XAS Studies of pMMO from *Methylococcus capsulatus* (Bath)

In this study, we have oxidized the particulate methane monooxygenase (pMMO) from Methylococcus capsulatus (Bath) to different levels of oxidation of the copper ions using dioxygen, hydrogen peroxide, ferricyanide, and dioxygen in the presence of the suicide substrate acetylene, and have characterized the oxidized copper centers using X-ray absorption spectroscopy (XAS). The results are consistent with the grouping of the copper ions into catalytic clusters (C-clusters) and electron transfer clusters (E-clusters). Quantification indicates that there are, indeed, two C-clusters. A model will be presented in which the C-clusters are activated by dioxygen starting from their fully reduced states and mediate the hydroxylation chemistry of methane.

Particulate methane monooxygenase (pMMO) is a membrane protein that mediates the conversion of methane to methanol in bacteria, a controlled oxidation process that is extremely difficult to carry out in the laboratory under ambient conditions of temperature and pressure. It is a multi-copper enzyme. Whereas pMMO is found in all methanotrophic bacteria, soluble methane mono-oxygenase (sMMO), the other methane mono-oxygenase found in nature, has only been isolated from certain strains of methanotrophs. The sMMO is a water-soluble cytoplasmic protein that is expressed only under copper deficient conditions. It is a multi-iron enzyme.

Copper is a metabolic activator in *Methylococcus capsulatus* (Bath) and stimulates the production of high concentrations of pMMO in the plasma membrane of the cell. The expression of the pMMO is accompanied by the formation of an extensive network of intracytoplasmic membranes, where the membrane-bound pMMO resides. The addition of more copper ions to the growth medium leads to the synthesis of additional intracytoplasmic membranes, appearance of the membrane proteins associated with the pMMO, increase of growth yields, and a loss of sMMO activity.

We recently have purified the pMMO from *M. capsulatus* (Bath) to homogeneity with high specific activity and showed that it is a multi-copper protein with 13-14 copper ions per 99 kDą monomer. Thus, copper ions not only regulate the expression of the pMMO, but also are crucial for pMMO activity. The solubilized pMMO-detergent complex is a protein monomer with a molecular mass of 220 kDa for the combined protein-detergent complex. Three polypeptides with molecular masses of 42, 29, and 28 kDa are associated with the monomer.

Previous studies of the X-ray absorption edge of pMMO from *M. capsulatus* (Bath) *in situ* revealed that the enzyme contained unusually high levels of copper ions with a significant portion existing as Cu(I) in the as-isolated membranes (70–80%). X-ray absorption near-edge spectroscopy (XANES) has been an important tool to monitor the oxidation state of the copper ions in pMMO, as Cu(I) is silent to all other common spectroscopic methods. We have exploited X-ray spectroscopy to monitor the levels and oxidation states of the copper ions in pMMO in whole cells, cell membranes, and the purified enzyme reconstituted into detergent micelles. Thus, access to the synchrotron radiation facilities at NSRRC has been indispensable for this project.

When fully reduced pMMO was exposed to air in the absence of methane, or when pMMO-enriched membranes were isolated from the cells under otherwise aerobic conditions, a fraction of the copper ions in the pMMO became oxidized. About 40% of the copper ions were oxidized in the "as-isolated" pMMO based on the near edge feature at 8984 eV in the Cu K-edge (Fig. 1). It was on this basis that the ~ 15 copper atoms in the protein were divided up into two groups: 2 catalytic

