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REVIEW OF MULTIFREQUENCY EPR OF COPPER IN PARTICULATE METHANE MONOOXYGENASE

SABASTIÃNO S. LEMOS[1], HUA YUAN[2], MARY LYNNE PERILLE COLLINS[3], WILLIAM E. ANTHOLINE[2]

[1]Instituto de Quimica, Universidade de Brasilia, Brasilia-DF 70919-970 Brazil;
[2]Biophysics Research Institute, Medical College of Wisconsin, Milwaukee, WI 53226 USA;
[3]Department of Biological Sciences, University of Wisconsin-Milwaukee, Milwaukee, WI 53201 USA.

There is substantial interest in methane monooxygenase because this enzyme converts methane to a potential fuel source, methanol, detoxifies trichloroethylene, and uses a green house gas as a reactant. Particulate methane monooxygenase, pMMO, is a copper enzyme. Of interest to EPR spectroscopists is the accumulation of high concentrations of a type 2 EPR-detectable cupric signal in *Methylococcus capsulatus* (Bath), *Methylomicrobium album* BG8 & *Methylosinus trichosporium* OB3b cells. In addition to the type 2 cupric signal, a broad signal is sometimes found in *M. capsulatus* (Bath) cells. This review focuses primarily on EPR studies and current models for the copper sites in pMMO.

INTRODUCTION

Methane monooxygenases (MMOs) are enzymes in methanotrophs that convert methane to methanol (Hanson, Netrusov & Tsuji, 1991; Hanson & Hanson, 1996; Anthony, 1986).

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

Methanotrophs grow on methane as their sole source of carbon and energy. Conversion of methane to methanol could provide a means to use methane as an energy source. Detoxification of trichloroethylene and the role of methane as a greenhouse gas have generated considerable interest in MMOs. It is estimated that methane contributes about 26 times that of carbon dioxide (mole for mole) to climate change (Hanson & Hanson, 1996). In addition, the understanding of methane hydroxylation by pMMO may shed some light on the C-H activation, a priority issue both in basic chemistry and in technology.

Two forms of MMO exist, soluble (sMMO) and particulate (pMMO) methane monooxygenase. The active center of sMMO has an oxo-bridged dinuclear iron center, is limited to some strains and is made under conditions where copper is limited (Prior & Dalton, 1985). In contrast, pMMO is present in all known methanotrophic bacteria with one possible exception (Dedysh, Liesack, Khmelenina, Suzina, Trotsenko, Semrau, Bares, Panikov & Tiedje, 2000). It is generally thought that pMMO is a copper enzyme, but a few have proposed Cu-Fe as the active site (Zahn & DiSpirito, 1996; Tukhvatullin, Gvozdev & Andersson, 2000). The pMMO from *Methylococcus capsulatus* (Bath) consists of three subunits of 47, 27 and 25 kDa. The genes encoding pMMO have been sequenced from several methanotrophs. It is also informative to interpret this sequence data in the context of the sequences available for genes encoding the related enzyme ammonia monooxygenase (AMO). To date no crystal structure of pMMO is available. Spectroscopic techniques are used to gain insight into the structure and mechanism of action of pMMO. An understanding of pMMO structure, developed over the last five years primarily from EPR studies, follows.

Stoichiometry of copper bound to pMMO

Early models of pMMO structure suggested 15-21 copper atoms arranged in E-clusters for the electron transfer sites and C-clusters for the catalytic sites and grouped into seven trinuclear copper clusters (Chan, Nguyen, Shiemke & Lidstrom, 1993; Nguyen, Nakagawa, Hedman, Elliott, Lidstrom, Hodgson & Chan, 1996). A major argument against this scheme is that there are not any repeating sequences to support the large number of E- and C-clusters. It may be that the copper bound to small molecules with high affinity binding inflated the number of coppers estimated to be bound to the three pMMO subunits of 47, 27 and 25 kDa. Others have reported EPR signals, some with EPR parameters consistent with mostly oxygen donor

