From micelles to bicelles: Effect of the membrane on particulate methane monooxygenase activity

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Running title: pMMO from micelles to bicelles

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**Keywords:** particulate methane monooxygenase, methane oxidation, methanotroph, bicelle, membrane protein, copper metalloenzyme, protein-lipid interaction, biofuel, biotechnology

Data deposition: The coordinates of pMMO from *Methylomicrobium alcaliphilum* 20Z have been deposited in the Protein Data Bank (PDB accession code 6CXH).

ABSTRACT

Particulate methane monooxygenase (pMMO) is a copper-dependent, integral membrane metalloenzyme that converts methane to methanol in methanotrophic bacteria. Studies of isolated pMMO have been hindered by loss of enzymatic activity upon its removal from the native membrane. To characterize pMMO in a membrane-like environment, we reconstituted pMMOs from *Methyllococcus (Mec.) capsulatus* (Bath) and *Methylmicrobium (Mm.) alcaliphilum* 20Z into bicelles. Reconstitution into bicelles recovers methane oxidation activity lost upon detergent solubilization and purification without substantial alterations to copper content or copper electronic structure as observed by electron paramagnetic resonance (EPR) spectroscopy. These findings suggest that loss of pMMO activity upon isolation is due to removal from the membranes rather than caused by loss of the catalytic copper ions. A 2.7 Å resolution crystal structure of pMMO from *Mm. alcaliphilum* 20Z revealed a mononuclear copper center in the PmoB subunit. These results underscore the importance of studying membrane proteins in a membrane-like environment, and provide valuable insight into pMMO function.

Introduction

Methanotrophic bacteria convert methane, the second most abundant greenhouse gas, to methanol in the first step of their metabolic pathway (1,2). As the main methane sink in nature, these microorganisms are promising biological tools for methane remediation and biofuel production (3-6). Methanotrophs activate a 105 kcal/mol C-H bond in methane using metalloenzymes called methane monooxygenases (MMOs), which are classified as soluble (sMMO) or membrane-bound (particulate, pMMO) (7). pMMO is the predominant methane oxidation catalyst in nature, but is less well characterized (8). A detailed understanding of methane oxidation by pMMO has the potential to guide synthetic catalyst design and to facilitate methanotroph engineering.

pMMO is a complex integral membrane enzyme that requires copper for activity (9-11). Crystal structures of pMMO from four different methanotrophs reveal a 300 kDa α3β3γ3 trimer, composed of subunits PmoA, PmoB, and PmoC (11-14). PmoA and PmoC are integral membrane subunits, whereas PmoB consists of two
periplasmic domains linked by two transmembrane helices. Present in all these structures is a copper site at the N-terminus of PmoB, with the N-terminal histidine of PmoB and two histidines from an HXH motif as ligands. This copper center, assigned as the active site (10), has been modeled with either one or two copper ions in the different structures. An additional PmoB monocopper site is found only in the structure of pMMO from Methylomicrobium (Mm.) alcalophilum 20Z (20Z-pMMO) and Mcc. capsulatus (Bath) (Bath-pMMO) into bicelles. Bicelle reconstitution recovers methane oxidation activity of both pMMOs without addition of exogenous copper ions or substantial alteration in the copper sites as observed by electron paramagnetic spectroscopy (EPR). A crystal structure of 20Z-pMMO provides some insight into how solubilization might affect protein stability. Finally, extended X-ray absorption fine structure (EXAFS) analysis of 20Z-pMMO does not indicate the short Cu-Cu interaction observed in previous samples, prompting further investigation of the active site nuclearity.

**Results**

**Recovery of $^{13}$C-methane oxidation activity by bicelle reconstitution**

To systematically investigate loss of pMMO activity, methane oxidation activity was measured for as-isolated, solubilized, purified, and bicelle-reconstituted pMMO samples. pMMO activity assays are typically performed using either NADH or duroquinol as a reductant. Duroquinol can directly reduce pMMO, whereas a type 2 NADH dehydrogenase (NDH-2) likely oxidizes NADH and reduces quinones for subsequent electron transfer to pMMO (9). Solubilization with the detergent n-dodecyl-$\beta$-D-maltopyranoside (DDM) separates pMMO from the membranes (solubilized pMMO), which abrogates NADH-driven activity (Fig. 1). Solubilized pMMO was then reconstituted in bicelles to mimic the lipid bilayer and to investigate membrane-dependent activity loss. Methane oxidation activity was measured for as-isolated membranes, solubilized and purified pMMO in detergent (DDM), and bicelle [3% (w/v) DMPC-CHAPSO] reconstituted pMMO using both reductants (Fig. 1). Due to $^{12}$C methanol contamination in many buffers and reagents, a new activity assay was developed in which conversion of $^{13}$C methane to $^{13}$C methanol is detected via GC-MS.

