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Particulate methane monooxygenase from *Methylosinus trichosporium* is a copper-containing enzyme

Jia-Ying Xin,[a,*] Jun-Ru Cui,[a] Xiao-Xue Hu,[a] Shu-Ben Li,[a] Chun-Gu Xia,[a] Li-Min Zhu,[b,1] and Yi-Qun Wang[b]

[a] *State Key Laboratory for Oxo Synthesis and Selective Oxidation, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou 730000, P.R. China*

[b] *School of Biological and Applied Sciences, University of North London, London N7 8DB, UK*

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Abstract

Particulate methane monooxygenase (pMMO) has been exfoliated and isolated from membranes of the *Methylosinus trichosporium* IMV 3011. It appears that the stability of pMMO in the exfoliation process is increased with increasing copper concentration in the growth medium, but extensive intracytoplasmic membrane formed under higher copper concentration may inhibit the exfoliation of active pMMO from membrane. The highest total activity of purified pMMO is obtained with an initial concentration of 6 μM Cu in the growth medium. The purified MMO contains only copper and does not utilize NADH as electron donor. Treatment of purified pMMO with EDTA resulted in little change in copper level, suggesting that the copper in the pMMO is tightly bound with pMMO.

Keywords: Methane-oxidizing bacterium; Intracytoplasmic membrane; Copper; Exfoliation; Purification; Stability; Activity; Growth medium; Particulate methane monooxygenase; EDTA; Electron donor

Methanotrophic bacteria use methane as the sole carbon and energy source. The oxidation of methane by methanotrophs is initiated by methane monooxygenase (MMO), which catalyzes the oxidation of methane to methanol [1].

There are two distinct types of MMO: a soluble, cytoplasmic enzyme complex (sMMO) and a membrane-bound, particulate enzyme system (pMMO). It has been found that at low copper:biomass ratios, the predominant form of the enzyme occurs in the soluble fraction of cell extracts (sMMO) and that at high copper:biomass ratios, the predominant form of the enzyme occurs in the membrane-bound fraction (pMMO) [2]. These two forms of MMO are distinguishable not only by their intracellular locations, but also by differences in structure, active site, inhibitor sensitivity, and electron donors.

Past attempts to isolate pMMO have resulted in some confusion regarding the nature of enzyme [3]. Currently, there is no clear consensus on the involvement of copper and iron in this enzyme. Published data to date consistently point to copper as being important for the activity of the enzyme [4,5] and suggest that pMMO might be a copper-containing protein. Some reports, however, have supported the view that the pMMO active site contains iron [6], despite overwhelming evidence suggesting that copper is the element responsible for supporting catalysis [7].

To resolve this problem, it is essential to preserve enzyme activity during isolation and purification. pMMO has not been well characterized due to the unusual instability of the enzyme. Activity is frequently lost upon detergent solubilization [5,8,9]. As a result, a highly active and purified preparation of the enzyme has proved difficult to obtain.

The results obtained in this work for *M. trichosporium* IMV 3011 show that bacterial culture conditions significantly affect both membrane yield and active

* Corresponding author. Fax: +86-931-827-7088.
E-mail address: xinjy1966@hotmail.com (J.-Y. Xin).
[1] Also corresponding author.

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pMMO exfoliation from the membrane. We describe here the purification and characterization of active pMMO. The results strongly support the contention that pMMO is a copper-containing enzyme system. We also report evidence that pMMO might bond less strongly with the membrane when cells are grown in a reduced copper environment.

Materials and methods

Cell breakage and membrane fraction obtained M. trichosporium. IMV 3011 cells were prepared as previously described [10]. The cells were broken by sonication (300 W, 396 S) at 0–4 °C under nitrogen (N_2). Cell debris and unbroken cells were removed by centrifugation at 6000*g* for 30 min, the supernatant containing membrane was filtrated through a membrane with MW cut-off of 1 MDa, and a 10 mM sodium phosphate buffer (pH 7.0) containing 1 mM $MgCl_2$, 300 μM $CuSO_4$, and 2 mM dithiothreitol was used to elute the soluble cytosolic component. The washed membrane was centrifuged at 15,000*g* for 85 min and the membrane pellet was resuspended in the same buffer.