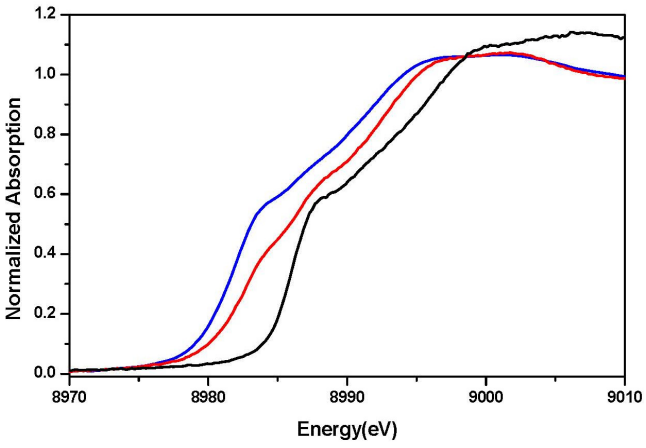

Fig. 1: *X-ray absorption K-edge spectra at 77K of pMMO-enriched membranes: "As-isolated" pMMO-enriched membranes (blue line); pMMO purged with pure dioxygen (red line); and pMMO-enriched membranes purged with pure dioxygen and treated with acetylene (black line).*

trinuclear copper clusters (C-clusters) of 6 copper ions, and 3 electron-transfer trinuclear copper clusters (E-clusters) of 9 copper ions. Since the motion of the spins appeared to be correlated when the copper ions in the C-clusters were oxidized, and the coupling among the spins was ferromagnetic with an S = 3/2 ground state on the basis of low temperature magnetization measurements, it was assumed that the copper ions were arranged into equilateral triads.

Nevertheless, the exact nature of the copper centers in the pMMO is very complex. Their geometric arrangements, ligand structures and their function during catalysis are still unclear. Evidently, even at this juncture, the number of copper ions per protein remains controversial. Toward clarifying some of these issues, we describe here the results of experiments designed to oxidize the copper ions in the protein to different levels in a variety of preparations and then characterize the nature of the oxygenated species by low-temperature X-ray absorption spectroscopy (XAS).

The level of oxidation of the copper ions in the "as-isolated" pMMO remained stable over time, indicating that there was a barrier to electron transfer from the E-clusters to the C-clusters in this non-physiological form of the enzyme. However, when the "as-isolated" samples were incubated in, or purged with, pure dioxygen for prolonged periods approaching 30 min to 1 hr, an additional 20%, or a total of about 60% of the copper ions in the pMMO became oxidized, according to X-ray absorption near-edge spectroscopy. It appeared that all the catalytic copper clusters and a small number of the electron transfer copper ions had become oxidized under these conditions. No further oxidation of the E-cluster copper ions occurred thereafter.

It has been known for some time that acetylene is a suicide substrate of the enzyme. The suicide substrate inhibited one or both of the two C-clusters when the activated copper cluster reacted with the acetylene to form ketene, which then modified a histidine residue in the hydrophobic alkane-binding pocket. According to EXAFS experiments, covalent modification of the enzyme by acetylene or other alkynes led to an overall tightening of coordination shell of the copper ions, providing direct evidence that at least one of the C-clusters was involved in the suicide substrate chemistry. However, unlike the "as-isolated" pMMO, this inhibited form of the enzyme was fully competent towards dioxygen chemistry. Upon exposure of this modified enzyme to excess dioxygen, an almost fully oxidized pMMO (12.9 ± 0.9 Cu(II) ions according to EPR intensity at 77 K, or 92 ± 10% of the total 14 copper ions assumed per protein), or a fully oxidized protein (as revealed by Cu K-edge X-ray absorption spectroscopy) was obtained (Fig. 1). Thus, by exposing the enzyme to dioxygen in the presence of acetylene, we show that it is possible to drain nearly all the reducing equivalents out of the protein and oxidize all 14–15 of the copper ions. These results suggest that (1) both the C-clusters and E-clusters are part of the same protein; (2) there are two C-clusters in the enzyme; (3) while both C-clusters participate in dioxygen chemistry, only one is directly involved in methane hydroxylation; (4) acetylene acts as a suicide substrate by covalent modification of the alkane hydroxylation site and blocks further access of substrate to the catalytic site; (5) dioxygen activation of the alkane hydroxylation C-cluster is apparently a prelude to the chemical modification of the active site by the suicide substrate; and (6) there must be some allosteric interaction between the two C-clusters so that alkane binding to the hydroxylation site activates the other C-cluster for facile dioxygen chemistry. In other words, the barrier to electron transfer from the E-clusters to the C-clusters postulated for the "as-isolated" enzyme is lowered or removed when substrate is bound to the alkane hydroxylation site.