Methane oxidation activities for Bath-pMMO and 20Z-pMMO were measured at 30 °C after 5 min, due to solidification of bicelles at higher temperatures and longer incubation times. NADH-driven activity (41.1 ± 1.7 and 14.5 ± 1.2 nmol $^{13}$C methanol·mg$^{-1}$ protein for Bath-pMMO
and 20Z-pMMO, respectively) is abolished upon solubilization and purification (Fig. 1, Table S1). For membrane-bound and solubilized samples, the activity measured using duroquinol was significantly lower than the NADH-driven activity for Bath-pMMO and not detected for 20Z-pMMO (Fig. 1). For both pMMOs, reconstitution into bicelles recovers methanol oxidation activity of solubilized and purified samples using duroquinol as a reductant (Fig. 1, Table S1). However, NADH-driven activity is only restored for Bath-pMMO. It may be that an NDH-2 or other components of the electron transport chain responsible for NADH-dependent methanol oxidation are not properly reassembled after solubilization and reconstitution of 20Z-pMMO.

Notably, for both pMMOs, duroquinol-driven activity is significantly higher for bicelle-reconstituted samples than for as-isolated membranes and is comparable to NADH-driven activity in membranes (Fig. 1, Table S1). The different properties of phosphatidylcholine (PC), the main lipid in DMPC bicelles, and phosphatidylethanolamines (PE), the predominant phospholipid found in these methanotrophs (29-31) provide a possible explanation for this observation. The amine head group of PC is less polar than that of PE and may increase solubility and access of duroquinol, as well as O₂ and methane. Additionally, DMPC is composed of saturated 14:0 PC whereas methanotroph PEs are primarily composed of a saturated and unsaturated mixture of 16:0 and 16:1 PE. The various head groups and acyl chain compositions can affect lipid packing, membrane fluidity, and even the structure of membrane proteins (32). Finally, in as-isolated membranes, it is possible that the native quinones occupy the binding site that duroquinol needs to access in order to reduce pMMO. Taken together, these results indicate that solubilized pMMOs are not irreversibly inactivated. Interestingly, solubilized or purified pMMO samples were reconstituted in bicelles without the addition of copper, suggesting that bicelles alone are responsible for the recovered activity.

**Effect of bicelle reconstitution on pMMO copper centers**

To further investigate the relationship between bicelle reconstitution and the pMMO copper sites, the copper concentrations of pMMO samples in as-isolated membranes, detergent, and bicelles were measured using ICP-OES. The presence of ~3 copper ions per protomer in purified Bath-pMMO (Fig. 2, Table S2) is consistent with previous studies (12). Purified 20Z-pMMO contains approximately 2.7 eq of copper per protomer (Table S2). The copper contents of the native membranes and solubilized pMMOs are batch dependent, accounting for the variability in copper stoichiometry values for these samples. Loss of some adventitiously bound copper is also typically observed during solubilization and purification (11).

The copper stoichiometry does not change between pMMO samples in detergent and in bicelles (Fig. 2, Table S2). This observation, in conjunction with the recovered activity, indicates that the catalytically essential copper ions are still present in detergent-solubilized pMMO samples. The differences in activity between as-isolated membranes, detergent-solubilized pMMO, and bicelle-reconstituted pMMO therefore cannot be attributed to changes in copper content. Consequently, the membrane, and not copper depletion, is a crucial factor contributing to activity loss upon solubilization.

To directly assess the Cu²⁺ electronic and geometric structure through the bicelle reconstitution process, we collected EPR spectra of Bath-pMMO and 20Z-pMMO before and after bicelle reconstitution (Fig. 3). A previous EPR analysis of purified Bath-pMMO revealed two distinct Type 2 Cu²⁺ signatures (33). The bicelle-reconstituted Bath-pMMO exhibits the same Cu²⁺ EPR spectrum as the purified Bath-pMMO, and is simulated with the same parameters as reported previously. However, the bicelle-reconstituted enzyme contains slightly more Cu²⁺ per protomer than the purified sample. Consequently, some of the Cu²⁺ observed in the bicelle-reconstituted sample is Cu¹⁺ in the purified sample and oxidizes to Cu²⁺ during the reconstitution procedure.

The purified 20Z-pMMO EPR spectrum exhibits the Cu²⁺ spectrum seen in both forms of Bath-pMMO as well as a small contribution from additional Cu³⁺ resonance (Fig. 3B, Table S2), suggesting adventitious Cu²⁺ binding to 20Z-pMMO in a site either unoccupied or containing Cu¹⁺ in Bath-pMMO. Similar to Bath-pMMO, incorporation of 20Z-pMMO into bicelles oxidizes
some Cu$^{1+}$ to Cu$^{2+}$, as evidenced by the slightly altered $g_\perp$ region and increased amount of Cu$^{2+}$ per protomer (Table S2), but the signal is otherwise the same as observed for the purified sample.