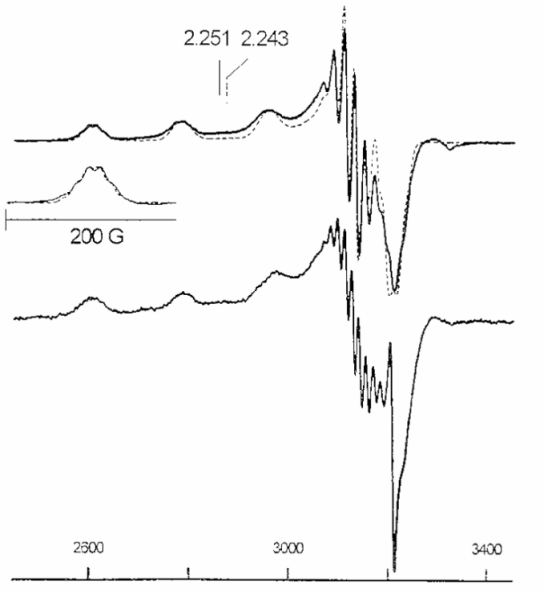

Fig. 1. X-band EPR spectra (upper trace) of *M. album* BG8 cells grown with ^{15}N and $63Cu^{2+}$. The upper spectrum is the average of four scans. The insert is the expansion of the -3/2 line in the $g_{\|}$ region after 15 scans. The dotted lines are simulations. Spectrometer conditions: microwave frequency, 9.089 GHz; modulation amplitude, 5G; microwave power, 5 mW; temperature, 77 K. Simulated spectra are the sum of two spectra, A and B. Simulation parameters are listed in Table 1. [This Table was not attached to the article]. Spectrum B: sample parameters as used for spectrum A except g_z = 2.251. X-band spectrum (lower trace) of M. Album BG8 cells grown with ^{14}N and $^{63}Cu^{2+}$. (From Yuan et al., 1999, with permission.)

atoms, from low molecular weight complexes (DiSpirito, Zhan, Graham, Kim, Larive, Derrick, Cox & Taylor, 1998), but in our studies with *M. album* BG8, we have not observed these signals.

Using membrane fractions, we estimated this molar ratio (copper: pMMO) is about four, of which one is EPR detectable (Yuan, Collins & Antholine, 1998). In our opinion, the concentration of the type 2 signal is a good measure of the concentration of pMMO under conditions where the Cu^{2+} type 2 binding sites of pMMO are saturated. The x-ray absorption and EPR-detectable concentration of Cu^{2+} in membrane fractions was 20 to 30% of the total copper (Nguyen *et al.*, 1996). After exposure to oxygen, 50% of the sample was Cu^{2+} and 50% was Cu^{+}.

In summary, edge analysis suggests that $70 \pm 10\%$ of the total copper exists as Cu^{+} in the preparations as isolated. Using both x-ray absorption and EPR data, it is concluded that there exists one EPR detectable type 2 Cu^{2+} site and two or three Cu^{+} sites in pMMO.

In a recent study, it was found that pMMO from *M. capsulatus* (Bath) contains three subunits of molecular masses 29, 45 and 31 kDa in a stoichiometry of $\alpha_2\beta_2\gamma_2$ containing up to six copper atoms per 200 kDa enzyme (Lieberman, Doan, Hoffman & Rosenzweig, 2001). This agrees with the two or three Cu^{+} per 100 kDa not detected by EPR in our work with membrane fractions (Yuan *et al.*, 1998), but raises questions about the EPR-detectable site. Specifically, is the type 2 EPR-detectable site attributable to pMMO? If not, how can these cells acquire such a large concentration of type 2 EPR-detectable sites and what is the origin and function of the EPR detectable Cu^{2+} site?

EPR of detectable Cu^{2+} in pMMO

Until recently, most investigators agreed that the enormous EPR signal detected in cells and membrane fractions is Cu^{2+} bound to pMMO. The most compelling evidence is that pMMO is the only component in these cells and in membrane fractions expressed at such a high concentration. In our studies of cells and membrane fractions, the EPR-detectable concentration was as high as 500 μM (Fig. 1, Yuan, Collins & Antholine, 1999). This means that total copper is 2 mM. The room temperature EPR signal has $g_{\|}$ and $A_{\|}$ features that are not averaged, indicating that the signal is not from a low molecular weight cupric complex (unpublished data). In further support of binding of copper to pMMO, only the bands on gels attributed to pMMO are concentrated enough to bind 2 mM copper. Finally, an effective purification is described in the work of Okura and collaborators using *Methylosinus trichosporium* OB3b, and they detect the type 2 copper signal in purified pMMO (Kamachi, Miyai & Okura, 2001).

