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Feature Article

The Copper Clusters in the Particulate Methane Monooxygenase (pMMO) from *Methylococcus capsulatus* (Bath)

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The particulate methane monooxygenase (pMMO) from *Methylococcus capsulatus* (Bath) is a multicopper protein that hydroxylates methane and other small n-alkanes to their corresponding alcohols with high regiospecificity and stereoselectivity. The copper ions appear to be arranged into ~5 trinuclear copper clusters. Two of these clusters are thought to be involved in the dioxygen chemistry and alkane hydroxylation mediated by the enzyme. Accordingly, these catalytic clusters (C-clusters) are typically oxidized, as the protein is isolated. The remaining copper ions are normally reduced, and it has been suggested that they provide a reservoir of reducing equivalents needed for the turnover of the enzyme. In this study, we have oxidized the protein 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 low temperature electron paramagnetic resonance (EPR) and X-ray absorption spectroscopy. The results are consistent with the grouping of the copper ions into catalytic clusters and electron transfer clusters (E-clusters). Quantification indicates that there are, indeed, two C-clusters. A model in which the two C-clusters are activated by dioxygen starting from their fully reduced states and mediate the hydroxylation chemistry of methane will be presented and discussed.

Keywords: Copper clusters; pMMO; Redox titrations; Suicide substrate; Low temperature EPR; X-ray absorption spectroscopy.

INTRODUCTION

Methanotrophic bacteria utilize methane both as a carbon and an energy source.[1] The oxidation of methane by aerobic methanotrophs is mediated by the methane monooxygenases (MMOs), the classical monooxygenases that utilize two reducing equivalents to split the O-O bond of dioxygen and transfer one of the oxygen atoms to a hydrocarbon substrate. In pMMO, the latter oxygen atom is incorporated into the C-H bond of methane to form methanol, while the other is reduced to form H_2O. The controlled conversion of methane to methanol in the chemical laboratory is extremely difficult to realize and requires the use of expensive catalysts in addition to high temperatures and pressures.[2] In contrast, the biological process in Nature is quite facile even under ambient temperatures and pressures.

There are two forms of the MMOs in methanotrophic bacteria. One form is found in the cytoplasm of the cell and is referred to as the soluble methane monooxygenase (sMMO).[3,4] The sMMO is an iron enzyme, and the hydroxylase protein is an $(\alpha\beta\gamma)_2$ dimer containing two non-heme binuclear iron clusters that activate the dioxygen and mediate the hydroxyl-

Dedicated to Professor Sunney I. Chan on the occasion of his 67th birthday and his retirement from professional life.

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ation chemistry.[3,4,5-7] The other MMO, called the particulate methane monooxygenase (pMMO), is a membrane-bound protein.[8] The pMMO is a multi-copper enzyme.[9] It has recently been purified from pMMO-enriched membranes and solubilized as a protein-detergent complex.[10] Whereas the pMMO is found in all methanotrophic bacteria, the sMMO has only been isolated from certain strains of methanotrophs.[1]

The two proteins are products of different genes. In fact, they are differentially expressed, with the expression controlled and regulated by the level of copper ions in the growth medium.[10,11] When the growth medium contains iron at sufficient levels and the level of copper ions is limiting, the cells express only the sMMO. Under high copper/biomass ratios, the cell switches from the expression of sMMO to pMMO. Normally, this occurs at copper ion concentrations > 10 μM in the growth medium. Aside from being a transcriptional switch, copper is also a metabolic activator and stimulates the production of high concentrations of pMMO in the plasma membrane.[12] 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, increased growth yields, and a loss of sMMO activity. The connection between pMMO activity and copper concentration was largely established by the pioneering work of Dalton and co-workers.[11]

More recently, Chan and co-workers[10] have purified the pMMO from *Methylococcus capsulatus* (Bath) to homogeneity with high specific activity and showed that it is a multi-copper protein with 13-14 copper ions per 99 kDa 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 $\alpha\beta\gamma$ monomer with a molecular mass of 220 kDa. Three polypeptides with molecular masses of 42, 29, and 28 kDa are associated with the monomer.

The recent results of the Chan laboratory are in agreement with the earlier work of Nguyen et al.[9] but are in substantial disagreement with the findings of DiSpirito and coworkers[13] and the more recent observations of Rosenzweig and coworkers.[14] DiSpirito and co-workers have generated a purified pMMO preparation from *Methylococcus capsulatus* (Bath),[13] in which they suggested that the active pMMO contained 2.5 iron and 14.5 copper atoms per protein unit. These authors also reported electron paramagnetic resonance (EPR) evidence for a type 2 Cu(II) center, a weak high-spin iron signal (g = 6.0) and a broad low-field signal at g = 12. In more recent experiments, Rosenzweig and coworkers[14] have purified a pMMO containing 4-6 copper ions per dimer but with relatively low specific activity. Moreover, these workers presented evidence for both type II copper as well as copper clusters. Antholine and co-workers[15] have also reported evidence for nearly identical type 2 Cu(II) EPR signals with resolved hyperfine coupling to four nitrogen atoms for *Methylococcus capsulatus* (Bath) and *Methylomicrobium* BG8.

Okura and co-workers[16] have focused their attention on the pMMO from *Methylosinus trichosporium* OB3b. This laboratory has also reported that the purified pMMO is a multi-copper protein containing 12.8 copper ions per molecule, but there is also one iron (0.9 iron atom), suggesting that iron might play a role in the pMMO active site. In more recent work, this group has purified the pMMO without any iron and concluded that the iron might be adventitious. Xin et al.[17] have also recently reported the purification and characterization of the pMMO from *Methylosinus trichosporium*. In the hands of this group, the pMMO is a copper only protein containing 17 copper ions per 100 kDa molecular mass.

The original work of Chan and co-workers[9] indicated that there were 12-15 copper ions bound per protein monomer. From low temperature magnetization and EPR measurements, Nguyen et al. suggested that the copper ions of the protein were somehow arranged into trinuclear copper clusters.[18] In subsequent work, the Chan group[19] utilized a combination of EPR and X-ray absorption spectroscopy (XAS) to probe the oxidation state of the copper ions in pMMO under various conditions. Based on these studies, they proposed a model of the enzyme with catalytic copper clusters (C-clusters) and electron transfer copper clusters (E-clusters). Indeed, subsequent studies have demonstrated a catalytic role for some of the copper ions in the protein.[20] Further studies have indicated that there must be at least two histidine residues at the active site of pMMO (C-clusters) by pulsed EPR and electron spin echo envelope modulation (ESEEM) experiments.[21]

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 report here experiments designed to oxidize the copper ions in the protein to different levels. We began with the as-isolated protein[19] obtained during the purification of the membranes or the protein in air and characterized the nature of the oxygenated species by low-temperature EPR and XAS. We then subjected the as-isolated protein to further oxidation of the copper ions by dioxygen in the presence of the suicide substrate inhibitor acetylene.[22] The as-isolated protein was also fully reduced with dithionite and the copper ions re-oxidized to varying levels of oxidation by hydrogen peroxide and ferricyanide. In each experiment, the copper ions were monitored by low-temperature EPR and XAS spectroscopy at the various levels of oxidation or reduction.

MATERIALS AND METHODS

The Culturing of *Methylococcus capsulatus* (Bath)

Methylococcus capsulatus (Bath) were maintained on Petri plates containing the NMS medium (ATCC medium: 1306 nitrate mineral salts medium) with 1.7% agar. Cultures were maintained in a closed anaerobic jar with an atmosphere of 20% methane in air and streaked onto fresh plates every 4-6 weeks. The organism was first transferred from Petri plates to 250-mL flasks and subsequently to 2-L Erlenmeyer flasks containing 30 and 300 mL, respectively, of the nitrate mineral salts medium with added $CuSO_4$ (10 μM), a 20% methane in air atmosphere, and continual shaking. The organism was grown for 48 h in these small-scale cultures. The 300 mL cultures were used to seed a Bioflo 3000 fermentor (New Brunswick Inc.) with a 5-L fermentor vessel containing 3 L of the above-described medium. After 24 h culturing, when the optical density of the culture media had attained 1.2-1.6, based on the UV-Visible absorption at 595 nm, additional culturing medium was added to increase the culture to 5.5 L. 12 h later, the culture vessel of the fermentor was connected to a hollow-fiber reactor (pore size 0.2 μm, 2790 cm^3 volume capacity, length 25 inch and diameter 1¼ inch, A/G Technology Inc.). The hollow fiber reactor consisted of a bundle of fiber capillary tubing. With the help of a peristaltic pump, it was possible to do continuous flow fermentation by draining out the used medium and replenishing with the fresh medium. With this modification, the cell capacity in the medium was no longer restricted by the regular stationary phase. A cell density up to 12-14 g/L could be obtained under 1:4 methane/air ratio after 24 h medium renewal. Methane feeding rate was controlled by the dioxygen content in the culture media to around 2-5% of the dissolved dioxygen at saturation. In addition to increasing the cell yield, the adjustment of the copper concentration could be precisely controlled in the culture media by the use of the hollow-fiber bioreactor technology. Gradually, we could refresh the medium by incrementing the $CuSO_4$ concentration up to 40 μM to stimulate the expression of pMMO.

Cell Breakage and Isolation of pMMO-Enriched-Membranes

The pMMO enriched-membranes were isolated from *M. capsulatus* (Bath) that were grown under NMS buffer at 30 μM copper ion concentration. Cytosolic and membrane fractions were separated by passing a cell suspension (~0.8 g wet weight of cells per mL) three times through a French pressure cell at 20,000 psi.

Un-lysed cells and cell debris were removed by centrifugation at 27,000 × g for 20 min. The supernatant was then ultra-centrifuged at 220,000 × g for 40 min to pellet the membrane fraction.

The clear supernatant obtained after ultra-centrifugation was saved as the cytosolic fraction. The membrane pellet was re-suspended in 25 mM Pipes buffer (pH 7.4) and washed by a Dounce homogenizer. The membrane fraction was re-pelleted by ultra-centrifugation and re-suspended in a volume of 25 mM Pipes buffer (pH 7.4) equivalent to the volume of the original cell suspension. This process was repeated once or twice until the supernatant was virtually free of heme-containing soluble proteins. The pMMO-enriched membranes were then obtained.

