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Evidence for Oxygen Binding at the Active Site of Particulate Methane Monooxygenase

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Supporting Information

ABSTRACT: Particulate methane monooxygenase (pMMO) is an integral membrane metalloenzyme that converts methane to methanol in methanotrophic bacteria. The enzyme consists of three subunits, pmoB, pmoA, and pmoC, organized in an $\alpha_3\beta_3\gamma_3$ trimer. Studies of intact pMMO and a recombinant soluble fragment of the pmoB subunit (denoted as spmoB) indicate that the active site is located within the soluble region of pmoB at the site of a crystallographically modeled dicopper center. In this work, we have investigated the reactivity of pMMO and spmoB with oxidants. Upon reduction and treatment of spmoB with O_2 or H_2O_2 or pMMO with H_2O_2, an absorbance feature at 345 nm is generated. The energy and intensity of this band are similar to those of the μ-η^2:η^2-peroxo-Cu^{II}_2 species formed in several dicopper enzymes and model compounds. The feature is not observed in inactive spmoB variants in which the dicopper center is disrupted, consistent with O_2 binding to the proposed active site. Reaction of the 345 nm species with CH_4 results in the disappearance of the spectroscopic feature, suggesting that this O_2 intermediate is mechanistically relevant. Taken together, these observations provide strong new support for the identity and location of the pMMO active site.

Methanotrophic bacteria oxidize methane to methanol using methane monooxygenases (MMOs). In contrast to costly and inefficient industrial catalysts, MMOs oxidize this inert hydrocarbon (C−H bond dissociation energy 104 kcal mol^{-1}) and major greenhouse gas under ambient conditions in an environmentally benign fashion. The soluble form of methane monooxygenase (sMMO) is produced by some methanotrophs under conditions of copper starvation[1] and contains a catalytic, carboxylate-bridged diiron center. The particulate form (pMMO) is an integral membrane protein expressed by almost all methanotrophs. Whereas the chemistry of methane oxidation by sMMO is well-understood,[2] mechanistic studies of pMMO have been hindered by controversy surrounding the identity and nature of the metal active site.

pMMO consists of three polypeptide chains, denoted as pmoB, pmoA, and pmoC (Figure S1 in the Supporting Information), that are arranged in an $\alpha_3\beta_3\gamma_3$ trimer.[3−5] Several active site models have been proposed in the context of this architecture.[6] In one model, a hydrophilic patch of residues within the transmembrane pmoA and pmoC subunits has been postulated to house a catalytic tricopper center.[7,8] This site is devoid of metal ions in the three available pMMO crystal structures, however.[3−5] A second model also suggests that the active site is within these subunits, but instead involves a diiron center similar to that in sMMO.[9,10] This diiron center is proposed to occupy a solvent-accessible site known to bind a single zinc or copper ion, depending on the crystallization conditions.[4,5]

Data from our laboratory indicate that the pMMO active site contains copper, not iron. In addition, a recombinant protein fragment corresponding to the soluble region of the *Methylococcus capsulatus* (Bath) pmoB subunit (spmoB) (Figure S1) exhibits methane oxidation activity, localizing the active site within spmoB rather than within the transmembrane pmoA and pmoC subunits.[11,12] Two distinct copper sites have been found in the pmoB subunit. The first, a mononuclear site, is present in *M. capsulatus* (Bath) pMMO[3], but not in pMMOs from *Methylosinus trichosporium* OB3b and *Methylocystis* species strain M.[4,5] The second, which is conserved in all three characterized pMMOs, has been modeled as dinuclear with a Cu−Cu distance of ∼2.6 Å on the basis of crystallographic and X-ray absorption spectroscopic data.[4,5,13] Site-directed variants of spmoB have allowed us to pinpoint the activity to this dicopper site, which is coordinated by His 33, His 137, and His 139 [*M. capsulatus* (Bath) pMMO numbering]. Although these data clearly show that pMMO activity derives from this specific location, the moderate resolution of the crystal structures (2.68 Å at best) and the low activity of spmoB[11] leave room for debate regarding both the nuclearity and the reactivity of the dicopper site.

