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Redox Potentiometry Studies of Particulate Methane Monooxygenase: Support for a Trinuclear Copper Cluster Active Site**

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Particulate methane monooxygenase (pMMO) is a complex membrane protein ($\alpha\beta\gamma$) that—in our hands (see below)—contains approximately 15 copper ions,[1,2] sequestered into catalytic trinuclear copper clusters (C clusters) and electron-transfer copper ions known as E clusters.[3,4] We have proposed that the C clusters mediate alkane hydroxylation and dioxygen reduction chemistry, while the E clusters provide a buffer of reducing equivalents to rapidly re-reduce the C cluster copper ions during turnover. Herein, we provide additional support for our hypothesis of a trinuclear copper cluster as the hydroxylation site of pMMO.[4]

The EPR spectrum for the as-isolated pMMO in pMMO-enriched membranes typically consists of a superposition of signals: a classical type 2 Cu^{II} signal at $g_{av} \approx 2.12$, with Cu hyperfine structure in the parallel region ($g_{\|} = 2.24$) and ^{14}N superhyperfine structure in the perpendicular region ($g_{\perp} = 2.059$), and an almost featureless isotropic signal centered at $g \approx 2.1$ that we have assigned to a trinuclear $Cu^{II}Cu^{II}Cu^{II}$ cluster.[4,5] Unlike the type 2 Cu^{II} signal, the signal of the cluster does not saturate at high microwave power.[5]

The number and type of metals in pMMO remains an area of intense controversy.[4,6–10] Others have implicated one or two iron atoms,[6–10] while the X-ray crystal structure of pMMO from *Methylococcus capsulatus* (Bath) reveals a $(\alpha\beta\gamma)_3$ trimer, with only one mononuclear copper center, one dinuclear copper cluster, and one zinc ion per $\alpha\beta\gamma$ monomer.[12] No trinuclear copper cluster is observed. We have speculated, however, that as the enzyme preparation on which the X-ray crystal structure is based does not display activity, it may lack the full complement of copper ions to be functional.

In principle, the debate over the existence of a trinuclear copper cluster could be settled by redox potentiometry. The copper sites in pMMO would be predicted to have distinct redox potentials, and thus by reducing the protein at different cell potentials the spectroscopic features for each site could be separated and individually assigned. With this goal in mind, EPR data for pMMO-enriched membranes (80–90% pMMO) have been obtained after equilibration in an electrochemical cell at fixed potentials.

Potentiometric titrations of the copper centers were performed by poising the electrochemical cell at +120 mV and adding small amounts of sodium dithionite to reduce the enzyme gradually in the presence of mediators. The E cluster copper ions remained reduced, as manifested by the appearance of only the signals for the C clusters in the EPR spectrum. The redox potential(s) of the E cluster copper ions are significantly higher than +120 mV. Reduction of the copper ions of the C clusters occurred at much lower potentials. The type 2 Cu^{II} EPR signal decreased gradually in intensity with increasingly more negative potentials beginning at +18.3 mV. At a cell potential of −121.3 mV, the spectrum eventually gave way to an isotropic signal centered at $g = 2.05$ (Figure 1).

The intensity of the signal at +120 mV corresponded to approximately two copper ions per protein monomer, as expected for contributions from one type 2 Cu^{II} site and one trinuclear $Cu^{II}Cu^{II}Cu^{II}$ cluster.[4,5,13] Simulation of the EPR signal at 4K of the C cluster copper ions of the pMMO samples poised at varying cell potentials yielded the following results: 1) at +18.5 mV, the measured EPR intensity corresponded to 1.40 Cu^{II} ions per protein, consisting of the superposition of signals from 0.99 type 2 Cu^{II} ions and 0.42 trinuclear $Cu^{II}Cu^{II}Cu^{II}$ clusters; 2) at −53.0 mV, the measured EPR intensity corresponded to 0.84 Cu^{II} ions per protein, consisting of the superposition of signals from 0.56 type 2 Cu^{II} ions and 0.29 trinuclear $Cu^{II}Cu^{II}Cu^{II}$ clusters; and 3) at −121.3 mV, only the trinuclear $Cu^{II}Cu^{II}Cu^{II}$ signal was evident, and the EPR intensity corresponded to 0.13 trinuclear $Cu^{II}Cu^{II}Cu^{II}$ clusters.