^{15}N-Labeled pMMO-Enriched Membranes

For isotope enrichment studies, cells were grown in the same medium by the same process, except that potassium nitrate was replaced by potassium nitrate-^{15}N (98% ^{15}N; Cambridge Isotope Laboratories, Inc.). The ^{15}N-labeled pMMO-enriched membranes were isolated from the cells as described above.

Modification of the Protein by the Suicide Substrate Acetylene

An as-isolated pMMO membrane stock solution was prepared by diluting pMMO-enriched membranes to a concentration of ~50 mg/mL, and 5 mL was added to a 10-mL serum bottle. The gas was then withdrawn, and acetylene was injected into the vial for 5 min at room temperature. The reaction mixture was kept at 45 °C for another 55 min.

As a control, a similar sample of pMMO-enriched membranes was first exposed to air for 5 min before incubation with acetylene at room temperature for another 15 min. Suitable samples of the reaction mixture were then withdrawn into an EPR tube or a XAS sample holder and frozen at 77 K for subsequent EPR and XAS observations.

Purging of the Acetylene-Modified pMMO with Pure Dioxygen

The acetylene-modified pMMO was purged with pure dioxygen violently for 20 min at room temperature and then incubated at 45 °C for 40 min. Suitable samples of the reaction mixture were then withdrawn into an EPR tube and a XAS sample holder. These samples were then frozen in a liquid-nitrogen chilled isopentane solution.

As a control, a similar sample of as-isolated pMMO-enriched membranes, without prior exposure to and incubation with the suicide substrate inhibitor acetylene, was subjected to a similar violent purging with dioxygen and frozen in a liquid-nitrogen chilled isopentane solution for EPR and XAS measurements.

Reductive Titration with Sodium Dithionite

Solutions of Pipes buffer (25 mM, pH 7.0) and sodium dithionite (100 mM) were both deoxygenated by successive freeze-thaw cycles of vacuum/dioxygen-free argon for 10 min. Different concentrations of the reductant were prepared by mixing 5 to 50 μL sodium dithionite (100 mM) in 5 μL steps with the deoxygenated Pipes buffer to a final volume of 50 μL. Samples of the as-isolated pMMO membranes (350 μL, 50 mg protein/mL) were purged with dioxygen-free argon and mixed with samples of the reductant solution at the varying concentrations prepared above in a glove box under a N_2 atmosphere at room temperature. Following thorough mixing for 1 min, the reductant-treated pMMO-enriched membrane samples were loaded into the EPR tubes and XAS sample holder anaerobically and plunged into a liquid-nitrogen chilled isopentane solution. The frozen samples were then analyzed by EPR and XAS experiments.

Oxidative Titration with Potassium Ferricyanide

Similar solutions of Pipes buffer (25 mM, pH 7.0) and potassium ferricyanide (100 mM) were prepared using the same procedure as described above. Different concentrations of the oxidant were prepared by mixing 0, 5, 10, 15 and 20 μL potassium ferricyanide at 100 mM with the deoxygenated Pipes buffer to a final volume of 20 μL. Equal volumes of the fully reduced pMMO membranes were then oxidized by the above potassium ferricyanide solutions at the varying concentrations under nitrogen atmosphere at room temperature. Following thorough mixing for 1 min, the partially or fully oxidized pMMO membranes (280 μL, 40 mg protein/mL) were loaded into EPR tubes anaerobically and plunged into a liquid-nitrogen chilled isopentane bath. The frozen samples were then analyzed by EPR and XAS measurements.

Oxidative Titration with Hydrogen Peroxide

Different concentrations of hydrogen peroxide were prepared by mixing 0, 1, 50, 100, 200 and 300 equivalent of 0.1% solution with Pipes buffer to a final volume of 100 μL. Equal volumes of the fully reduced pMMO membranes were then oxidized by the above concentrations of hydrogen peroxide at room temperature. Following thorough mixing for 1 min, the partially or fully oxidized pMMO membranes were loaded into EPR tubes anaerobically and plunged into a liquid-nitrogen chilled isopentane bath. The frozen samples were then analyzed by EPR and XAS measurements.

EPR Spectroscopy

EPR spectra were obtained at X-band (9.5 GHz) by using a Bruker E500 spectrometer equipped with a Bruker TE102 cavity. During EPR experiments, the sample temperature was maintained at 77 K by immersion of the EPR tube in a liquid-nitrogen containing finger dewar, or at 3 K or 4.5 K by using an Oxford Instruments continuous liquid-helium cryostat equipped with a helium pump to lower the vapor pressure of the liquid helium when desired. A 500 μM of $CuSO_4$ in 1:1 v/v pure water and glycerol was employed as a standard sample for spin counting purposes. 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 samples.

X-ray Absorption Spectroscopy

X-ray absorption spectroscopy data were collected at the National Synchrotron Radiation Research Center (NSRRC) in Hsinchu using the Si (111) double crystal monochromator on beamline Wiggler 17C. All samples were loaded into sample holders (1.4 cm × 1.4 cm × 0.2 cm) covered with sheets of Kapton. During the measurements, the samples were kept at 10 K by using an Oxford Instruments continuous liquid-helium cryostat. Fluorescence data were obtained using an Ar-filled ionization chamber detector equipped with a Ni filter and Soller slits. Typically, an average of 10-16 scans were

taken. Data reduction included energy calibration followed by pre-edge subtraction using a polynomial function spline. The first inflection points of the K-absorption edges of Fe and Cu foils were assigned energies of 7112.0 and 8980.3 eV, respectively.

FTIR Spectroscopy

FTIR spectra were recorded at 0.5 cm^{-1} resolution with a Mattson Sirius 100 FTIR interferometer, which was fitted with a liquid nitrogen cooled InSb detector. The detector was fitted with appropriate low-pass optical filters to limit the optical bandpass to the spectral region of interest and to improve the signal-to-noise. pMMO membranes were pressed between BaF_2 windows with an optical path of 8.8 μm.

RESULTS

3 K or 4 K EPR of the As-Isolated pMMO-Enriched Membranes

Fig. 1(a) shows the EPR spectra of the as-isolated pMMO-enriched membranes from *Methylococcus capsulatus* (Bath) cells recorded at 4.2 K under various microwave powers. The spectrum observed under the lowest microwave power (2 μW) is shown at the top. As noted in the earlier work of Nguyen et al.,[18] this spectrum is a composite of a type 2 Cu(II) signal and an isotropic signal centered at g ~ 2.1. With increasing microwave power, the type 2 Cu(II) signal gradually became saturated. At the microwave power in excess of 20 mW, essentially only the isotropic EPR signal remained. Nguyen et al.[18] have assigned this isotropic signal to an oxidized trinuclear copper cluster wherein the three Cu(II) ions are ferromagnetically coupled in the ground state.

The spectral features of the type 2 Cu(II) EPR are: $g_{\|}$ = 2.24 with $A_{\|}$ = 185 G, and $g_{\perp}$ = 2.059. Additionally, superhyperfine structure was also evident in the $g_{\perp}$ = 2.059 region under low microwave powers (Fig. 1(b)). A weak radical signal was also observed at g = 2.007. The superhyperfine structure was associated with the type 2 Cu(II), as the Cu(II) hyperfine and the superhyperfine features observed in the parallel and perpendicular regions, respectively, saturated together with increasing microwave powers.

Neither the type 2 Cu(II) EPR signal nor the isotropic trinuclear Cu(II) cluster signal appeared to be saturated at the

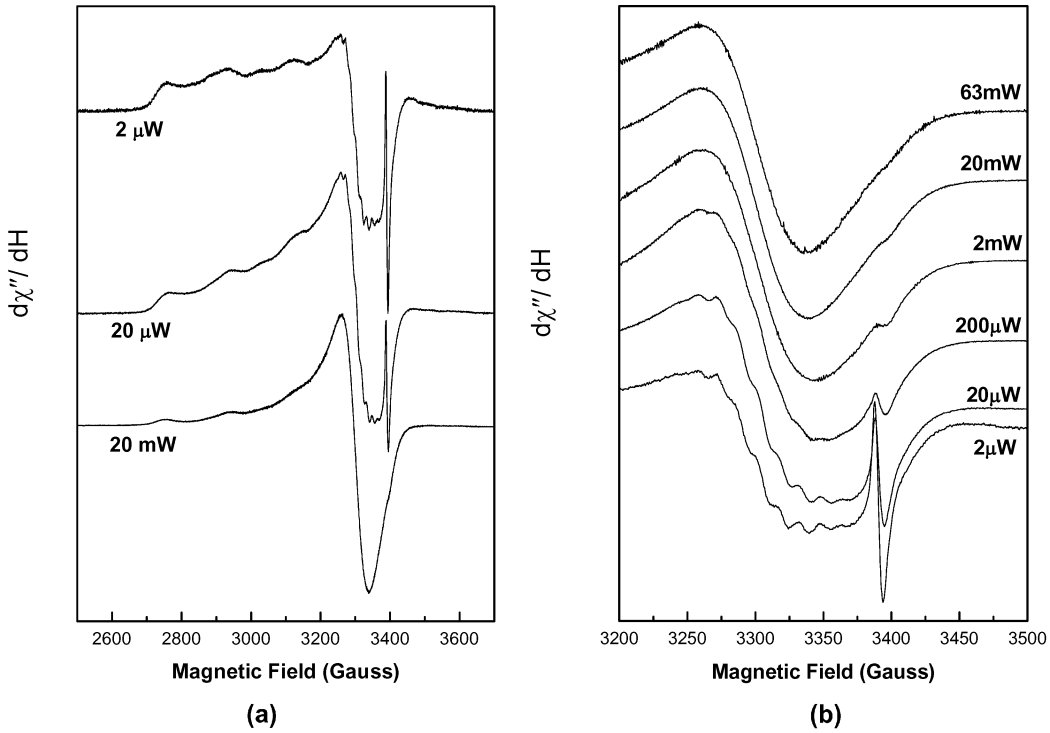


Fig. 1. 4.2 K X-band EPR spectra of as-isolated pMMO in pMMO-enriched membranes. The spectra were recorded by varying the microwave power: (a) 2 μW; 20 μW; and 20 mW. (b) Expanded $g_{\perp}$ region containing superhyperfine structure was examined at microwave power levels from 2 μW to 63 mW. Other spectral parameters: microwave frequency 9.517 GHz; modulation frequency 100 kHz; and modulation amplitude 5 Gauss.

microwave power of 20 μW. From a detailed study of the power saturation behavior of the type 2 Cu(II) signal (Fig. 1(b)), we estimated that P½ ~ 200 μW at 4.2 K. Thus, the spectrum observed at the microwave power of 20 μW should be a composite of the spectral components for the two types of copper ions without distortion from power broadening. A deconvolution of the composite spectrum into the two components indicated that the type 2 and the trinuclear Cu(II) cluster EPR signals are made up of roughly equal proportions.