The best resolved EPR signal with fewer additional minor (less intense) signals is the type 2 Cu^{2+} signal from *M. album* BG8 cells (Fig. 1). Normally the signal would be attributed to a single cupric site (Yuan, Collins & Antholine, 1997), but enhanced resolution of the EPR signal from cells grown on ^{15}N-potassium nitrate and ^{63}Cu gave resolved lines that were shown to arise from two very similar signals. Expansion of the $M_I = -3/2$ line at X-band gave an even line pattern (Fig. 1). At low frequency (3.45 GHz) the patterns from the two sites collapsed to give a five-line pattern on the $M_I = -1/2$ line of the g_{II} region attributed to four approximately equivalent nitrogen donor atoms (Yuan *et al.*, 1999). Thus, the X-band spectrum is a superposition of two spectra, each from a cupric ion bound to four approximately equivalent nitrogen donor atoms. The biggest change in EPR

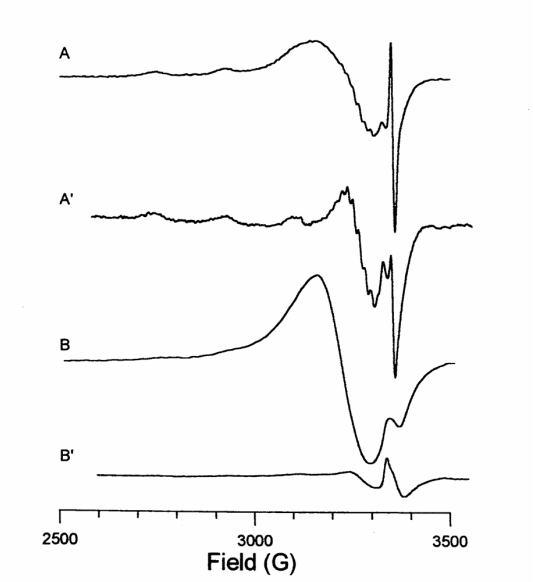

Fig. 2. X-band EPR spectra of whole cells. (A) Temperature 12 K, microwave power 0.1 mW, *M. capsulatus* (Bath). (A′) Temperature 9 K, microwave power 0.2 mW, *M. album* BG8. (B) Temperature 12 K, microwave power 200 mW, *M. capsulatus* (Bath). (B′) Temperature 9 K, microwave power 200 mW, *M. album* BG8.

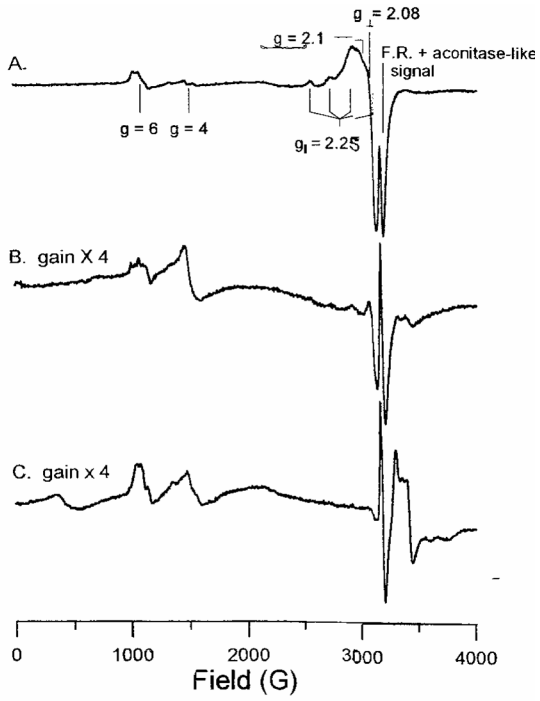

Fig. 3. X-band EPR spectra of whole cells from *M. capsulatus* (Bath) and *M. album* BG8 at 12 K. (A) [Cu^{2+}] 5 mM, (Bath); (B) [Cu^{2+}] 5 mM (BG8); (C) no Cu^{2+} added, *M. capsulatus* (Bath).