If this dicopper center is the site of methane oxidation, it should bind O_2. The binding of O_2 to dicopper centers in both proteins and model compounds has been studied extensively. Potentially relevant biological systems include the oxygen carrier hemocyanin and the enzymes tyrosinase and catechol oxidase.[14] Although pMMO has a shorter Cu−Cu distance and fewer coordinating histidine residues (Figure S2),[15−17] these type 3 copper proteins represent a reasonable starting point for considering the interaction of O_2 with the pMMO active site. In these proteins, the reaction of O_2 with the Cu^{I}_2 state or of H_2O_2 with the met-Cu^{II}_2 state yields a well-defined μ-η^2:η^2-peroxo-Cu^{II}_2 species with $\varepsilon_{345\ nm} \approx 20\,000\ M^{-1}\ cm^{-1}$.[14,18] This species can also be formed in a number of synthetic complexes, and in some of these, it isomerizes into a bis-μ-oxo-Cu^{III}_2 core, which is spectroscopically distinct and has not been detected in biological systems.[19−22] Also of particular relevance to pMMO

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are recent studies of a Cu-ZSM-5 zeolite that converts methane into methanol. In this system, the active species is believed to be a mono-μ-oxo-Cu^{II}_2 species that is formed from a μ-η^2:η^2-peroxo-Cu^{II}_2 precursor.[23−25] Although the O_2 reactivity of the pMMO copper sites has been explored computationally,[26,27] the possibility of a detectable interaction between copper and O_2 has not been addressed experimentally. To probe further the location of the active site and as a first step toward mechanistic studies, we have investigated O_2 binding to the active sites of both pMMO and spmoB.

After generating the oxy forms of tyrosinase and hemocyanin[28,29] and observing the 345 and 580 nm optical features of the μ-η^2:η^2-peroxo-Cu^{II}_2 species (Figure S3), we performed similar experiments using *M. capsulatus* (Bath) pMMO solubilized with *n*-dodecyl-β-D-maltopyranoside. Solubilized pMMO was used instead of purified sample to preserve the enzymatic activity.[30] No spectral changes were observed upon addition of H_2O_2 to the as-isolated pMMO, which contains a mixture of Cu^I and Cu^{II}.[13] Reduction with ascorbate followed by O_2 addition did not produce any observable spectral changes either.[31] Reduction was then performed more rigorously by repeated argon/vacuum cycling of the sample on a Schlenck line and incubation with excess ascorbate in an anaerobic chamber. This procedure yields reduced pMMO, as shown by electron paramagnetic resonance (EPR) spectroscopy (Figure S4). After 30 min, the reduced pMMO was removed from the chamber in a sealed cuvette and treated with excess H_2O_2. After incubation at room temperature overnight, an optical feature with λ_{max} = 345 nm was observed (Figure 1). A

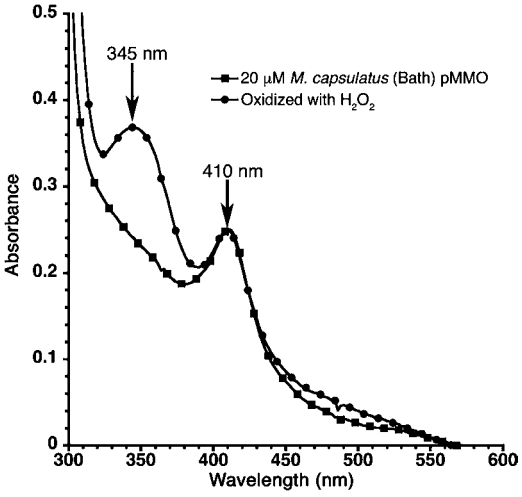

Figure 1. Reaction of H_2O_2 with solubilized pMMO from *M. capsulatus* (Bath). Upon addition of H_2O_2 to anaerobically reduced pMMO, the optical feature at 345 nm slowly appears and is detectable after ∼12 h. The peak at 410 nm derives from heme contaminants.

second peak at 410 nm derived from heme contaminants was present both before and after treatment.[30] We were not able to generate the 345 nm feature using oxygenated buffer instead of H_2O_2.

The experiments were then carried out using spmoB. Although its methane oxidation activity is much lower than that of intact pMMO,[11] spmoB is free of heme and other potential contaminants from the *M. capsulatus* (Bath) membranes and thus is useful for spectroscopic analysis. There was again no observable absorbance change upon addition of H_2O_2 to as-isolated spmoB or O_2 to ascorbate-reduced spmoB. However, careful deoxygenation and reduction in the anaerobic chamber (Figure S4) followed by treatment with either H_2O_2 or O_2 yields a peak at 345 nm (Figure 2).