MMO exfoliation from membrane fractions. Membrane protein fractions containing MMO were exfoliated from the membrane fractions obtained above using more violent strength sonication (800 W, 792 S). The procedure was carried out at 0–4 °C under N_2. The suspension was filtrated through a membrane with MW cut-off of 1 MDa, using a 10 mM sodium phosphate buffer (pH 7.0) containing 1 mM $MgCl_2$, 300 μM $CuSO_4$, and 2 mM, dithiothreitol as before. The exfoliated membrane protein obtained by the elution was concentrated by PEG-2000.

Purification of pMMO. A DEAE–Sepharose Cl-6B column was first degassed and then equilibrated with sodium phosphate buffer containing 1 mM $MgCl_2$, 300 μM $CuSO_4$, and 2 mM dithiothreitol (pH 7.0). All manipulations were performed in a nitrogen chamber at 0–4 °C. Exfoliated membrane protein was added to the DEAE–Sepharose Cl-6B column. NaCl (0.1 M) was then applied in the same buffer to elute pMMO. Effluent fractions (10 ml) were collected and tested for MMO activity.

pMMO activity assay. pMMO activity was measured by propene oxidation. The sample solution (1.0 ml) and 1 mM quinol (or other reductant) were added to 10 mM sodium phosphate buffer containing 1 mM $MgCl_2$, 300 μM $CuSO_4$, and 2 mM dithiothreitol (pH 7.0). The flask (ca. 10 ml) was sealed with a Teflon-sealed septum and then incubated for 5 min at 32 °C. The reaction was initiated by injecting 2.5 ml propene into the flask using a gas-tight syringe. Epoxypropane formation from propene was determined chromatographically using a gas chromatograph equipped with a capillary GC column (ϕ 0.23 mm×30 m; stationary phase, SE-54) and a flame ionization detector (FID).

Specific activities (U) were expressed as nanomoles of epoxypropane formed per minute per milligram of dry weight cell or protein in the sample, determined by the Lowery method.

Metal ion analysis and molecular weight estimation. Metal ion analysis was performed by atomic emission spectroscopy using the ICP-AES (ARL3520). The molecular weight of the pMMO system was obtained by HPLC on BIOSEP SEC-S3000 column ($7.8 \times 300\,mm^2$) using 50 mM sodium phosphate buffer (pH 6.8) as elution buffer (flow rate:0.3 ml/min). Standard proteins used were 12.5 kDa (cytochrome c), 30 kDa (alcohol dehydrogenase), 43 kDa (ovalbumin), 67 kDa (bovin serum albumin), 160 kDa (aldolase), and 250 kDa (catalase).

Results and discussion

Effect of copper ion concentration in the growth medium

M. trichosporium IMV 3011 was cultivated in mineral salt medium with various concentrations of $CuSO_4$. As shown in Table 1, high concentrations of copper give a substantially higher membrane yield and whole cells of *M. trichosporium* IMV 3011 exhibited a greater pMMO specific activity as copper concentration was increased. The correlation of membrane yield and MMO specific activity in whole cells with copper concentration suggests the possibility of a causal relationship. It is possible that in methanotrophs, pMMO is involved in intracytoplasmic membrane formation and the formation of a network of internal membrane fractions depends on copper concentration, although our results do not distinguish between intracytoplasmic membrane and outer membranes.

As shown in Table 1, the level of pMMO expression in the membrane fractions apparently does not appear to change with increasing copper concentration. The specific activity of purified pMMO was increased but the content of purified pMMO in the total membrane protein obviously decreased with increasing copper concentration. It appears that the stability of pMMO in the exfoliation process was greater with elevated copper concentration in the growth medium. But extensive intracytoplasmic membrane formed under higher copper concentration or copper itself might inhibit the exfoliation of active pMMO from membrane fraction in this strain.

Table 1
Effect of copper concentration in the culture medium on pMMO activity and membrane yield

[Cu in growth medium (μM)]	Membrane yield (mg wet weight membrane/g wet weight cell)	pMMO specific activitys				pMMO in total membrane protein (%)	Total purified pMMO activity obtained
		Whole cell (nmol/mg dry weight cell min)	Membrane (nmol/mg wet weight membrane min)	Exfoliation fraction (nmol/mg protein min)	PMMO (nmol/mg protein min)		
2	7	0.73	7.6	0.04	0.30	8.1	3.6
4	59	5.12	7.2	1.23	2.10	5.2	16.0
6	108	8.05	7.3	1.98	4.06	3.0	20.8
8	123	9.23	7.5	0.11	4.82	0.3	1.7

Since copper ions doubled the reaction rate, assays of MMO were routinely performed in the presence of 300 μM copper sulfate (data not shown).