parameters in the two very similar spectra is a difference of 0.008 in $g_{\parallel}$. This is often attributed to a difference in axial ligation, for which the ratio of the signals might change in different solvents. But, there are always approximately equal amounts of the two signals in our samples, which might be more consistent with two configurations like *cis* and *trans*. While these signals are not as well resolved in *M. capsulatus* (Bath), given the signals in *M. album* BG8 as a template, both signals are also apparent in *M. capsulatus* (Bath) (Lemos, Collins, Eaton, Eaton & Antholine, 2000). Not only are there four donor atoms forming a Cu^{2+} complex, three or four of these donor atoms are from imidazole nitrogens from histidines. The evidence for more than two bound histidines is due to the substantial intensity of quadrupolar combination frequencies from samples of cells from both *M. album* BG8 and *M. capsulatus* (Bath) (Lemos *et al.*, 2000).

Another reason to examine the EPR signals from *M. capsulatus* (Bath) is that a broad EPR signal was observed in these cells (Nguyen, Shiemke, Jacobs, Hales, Lidstrom & Chan, 1994), but has not as yet been observed in *M. album* BG8 cells. X-band spectra of *M. capsulatus* (Bath) cells were obtained at 77 K as the concentration of cupric ion added to the growth medium varied from 0 μM to 20 μM (unpublished results). In addition to the type 2 Cu^{2+} signals, a broad signal at $g = 2.1$ is observed. This broad signal is emphasized at lower temperatures (Fig. 2B), because the broad signal does not saturate as readily as the type 2 Cu^{2+} signal. In addition to the broad signal at $g = 2.1$, there is a signal at $g = 2.00$ attributed to a free radical (Fig. 2A) and a signal at $g = 2.01$ attributed to an iron cluster (Fig. 2B). The signal at $g = 2.01$ is more readily observed in spectra from *M. album* BG8 because the $g = 2.1$ signal is absent from this preparation (Fig. 2B). Upon expansion of the magnetic field range from 1000 G to 4000 G, signals at $g = 6$, most likely signals from hemes and signals at $g = 4$, most likely non-heme iron signals, are also detected in cells from both *M. capsulatus* (Bath) and *M. album* BG8 that had Cu^{2+} added to the growth medium (Fig. 3A and 3B). If Cu^{2+} is not added to the growth medium for *M. capsulatus* (Bath) cells, few type 2 Cu^{2+} EPR signals are obtained, but signals at $g = 15$ and at $g = 1.94$, 1.86 and 1.75 are obtained and tentatively assigned to the μ-oxo-bridged binuclear iron cluster in sMMO (Fox, Jurerus, Münck & Lipscomb, 1998) (Fig. 3C). The absence of signals from sMMO in cells for which Cu^{2+} is added to the growth medium is a good indication that predominantly pMMO and little sMMO is present when Cu^{2+} is added to the medium. In summary, all the EPR signals in cells have been tentatively identified except the broad $g = 2.1$ signal.

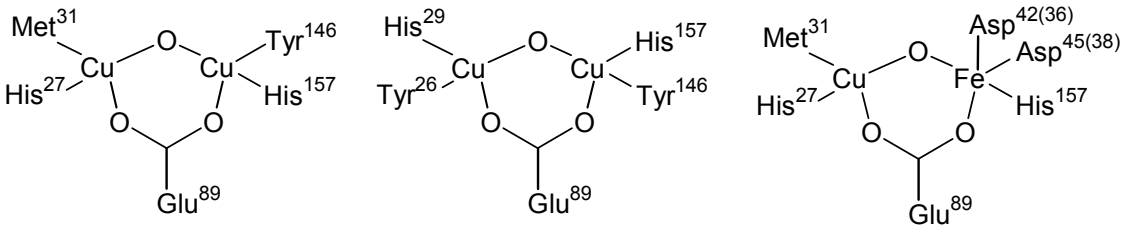

Fig. 4. Three of the derived structures using copper from the 27 kDa subunit of the active center for pMMO (adapted from Tukhvatullin *et al.*, 2000).