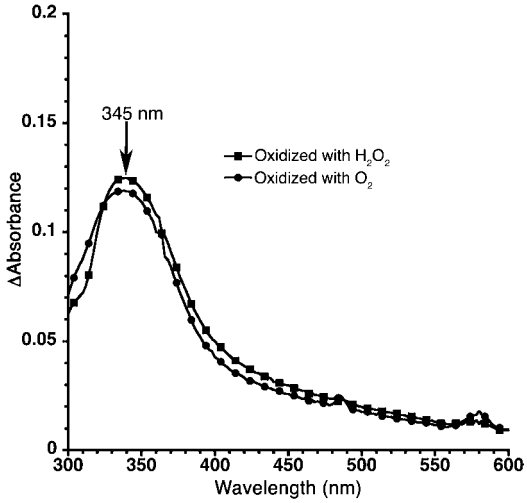

Figure 2. Difference spectra showing the reactions of O_2 and H_2O_2 with 20 μM spmoB. The feature at 345 nm appears immediately, and the value of $\varepsilon_{345\ nm}$ was estimated to be 10 000 M^{-1} cm^{-1}.

This feature appeared immediately. On the basis of the estimated $\varepsilon_{345\ nm}$ value of 10 000 M^{-1} cm^{-1}, the occupancy of the 345 nm species in these samples is ∼50%. The feature was not observed in control experiments using the apo form of spmoB.

It is not clear why the 345 nm feature can be generated with both H_2O_2 and O_2 for spmoB, but only with H_2O_2 for pMMO. The dicopper center is presumably far more accessible in spmoB, and it may be more difficult to deliver a sufficient concentration of O_2 within the intact membrane-bound pMMO, especially given the limited solubility of O_2 in buffer.[32] However, the observation of the same copper and oxygen-dependent optical feature in both pMMO and spmoB strongly supports our previous conclusions[11] that the active site resides in spmoB.

To determine whether the 345 nm peak indeed originates from O_2 binding at the proposed pMMO active site, we took advantage of the previously characterized spmoB variants.[11] These variants, which were critical in locating the active site, include spmoB_H48N, which disrupts the monocopper site but still exhibits enzyme activity, spmoB_H137,139A, which disrupts the dicopper site and exhibits no activity, and spmoB_H48N_H137,139A, which is also inactive. Samples of all three variants were subjected to the same reduction procedure followed by reaction with O_2 or H_2O_2. Only spmoB_H48N exhibits the absorbance feature at 345 nm (Figure 3 and Figure S5). Thus, oxidant binding occurs only when the copper site ligated by His 33, His 137, and His 139 is intact.

Further experiments were then performed in the presence of the substrate CH_4. The 345 nm optical feature was generated in samples of spmoB using either O_2 or H_2O_2, and the samples were incubated with CH_4 for 1 h at 45 °C. The samples were then examined by optical spectroscopy. Protein precipitation prevented monitoring of the 345 nm feature as a function of time at 45 °C, so instead, samples were centrifuged, and the end-point spectrum was recorded (Figure 4). After 1 h, the

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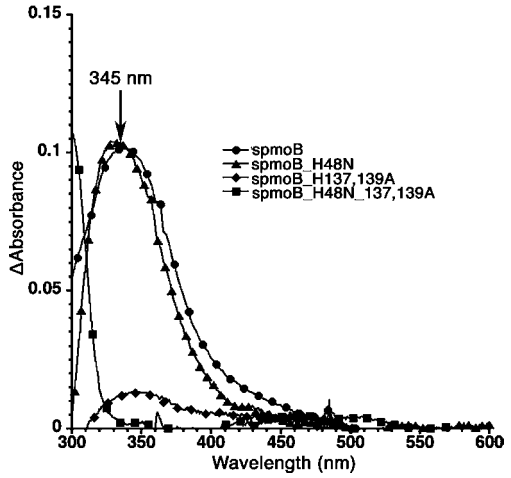

Figure 3. Difference spectra showing the reaction of O_2 with wild-type and variant spmoB proteins. The 345 nm peak is observed only for spmoB and spmoB_H48N, the two forms that retain the dicopper center and exhibit methane oxidation activity.

absorbance at 345 nm is significantly diminished. In the absence of CH_4, this feature retains its intensity in an anaerobic cuvette for ~12 h. This finding is consistent with the 345 nm species being on the pMMO reaction pathway.

