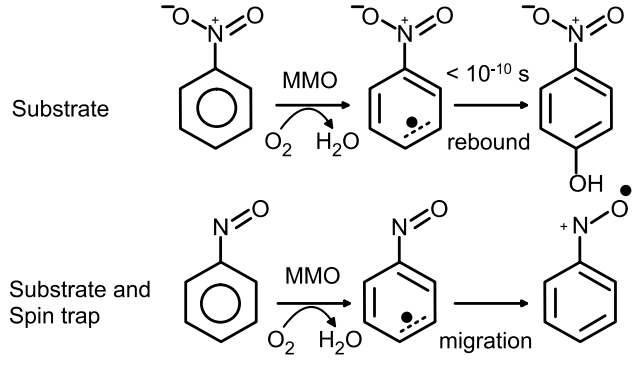
The failure to observe DMPO- or POBN-trapped radicals from substrate turnover reactions using the complete *M. trichosporium* OB3b MMO system could have many origins, the most obvious of which is that substrate radicals may not have been made. However, some of the substrates investigated here, such as ethane and 1,1,2,2-tetramethylcyclopropane, are thought to make radical intermediates based on the formation of rearranged products [14,23], yet do not produce any spin adducts. These observations make the alternative possibility that radical intermediates formed, but do not escape from the active site to contact the radical-trap more attractive. In order to examine this possibility, a radical-trap is needed that is functional at the site where the radical is made. The results reported here suggest that NOB may be such a molecule.

Three results suggest that NOB reacts in the active site to generate a radical species. First, NOB is among the strongest inhibitors known for the MMO reaction. The results show it to be a noncompetitive rather than a competitive inhibitor for the nitrobenzene oxidation reaction. This result indicates that NOB either has a special site separate from the nitrobenzene binding site where it acts as an inhibitor, or that it can bind along with nitrobenzene in the active site and compete successfully for reaction with **Q**. The structural similarity of NOB and nitrobenzene, and the lack of any previous indication of a special inhibitor site for any other molecule support the latter possibility. Also, unlike the typical enzyme, the MMOH active site appears to be simply a hydrophobic pocket without discrete binding determinants. Consequently, substrates are unlikely to occupy a single position, making observation of classical competitive inhibition unlikely. Second, the NOB radical is generated without any known substrate present, yet its formation is completely dependent on the presence of each of the MMO system components, oxygen, and NADH. This suggests that NOB can directly contact the only species in MMO likely to generate radical intermediates, compound **Q**. Third, oxygen uptake is increased when NOB is added at low concentrations in the complete system with or without furan present. Furan is a good substrate for MMO that is turned over at a rate only slightly slower than that of methane. The increase in O_2 uptake at low NOB concentrations may mean that NOB is also serving as a substrate. This may also be true when nitrobenzene is the substrate, but in that case, the only the product *p*-nitrophenol is monitored so the increased turnover due to NOB oxidation would not be detected. Together these three observations raise the intriguing possibility that one molecule of NOB can act simultaneously as the substrate and the radical-trap.

One alternative route to formation of an NOB radical is direct 1-electron reduction. However, the redox potential for reduction of NOB to the neutral radical has been reported to be −910 mV vs SHE [44] which is much lower than the redox potential of any of the MMO components. The intermediates of the MMOH reaction cycle after the diferrous state are progressively more oxidizing with increasing potentials. Consequently, it is unlikely that direct reduction of NOB is occurs.

The hypothesized ability of NOB to uniquely probe the formation of a substrate radical can be appreciated by comparing the reactions of nitrobenzene and NOB as illustrated in Scheme 1. Under our mechanistic scenario, an electron is removed from nitrobenzene by **Q** in the first step of the reaction and then the bound hydroxyl radical formed in this process rebounds to form nitrophenol [12,45]. The same first step can be proposed for the reaction with NOB, but in this case, the electron is immediately delocalized to the nitroso-moiety, greatly decreasing the driving force for the rebound reaction.

The fact that a radical is observed strongly supports our mechanistic proposal that the reaction of MMO is similar to that of P450. Both systems appear to activate substrate by an initial electron or hydrogen atom abstraction. However, it is unlikely that the observed NOB radical is that initially derived from the electron abstraction reaction, because its spectroscopic signature shows it to be the neutral rather than the cationic radical [39]. This is not



Scheme 1. Hypothesis for the role of NOB as both a substrate and a spin-trap.

surprising given the several minutes required to obtain an EPR spectrum. Nevertheless, the fact that the radical is observed for more than 1 h as opposed to the few minutes for the typical spin-trapped species may mean that the NOB radical remains at least partially sequestered. Transient kinetic techniques can now be employed to search for short-lived intermediates using this new mechanistic probe and these studies are in progress.

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