However, cell growth in a low copper medium (2 μM) followed by preservation in a high copper medium at 30 °C for 30 min just prior to sonication did not show the same results. It has been suggested that endogenous copper (II) does not stabilize pMMO. Other forms of copper, e.g., Cu(I), might bind with pMMO during cell growth and stabilise the pMMO.

The effect of copper on the specific activity of MMO in exfoliate fractions was dependant upon the concentration incorporated in to growth medium and intracytoplasmic membranes. Specific activity increased with increasing copper level at lower concentrations, but decreased with increasing copper additions at higher concentrations. The highest total activity of purified pMMO was obtained using an initial concentration of 6 μM Cu in the growth medium. Under these culture conditions, although the recovered specific activity of purified pMMO was less than that found using higher copper concentrations, the amount and total activity of purified pMMO were significant enough to enable some conclusions to be drawn about the nature of the pMMO. The results suggested that copper ions were needed for the expression of stable pMMO from *M. trichosporium* IMV 3011 and copper in the growth medium increased stability of pMMO, possibly due to an effect on synthesis or assembly. However, higher copper concentrations appeared to stimulate proliferation of intracytoplasmic membranes. The formation of extensive intracytoplasmic membranes under higher copper concentrations might inhibit exfoliation of active pMMO from membrane fraction.

Purification and characterization of pMMO

The results of purifying pMMO from membrane protein are shown in Fig. 1. pMMO was eluted by 0.1 M NaCl from a DEAE–Sepharose Cl-6B column. Purified pMMO was able to catalyze propene oxidization using quinol as an electron donor. The UV–visible absorption spectrum of purified pMMO demonstrated the absence of other common biological co-factors; only adsorption due to the protein was observed (280–300 nm). To determine if purified pMMO was contaminated by other proteins in the membranes (e.g., heme proteins), the UV–visible absorption spectrum range from 400 to 420 nm was examined carefully (heme proteins have an absorption band at 410 nm [9]). No obvious absorption band in this range was found and heme proteins were therefore considered to be effectively absent.

The molecular weight of purified pMMO was measured by using HPLC. The pMMO component purified from membrane protein by the DEAE–Sepharose column showed the main peak, corresponding approximately to 96 kDa (80%). However, pMMO treated by detergent (10% lauryl maltoside (w/V)) gave four peaks corresponding approximately to 96–92, 72–68, 45–49, and 23–26 kDa and was completely inactive under quinol as an electron donor. This result suggested that subunits of pMMO and the electron transport chain from quinol to pMMO hydroxylase might be disrupted by detergent treatment pMMO contains at least 3 subunits, which might be co-eluted in the differently bound forms by HPLC, following detergent treatment. This result provides an explanation for the lack of activity in pMMO exfoliated from the membrane fraction by the detergent in this strain.

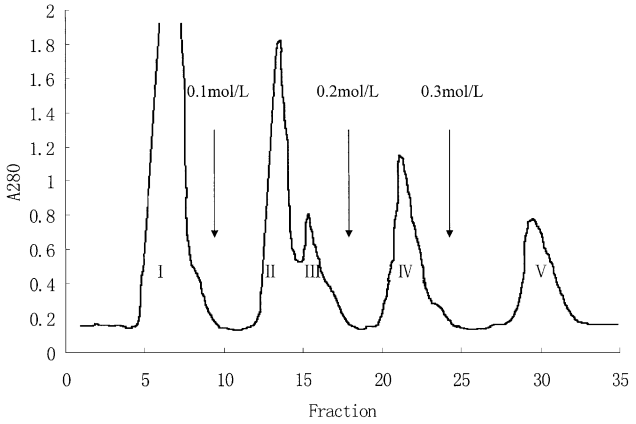

Fig. 1. Fractionation of the membrane protein on DEAE-Cl 6B column. Peak I: the unbound fraction; peak II: pMMO fraction; peaks III–V: fractions showing almost negligible MMO activity

Metal ions analysis

Although the membrane fractions contained iron (the copper/iron ratio ranges from 1.4 to 10.0), the purified active pMMO contained only copper ions. We measure 16.8–17.5 nmoles of copper per mg protein, which corresponds to 17 copper ions per pMMO molecular (MW of pMMO was 96 kDa). This copper content is close to 20 ± 3 copper per pMMO molecule reported by Nguyen et al. [7]. On the basis of high Cu/protein ratio, it seems likely that the pMMO possesses multiple copper sites. Since only copper ions were detected in this active preparation, it was concluded that copper, and not iron, is the metallic co-factor of pMMO and is a constituent of the enzyme's active site in M3011.