In a series of experiments, we show that it is possible to completely oxidize all the copper ions in the pMMO with hydrogen peroxide or ferricyanide as well.

We began with a reductive titration of the "as-isolated" protein to obtain preparations of the fully reduced enzyme. Varying amounts of deoxygenated sodium dithionite were added slowly to different aliquots of the "as-isolated" pMMO-enriched membranes. The final volume of the different samples was the same to ensure that the protein concentration was constant for all the measurements. All the samples were prepared at room temperature in a glove box under N_2 atmosphere.

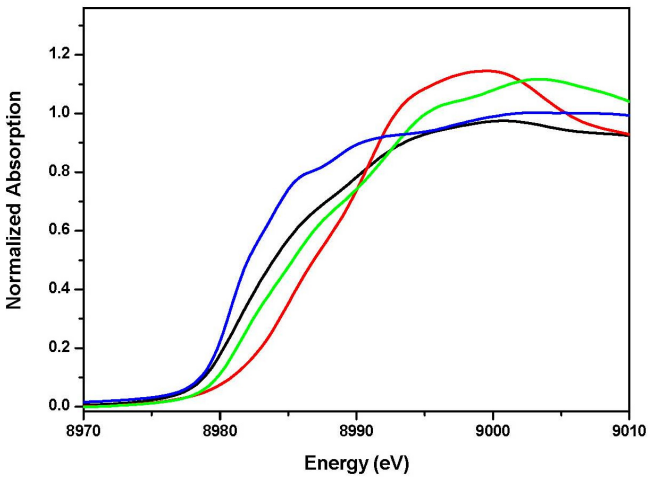

Fig 2: Oxidation of pMMO-enriched membranes by hydrogen peroxide. X-ray absorption K-edge spectra of pMMO-enriched membranes: pMMO fully reduced by sodium dithionite (blue line); pMMO oxidized by 100 equivalents (black line); 200 equivalents (green line); and 300 equivalents (red line, fully oxidized) H_2O_2.

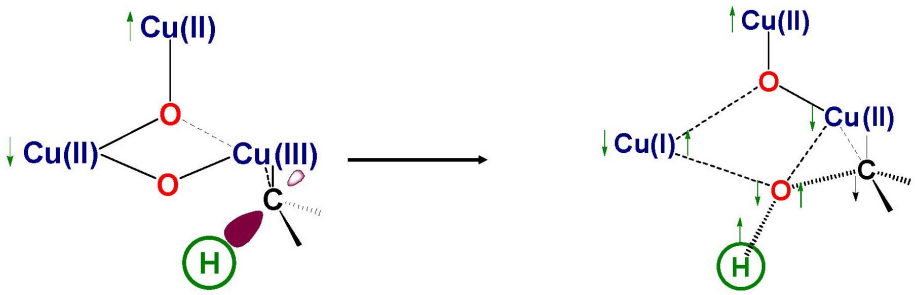

Fig. 3: Details of the adiabatic singlet "oxene" transfer from a dioxygen activated trinuclear copper cluster.

X-ray K-edge spectroscopy was used to monitor the oxidation levels of the same pMMO samples as they were reoxidized by varying amounts of hydrogen perioxide. These results are summarized in Fig. 2. The Cu (I) absorption feature at 8984 eV decreased with increasing concentration of hydrogen peroxides as all the copper ions in the pMMO were oxidized to Cu (II). Up to 95 ± 10% of all the copper ions were oxidized according to X-ray spectroscopy.

