Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

69451 Weinheim, Germany

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

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Experimental Section

pMMO-enriched membranes - pMMO-enriched membranes were derived from *Methylococcus capsulatus (Bath)* cells cultured under high copper concentrations (up to 40 μM) in a Bioflo 3000 fermentor adapted with a hollow-fiber membrane bioreactor as previously described [1]. Metal analysis of the membranes by ICP-AES revealed a copper content of ~12 copper ions per 100 kDa of total membrane proteins, or ~14 coppers per pMMO. The Fe/Cu ratio was < 0.1, and there was no evidence of Zn ion. The purified pMMO in detergent micelles also exhibited similar metal content. EPR of the "as-isolated" pMMO in the enriched membranes were

identical to that of the purified protein in dodecyl-β-D maltoside micelles [1,4]. To obtain the purified protein, the pMMO-enriched membranes were solubilized in dodecyl-β-D maltoside, followed by a single-step of S-300 size exclusion chromatography using FPLC.

The activity of the purified pMMO-enriched membrane fragments was *ca*. 90 nmoles/min/mg of protein at 45˚C, as assayed by the propene epoxidation method. However, the specific activity of the purified enzyme in dodecyl-β-D maltoside micelles was reduced by a factor of 3–4. The difference in specific activity of pMMO between whole cells, purified pMMO-enriched membranes, and the purified pMMO in detergent is related to the level of hemerythrin associated with the pMMO complex. When pMMO is over-produced in *Methylococcus capsulatus* (Bath), the bacterium also over-produces a hemerythrin [W.-C. Kao, Y.-R. Chen, E.-C.Yi, H. Lee, Q. Tian, K.-M. Wu, S.-F. Tsai, S. S.-F.Yu, R. Aebersold, S. I. Chan, *J. Biol. Chem*. **2004.** *279*, 51554-51560]. This bacterial hemerythrin acts as a carrier of dioxygen to ensure an adequate level of dioxygen in the membranes in order to optimize the activation of the enzyme at the high concentration produced. The pMMO activity is very high in whole cells. As the hemerythrin is removed during detergent solubilization of the membrane fragments, the dioxygen activation of the pMMO ultimately becomes limited by the accessibility of dioxygen and the enzyme activity is reduced to a basal level determined by the solubility and diffusion of dioxygen in the membrane fragment or detergent micelle.

Repeat of the protein purification protocol developed by Lieberman et al. – We have repeated the protein purification protocol developed by Lieberman *et al.* [8,11] to obtain the protein sample used in their crystallographic x-ray analysis. The results obtained are summarized in Table 1, where we have compared the specific activity and the metal contents of the pMMO obtained at various stages of the purification procedures. The method of Lieberman *et al*. started with solubilization of the purified membranes in dodecyl-β-D maltoside, which was followed by (1) $(NH_4)_2SO_4$ precipitation, (2) detergent re-solubilization, and (3) a final step of anion exchange chromatography. From Table 1, we see that, in our hands, copper ions were removed subsequent to the $(NH_4)_2SO_4$ precipitation with concomitant loss of enzymatic activity. The final preparation contained 2.5 copper ions per protein and was inactive, as reported by Lieberman and Rosenzweig [8,11]

Redox potentiometry - The electrochemical cell used in the redox potentiometry was constructed according to the design of Dutton [P. L. Dutton, *Methods in Enzymology* **1987**, *54*, 411-435]. Gold electrodes (2 mm in diameter) from CH Instruments were deployed as the working electrodes. The Ag/AgCl electrode was used as a reference. Cell potentials were monitored by a Suntex SP-2000 pH meter.