Reduction of pMMO by electron donors

As in all monooxygenase enzymes, pMMO requires reducing equivalents for activity. Methanotrophs are known to generate NADH from the oxidation of formaldehyde and formate. On the other hand, pMMO in membranes from some bacteria can obtain reducing equivalents from succinate. According to Shiemke et al. [11], NADH can provide reducing equivalents for pMMO in whole cells and membranes, but not after detergent solubilization. However, qui-

Table 2
Comparison of pMMO specific activity in various forms with various reductants

Reductant	Specific activity of pMMO		
	Whole cells (nmol/min mg dry cell)	Membrane fractions (nmol/min mg protein)	Purified pMMO (nmol/min mg protein)
No addition	8.1	2.7	0.4
NADH	18.2	3.8	0.4
quinol	9.2	7.3	4.1
succinate	22.1	6.2	0.4
formate	11.0	2.8	0.4

Assays of MMO activity were performed in the presence of 0.3 mM copper sulfate.

nols can provide reducing equivalents for pMMO in both membrane-bound and detergent solubilized forms. Akentieva et al. [12] reported that purified pMMO containing iron did not use NADH as the electron donor. We also tried several variations on the standard propene oxide production assay for MMO activity on various pMMO fractions (Table 2). In these studies, we showed that quinol provides reducing equivalents for MMO activity in whole cells and membrane fractions, as well as for purified pMMO, while NADH and succinate produced MMO activity in whole cells and membrane fractions. Formate provided reducing equivalents for MMO activity in whole cell only. Quinol was more effective than NADH in membrane fractions and the purified pMMO. pMMO in membranes from this bacterium can obtain reducing equivalents from succinate, suggesting that a succinate dehydrogenase similar to mitochondria complex (II) catalyzes reduction of pMMO by succinate. The relative activities of electric donors with purify pMMO are different from those observed with whole cell, membrane, and exfoliated fractions, indicating that the electron-transport chain for reduction of pMMO is altered by isolation and purification.

Effect of EDTA on pMMO activity and copper content

According to primary reports, EDTA inhibited pMMO activity in whole cell and cell membranes. To understand why EDTA inhibits pMMO, we have undertaken an investigation into the effect of EDTA on varying forms of pMMO in an attempt to describe how EDTA reacts with pMMO. Attention has been focused on copper content and pMMO activity because copper ion appears to be the metallic cofactor of the pMMO (see Metal ion analysis part). As 6 μM copper is contained in growth medium, the effect of EDTA on pMMO activity was investigated. The effects of EDTA on pMMO activity are shown in Table 3. pMMO activity in whole cells, membrane fractions, and purified forms was inhibited by EDTA to various degrees. Following treatment with EDTA, copper levels in membranes decreased by nearly 50% compared to those found in as-isolated membranes and specific activity decreased by nearly 40% compared with that of the as-isolated membrane. EDTA may remove copper ions bound loosely to the membrane.

Table 3
Effect of EDTA on pMMO activity and copper content

Sample	Addition	pMMO relative activity (%)	Content of copper (umol/mg pr)
Whole cell	None	100[a]	/[d]
	1 mM EDTA	70	/[d]
Membrane	None	100[b]	84
	1 mM EDTA	61	43
Purified pMMO	None	100[c]	0.168–0.175
	1 mM EDTA	83	0.159–0.190

Assays of MMO activity were performed in the presence of 1 mM quinol as electron donor.
[a] 100% = 4.3 nmol/min mg dry weight cell.
[b] 100% = 3.6 nmol/min mg protein.
[c] 100% = 1.9 nmol/min mg protein.
[d] Not determine.

EDTA treatment of purified pMMO, however, resulted in little change in copper levels. This suggests that copper in pMMO is tightly bound to pMMO protein. EDTA only extracted copper ion from membranes.

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