The EPR data at −121.3 mV confirms the ability of redox potentiometry to isolate the isotropic $g = 2.05$ signal from the composite spectrum of the oxidized C cluster copper ions. This result provides a straightforward, if not direct, verification of our earlier assignment of the EPR spectrum of as-isolated pMMO.[4,5] An essentially identical EPR spectrum has been recorded for a model ferromagnetically coupled

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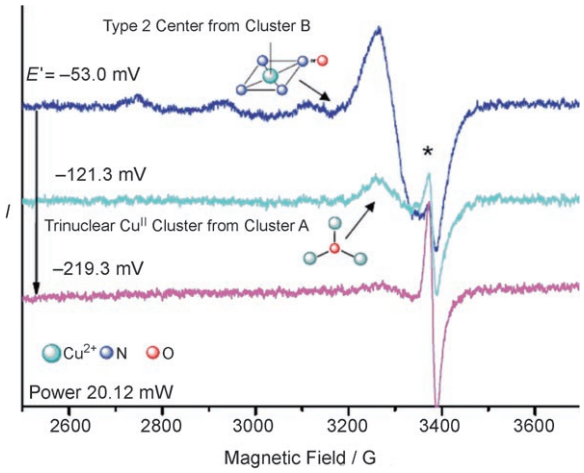

Figure 1. 4K EPR spectrum of the copper centers of pMMO samples poised at varying cell potentials by varying the amounts of sodium dithionite in an anaerobic electrochemical cell in the presence of redox mediators at room temperature. Potentials were measured relative to the standard hydrogen electrode (SHE). Asterisks in the spectra at $g \approx 2.002$ denote signals originating from free radicals associated with the dithionite and the redox mediators.

trinuclear $Cu^{II}Cu^{II}Cu^{II}$ cluster that we have synthesized recently (see the Supporting Information).[14]

While the results presented here provide compelling support for our previous proposal of a trinuclear $Cu^{II}Cu^{II}Cu^{II}$ cluster in pMMO, it remains for us to reconcile the biophysical/biochemistry observations compiled here and elsewhere[2–5] with the crystal structure recently reported by Lieberman and Rosenzweig,[11,12] for which no such clusters were observed. As their protein was essentially inactive, the possibility that some copper ions were lost during the sample preparation immediately suggested itself. To assess this possibility, we repeated their purification procedures[8,11] and indeed observed a loss of about 12 copper ions and concomitant decrease in hydroxylation activity.

If, as suggested by our experiments, copper ions forming the hydroxylation site were lost prior to structure determination, the question at this juncture would be whether or not we could locate potential sites in the crystal structure that could accommodate the trinuclear copper cluster identified in our studies. While the location of such a site would be challenging, specific criteria could be invoked to facilitate this search. Perhaps the most important of these criteria would be the need to have the requisite ligands (acidic residues, methionines, and histidines) necessary to promote binding of the copper atoms of the cluster in a localized area. Another criterion was the possibility that if a putative alkane hydroxylation site existed, there might be a hydrophobic channel leading up to it in order to facilitate binding of the methane substrate molecules. Finally, perhaps, one would also expect this active site to be in the transmembrane hydrophobic domain.

The application of these criteria to search for a putative alkane hydroxylation site yielded one promising lead. This putative site (D site), shown in Figure 2, is bound by potential ligating residues His38, Met42, Met45, Asp47, Trp48, Asp49,