Importantly, the EPR spectra of both pMMOs show that the Cu$^{2+}$ ligation is not substantially altered by the bicelle incorporation procedure. Therefore, the appreciable recovery of pMMO activity upon insertion of Bath-pMMO into the bicelle is not due to differences in the active site copper structure, consistent with the notion that the membrane environment plays a critical role in modulating activity.

Crystal structure of 20Z-pMMO

To further characterize 20Z-pMMO, a crystal structure was determined to 2.7 Å resolution (Table 1). The protein was purified in the presence of n-dodecyl-β-D-maltopyranoside (DDM), exchanged into the detergent Cymal-5, and then crystallized with ammonium sulfate as the precipitant. Varying the concentration of this precipitant was crucial for obtaining well diffracting crystals. The 20Z-pMMO structure exhibits similar overall architecture to Bath-pMMO, with an $\alpha_3\beta_3\gamma_3$ trimeric structure. Unlike previous pMMO structures (11-14), there is a single protomer in the asymmetric unit (Fig. 4A).

Despite the overall structural similarity, the PmoC subunit of 20Z-pMMO is significantly disordered compared to the previous structures (11-14) (Fig. 4B). Electron density is not observed for 60% of the PmoC subunit, including residues 1-89, 123-156, and 193-218. These disordered regions include the variable metal binding site (Asp 128, His 132, His 145) and surrounding residues. This significant disorder may result from destabilization of PmoC in detergent, and could be related to the complete loss of activity upon detergent solubilization and purification (Fig. 1B). PmoC, at least in 20Z-pMMO, is thus more flexible than suggested by previous structures.

The metal binding sites of 20Z-pMMO also differ from those observed in previous pMMO structures (11-14). In the Bath-pMMO PmoB subunit, there is a monocopper site coordinated by His 48 and His 72 (12). Although both residues are conserved in 20Z-pMMO, electron density attributable to copper or any other metal ion is not present (Fig. 4C). It is unclear why this site is only occupied in Bath-pMMO, but the metal binding residues are not conserved in all pMMOs, with His 48 substituted by Asn and Gln in Type II methanotrophs, indicating that this metal center is not essential for methane oxidation. The PmoB subunit also contains a bound copper that is coordinated by residues His 33, His 137, and His 139, and has been assigned as the active site. In some pMMO structures, this site has been modeled with two copper ions, including Bath-pMMO (12-14). The dicopper site model is based on EXAFS data that consistently indicate the presence of a short Cu-Cu distance as well as the measured copper stoichiometry upon purification (10,11,13,14,34). However, in other structures, the site has been modeled with a single copper ion (11,14).

In the 20Z-pMMO structure, this PmoB site is also best modeled with one copper ion (Fig. 4D). The site is square planar with Cu-N distances of 2.1 Å for the His 137 δN, 2.1 Å for the His 139 εN, 2.5 Å for the His 33 δN, and 1.9 Å for the amino terminal nitrogen of His 33. The electron density for His 33 is not as well defined as that for other two histidine residues. Interestingly, very strong additional electron density is observed for PmoB residue Lys 155 in PmoB appended to the side chain ζN atom. We could not conclusively model this density, but it could potentially arise from post-translational modification of this residue.

XANES and EXAFS analysis of 20Z-pMMO

Cu XANES spectra measured for 20Z-pMMO indicate a mixed Cu(I) and Cu(II) metal environment. A subtle transition, observed at 8978.8 eV (Fig. 5A), is consistent with the forbidden 1s→3d transition for Cu(II) (35). Additional edge transitions, observed at 8983 and 8986.3 eV, and illustrated in the first derivative of the edge at 8982.3 and 8985.5 eV in Fig. 5A (inset), are characteristic of the 1s→4p transitions often observed for systems containing a mixture of Cu(I) and Cu(II) (35).

Analysis of the Cu EXAFS spectra for 20Z-pMMO suggest a mononuclear Cu-ligand environment constructed by only oxygen and nitrogen within the first ligand sphere (Fig. 5B). Simulations of Cu-O/N nearest neighbor ligand scattering suggest a disordered ligand environment.
composed of ca. 2.5 to 3.5 O/N ligands at an average bond length of 1.96 Å (Table 2). Inclusion of a direct Cu-Cu scattering vector was not justified in our simulations. Long-range scattering could be simulated using low Z (carbon/nitrogen) scattering at bond lengths of 2.97, 3.36 and 3.97 Å, reminiscent of patterns observed due to imidazole scattering interactions from coordinated histidines (36). In support of imidazole scattering, the pronounced camelback feature at 4 Å⁻¹, characteristic of metal-histidine ligation (37), is also observed.