Electron paramagnetic resonance - EPR spectra of the various samples were recorded at X-band at 9.6 GHz; modulation frequency 100 kHz; and modulation amplitude 5 Gauss) on a Bruker E580 spectrometer equipped with a Bruker dual-mode ER 4116DM cavity. The sample temperature was maintained at 4K or 6K by using an Oxford Instruments continuous liquid-helium cryostat equipped with a turbo pump to lower the vapor pressure of the liquid helium. A capillary containing 0.5 mM $CuCl_2$ in 1:1 v/v pure water/glycerol was employed as a standard sample for spin counting. EPR spectra of the background were also recorded and subtracted from the sample spectra prior to double integration to determine the Cu(II) concentration in the sample. When the Cu(II) signals monitored were weak due to low concentrations, an EPR spectrum of the membranes were also recorded after complete reduction with dithionite to correct for possible interference from low levels of background signals arising from the membranes. Simulations of the EPR spectra were carried out by using the WinEPR Simfonia and Easyspin programs [Stoll, S., Schweiger, A. E. (2006) *J. Magn. Reson.* **178,** 42–55] in the Matlab package.

Preparation of a biomimetic model trinuclear copper cluster - We have prepared a biomimetic model trinuclear copper cluster using the ligand 3,3'-(1,4-diazepane-1,4-diyl)bis(1-((2-(diethylamino)ethyl)(ethyl) amino)propan-2-ol (Figure 4 A). This ligand is capable of trapping three Cu(II) ions, yielding the trinuclear $Cu^{II}Cu^{II}Cu^{II}$ cluster, whose crystal structure and 4K EPR are shown in Figure 4 B and C, respectively. When the trinuclear $Cu^{I}Cu^{I}Cu^{I}$ cluster is activated by dioxygen, the complex mediates facile oxo-transfer chemistry.

Methods

Trinuclear cluster model design and optimization - Once the putative location of the hydroxylation site was identified, attempts were made to model a trinuclear cluster into the proposed cavity using the available residues. The first step was a sequence analysis to examine

the conservation of the putative ligand binding residues. This process was informative as Met45 could be ruled out as a ligand given its low sequence conservation. The cluster was then manually fit into the active site using the remaining residues (His38, Met42, Asp47, Trp48, Asp49, and Glu100 from PmoA, and Glu154 from PmoC) with potential to serve as copper binding ligands. The histidine and its relatively constrained geometry and planarity could be used to fix one copper, while the remaining coppers were fit based on the geometric constraints of the remaining carboxylate and methionine ligands. During this model building step, attempts were made to reduce major side chain conformational changes and to take into account potential hydrogen bonding interactions. The final model was obtained following use of the model minimization (without experimental energy terms) routine of CNS to optimize the geometry of the copper ions, and residues forming the active site (residues 37-52 and 94-102 from PmoA, and residues 152-156 from PmoC). The remaining residues of the pMMO structure were fixed, and thus their interactions with the active site residues could be used to anchor the refinement.

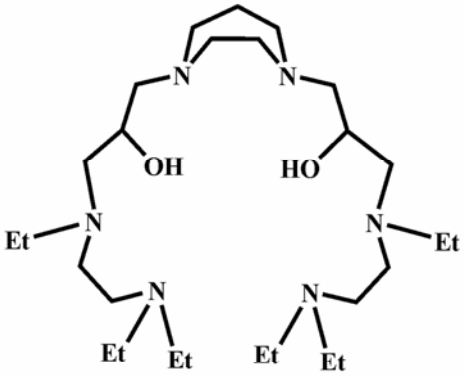
Table 1. Comparison of enzymatic activity and metal contents of the purified pMMO samples reported by Lieberman *et al* [8,11] in their preparation of the protein sample for crystallographic x-ray analysis and repeated here in this work according to the same protein purification procedures..