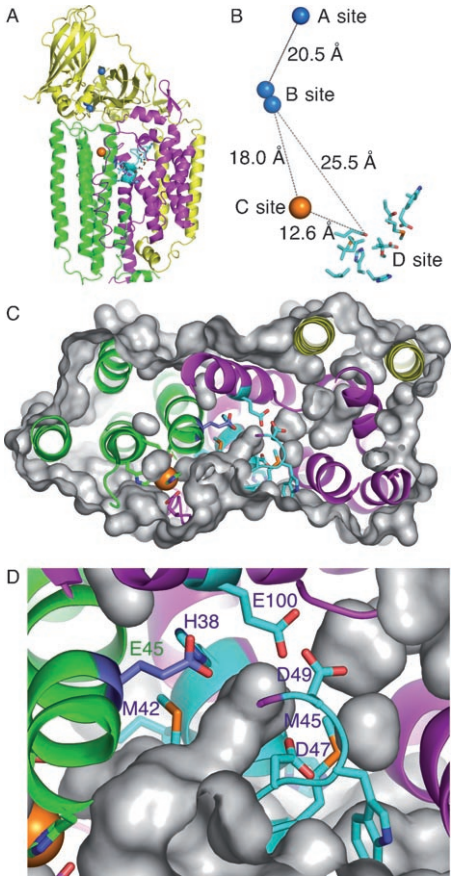

Figure 2. Proposed location of a trinuclear cluster within the framework of the pMMO crystal structure.[11,12] A) Ribbon diagram with PmoA, PmoB, and PmoC subunits colored in magenta, yellow, and green, respectively, with exception of the proposed trinuclear binding-site D site residues which are colored in cyan (from PmoA) and light blue (from PmoC). The side-chain atoms of the putative metal-binding residues are shown in stick representation with the carbon atoms colored as in the ribbon, and the remaining atoms are shown in the Corey–Pauling–Kultin (CPK) color scheme. Functionally relevant copper and zinc ions are shown as blue and orange spheres, respectively. B) Distances between the functionally relevant clusters of pMMO and the proposed D site. C) Ribbon and surface diagram showing a slice through the pMMO structure that includes the zinc C site and putative trinuclear copper-binding site (D site). The surface diagram not only reveals a cavity for binding the proposed trinuclear cluster but also a channel leading through the PmoA subunit that could accommodate a methane substrate. D) Close-up view of the putative D site with potential metal-binding residues labeled. Molecular graphics were produced with the assistance of the PyMOL molecular graphics and modeling package.

and Glu100 from subunit A (PmoA) and residue Glu154 from subunit C (PmoC) in the crystal structure. Lieberman and Rosenzweig[12] briefly noted this "cluster of hydrophilic residues" but discounted its role as a metal site, favoring instead a role in intersubunit stabilization. Given the constraints from the biochemical/biophysical data, and our own analysis, however, we computationally modeled a trinuclear $Cu^{II}Cu^{II}Cu^{II}$ cluster into the site taking into account favorable side-chain rotomers, potential hydrogen-bonding interactions, and the metal–ligand bond and angle geometries. The model minimization with no experimental energy terms routine of

the CNS (Crystallography and NMR System) program[15] was then used to optimize the geometry of the residues and metal ions of the modeled trinuclear copper site (Figure 3). The coordinated ligands and the geometry of the cluster, including

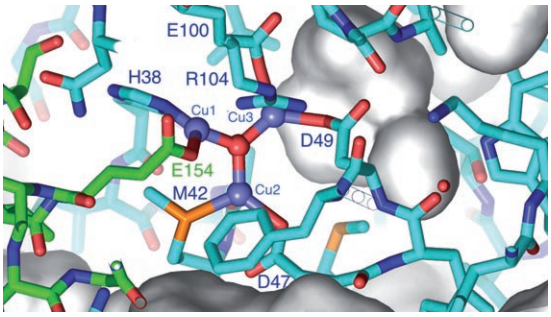

Figure 3. Trinuclear copper site modeled into the putative cavity. The ligands to the copper atoms in this model are as follows: PmoC Glu154 and PmoA His38 for Cu1; PmoA Met42 and Asp47 for Cu2; and PmoA Asp49 and Glu100 for Cu3. The carbonyl group of PmoA Ala41 refines to a position where it can also bind Cu2. The carbon atoms of the PmoA subunit are colored in cyan, and those of the PmoC subunit are shown in green. The remaining atoms are colored in CPK. As in Figure 2, the putative methane-binding channel lies to the upper right.

the Cu–Cu and Cu–O distances, are all reasonable and demonstrate the feasibility of the pMMO protein to accommodate a trinuclear copper cluster. The trinuclear $Cu^{II}Cu^{II}Cu^{II}$ structure modeled here corresponds to that of the fully oxidized cluster after turnover by dioxygen in the absence of alkane.[4]

The combination of 1) our new spectroscopic data, which confirm the existence of a trinuclear copper site, 2) our ability to computationally model a trinuclear copper cluster into a cavity within the pMMO crystal structure site, and 3) our model compound studies which demonstrate that a trinuclear copper cluster can mediate facile oxo-transfer activity[14] (see the Supporting Information) provides strong support for the existence of a trinuclear copper cluster as the site of hydroxylation within the pMMO protein.

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