Details of this deconvolution of the 3 K EPR spectrum of the C-clusters are as follows. Basically, the composite spectrum was taken as a linear combination of a type 2 Cu(II) signal and a trinuclear Cu(II) cluster signal (principal components). The type 2 signal was simulated from the g-values, the Cu(II) hyperfine tensor, and the ^{14}N superhyperfine interactions expected for a tetragonal Cu(II) center. The hyperfine features in the $g_{||}$ region were used to guide the spectral simulations, and the full type 2 Cu(II) spectrum was simulated using the EPR parameters given in the figure caption. The cluster signal was calculated by the method described in our companion paper.[23] A spectrum for the trinuclear Cu(II) cluster obtained by subtracting the simulated type 2 Cu(II) spectrum from the observed EPR signal was used as a starting point for the cluster calculations. The various spin Hamiltonian parameters for the two types of copper centers as well as their relative proportions were then adjusted until a best fit was obtained between the simulated overall EPR spectrum and the EPR spectrum observed experimentally.

The component EPR spectra for the type 2 center and the cluster Cu(II) center that provided the best fit to the overall composite EPR spectrum of the as-isolated pMMO at 3 K are depicted in Fig. 2. Double-integrations of the two components gave an intensity ratio of 0.9 ± 0.2 for cluster signal to type 2 signal. Spin counting of the composite EPR signal against $CuSO_4$ standards gave a total overall Cu(II) EPR intensity of 1.7 ± 0.4 copper ions per protein.

77 K EPR of the As-Isolated pMMO-Enriched Membranes and the Purified pMMO-Detergent Complex

Fig. 3 shows the EPR spectrum of the same pMMO-enriched membranes at 77 K. The spectrum resembles more like that of a type 2 Cu(II) center, although the high-field half of the broad cluster component centered at g ~ 2.1 is still apparent. Again, the composite spectrum could be simulated by roughly equal proportions of the type 2 Cu(II) and the trinuclear Cu(II) cluster EPR signals. A deconvolution of the composite spectrum into the two component parts is shown on the lower half of the panel. The EPR parameters used to simulate the type 2 center were the same as those used in the simulation of the corresponding spectrum at 3 K. Essentially the same spin Hamiltonian parameters as used in the simulation of the 3 K cluster signal were also used to obtain the best fit of the composite EPR spectrum at 77 K, except for an adjustment of the zero-field splitting parameter D from 175 Gauss to 200 Gauss.[23] Double integrations of the two component signals that best fitted the observed 77 K EPR spectrum gave an intensity ratio of 0.8 ± 0.2 for the cluster Cu(II) to the type 2 Cu(II) signals, and spin counting against $CuSO_4$ standards yielded a total EPR intensity for the composite spectrum corresponding to 1.6 ± 0.2 Cu(II) per protein molecule.

The 77 K EPR spectrum of the pMMO-enriched membranes are compared with that of the pMMO-detergent complex fractionated from the pMMO-enriched membranes solubilized in dodecyl-β-D-maltoside in Fig. 4. The two spectra are essentially identical, except that the purified pMMO-

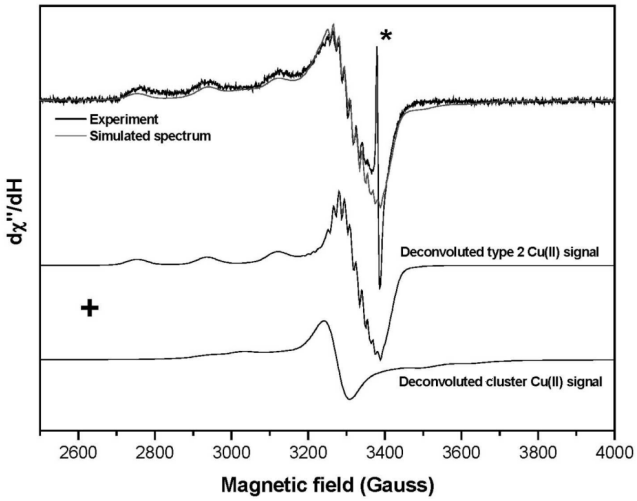


Fig. 2. The 3 K X-band EPR spectrum of as-isolated pMMO in pMMO-enriched membranes recorded at a low microwave power of 2 μW (solid line) is compared with the simulated spectrum (red). Microwave frequency 9.487 GHz; modulation frequency 100 kHz; and modulation amplitude 5 Gauss. The type 2 Cu(II) signal was simulated with the following spectral parameters: $g_z = 2.243$; $A_{zz} = 185$ Gauss; $g_x = 2.061$; $g_y = 2.033$; $A_{xx} = A_{yy} = 8.1$ Gauss; line widths used to simulate the spectrum are 50 Gauss and 40 Gauss for the parallel and perpendicular components, respectively. The cluster signal was calculated with D = + 0.017 cm^{-1} (175 Gauss) and E/D = 0.15.

detergent complex exhibited better resolved superhyperfine structure in the $g_{\perp}$ region. In a recent study,[10] we showed that both preparations of the enzyme are functional and contained the same number of copper ions. As expected, the enzymes in the pMMO-enriched membrane are somewhat more active toward propylene epoxidation when either NADH or duroquinol is used as the reducing substrate. The lipid bilayer is a more natural environment to assemble a multi-subunit membrane protein than a detergent micelle so that the lower specific activity of the enzyme in the detergent micelle is understandable.

^{15}N-Labeling of the pMMO

To clarify the origin of the observed multi-line pattern observed in the $g_{\perp}$ region of the type 2 Cu(II) signal, we have compared the EPR spectrum of a sample of the as-isolated ^{15}N-labeled-pMMO in pMMO-enriched membranes, wherein all the nitrogens have been replaced by the ^{15}N isotope (98%), with that of the native pMMO in similarly pMMO-enriched membranes (Fig. 5). The observed spectrum for the type 2 center in the native pMMO consisted of parallel features at $g_{\|} = 2.24$ with $A_{\|}(Cu) = 185$ G and perpendicular features at $g_{\perp} \approx 2.04$ with nine resolved superhyperfine components. A similar spectrum was obtained for the ^{15}N-labeled-pMMO, except that the 9-line pattern centered at $g_{\perp} \approx 2.04$ had given way to an apparent quintet. These results clearly indicate that the electron spin of the type 2 Cu(II) is coupled to nitrogen ligands. The apparent hyperfine coupling constants of 16 G and 23 G measured from the line spacings observed for the ^{14}N-pMMO and ^{15}N-pMMO, respectively, are consistent with this interpretation.

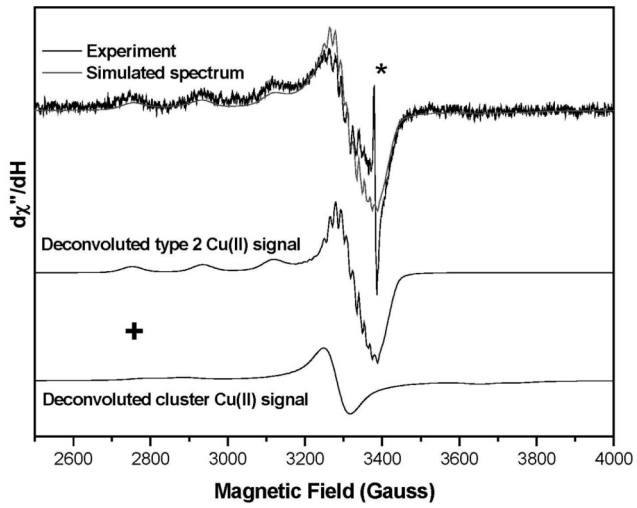


Fig. 3. The 77 K X-band EPR spectrum of as-isolated pMMO in pMMO-enriched membranes recorded at the microwave power of 0.2 mW (solid line) is compared with the simulated spectrum (red). Microwave frequency 9.482 GHz; modulation frequency 100 kHz; and modulation amplitude 5 Gauss. The type 2 Cu(II) signal was simulated with the following spectral parameters: $g_z = 2.243$; $A_{zz} = 185$ Gauss; $g_x = 2.061$; $g_y = 2.033$; $A_{xx} = A_{yy} = 8.1$ Gauss; line widths used to simulate the spectrum are 50 Gauss and 40 Gauss for the parallel and perpendicular components, respectively. The 77 K cluster signal was calculated with D = + 0.019 cm^{-1} (200 Gauss) and E/D = 0.15.