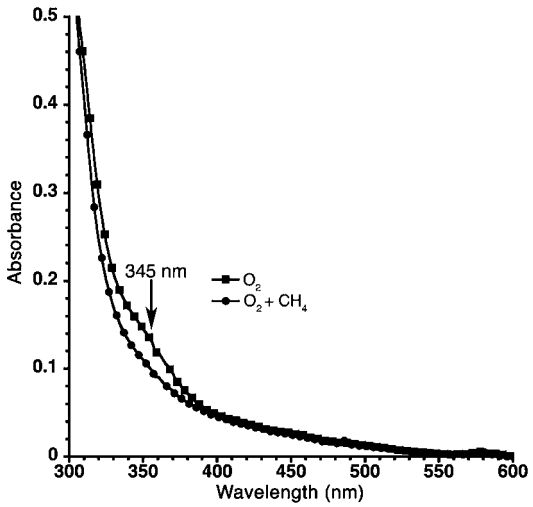

Figure 4. Reactivity of the spmoB 345 nm species with CH_4. Addition of CH_4 to the 345 nm species results in reduction of the absorbance peak.

Taken together, these data provide key new support for our pMMO active site model. First, the optical feature observed upon reaction with H_2O_2 or O_2 was observed only in samples containing the pmoB copper center that we assigned as the active site.[11] Second, the data are consistent with this site being a dicopper center. The energy and extinction coefficient are very similar to those of the hemocyanin and tyrosinase μ-η^2:η^2-peroxo-Cu^{II}_2 species.[18] Generation of this species by H_2O_2 treatment would require oxidation of the deoxy-Cu^{I}_2 form to a met-Cu^{II}_2 form followed by conversion to the μ-η^2:η^2-peroxo-Cu^{II}_2 species by reaction with a second molecule of H_2O_2, similar to what has been proposed for hemocyanin[33,34] and tyrosinase.[35,36] Titration with H_2O_2 gave results consistent with the binding of two molecules of H_2O_2 per spmoB (or per dicopper center) (Figure S6). The 345 nm feature may alternatively correspond to a met-Cu^{II}_2 form, similar to that generated in hemocyanin.[34,37] Finally, the hydroxo-bridged Cu^{II}_2 type 3 site in multicopper oxidases exhibits a feature at 330 nm ($\varepsilon_{330\ nm}$ = 2−5000 M^{-1} cm^{-1})[14,38,39] that is also consistent with the present observations. These scenarios are all compatible with a dicopper center model, and the disappearance of the 345 nm species after incubation with CH_4 demonstrates its relevance to pMMO catalysis. Definitive assignment of the 345 nm species would be facilitated by resonance Raman spectroscopic data. However, refolded spmoB precipitates at micromolar concentrations, and it has not been possible to generate an appropriately concentrated sample despite extensive efforts. Such experiments with pMMO are complicated by the presence of heme contaminants.

Both density functional theory (DFT) calculations on pMMO and spectroscopic and DFT studies of the Cu-ZSM-5 zeolite suggest that a μ-η^2:η^2-peroxo-Cu^{II}_2 species may be a precursor to an intermediate that reacts with methane. According to DFT calculations, a mixed-valent bis-μ-oxo-$Cu^{II}Cu^{III}$ species may be able to activate the C−H bond in methane.[26,27,40] This species could be generated from the μ-η^2:η^2-peroxo-Cu^{II}_2 or bis-μ-oxo-Cu^{III}_2 core by injection of an electron from an exogenous source, from another metal ion, or from a protein residue.[19,41] In the Cu-ZSM-5 zeolite, spectator Cu^{I} ions in the zeolite lattice are suggested to provide two electrons to convert a μ-η^2:η^2-peroxo-Cu^{II}_2 precursor into a bent mono-μ-oxo-Cu^{II}_2 active species[23,25] that can be modeled into the pMMO active site.[18] However, additional spectroscopic data, optimally on a more tractable soluble protein model system, will be critical to understanding the relevance of these oxygen intermediates to pMMO catalysis.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Full description of experimental procedures; figures showing pMMO and the pMMO metal centers; figures showing the active sites of pMMO, hemocyanin, and tyrosinase; absorption spectra of oxyhemocyanin and oxytyrosinase; EPR spectra of reduced pMMO and spmoB; difference spectra showing reaction of H_2O_2 with wild-type and spmoB variant proteins; and titration of spmoB with H_2O_2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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