Two pieces of data support the hypothesis that the broad $g = 2.1$ signal is a pMMO signal. First, the broad $g = 2.1$ signal increases as the Cu^{2+} concentration in the medium increases (unpublished data). It is known that pMMO activity increases as the Cu^{2+} concentration in the medium increases and pMMO is the dominant protein in these cells, so the best candidate for the broad $g = 2.1$ signal is pMMO. The concentration of spins from type 2 Cu^{2+} and the broad signal is 600 μM, of which 500 μM is from type 2 Cu^{2+} and 100 μM is from the broad $g = 2.1$ signal. Again, pMMO is the only protein that matches these high concentrations. Second, the broad signal, as well as the signals for type 2 Cu^{2+}, is found in the membrane fraction, where the dominant protein is pMMO.

Assuming that the origin of this broad $g = 2.1$ signal is pMMO, the signal most likely arises from a multinuclear site. The signal is difficult to saturate at 12 K (Fig. 2), an indication that it arises from a multinuclear site. The broad $g = 2.1$ signal disappears over time after addition of hypochlorite (unpublished results). This suggests that the site for the broad signal arises from a site with multiple spins, or the signal arises from a mixed valence multinuclear site, like, for example, the dinuclear Cu_A site in cytochrome *c* oxidase or even a trinuclear copper site (see models, discussion following).

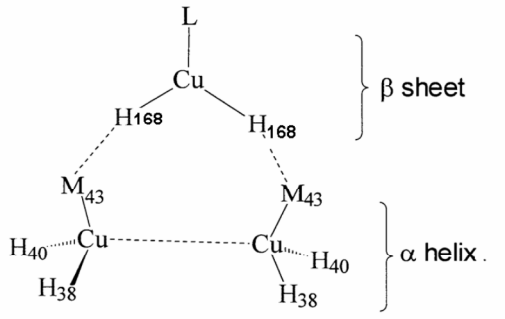

Fig. 5. Proposed model for one of two possible symmetrical active sites in pMMO invoking β2, i.e., two 27 kDa subunits.

Structures for the copper sites

Normally a Cu^{2+} site with three or four nitrogen donor atoms from imidazoles from histidine would allow one to suggest the binding site for type 2 Cu^{2+} from the amino acid sequence, but the site for type 2 Cu^{2+} in pMMO has been elusive. We used a synthetic fragment of the first 20 amino acids from the N-terminal and added Cu^{2+} to model the site in pMMO (Yuan, *et al.*, 1999). EPR parameters were similar to EPR parameters for Cu^{2+}-pMMO when the ratio of fragment to Cu^{2+} was 2:1. Cu^{2+} bound two fragments, presumably using the N-terminal NH_2 and a nitrogen donor atom from imidazole of the N-terminal histidine from each fragment. The N-terminal nitrogen from the amine could be involved if nitrogen donor atoms from three histidines are bound to Cu^{2+} and the N-terminal amine is excluded if nitrogen donor atoms from four histidines bind Cu^{2+}.

Assuming the two resolved signals for type 2 Cu^{2+} are small variations of a single site, all three or four histidines originate from one subunit and all histidines involved in binding Cu^{2+} are conserved or semi-conserved, the following is argued. The 27 kDa subunit of pMMO of *M. capsulatus* (Bath) has three histidines, His 38, His 40 & His 168, that are conserved in both pMMO (GenBank #U94337, U31650, U81596) and AMO (GenBank #96611, AF047705, AF153344). This subunit is thought to be the site of the active center because it covalently binds the suicide substrate acetylene (Prior & Dalton, 1985; Zahn & DiSpirito, 1996; Tukhvatullin *et al.*, 2000). Most likely the active site involves Cu^{+} ions, which are likely to bind to one or more histidines, as discussed in a following paragraph. This would not leave the three or four histidines free to bind to type 2 Cu^{2+}.