We have attempted to simulate the spectra of the type 2 Cu(II) for both the ^{14}N-pMMO and ^{15}N-pMMO based on axial and rhombic models for the type 2 Cu(II) center with 2, 3, or 4 coordinating nitrogens. As shown in Fig. 5, a simultaneous best fit to both the ^{14}N-pMMO and ^{15}N-pMMO data was only obtained for a rhombic model with 2 coordinating nitrogens: $g_{\|} = 2.240$, $A_{\|}(Cu) = 185$ G, $A_{\|}(^{14}N) = 0$ G, $g_{\perp} = 2.060$, $g_{\perp'} = 2.030$, $A_{\perp}(Cu) = A_{\perp'}(Cu) = 9.0$ G, $A_{\perp}(^{14}N) = A_{\perp'}(^{14}N) = 15$ G for ^{14}N-pMMO; and $g_{\|} = 2.240$, $A_{\|}(Cu) = 187$ G, $A_{\|}(^{15}N) = 0$ G, $g_{\perp} = 2.065$, $g_{\perp'} = 2.031$, $A_{\perp}(Cu) = A_{\perp'}(Cu) = 8.1$ G, $A_{\perp}(^{15}N) = A_{\perp'}(^{15}N) = 21$ G for ^{15}N-pMMO. It was also possible to fit both the ^{14}N-pMMO and ^{15}N-pMMO spectra with an axial model with 4 coordinating nitrogens, but the quality of the fit was less satisfactory. On the other hand, we were unable to obtain a good fit to the superhyperfine interac-

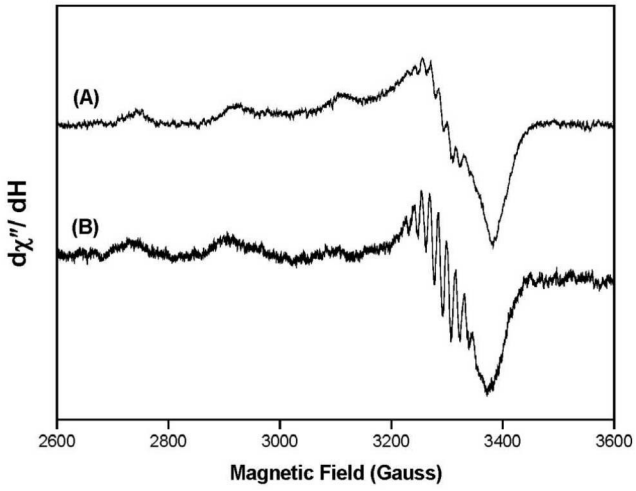


Fig. 4. 77 K EPR spectra of the as-isolated pMMO in pMMO-enriched membranes (A); and the purified pMMO-detergent complex (B). Microwave frequency 9.472 GHz; microwave power 5 mW; modulation frequency 100 kHz; and modulation amplitude 5 Gauss.

tion with a ligand-structure model involving 3 coordinating nitrogens.

EPR and XAS Spectroscopy of pMMO-Enriched Membranes. Effects of the Suicide Substrate

Our previous studies of the X-ray absorption edge and EPR spectroscopy of the 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%).[19]

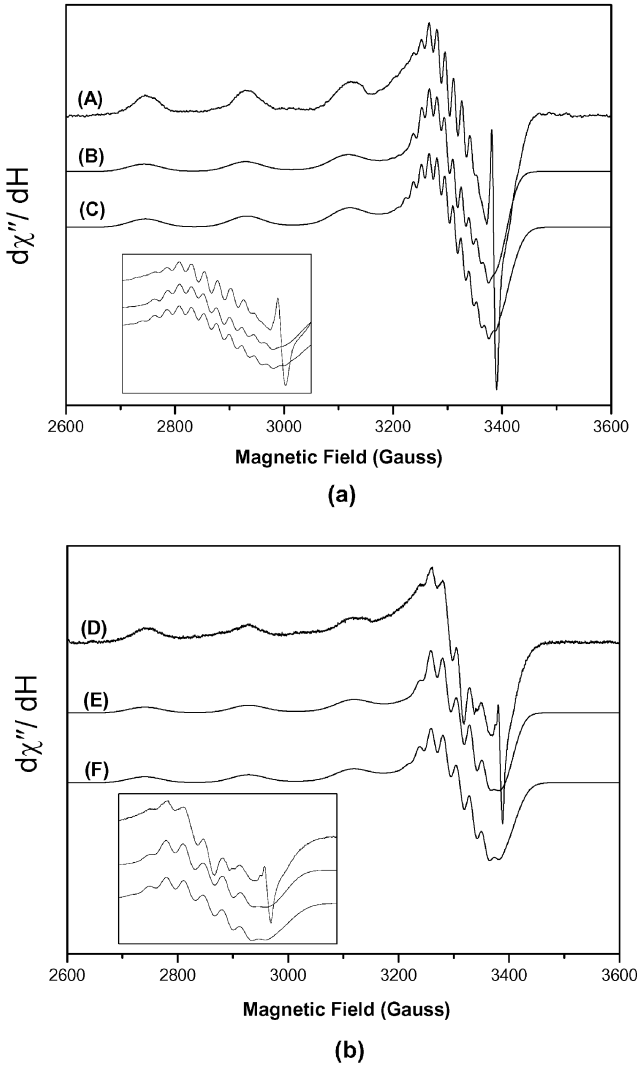


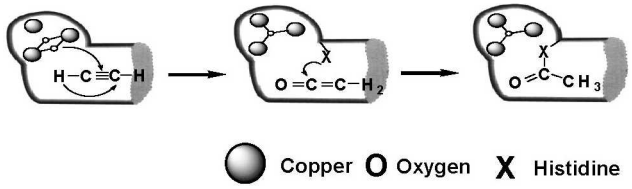
Fig. 5. 77 K X-band EPR spectrum of ^{14}N-pMMO (A); theoretical spectra simulated with coupling to two nitrogens (B) and to four nitrogens (C). (b) 77 K X-band EPR spectrum of ^{15}N-pMMO (D); theoretical spectra simulated with coupling to two nitrogens (E) and to four nitrogens (F). Other parameters used in the spectral simulations are given in the text. In the insets of (a) and (b), the $g_{\perp}$ region has been expanded to allow for detailed comparison between theory and experiment. EPR spectral parameters are the same as for Fig. 3.

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 or according to the Cu(II) EPR intensity in the EPR spectrum (Fig. 6). It was on this basis that Nguyen et al.[19] divided up the ~15 copper atoms in the protein into two groups: 2 catalytic 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 on the basis of low temperature magnetization measurements, it was assumed that the copper ions were arranged into equilateral triads. A similar assumption was made for the copper atoms associated with the E-clusters, although it was evident that the extent of the ferromagnetic coupling among the Cu(II) ions was much smaller for the E-clusters than for the C-clusters.

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 EPR and 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.[22,24,25] 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 (Scheme I).[22] 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

Scheme I



least one of the C-clusters was involved in the suicide substrate chemistry.[25] 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 the protein was essentially totally oxidized, according to X-ray absorption edge data) was obtained, as revealed by Cu(II) EPR and Cu K-edge X-ray absorption spectroscopy (Fig. 6). 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.

Reductive Titration of the As-Isolated pMMO. Oxidative Titration of the Fully Reduced pMMO by Hydrogen Peroxide

In this series of experiments, we show that it is possible to completely oxidize all the copper ions in the pMMO with hydrogen peroxide 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. As shown in Fig. 7, the reductive titration of samples of the as-isolated pMMO resulted in

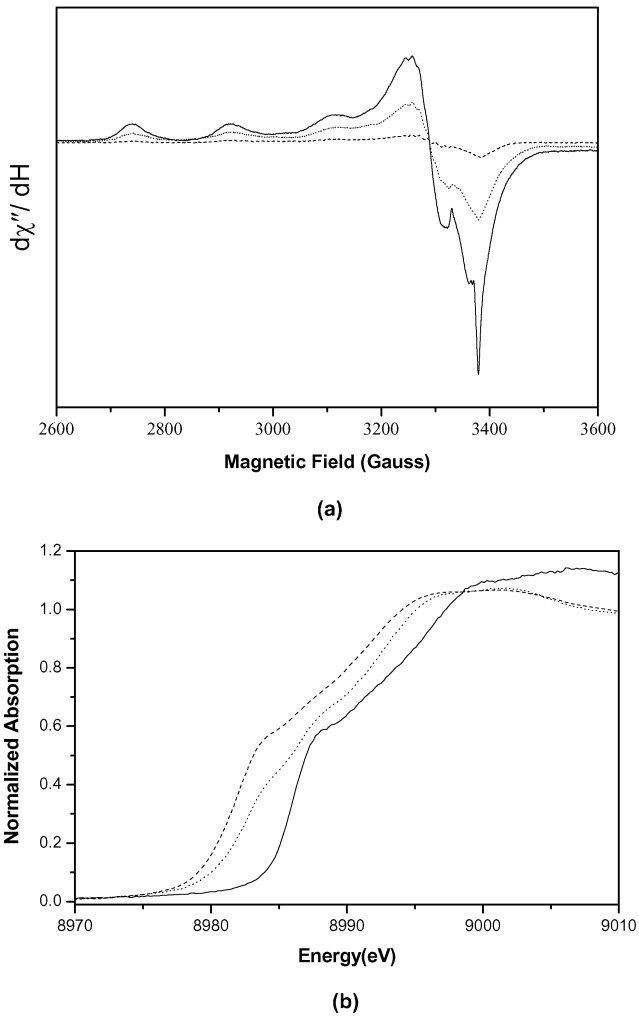


Fig. 6. Treatment of pMMO-enriched membranes with dioxygen and acetylene. 77 K X-band EPR spectra (a) and X-ray absorption K-edge spectra (b) of pMMO-enriched membranes. As-isolated pMMO-enriched membranes (dashed line); pMMO purged with pure dioxygen (dotted line); and pMMO-enriched membranes purged with pure dioxygen and treated with acetylene (solid line). EPR spectral parameters are the same as for Fig. 3. Details of the XAS experiments are given in the text.

gradual decrease of the Cu(II) EPR signal with increasing amounts of reductant. Throughout the reductive titration, the appearance of the multi-line pattern remained apparent. In other words, the ^{14}N superhyperfine structure centered at $g_{\perp} \approx 2.04$ region did not behave in conformity with the changes in the Cu(II) EPR signal intensity. This observation reflects the heterogeneity of the EPR signal, namely the observed EPR signal is a composite spectrum of contributions from a number of Cu(II) centers. Since we began this reductive titration with the "as-isolated" protein, where essentially only the copper ions of the C-clusters were oxidized, the EPR signal is comprised of contributions from both a trinuclear Cu(II) cluster as well as the type 2 Cu(II) center with its ^{14}N-superhyperfine structure, as we have already mentioned. It follows from these observations that the Cu(II) center with the ^{14}N-superhyperfine structure must possess the lowest reduction potential of all the copper ions at the catalytic site. Apparently, no ^{14}N-superhyperfine structures were associated with the EPR of the E-cluster copper ions when they became oxidized, as was previously concluded from ESEEM studies.[21]

The amount of dithionite required to obtain the protein sample with the "null" EPR signal was used to scale up the production of the fully reduced pMMO. Aliquots of the fully reduced protein were then prepared, and the pMMO samples were re-oxidized by adding varying amounts of a 0.1% hydrogen peroxide solution. Again, the protein concentration was kept constant by adding a fixed volume of the reductant (varying concentration) to equal volumes of the fully reduced pMMO sample. EPR spectra of the pMMO samples re-oxidized using different equivalents of hydrogen peroxide are shown in Fig. 8(a). As expected, the type 2 Cu(II) center with the ^{14}N-superhyperfine structure manifested itself in the EPR without interference from the copper ions of the trinuclear Cu(II) cluster upon the addition of the smallest number of equivalents of the oxidant to the sample. Thus, not surprisingly, the copper center with the lowest reduction potential was among the first to be oxidized by the hydrogen peroxide. Samples to which additional equivalents of hydrogen peroxide were added led to EPR Cu(II) spectra with increasing intensity until over 13 ± 1 copper ions of the protein were observed in the EPR, or over 93 ± 10% of the expected intensity. At the low concentrations of hydrogen peroxide used in this series of experiments, there was no evidence of degradation of the protein over the time course of the study (of the order of one minute for each addition of hydrogen peroxide). No radical EPR signals were seen during the re-oxidation of the protein by hydrogen peroxide. Comparison of the EPR of the protein re-oxidized by hydrogen peroxide and that re-oxidized by dioxygen in the presence of acetylene indicated that the pMMO had remained intact during the short time that the protein had been exposed to hydrogen peroxide.