pMMO sample	**Specific activity**		**Copper content**		**Iron content**	
Status of purification	(nmol/min/mg protein)		(atoms/protein)		(atoms/protein)	
	This work	Ref. 8, 11	This work	Ref. 8, 11	This work	Ref. 8, 11
Membranes	88.9	19.0	12-15	–[a]	0.9	–[a]
Membrane fragments (solubilized in detergent)	93.5	3.9	13.3	10.4	nd[b]	1.1
Purified detergent-protein complex (prior to $(NH_4)_2SO_4$ precipitation)	21.5	–[a]	10.0	–[a]	nd[b]	–[a]
Purified detergent-protein complex (following $(NH_4)_2SO_4$ precipitation, detergent re-solubilization, and anion exchange chromatography)	inactive	inactive	2.5	2.4	nd[b]	0.8

[a] no data provided or available

[b] not detected

A.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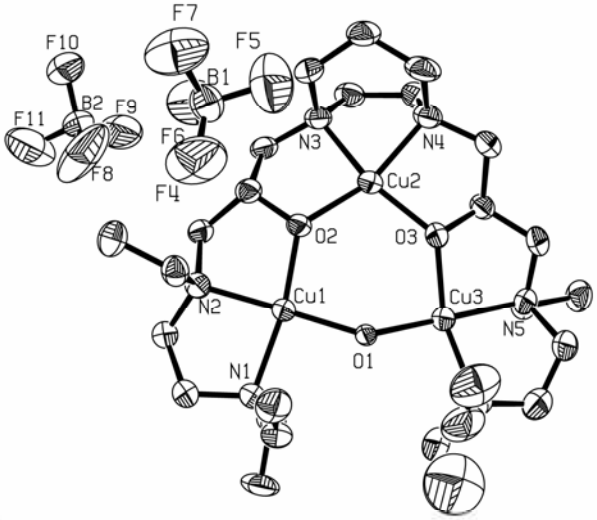
B.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F9
F11
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O2
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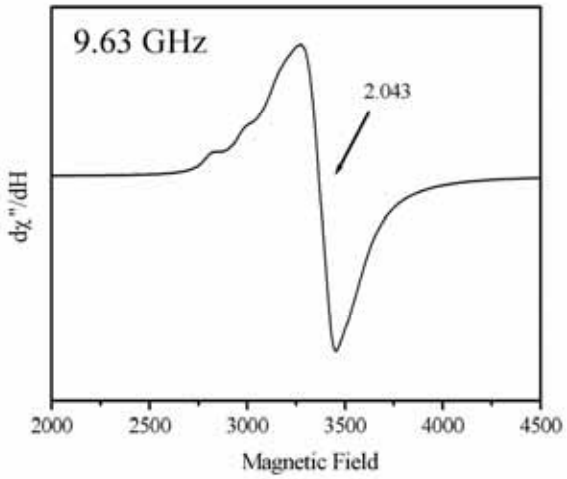
Cu1
Cu3
N5
O1
N1

C.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2.043
dχ"/dH
2000
2500
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4000
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Magnetic Field

Figure 4. (A) The ligand, 3,3'-(1,4-diazepane-1,4-diyl)bis(1-((2-(diethylamino)ethyl)(ethyl) amino)propan -2-ol)(7-Et) contains six neutral amines and two hydroxyl groups that are capable of trapping three copper ions simultaneously. (B) ORTEP representation of the $[Cu^{II}Cu^{II}Cu^{II}(7\text{-}Et)(O)](BF_4^-)_2$ showing atom labeling scheme together with 30 % probability ellipsoids (H-atoms omitted for clarity). Selected bond distances (Å) and angles (^{o}) are as follows: Cu1-O1, 1.934(4); Cu1-O2, 1.934(5); Cu2-O2, 1.927(5); Cu2-O3, 1.926(5); Cu3-O1, 1.925(5); Cu3-O3, 1.926(5); O1-Cu1-O2 96.64(19); O3-Cu2-O2, 108.3(2); O1-Cu3-O3, 92.72. (C) 4 K EPR spectrum of the $[Cu^{II}Cu^{II}Cu^{II}(7\text{-}Et)(O)](BF_4^-)_2$ in the solvent of CH_3CN.