Discussion

The recovery of methane oxidation activity upon pMMO reconstitution into bicelles underscores the importance of studying membrane proteins in native-like environments. Although studying membrane proteins in a membrane context seems obvious, detergent micelles are still typically used instead. Besides their amphipathic nature, detergent micelles lack important lipid bilayer characteristics that provide structural support (19). Reconstitution of pMMO into bicelles restores methane oxidation activity of inactive detergent-solubilized pMMOs close to levels measured for membrane-bound pMMO (Fig. 1). The copper stoichiometries and EPR spectroscopic features are nearly identical for inactive detergent-solubilized and active bicelle-reconstituted pMMO samples and are consistent with previous observations (12,33).

These data indicate that the copper centers detected in detergent-solubilized pMMO are functionally relevant. In previous pMMO crystal structures, 1-3 copper ions were modeled per protomer, found only in the PmoB and PmoC metal centers, and only the PmoB site coordinated by His 33, His 137, His 139 consistently houses copper ions (11-14). Chan and coworkers have proposed that Bath-pMMO instead contains ∼15 copper ions, including a tricopper active site in PmoA and 6-7 Cu¹⁺ ions bound to the C-terminus of PmoB, and have suggested that copper loss from these sites upon membrane solubilization is responsible for reduced activity of purified Bath-pMMO (15,16). However, the recovered activity of bicelle-reconstituted pMMO samples indicates that large numbers of essential copper ions are not lost during isolation from the membranes.

The crystal structure of 20Z-pMMO provides some insight into how removal from the membrane could affect activity. PmoC is largely disordered, suggesting destabilization upon solubilization and resultant activity loss. PmoB only contains two transmembrane helices, and PmoA is sandwiched between PmoB and PmoC, features that may contribute to their structural stability in detergent micelles. By contrast, only the PmoC helices near PmoA are ordered (Fig. 4A) while the disordered regions are exposed to the lipid membrane and perhaps more susceptible to perturbations upon reconstitution into detergent micelles. Without lateral pressure or specific lipid binding, PmoC may be structurally less stable in micelles. PmoC is positioned directly adjacent to the proposed PmoB active site, and could be involved in stabilization of the active site or copper binding that may be essential for activity. In addition, for a hydrocarbon monoxygenase (HMO) homolog of pMMO, mutation of the PmoC metal binding residues reduces activity, suggesting an important functional role (38). Previous efforts have mainly focused on characterizing perturbations in PmoB to explain activity loss. Some of this attention should be shifted to understanding how the transmembrane subunits, particularly PmoC, play an essential role in methane oxidation.

Finally, a mononuclear copper active site remains a viable possibility (6,8). The 20Z-pMMO PmoB site is best modeled as monocopper (Fig. 4). Additionally, the short Cu-Cu distance detected in the EXAFS analysis of other pMMOs (11,14,35) is not present in 20Z-pMMO (Fig. 5). Its absence in 20Z-pMMO could be due to lower protein concentrations, a heterogeneous distribution of Cu-Cu vectors in the samples that cancel out the overall signal, or even the reduced presence of other copper contaminant proteins that could contribute to the observed feature. This result is consistent with a recent quantum refinement of the Bath-pMMO PmoB copper site (39). Most relevant to a pMMO monocopper active site are the lytic polysaccharide monoxygenases (LPMOs), which utilize a monocopper active site for oxidative cleavage of glycosidic bonds. Both the PmoB copper site and the LPMO active site contain a histidine brace metal-binding motif. However, LPMOs lack a third histidine ligand and additional metal binding sites. In addition, some
contain a methylated histidine ligand. Moreover, the substrates of pMMO and LPMO are drastically different (40-42). Overall, studying pMMO in a membrane-bound context has validated past characterizations and provides new insights into the importance of the PmoC subunit and the nature of the active site. It will be important to continue this approach in future studies of pMMO activity and mechanism.

**Experimental procedures**

**Methanotroph cell growth**

*M. alcaliphilum* 20Z was cultured as described previously (43,44). Briefly, cells were grown in 1X modified nitrate mineral salts medium, 0.5 M NaCl, 2.3 mM phosphate buffer, 50 mM carbonate buffer, pH 9.5, supplemented with 40 µM CuSO₄•H₂O and trace elements solution under a 1:3 methane-to-air ratio in 12 L bioreactors. *M. capsulatus* (Bath) cells were grown in 1X nitrate mineral salts medium, 3.9 mM phosphate buffer, pH 6.8, supplemented with 50 µM CuSO₄•H₂O, 40 µM iron NaFe(III)EDTA, and trace element solution under a 1:4 methane-to-air ratio in 12 L bioreactors (12). All bioreactor cell growths were harvested at an OD₆₀₀ of 8-10 and centrifuged at 8,000 x g for 30 min. Cell pellets were flash frozen in liquid nitrogen and stored at -80 °C for future use.