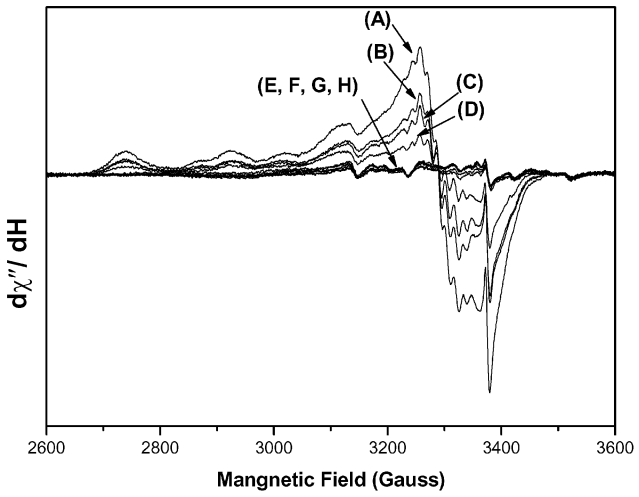


Fig. 7. Effects of gradual dithionite treatment on the 77 K X-band EPR spectra of as-isolated pMMO-enriched membranes. Dithionite concentrations: (A) 0.00 mM; (B) 1.25 mM; (C) 2.50 mM; (D) 3.75 mM; (E) 5.00 mM; (F) 7.50 mM; (G) 12.50 mM; and (H) 17.50 mM. pMMO is fully reduced in (E). Protein concentration: 0.5 mM. Reaction incubation time ~1 min after the addition each aliquot of reductant. EPR spectral parameters are the same as for Fig. 3.

X-ray K-edge spectroscopy was also used to monitor the oxidation levels of the same pMMO samples. These results are summarized in Fig. 8(b), and they fully corroborate the conclusions derived from the EPR experiments highlighted above. Up to 95 ± 10% of all the copper ions were oxidized according to X-ray spectroscopy.

Oxidative Titration of the Fully Reduced pMMO by Ferricyanide

As demonstrated earlier by Nguyen et al.,[19] as well as by others,[16] the as-isolated protein could also be fully oxidized by ferricyanide. The process was, however, complicated by the formation of ferrocyanide-adducts with two of the E-clusters.

In the present study, samples of the fully reduced enzyme was re-oxidized by slowly adding varying amounts of deoxygenated potassium ferricyanide to different equal aliquots of the fully reduced pMMO-enriched membranes in a N_2 atmosphere. In each case, a fixed volume of the ferri-

cyanide solution containing varying amounts of the oxidant was added to equal volumes of the fully reduced pMMO-enriched membranes so that the total pMMO concentration in the final solutions remained constant. The membranes were not washed with oxidant-free buffer after treatments with ferricyanide, because the oxidant was added slowly, and we assumed that no excess ferricyanide remained in the system in any of the samples. The protein samples were then examined by low-temperature EPR and X-ray absorption spectroscopy.

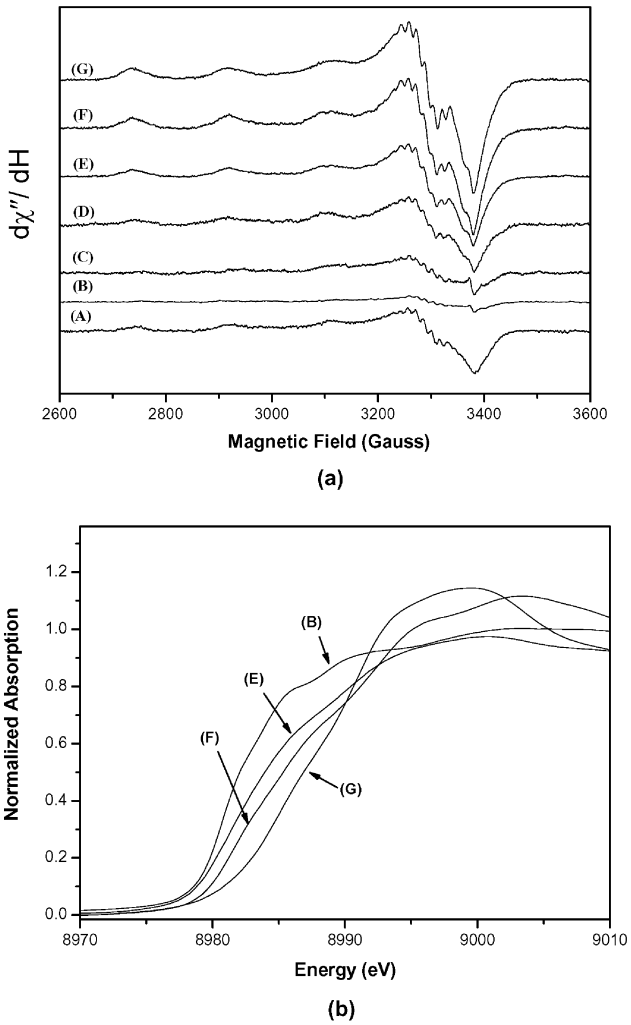


Fig. 8. Oxidation of pMMO-enriched membranes by hydrogen peroxide. 77 K X-band EPR spectra (a) and X-ray absorption K-edge spectra (b) of pMMO-enriched membranes. (A) As-isolated pMMO-enriched membranes; (B) pMMO fully reduced by sodium dithionite; (C) pMMO oxidized by 1 equivalent H_2O_2; (D) pMMO oxidized by 50 equivalents H_2O_2; (E) pMMO oxidized by 100 equivalents H_2O_2; (F) pMMO oxidized by 200 equivalents H_2O_2; and (G) pMMO oxidized by 300 equivalents H_2O_2 (fully oxidized). Reaction incubation time ~1 min after the addition of each aliquot of oxidant. Protein concentration: 0.4 mM. 1 equivalent H_2O_2 corresponds to 0.4 mM. EPR spectral parameters are the same as for Fig. 3. Details of XAS experiments are given in the text.

In Fig. 9(a), we compare the EPR spectra recorded after the addition of different levels of ferricyanide (C, D, and E) with that of the fully reduced (B) and the as-isolated preparations (A). With increasing ferricyanide, the trinuclear-Cu(II) cluster signal at g ~ 2.1 and the type 2 Cu(II) signal with the ^{14}N-superhyperfine structure gradually crept in, indicating that the copper ions of the C-clusters were the first to become oxidized. However, the details of the EPR (see spectra C and D) did not exactly match those of the as-isolated enzyme, suggesting different chemistry at the active site when the C-clusters are oxidized by dioxygen and ferricyanide. At higher ferricyanide levels added to the reduced protein, the spectrum grew mostly in the low field region toward g > 2.1, reflecting formation of the ferricyanide-Cu_3 adducts. Double integration of spectrum G indicated that 92 ± 10% of the expected EPR intensity had been realized.

Earlier, Nguyen et al.[19] attempted to oxidize the copper ions of the as-isolated enzyme further by ferricyanide to include oxidation of the E-clusters. At essentially stoichiometric proportions of ferricyanide to protein, 2:1 ferrocyanide-pMMO adducts were formed. Structural evidence for the adduct came from Fe and Cu EXAFS data, where Cu-backscattering was observed in the Fe EXAFS, and Fe-backscattering was observed in the Cu EXAFS (Fig. 10).[26] Assignment of the iron oxidation state as Fe(II) came from FTIR of the adduct from the vibration frequencies of the coordinated cyanides (Fig. 11). The strong band at 2096 cm^{-1} observed for the C≡N was consistent with Fe(II), as the C≡N stretch appeared at 2095 cm^{-1} for $Cu(II)_2[Fe(II)(CN)_6]$ and 2177 cm^{-1} for $Cu(II)_3[Fe(III)(CN)_6]_2$. No bands due to free ferrocyanide or ferricyanide were observed for the protein solution verifying that the ferrocyanide was associated with the protein as an adduct and there was no free ferricyanide in the solution.

The EPR of the ferrocyanide-copper adduct formed under stoichiometric ferricyanide-pMMO conditions as well as the limiting adduct obtained upon complete oxidation of all the copper ions in the E-clusters gave a broad signal at g ~ 2.14, in agreement with related synthetic model compounds.[25] Excess ferricyanide was required to fully oxidize all the copper ions in the protein. Presumably the E-cluster copper ions

and the ferrocyanide-adducts serve as a conduit to transfer the remaining reducing equivalents from within the protein to the excess ferricyanide in the solution via an outer sphere mechanism, and the stoichiometry of the adduct remained unchanged, namely 1 Fe to 8 Cu, or 2 Fe to 14-15 Cu.