There are seven histidines in the 47 kDa subunit of *M. capsulatus* (Bath) pMMO. Of these seven, four are conserved in both pMMO and AMO. Four of the five histidines in the *M. capsulatus* (Bath) 25 kDa subunit are conserved. Typical patterns for strong binding of Cu^{2+} such as …HXXH… are absent from pMMO. Copper-containing amine oxidases bind Cu^{2+} using …HXH… plus a third

histidine and two water molecules in a square pyramidal arrangement (Wilce, Dooley, Freeman, Guss, Marsunami, McIntire, Ruggiero, Tanizawa & Yamaguchi, 1997). There is a …HXH… sequence in both the 47 kDa and 27 kDa subunits. Both sites would need to be completed with two additional nitrogen donor atoms, with one or two of these nitrogens from imidazole. It is hypothesized that two His help form the active site and are not involved in the EPR detectable site (Tukhvatullin *et al.*, 2000). A second hypothesis is that the type 2 Cu^{2+} binds to the 47 kDa site and utilizes the …HXH… and possibly the N-terminal His and the terminal amine or two of the remaining histidines to form the binding site for type 2 Cu^{2+}. If two or more subunits of pMMO supply histidines to form a cluster, many more possibilities for the arrangement of histidines are possible. For now, it is assumed that the copper is bound to two sites, one a structural or possibly an electron transfer site in the 47 kDa subunit & the other the catalytic site in the 27 kDa subunit.

Computer analysis was used to predict the structure of the second site (Tukhvatullin *et al.*, 2000). It was assumed that the active center is formed by conserved residues of only one peptide, the 27 kDa subunit and the only potential ligands of the metal ions from the 27 kDa peptide active center are the conserved residues H, M, C, Y, E and D. Computer analysis of the amino acid sequence predicted a secondary structure with seven transmembrane helices. A model is proposed in which four helices contribute to the binding of the active site and the C-D loop blocks access to the active site from one side. Using most of the conserved amino acid residues, several structures are proposed, of which a dinuclear center is preferable to catalyse the reaction of oxygen to methane. One advantage of this model for the catalytic site is that the site is similar to the μ-oxo-bridged dinuclear iron site in sMMO. We prefer the Cu-Cu site to explain the EPR-silent copper and the broad g = 2.1 signal (Fig. 4). Whether the catalytic site is dinuclear or trinuclear is not proven and certainly the presence of iron in pMMO is controversial (Nguyen, Elliott, Yip & Chan, 1998). Clearly there are novel sites in pMMO for both the type 2 EPR-detectable site and the broad g = 2.1 signal. These sites should be observed in purified pMMO and subunits of pMMO to verify the hypotheses, but, since purification has been difficult to date, work on cells, membrane fractions and computer-derived structures have been provocative.

Hypothetical structure for the active site of pMMO

On a strictly inorganic chemical basis by analogy to Cu^{+} complexes, it is proposed that the active site is similar to the one depicted in Fig. 5. This site is composed of two β subunits, i.e. two 27 kDa subunits, consistent with the inhibitor acetylene binding to the 27 kDa active site. Two β subunits could bind six Cu^{+} ions at two equivalent sites. It must be verified whether a 54 kDa band on SDS-PAGE arises from the β_2 dimer. This hypothetical site would be expected to be fully reduced in agreement with the three Cu^{+} per 100 kDa.

Regulation of MMO

It has been suggested that a unique metabolic switch mediated by copper ions occurs to regulate pMMO and sMMO (Murrell, Gilbert & McDonald, 2000). It is proposed that copper ions irreversibly inhibit the activity of sMMO by inactivating the reductase compound (Nielson, Gerdes, Degn & Murrell, 1996). Additionally, transcriptional regulation occurs. A regulatory protein is proposed to bind copper, resulting in a conformational change. As a result, it directly or indirectly induces transcription of genes encoding pMMO and represses transcription of those encoding sMMO. Spectroscopy should be a useful tool to gain understanding of the structure and molecular mechanism of pMMO, as well as regulatory events.

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