**Membrane isolation**

*M. alcaliphilum* 20Z cell pellets (10 g) were resuspended in 100 ml of 25 mM PIPES, 500 mM NaCl, pH 7, supplemented with EDTA-free protease inhibitor tablets (Roche). The cells were manually stirred for resuspension on ice. The cell resuspension was sonicated at 4 °C for 1.5 min with an on/off interval of 1 sec/ 3 sec at 25% amplitude and centrifuged at 8,000 x g for 1 hr to remove cell debris. The supernatant was centrifuged at 100,000 x g for 1 hr to isolate the pelleted membranes containing pMMO. The membrane pellet was washed 2 times with a Dounce homogenizer in 25 mM PIPES, 250 mM NaCl, pH 7. 1 ml aliquots of pMMO-containing membranes at total protein concentrations of 10 mg/ml (measured by Bio-Rad DC Assay using BSA as a standard) were flash frozen in liquid nitrogen and stored at -80°C. *M. capsulatus* (Bath) membranes were isolated as described previously (45).

**pMMO purification and bicolle reconstitution**

Membranes were solubilized using 1.2 mg of DDM (Anatrace) per 1 mg of crude protein at 4 °C for 1 hr. The solubilized protein was centrifuged at 100,000 x g for 1 hr, and the supernatant was collected for purification. Solubilized 20Z-pMMO was buffer exchanged into 25 mM PIPES, 50 mM NaCl, pH 7, 0.02% (w/v) DDM using a 100 kDa MW cutoff Amicon (Millipore). 20Z-pMMO was purified using a 15Q anion exchange column (GE Healthcare) and eluted using a 50-800 mM NaCl gradient (Figs. S1, S2). Solubilized Bath-pMMO was concentrated to 1 ml using a 100 kDa MW cutoff Amicon and loaded onto a 120 ml Superdex 200 size exclusion column (Fig. S3). All eluted pMMOs were concentrated using a 100 kDa MW cutoff Amicon to 10 mg/ml in 25 mM PIPES, 250 mM NaCl, pH 7, 0.02% (w/v) DDM. Freshly solubilized or purified pMMO at 10 mg/ml was reconstituted with a 30% (w/v) DMPC:CHAPSO 2.8:1 bicolle solution (Molecular Dimensions) using a 4:1 volumetric ratio and incubated on ice for at least 30 min to prepare pMMO samples at 8 mg/ml reconstituted in 6% (w/v) bicelles. The copper concentration was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES) at the Quantitative Bio-element Imaging Center (QBIC) at Northwestern University.

**¹³C methane oxidation activity assay**

To measure methane oxidation activity of membrane-bound, solubilized, purified, and 6% (w/v) bicolle-reconstituted pMMOs, samples were diluted to 4 mg/ml [or 3% (w/v) bicelles] in 100 µl reactions consisting of reductant (280 µM NADH (Sigma-Aldrich) or excess duroquinol) in 2 ml screw top vials with septa tops (Agilent). A 1 ml volume of headspace gas was withdrawn from the and replaced with 1.5 ml of ¹³C methane (Sigma-Aldrich). All reactions were performed at 30 °C (bicelle samples solidify at 45 °C, the temperature typically used for Bath-pMMO activity assays). pMMO reconstituted in 3% or 1.5% (w/v) bicelles gave the highest activity, which decreased with lower bicelle concentrations (Fig. S4). Reactions were incubated at 30 °C, 200 rpm, for 5 min, put on ice for 5 min, and then quenched with 500 µL of chloroform spiked with 1 mM dichloromethane.
The reaction was vortexed at 2000 rpm for 10 min and centrifuged at 2,000 x g for 30 min to separate precipitate from the chloroform mixture. 2.5 µL of sample was injected onto a PoraBOND Q column (25 m x 250 µm x 3 µm) on an Agilent 7890B/5977A MSD GC/MS instrument with a split ratio of 10:1. The GC was maintained under a constant flow of 1.2 ml/min of helium gas. The initial oven temperature was maintained at 80 °C for 3.5 min, followed by an increase of 50 °C/min to 150 °C and held for 1.5 min. A second ramp rate of 15 °C/min was used to reach the final temperature of 300 °C held for 1 min. The mass spectrometer was maintained under ion source temperature of 230 °C, quad temperature of 150 °C, 70 eV, and a detector voltage of 2,999 V. Masses 31, 33, and 49 were monitored for detection of 12C methanol, 13C methanol, and dichloromethane (dwell times of 10 ms, 100 ms, 10 ms, respectively). The 13C methanol peak area was integrated, quantified from a standard calibration curve, and normalized to the concentration of the internal standard dichloromethane. The lower limit of detection was determined to be 10 µM 13C methanol, and a stringent cutoff for minimum concentration was set at 30 µM. Methane oxidation activity values using 13C methanol detection by GC-MS in comparison to 12C methanol detection using the GC-FID are shown in Table S3.