The various ferricyanide-oxidized preparations were also examined by Cu K-edge X-ray spectroscopy. As depicted in Fig. 9(b), the feature at 8984 eV due to Cu(I) was gradually reduced in intensity as the fully reduced 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

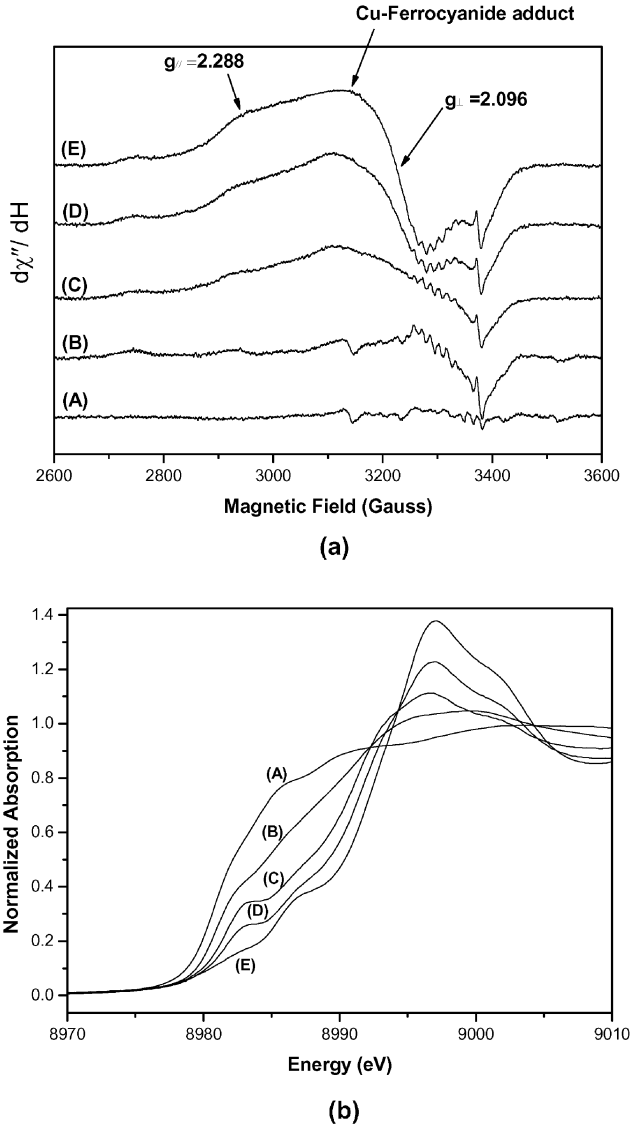


Fig. 9. Ferricyanide oxidation of pMMO-enriched membranes. 77 K X-band EPR spectra (a) and X-ray absorption K-edge spectra (b) of pMMO-enriched membranes. (A) pMMO fully reduced by sodium dithionite; (B) pMMO oxidized by 1.67 mM ferricyanide; (C) pMMO oxidized by 3.33 mM ferricyanide; (D) pMMO oxidized by 5.00 mM ferricyanide; and (E) pMMO oxidized by 6.67 mM ferricyanide (fully oxidized). Reaction incubation time ~1 min after the addition each aliquot of oxidant. Protein concentration: 0.4 mM. EPR spectral parameters are the same as for Fig. 3. Details of XAS experiments are given in the text.

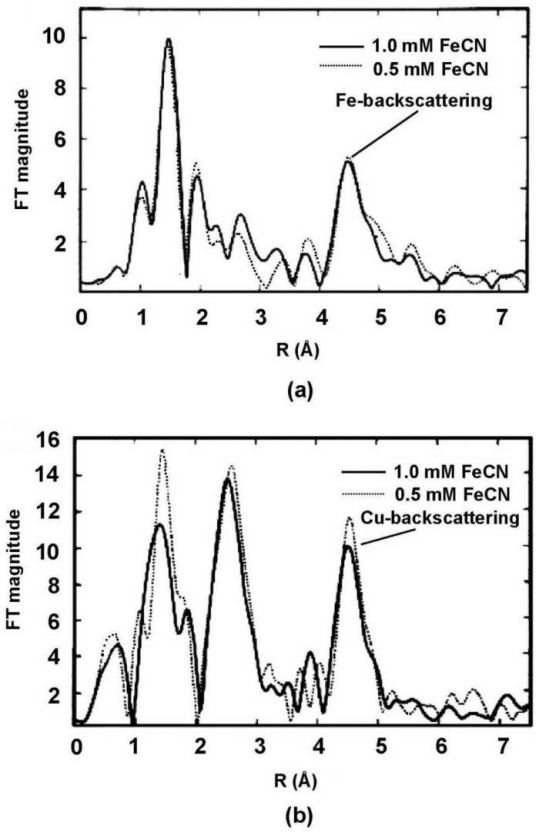


Fig. 10. Fourier transform (FT) (k-range = 3-13 $Å^{-1}$) of filtered EXAFS data for the ferrocyanide-pMMO adduct after ferricyanide treatment of pMMO-enriched membranes and extensive washing of membranes to remove unbound ferrocyanide or ferricyanide. (a) Cu EXAFS with Fe-backscattering highlighted at R = 4.5 Å; and (b) Fe EXAFS with Cu-backscattering highlighted at R = 4.5 Å. Protein concentration: ~0.5 mM. Fe/Cu stoichiometry (1:8 or 2 Fe to ~15 Cu) was determined from comparison of the calibrated intensity of Cu and Fe edges. Data taken from Ref. 26.

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).

DISCUSSION

Recently, we have devised an improved method for culturing and harvesting cells of *M. capsulatus* (Bath) to obtain high quality pMMO-enriched membranes with high specific activities in high yields.[10] With this procedure, the plasma membrane of these cells could be enriched in pMMO to the extent of 80-90% of the total membrane proteins. These pMMO-enriched membranes exhibited sensitivity to both NADH and duroquinol. Also, a pMMO-detergent complex was readily obtained by solubilization of the pMMO-enriched membranes with dodecyl-β-D-maltoside followed by fractionation of the solubilized proteins on a size-exclusion column. This pMMO-detergent complex was shown to be a αβγ monomer, containing ~14 gram atoms of copper ions per monomer, but no iron. Thus, the pMMO in *M. capsulatus* (Bath) is a multi-copper protein.

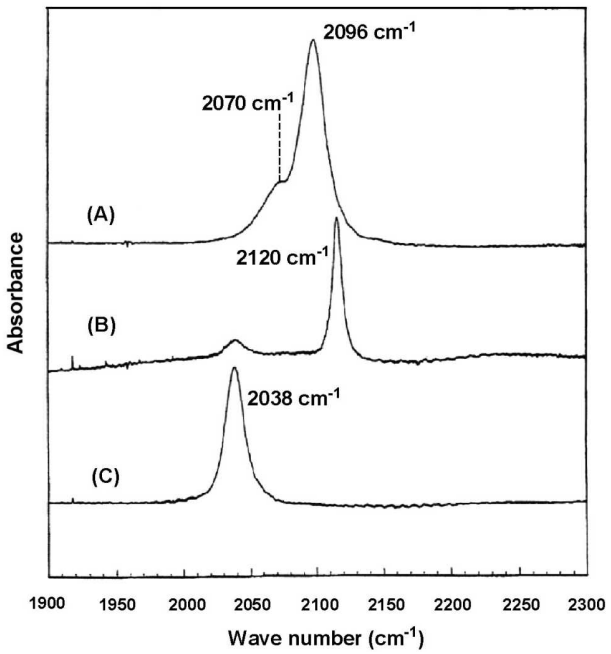


Fig. 11. Comparison of the FTIR spectrum of the ferricyanide-oxidized pMMO membranes with the corresponding spectra for ferrocyanide and ferricyanide in solution. Only the region depicting the C≡N stretching vibration is shown. Ferricyanide-oxidized pMMO (A); ferricyanide (B); ferrocyanide (C).

These results are in total agreement with the earlier work of Nguyen et al.[9] who also proposed that the ~15 copper ions in pMMO could be grouped into 2 C-clusters and 3 E-clusters, each consisting of a triad of copper ions.[19] It was hypothesized that the C-clusters participate in the catalytic chemistry of the enzyme, namely, dioxygen activation and alkane hydroxylation. The E-clusters copper ions, which were normally reduced, were suggested to provide a reservoir of reducing equivalents for the C-clusters during turnover. When all the copper ions were oxidized, low temperature magnetization measurements on the protein suggested that the coppers are grouped into trinuclear clusters with quartet ground states. With the exchange interaction written as $-2J[S_1 \bullet S_2 + S_2 \bullet S_3 + S_3 \bullet S_1]$, J was estimated to be 15-20 cm^{-1} from the temperature dependence of the bulk magnetic susceptibility. An average zero-field splitting parameter, D, was also estimated to be of the order of + 0.05 cm^{-1} (500 Gauss), or less, for the quartet states from a Curie plot of the data at the lowest temperatures.

The energy levels for such a trinuclear Cu(II) cluster are illustrated in Fig. 6 of our companion paper.[23] Here we have assumed an equilateral triad. The two excited doublet states are then degenerate. At 3 K, the lowest temperature in our EPR measurements, only the quartet manifold is significantly occupied for J ~ 15-20 cm^{-1} (the doublet states lie 3J above the quartet state). Since D is small, a central feature dominates the spectrum of the cluster. The principal contribution arises from the $-½ \rightarrow ½$ transition, which is essentially isotropic for small D's. While the $-3/2 \rightarrow -½$ and $-½ \rightarrow 3/2$ transitions are highly anisotropic and their resonance fields occur at lower and higher g-values, for sufficiently small D's, these transitions contribute mainly to asymmetric broadening of the central resonance. The EPR of the C-cluster recorded for the as-isolated pMMO manifests these features. We refer the interested reader to our companion paper[23] for a detailed discussion of the issues relating to this overall problem.

Evidence for a Trinuclear Cu(II) Cluster and a Type 2 Cu(II) Center in As-Isolated pMMO

In our earlier work, Nguyen et al.[18] exploited the differ-

ent EPR saturation properties of a quartet species compared to that of a doublet species that is typical of an isolated type 2 Cu(II) center, to obtain the quartet spectrum. Because of the existence of low lying doublet states for the trinuclear Cu(II)-cluster, it is much more difficult to power saturate the -½ → ½ transition within the quartet manifold of the cluster species compared to the EPR transition of the typical isolated type 2 Cu(II) center. In the present work, we elected to deconvolute the composite 3 K spectrum observed for the as-isolated pMMO at the lowest microwave power into a sum of the component spectra for the putative cluster species and the corresponding type 2 Cu(II) center. The results are shown in Fig. 2 at 3 K, with the type 2 Cu(II) center featured by its Cu(II) hyperfine structure at $g_{||} \sim 2.2$ and the trinuclear Cu(II) cluster by the powder spectrum of the quartet with $D \approx + 0.017$ cm^{-1} (175 Gauss) and E/D = 0.15. Double integration of the two components gave an EPR intensity ratio of 1.0 ± 0.2 (cluster to type 2). The corresponding intensity ratio at 3 K was predicted to be 0.96 ± 0.1 if the C-clusters of the as-isolated pMMO are supposed to be made up of an equilateral triad with J ~ 15-20 cm^{-1} together with a type 2 Cu(II) center.