The various ferricyanide-oxidized preparations were also examined by Cu K-edge X-ray spectroscopy. The feature at 8984 eV due to Cu(I) was gradually reduced in intensity as the fully red-uced enzyme was re-oxidized to the partially and fully oxidized form by the increments of ferricyanide added. As in the case of hydrogen peroxide, it was possible to fully re-oxidize all the copper ions within the enzyme with ferricyanide starting with the fully reduced protein. Unlike hydrogen peroxide, however, the reducing equivalents were drained out of the protein via the E-clusters and the ferrocyanide-Cu_3 adducts to the ferricyanide in the external solution. In the case of hydrogen peroxide, the electrons were shuttled out of the enzyme through the C-clusters buried in the membrane domain of the protein to the oxidant, presumably using the same pathway as when dioxygen is used as the oxidant (in conjunction with acetylene).

The controlled oxidation of the C-H bond in small alkanes is hard to achieve by standard synthetic methods. In the case of sMMO, the conversion of methane to methanol is accomplished by hydrogen-abstraction of the CH_4 by a dioxyferryl cluster, followed by radical recombination of ·OH and $·CH_3$ to form the product alcohol. pMMO does not contain Fe, according to elemental analysis of the metal contents in the purified enzyme by several methods including ICP-MS, inductively coupled plasma optical emission spectroscopy, atomic absorption, and X-ray absorption spectroscopy at the Cu and Fe K_α–absorption edges. Instead the hydroxylation is mediated by a copper cluster. Chan et al. have proposed that a trinuclear Cu(I) cluster could be activated by dioxygen to generate a "singlet oxene", which could undergo facile direct concerted insertion across a C-H bond with total retention of the configuration of carbon center oxidized. According to Density Functional Theory calculations, a trinuclear copper cluster with two anti-ferromagnetically coupled Cu (II) ions and one Cu (III) ion could harness such a "singlet oxene" (Fig. 3).

This "putative" high valent Cu (III) intermediate is difficult to detect experimentally. However, preliminary evidence was provided by the 1s → 3d Cu (III) pre-edge feature centered at 8981 ~ 2 eV. This feature was enhanced when we treated the "as isolated" pMMO with the suicide substrate acetylene, which lowered the barrier to electron transfer from the E-clusters to the C-clusters. Oxidation of the E-cluster copper ions reduced the interference from the strong Cu(I) absorption edge feature at 8984 eV, "unmasking" the much weaker 1s → 3d transition of Cu (III) pre-edge feature arising from the residual oxidized C-clusters copper ions that had not reacted with the suicide substrate (Fig. 4).

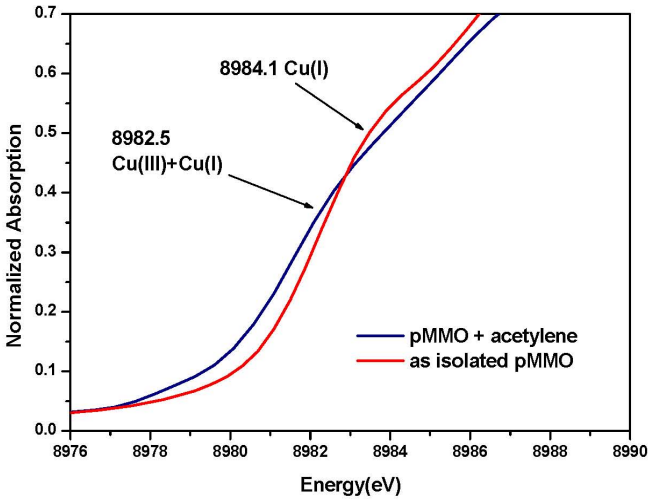

Fig. 4: The appearance of the Cu (III) pre-edge feature when the "as isolated" pMMO was partially treated with the suicide substrate acetylene to reduce the interference from the strong Cu(I) feature at 8984 eV from the E-cluster Cu(I) ions.

BEAMLINE
17C1 W20 EXAFS beamline

EXPERIMENTAL STATION
X-ray Absorption Spectroscopy end station

AUTHORS
K. H.-C. Chen, S. S.-F. Yu, and S. I. Chan
Institute of Chemistry, Academia Sinica, Taipei, Taiwan

J.-F. Lee
National Synchrotron Radiation Research Center, Hsinchu, Taiwan

PUBLICATIONS

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CONTACT E-MAIL
chans@chem.sinica.edu.tw