**EPR Spectroscopy**

EPR samples were prepared by aliquoting 100 µM (DDM samples) or 80 µM pMMO (bicelle samples) in 25 mM PIPES, 250 mM NaCl, pH 7, 0.02% (w/v) DDM or 6% (w/v) bicelles into Wilmad quartz EPR tubes (Sigma Aldrich). Measurements were collected on a continuous wave (CW) X-band Bruker ESP-300 spectrometer using a liquid helium flow Oxford Instruments ESR-900 cryostat. Spectra were corrected for background resonance by subtraction of a spectrum of 50 mM Tris pH 8.0, 150 mM NaCl collected under the same conditions. Cu2+ spin quantitation was performed by double integral area comparison of pMMO spectra to Cu2+-EDTA standards containing 25-500 µM Cu2+. All EPR simulations were performed using EasySpin (46).

**Crystallization and structural determination of 20Z-pMMO**

Purified pMMO in 0.02% (w/v) DDM was buffer exchanged into 0.12% (w/v) Cymal 5 using a 100 kDa MW cutoff Amicon. pMMO crystals were obtained from sitting drops containing 1 µl of 10 mg/ml protein in 25 mM PIPES, 250 mM NaCl, pH 7, 0.12% (w/v) Cymal-5, and 1 µl of 2.8 M AmSO4, 0.2 M MES, pH 6. Crystals were harvested in saturated LiSO4 cryoprotectant solution and flash frozen in liquid nitrogen.

Crystals were screened for diffraction at the LS-CAT and GM/CA-CAT beamlines at the Advanced Photon Source at Argonne National Laboratory. Data sets were processed using HKL2000 (47) (Table 1). Anisotropic processing using the UCLA anisotropy server (48) was found to improve the electron density maps. Phenix (49) was used for molecular replacement with the Bath-pMMO coordinates as a starting model (PDB accession code 3RGB) to solve the structure of 20Z-pMMO. 20Z-pMMO has 72%:78%:77% identity to the Bath-pMMO PmoB, PmoA, and PmoC subunits, respectively. Structure modeling and refinement were performed using Coot (50) and Phenix, and model quality was assessed using MolProbity (51). The final model for the 20Z-pMMO structure includes PmoB residues 33-414, PmoC residues 90-122, 157-192, and 219-250, PmoA residues 4-244, 1 copper ion, and 2 Cymal 5 molecules.

**X-ray Absorption Spectroscopy**

Purified 20Z-pMMO samples were concentrated to 385 µM using a 100 kDa MW cutoff Amicon centrifugal concentrator and resuspended in 30% (v/v) glycerol. The copper concentration of the 20Z pMMO samples was 732 µM. These samples were loaded into Lucite XAS cells wrapped with Kapton tape, flash frozen in liquid nitrogen, and stored at –80 °C. XAS data were collected at the Stanford Synchrotron Radiation Lightsource on beamline 9-3, equipped with a Si[220] double-crystal monochromator that contains an upstream mirror used for focusing and harmonic rejection. Fluorescence spectra were collected using a 100-element Ge solid-state Canberra detector. During data collection, the Oxford Instruments continuous-flow liquid helium cryostat was stabilized at 10 K. Copper excitation data were collected using a 6 µm nickel Lytle filter and solar slits placed between cryostat and...
detector to reduce scattering fluorescence. XAS spectra were measured using 5 eV steps in the pre-edge region (8750-8960 eV), 0.25 eV steps in the edge region (8986-9050 eV), and 0.05 Å⁻¹ increments in the extended X-ray absorption fine structure (EXAFS) region (to \( k = 13.3 \) Å⁻¹), integrating from 1 to 25 seconds in a \( k^3 \) weighted manner for a total scan length of approximately 40 minutes. A Cu foil spectrum was collected simultaneously with each protein spectrum for real-time spectral energy calibration, with an assigned first inflection point for the Cu foil spectrum at 8980.3 eV. Spectra were closely monitored for any photodamage and slight photoreduction was observed. To diminish the extent and impact of photoreduction, six individual spectra were collected at unique positions on the sample surface following a matrix positioning grid to ensure a new radiation exposure surface and only the initial exposure spectrum at each position was used during overall data analysis. Spectra were collected on duplicate independent samples, and data presented in this report represent the average of 6 scans.