It was also possible to deconvolute the 77 K EPR spectrum of the as-isolated enzyme into a type 2 Cu(II) signal and a trinuclear Cu(II) cluster signal with D increased slightly to + 0.019 cm^{-1} (200 Gauss) (Fig. 4). Double integration of the component signals gave an intensity ratio of 0.8 ± 0.2 (cluster to type 2), again in good agreement with prediction of 1.0 ± 0.1 based on the equilateral triad model with J = 20 cm^{-1}. The latter signal is now more complex, as we must include contributions from the transitions within the two doublet manifolds, in addition to the contributions from the quartet manifold.

Thus, we conclude from these results that there are two trinuclear copper clusters at the catalytic site of pMMO, which during quick turnover in air in the absence of the hydrocarbon substrate, yield a fully oxidized trinuclear Cu(II) cluster at one site and a type 2 Cu(II) center at the other.

Proposed Oxidation Chemistry Mediated by the C-Clusters of pMMO in the Absence of Hydrocarbon Substrate

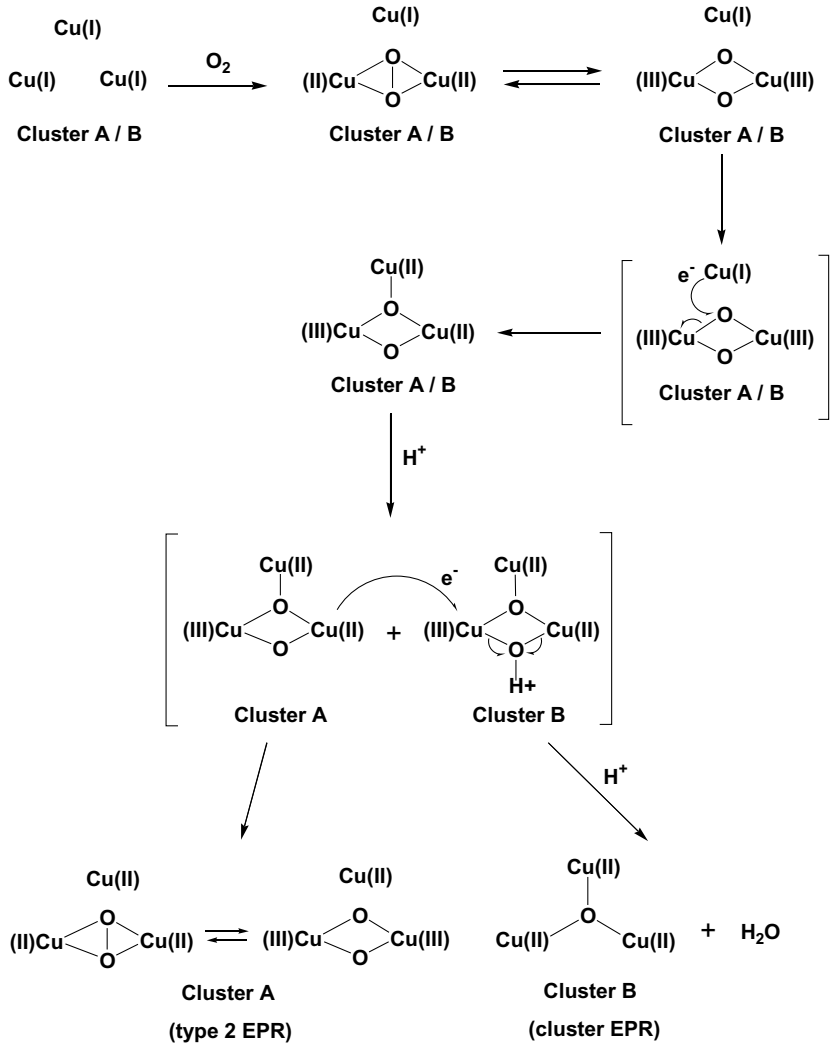
A scenario that leads to the above exact outcome for the hypothesized C-clusters in the absence of hydrocarbon substrate is summarized in Scheme II. Imagine two fully reduced trinuclear copper clusters A and B, and allow them to react with dioxygen. Assume that the oxidation at both sites proceeds beyond the Cu(I)-(μ-η^2:η^2-peroxo)Cu^{II}_2 tricopper core, initially to the Cu(I)-bis(μ-oxo)Cu^{III}_2 tricopper core, and then ultimately to the Cu(II)-mixed valence bis(μ-oxo)$Cu^{II}Cu^{III}$ tricopper core. The first part is known chemistry for a number of dicopper models.[27,28] In connection with the second, Fukuzumi and coworkers have reported evidence for reductive activation of a model bis(μ-oxo)Cu^{III}_2 dicopper core to promote oxo-transfer in the C-H bond activation of external substrates.[29] Now if the two C-clusters are sufficiently close to each other in the membrane domains of the protein to allow intercluster electron transfer, say from cluster A to cluster B, this electron transfer reaction would culminate in the formation of a fully oxidized trinuclear Cu(II) cluster at site B, and at site A, a type 2 Cu(II) center together with a proposed equilibrium between a bis(μ-oxo)Cu^{III}_2 dicopper core and (μ-η^2:η^2-peroxo)Cu^{II}_2 dicopper core. The EPR of the enzyme following this oxidation scenario would then consist of a trinuclear Cu(II) cluster from cluster B, and a type 2 Cu(II) center from cluster A, in accordance with our 3 K and 77 K EPR results on the as-isolated enzyme (all the other copper ions in the protein are reduced!).

Note that the dioxygen chemistry that takes place at the C-centers dictates the formation of a bis(μ-oxo)Cu^{III}_2 dicopper core or a (μ-η^2:η^2-peroxo)Cu^{II}_2 dicopper core at cluster B, or an equilibrium between these two species. This is not a speculation but a logical deduction from the chemistry if the oxidation chemistry involves two C-clusters. Starting with 6 Cu(I) ions, and two dioxygen molecules, all the copper ions must necessarily be oxidized following this chemistry. Since one type 2 Cu(II) center and one trinuclear Cu(II) copper cluster are detected in the EPR following the dioxygen chemistry, the remaining two Cu(II) ions are apparently EPR invisible. Accordingly, the two remaining Cu(II) ions must participate in the formation of an EPR silent antiferromagnetically coupled bis(μ-oxo)Cu^{III}_2 dicopper core or a (μ-η^2:η^2-peroxo)Cu^{II}_2 dicopper core. There is simply no other possible scenario.

Proposed Oxidation Chemistry Mediated by the C-Clusters of pMMO in the Presence of Hydrocarbon Substrate

The scenario summarized in Scheme II is, of course, not physiological, as the enzyme is designed to mediate the transfer of one of the oxygen atoms from one of the dioxygen molecules to an alkane. As shown in Scheme III, this feature of the chemistry is readily incorporated into the reaction scheme by including the oxo-transfer chemistry at C-cluster site A. Again, following the hydroxylation of the alkane, the extra

Scheme II



electron at site A could be transferred to site B to complete the reduction of the second dioxygen molecule to two water molecules and complete the oxidative phase of the turnover cycle of the enzyme. If this reaction scheme correctly describes the chemistry mediated by pMMO, then the chemical equation should be written as

$$CH_4 + 2\,O_2 + 3\,NADH + 3\,H^+ \rightarrow CH_3OH + 3\,H_2O + 3\,NAD$$

rather than the one generally assumed, namely,

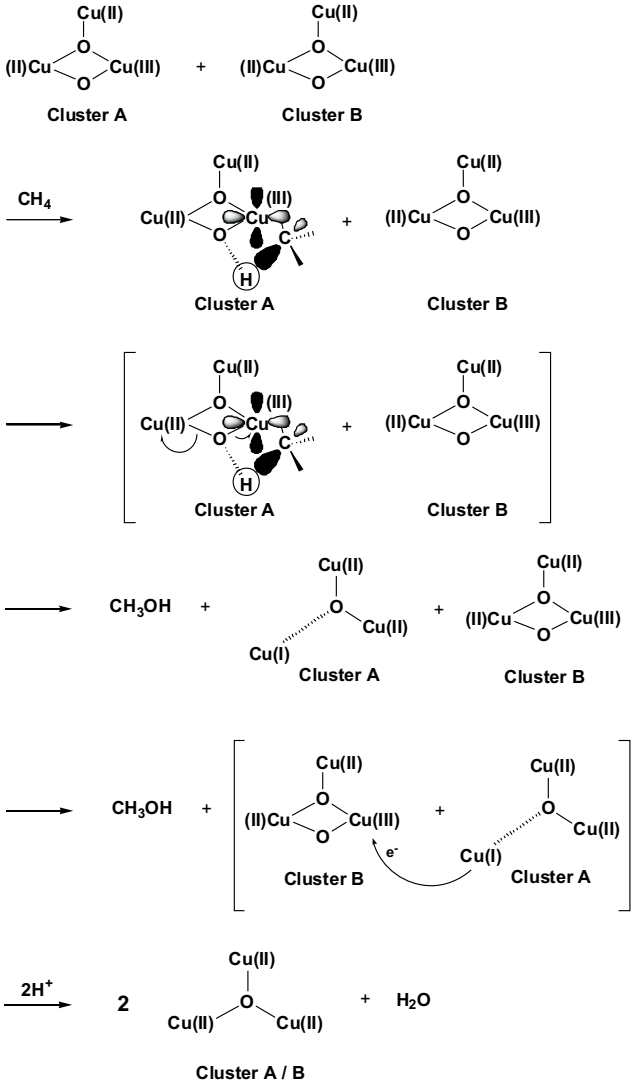
$$CH_4 + O_2 + NADH + H^+ \rightarrow CH_3OH + H_2O + NAD.$$

In other words, pMMO mediates the transfer of 6 reducing equivalents from 3 NADH (or other reductants) to 2 dioxygen molecules to carry out the dioxygen chemistry and the alkane hydroxylation. The requirement for 6 reducing equivalents would provide a rationale for the two C-clusters in the enzyme.