XAS spectra were processed and analyzed using the EXAFSPAK program suite written for Macintosh OS-X (52) integrated with the Feff v8 software (53) for theoretical model generation. EXAFS fitting analysis was performed on raw/unfiltered data. Single scattering models were calculated for oxygen, nitrogen, sulfur, copper and carbon coordination to simulate possible copper ligand environments, with values for the scale factors (Sc) and \( E_0 \) calibrated by previous fittings of characterized Cu(I)/Cu(II) crystallographic copper model compounds (35). Standard criteria for judging the best-fit EXAFS simulations included: 1) a reasonable Debye-Waller factor for the fit (\( \sigma^2 < 0.006 \) Å²) (54) 2) the spectral resolution of the data, calculated based on the energy range extent of usable data (55) and 3) the lowest mean square deviation between data and fit with, corrected for the number of degrees of freedom (\( F' \)) (55). During the standard criteria simulations, only the bond length and Debye-Waller factor were allowed to vary for each ligand environment. A dimensionless \( \text{Sc} = 1 \) and \( E_0 \) values of -12, -14 and -16 eV were used for Cu(I,II)-C/N/O, -S and -Cu theoretical model calibrations respectively during simulations (35).

ACKNOWLEDGEMENTS
This work was supported by NIH grants GM118035 and GM070473 (A.C.R.), GM111097 (B.M.H.), and DK068139 (T.L.S.). S.Y.R. and M.O.R. were supported in part by NIH grant 5T32GM008382. This work used the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract No. DE-AC02-06CH11357. Use of the LS-CAT Sector 21 was supported by the Michigan Economic Development Corporation and the Michigan Technology Tri-Corridor (Grant 085P1000817). Data were collected at the LS-CAT beamline 21-ID-D. Use of the Stanford Synchrotron Radiation Lightsource, SLAC National Accelerator Laboratory, is supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences under Contract No. DE-AC02-76SF00515. The SSRL Structural Molecular Biology Program is supported by the DOE Office of Biological and Environmental Research, and by the National Institutes of Health, National Institute of General Medical Sciences (including P41GM103393). The authors also thank Dr. Marina Kalyuzhnaya at San Diego State University for providing \textit{Mm. alcaliphilum} 20Z cultures.

CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest with the contents of this article.
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Abbreviations
The abbreviations used are: pMMO, particulate methane monooxygenase; MMO, methane monooxygenase; Mm., Methylomicrobium; Mcc., Methylococcus; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; NDH-2, type 2 NADH dehydrogenase; DDM, n-dodecyl-β-D-maltopyranoside; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; ICP-OES, inductively coupled plasma optical emission spectroscopy; Cymal-5, 5-cyclohexyl-1-pentyl-β-D-maltoside.
Table 1
Data collection and refinement statistics for 20Z-pMMO
Values in parentheses refer to the highest resolution shell.

|                      | 20Z-pMMO          | 20Z-pMMO (copper anomalous) |
|----------------------|-------------------|-----------------------------|
| **Data collection**  |                   |                             |
| Space group          | P63               | P63                         |
| Cell dimensions      | a, b, c           |                             |
|                      | 143.84, 143.84,   |                             |
|                      | 146.15            |                             |
| Resolution           | 2.70 Å            | 3.00 Å                      |
| Wavelength           | 1.03329 Å         | 1.37760 Å                   |
| R_pim                | 0.025 (0.220)     | 0.022 (1.32)                |
| R_meas               | 0.07 (0.588)      | 0.06 (0.337)                |
| CC_1/2               | 0.999 (0.953)     | 0.998 (0.984)               |
| I/σI                 | 40.6 (2.5)        | 113.5 (11.2)                |
| Completeness         | 99.1% (92%)       | 99.7% (97.7%)               |
| Redundancy           | 9.0 (6.4)         | 6.9 (5.7)                   |
| **Anisotropy correction** |               |                             |
| Truncation limit     | 3.1, 3.1, 2.7 Å   |                             |
| Completeness         | 74.9%             |                             |
| **Refinement**       |                   |                             |
| No. of reflections   | 35,187            |                             |
| R_work/R_free        | 0.2133/0.267      |                             |
| Average B-factor (Å²)| 39.69             |                             |
| Root mean square deviations |           |                             |
| Bond lengths (Å)     | 0.010 Å           |                             |
| Bond angles (°)      | 1.313°            |                             |
| Ramachandran favored | 87.39%            |                             |
| Ramachandran allowed | 10.64%            |                             |
Table 2
Summary of the best fit Cu EXAFS simulations for 20Z-pMMO sample
EXAFS were fit over the $k$-range of 1.0-12.85 Å$^{-1}$, for a spectral resolution of 0.13 Å