Ligand Structure of the Type 2 Center

The ^{14}N-superhyperfine structure in the $g_\perp$ region of the type 2 Cu(II) signal indicates that the type 2 Cu(II) center in C-cluster A is coordinated to nitrogen ligands. ^{15}N-labeling of the enzyme confirmed that the superhyperfine structure is derived from nuclear coupling to nitrogens. From ESEEM and ENDOR experiments,[21] we have previously concluded that these nitrogens are from the imidazoles of histidines. Three and four nitrogen-bearing ligands have been suggested in the literature;[15] however, our own spectral simulations of the ^{14}N-pMMO and ^{15}N-pMMO EPR indicated that the data are best fitted with a rhombic model with only two nitrogens

Scheme III



or imidazoles involved. At this juncture, it is not unreasonable to speculate that one of these histidines is involved in the covalent labeling of the protein during the acetylene suicide substrate reaction.

Electron Transfer

An interesting, if not puzzling, feature of the as-isolated pMMO is why the transfer of reducing equivalents from the E-clusters to the C-clusters is so sluggish in this form of the enzyme. Apparently, when the enzyme is inhibited by acetylene or other small alkynes, the barrier to this electron transfer becomes sufficiently lowered to allow the reducing equivalents stored in the E-clusters to be quickly drained out of the enzyme in the presence of dioxygen. This important observation suggests that the oxo-transfer chemistry at C-cluster A is allosterically linked to re-reduction of the C-clusters by the E-clusters. Hydrogen peroxide has the same effect, re-oxidizing all the copper ions in the enzyme and turning over the enzyme completely. Presumably, hydrogen peroxide interacts with the enzyme at the site of the C-clusters as does dioxygen. Although it is also possible to re-oxidize the enzyme using ferricyanide, the chemistry is different because of two ferrocyanide-Cu_3 adducts that are formed on the aqueous-exposed domains of the 45 kDa subunit. Here, the flow of the electrons is in the opposite direction from that during normal turnover of the enzyme.

E-Clusters

While EPR of the fully oxidized protein at 3 K indicate that the copper ions in the E-clusters are most likely also arranged in triads, these copper clusters are fundamentally different from the C-clusters. First, there is no evidence that the E-clusters react with dioxygen directly. Presumably, these clusters are coordination saturated. Second, even though all the available data so far indicate that the motions of the Cu(II) spins in the oxidized E-clusters are also correlated, and the exchange coupling is also ferromagnetic, the exchange interaction (J) and the zero-field splitting parameter (D) appear to be significantly smaller for these oxidized E-cluster triads than for the C-clusters so that they are effectively thermally decoupled at 77 K and manifest themselves essentially as-isolated type 2 Cu(II) ions in the EPR at the higher temperatures.

SUMMARY

In this study, we have extended the earlier work of Nguyen et al.[9,18,19] to clarify the structure of the copper clusters in pMMO. The present studies have been undertaken on pMMO-enriched membranes that have been prepared by an improved method that we have devised for culturing cells of *Methylococcus capsulatus* (Bath).[10] This improved technology afforded high-quality pMMO-membranes with high specific activity in high yields. A detailed study by parallel EPR and X-ray Cu K-edge absorption spectroscopy of the as-isolated pMMO in these pMMO-enriched membranes not only confirmed the earlier proposal by the Chan group at Caltech that six of the copper ions are sequestered into two trinuclear copper clusters,[19] but also shed new insights into how the two reduced C-clusters react with dioxygen to give the oxygen-

ated species at the C-clusters that we have attempted to delineate in this work. Models of the dioxygen chemistry at the C-clusters both in the absence and presence of alkane substrate have been presented and shown to be consistent with the turnover chemistry of the enzyme as well as the results of the physicochemical measurements that we are reporting here. A characteristic feature of the C-clusters in the as-isolated pMMO is that one of them exists as an almost equilateral triad of oxidized Cu(II) ions, while the other comprises a type 2 Cu(II) ion together with a proposed bis(μ-oxo)dicopper(III) or (μ-η^2:η^2-peroxo)Cu^{II}_2 dicopper core. Evidence was presented for coordination of the type 2 center to 2 nitrogens of imidazoles or histidines. In addition to the C-clusters, we have also shown that it is possible to re-oxidize the remaining 8 or 9 copper ions or the reduced E-clusters by ferricyanide, hydrogen peroxide, as well as dioxygen in the presence of the suicide substrate acetylene. These redox titrations were found to be facile, indicating that the E-clusters are also part of the protein, as suggested earlier by Nguyen et al.[19] However, although the copper ions in the E-clusters are most likely also arranged in triads, these copper clusters are fundamentally different from the C-clusters. Most significantly, there is no evidence to suggest that the E-clusters react with dioxygen directly.

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REFERENCES

1. Hanson, R. S.; Hanson, T. E. *Micorbiol. Rev.* **1996**, *60*, 439.
2. Periana, R. A.; Taube, D. J.; Gamble, S.; Taube, H.; Satoh, T.; Fujii, H. *Science* **1998**, *280*, 560.
3. Dewitt, J. G.; Rosenzweig, A. C.; Salifoglou, A.; Hedman, B.; Lippard, S. J.; Hodgson, K. O. *Inorg. Chem.* **1995**, *34*, 2505.
4. Lee, S. K.; Nesheim, J. C.; Lipscomb, J. D. *J. Biol. Chem.* **1993**, *268*, 21569.
5. Merkx, M.; Kopp, D. A.; Sazinsky, M. H.; Blazyk, J. L.; Müller, J.; Lippard, S. J. *Angew. Chem. Int. Ed. Engl.* **2001**, *40,* 2782.
6. DeRose, V. J.; Liu, K. E.; Lippard, S. J.; Hoffman, B. M. *J. Am. Chem. Soc.* **1996**, *117*, 121.
7. Liu, Y.; Nesheim, J. C.; Lee, S. K.; Lipscomb, J. D. *J. Biol. Chem* **1995**, *270*, 24662.
8. (a) Chan, S. I.; Nguyen, H.-H. T.; Shiemke, A. K.; Lidstrom, M. E. In *Bioinorganic Chemistry of Copper*; Karlin, K. D., Tyeklar, Z., Eds.; Chapman and Hall: New York, 1993. (b) Chan, S. I.; Nguyen, H.-H. T.; Shiemke, A. K.; Lidstrom, M. E. In *Microbial Growth on C1 Compounds*; Murrell, J. C., Kelly, D. P., Eds.; Intercept: Andover, Hampshire, U.K., 1993.
9. Nguyen, H.-H. T.; Elliott, S. J.; Yip, J. H.-K.; Chan, S. I. *J. Biol. Chem.* **1998**, *273*, 7957.
10. Yu, S. S.-F.; Chen, K. C.-H.; Tseng M. Y.-H.; Wang, Y.-S.; Tseng, C.-F.; Chen, Y.-J.; Huang, D.-H.; Chan, S.-I. *J. Bacteriol.* **2003**, *185*, 5915.
11. Stanley, S. H.; Prior, S. D.; Leak, D. J.; Dalton, H. *Biotechnol. Lett.* **1983**, *5*, 487.
12. Madigan, M. T.; Martnko, J. M.; Parker, J. *Biology of Microorganisms.* 8th Ed.; Simon & Schuster: USA, 1997.
13. Zahn, J. A.; DiSpirito, A. A. *J. Bacteriol.* **1996**, *178*, 1018.
14. Lieberman, R. L.; Shrestha, D. B.; Doan, P. E.; Hoffman, B. M.; Stemmler, T. L.; Rosenzweig, A. C. *Proc. Natl. Acad. Sci. USA,* **2003**, *100*, 3820.
15. Lemos, S. S.; Collins, M. L. P.; Eaton, S. S.; Eaton, G. R.; Antholine, W. E. *Biophys. J.* **2000**, *79*, 1085.
16. Takeguchi, M.; Miyakawa, K.; Okura, I. *J. Mol. Catal.* **1999**, *137*, 161.
17. Xin, J. Y.; Cui, J. R.; Hu, X. X.; Li, S. B.; Xia, C. G.; Zhu, L. M.; Wang, Y. Q. *Biochem. Biophys. Res. Comm.* **2002**, *295*, 182.
18. Nguyen, H. H. T.; Shiemke, A. K.; Jacobs, S. J.; Hales, B. J.; Lidstrom, M. E.; Chan, S. I. *J. Biol. Chem.* **1994**, *269*, 14995.
19. Nguyen, H. H. T.; Nakagawa, K. H.; Hedman, B.; Elliott, S. J.; Lidstrom, M. E.; Hodgson, K. O.; Chan, S. I. *J. Am. Chem. Soc.* **1996**, *118*, 12766.
20. Semrau, J. D.; Zolandz, D.; Lidstrom, M.; Chan, S. I. *J. Inorg. Biochem.* **1995**, *58*, 235.
21. Elliott, S. J.; Randall, D. W.; Britt, R. D.; Chan, S. I. *J. Am. Chem. Soc.* **1998**, *120*, 3247.
22. Prior, S. D.; Dalton, H. *FEMS Microbiol. Letts.* **1985**, *29*,

105.
23. Hung, S.-C.; Chen, C.-L.; Chen, K. H.-C.; Yu, S. S.-F.; Chan, S. I. *J. Chin. Chem. Soc.* **2004**, *51*, 1229.
24. Cook, S. A.; Shiemke, A. K. *J. Inorg. Biochem.* **1996**, *64*, 273.
25. Elliott, S. J. Ph D. Dissertation, California Institute of Technology, Pasadena, CA, 2000.
26. Nguyen, H. H. Ph D. Dissertation, California Institute of Technology, Pasadena, CA, 1998.
27. Tolman, W. B. *Acc. Chem. Res*. **1997**, *30*, 227.
28. Mahadevan, V.; Henson, M. J.; Solomon, E. I.; Stack, T. D. P. *J. Am. Chem. Soc*. **2000**, *122*, 10249.
29. Taki, M.; Itoh, S.; Fukuzumi, S. *J. Amer. Chem. Soc*. **2001**, *123*, 6203.