| Atom | R(Å) | CN | σ² | F' |
|------|------|----|----|----|
| O/N  | 1.96 | 2.5| 5.23|    |
| C    | 2.97 | 1.5| 3.73| 0.4|
| C    | 3.36 | 0.75| 4.25|    |
| C    | 3.96 | 3.0| 4.22|    |

a Independent metal-ligand scattering environment
b Scattering atoms: O (oxygen), N (nitrogen), Cu (copper)
c Average metal-ligand bond length from two independent samples
d Average metal-ligand coordination number from two independent samples
e Average Debye-Waller factor in Å² x 10³ from two independent samples
f Number of degrees of freedom weighted mean square deviation between data and fit.
Figure 1. Methane oxidation activity of pMMOs in native membranes, detergent, and bicelles. Activity values (nmol $^{13}$C methanol·mg$^{-1}$ protein) are shown for (A) Bath-pMMO and (B) 20Z-pMMO in as-isolated membranes, solubilized in detergent, solubilized in bicelles, purified in detergent, and purified in bicelles using both duroquinol (gray) and NADH (white) as reductants. Error bars represent standard deviations of 3 measurements, and the black and white dots represent the individual measurements.
Figure 2. Copper content of pMMOs in native membranes, detergent, and bicelles. Copper stoichiometry (copper equivalents per 100 kDa pMMO protomer) of (A) Bath-pMMO and (B) 20Z-pMMO in as-isolated membranes, solubilized in detergent, solubilized in bicelles, purified in detergent, and purified in bicelles. Error bars represent standard deviations of 3 measurements, and the black dots represent individual measurements.
Figure 3. X-band EPR spectra of (A) Bath-pMMO and (B) 20Z-pMMO. Spectra of pMMO purified in detergent (gray, dotted lines) and after bicelle reconstitution (black, solid lines) are normalized to protein concentration. For purified and bicelle reconstituted Bath-pMMO, EPR intensity was increased three-fold for ease of comparison. Red solid lines depict simulations using the (very slightly adjusted) previously defined $\text{Cu}^{2+}$ spin Hamiltonian parameters (33): equal contributions of (His)$_2$-$\text{Cu}^{2+}$ ($g_\| = 2.235$, $g_\perp = 2.047$, $A_\| = 585$ MHz, $A_\perp = 65$ MHz) and (His, Amine)-$\text{Cu}^{2+}$ ($g_\| = 2.295$, $g_\perp = 2.047$, $A_\| = 450$ MHz, $A_\perp = 40$ MHz). Collection conditions: 9.36-9.37 GHz microwave frequency, 20 K temperature, 160 ms time constant, 16 G modulation amplitude, 60 second scans, average of at least 10 scans.
Figure 4. Crystal structure of 20Z-pMMO. (A) The 20Z-pMMO protomer with PmoB, PmoA, PmoC shown in gray, salmon, and purple, respectively. The copper ion is depicted as a teal sphere. (B) Superposition of the 20Z-pMMO PmoC subunit (purple) with the Bath-pMMO PmoC subunit (PDB accession code 3rgb, green). The zinc ion in the Bath-pMMO variable metal binding site is shown as a sphere (gray) and is coordinated by two histidines and an aspartic acid. This region is disordered in 20Z-pMMO. (C) The site of the monocopper center in Bath-pMMO is unoccupied in 20Z-pMMO. (D) The 20Z-pMMO copper site. The copper anomalous difference density map (magenta, 20σ) is superimposed on the 2F_o-F_c map (gray, 1.0σ).
Figure 5. XANES and EXAFS analysis of 20Z-pMMO. Left, Cu XANES spectra for 20Z-pMMO. Inset: first derivative of near edge and edge features is displayed to more clearly highlight the features. Right, raw Cu EXAFS for 20Z-pMMO. Simulations were fit using a standard conservative approach that follows rules governing both spectral resolution relative to acceptable intra-ligand scattering interaction bond lengths and acceptable bond lengths (55). The EXAFS spectrum is shown in panel B, and the Fourier transform of the EXAFS is shown in panel C. Raw unfiltered data are in black, and the best fit simulations are in gray. EXAFS were fit over a $k$-range of 1.0-12.85 Å$^{-1}$. 
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monooxygenase activity
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J. Biol. Chem. published online May 8, 2018

Access the most updated version of this article at doi: 10.1074/jbc.RA118.003348

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