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Hydrogen peroxide as an effecter on the inactivation of particulate methane monooxygenase under aerobic conditions

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ABSTRACT

Particulate methane monooxygenase (pMMO), a copper-containing membrane protein, catalyzes methane hydroxylation under aerobic conditions. We found that the activity of pMMO was increased by catalase, implying that hydrogen peroxide (H_2O_2) is generated by pMMO with duroquinol, an electron donor for pMMO, and that the generated H_2O_2 inhibits pMMO activity. In addition, reversible inhibition of pMMO with H_2O_2 was observed upon treatment of pMMO with H_2O_2 followed by the addition of catalase, and H_2O_2 formation by pMMO with duroquinol was detected using a fluorescence probe. The redox behavior of type 2 copper in pMMO measured by the electron paramagnetic resonance revealed that H_2O_2 re-oxidizes the type 2 copper in pMMO reduced with duroquinol.

1. Introduction

Methane monooxygenase (MMO) catalyzes selective methane oxidation at ambient conditions. The enzyme is expressed in methanotrophic bacteria, which can grow aerobically with methane as the sole source of carbon and energy. All known methanotrophic bacteria with the exception of *Methylocella* express membrane-embedded particulate MMO (pMMO) in growth medium with a high copper concentration, and some but not all methanotrophic bacteria express soluble MMO (sMMO) in the cytoplasm under copper-limited conditions [1–3].

Selective catalytic oxidation of methane to methanol under mild conditions is one of the most attractive chemical reactions of natural gas exploitation. Several attempts have been made to use methanotrophic bacteria or isolated MMO as the biological catalyst for selective methanol synthesis from methane. Success of methanol biosynthesis from methane using the cells of methanotrophic bacteria was reported by some groups [4–9]. Briefly, the methanol biosynthesis is established by the inhibition of further methanol oxidation in the metabolic pathway (Scheme 1) using phosphate [4], cyclopropanol [5–7], or NaCl [8], along with the supplement of formate to regenerate NADH in the cells. Also, Xin et al. reported the methanol biosynthesis from methane and carbon dioxide [9]. On the other hand, success using isolated MMO has been elusive. One of the reasons for the difficulty of the enzymatic application is the instability of MMO isolated from the bacteria. In particular, pMMO activity is much less stable than sMMO, which hinders progress of the structural and functional analysis of pMMO.

The reason for the instability of pMMO activity has not been clarified. One of the reasons is believed to be related to structural disruption of pMMO during purification. pMMO is embedded in the bacterial lipid bilayer membrane and thus contains a hydrophobic part [10]. Therefore, purification of pMMO requires solubilization of pMMO from membrane fractions using detergent [11]; however this solubilization step is most likely to result in structural disruption of pMMO to some extent.

Another reason for the instability of pMMO may be a reactive oxygen species (ROS) generated during the isolation or activity assay of pMMO that inhibits pMMO activity. Inhibition of pMMO by ROS has not been directly examined but has been speculated from the results of sMMO research. Astier et al. established an electrochemical sMMO system, in which sMMOH was immobilized on a gold electrode [12]. In this system, catalase stimulates the hydroxylation activity of sMMOH, implying the generation of H_2O_2 from sMMOH by donation of electrons and the inhibition of sMMOH by the generated H_2O_2 activity. H_2O_2 may also be formed by pMMO because these two enzymes catalyze the same reaction. In fact, some researchers have suggested the importance of anaerobic conditions for purifying highly active pMMO [13,14], which may be due to the formation of H_2O_2 under aerobic conditions. Elucidation of the inhibitory effect of H_2O_2 on pMMO activity and the formation

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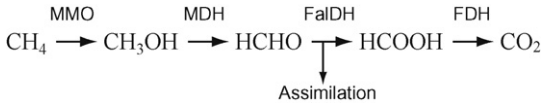

Scheme 1. Methane oxidation in the metabolic pathway of methanotrophic bacteria. MMO, methane monooxygenase; MDH, methanol dehydrogenase; FalDH, formaldehyde dehydrogenase; FDH, formate dehydrogenase.

of H_2O_2 by pMMO would clarify the mechanism of the instability of pMMO activity.

Of the ROS, H_2O_2 has relatively lower reactivity, but is nevertheless capable of damaging proteins. For instance, H_2O_2 oxidizes the sulfide group in cysteine of the proteins [15]. H_2O_2 also oxidizes metal center of enzyme such as ferrous ion in catecol-2,3-dioxygenase [16]. In addition, in the case of some copper enzymes, H_2O_2 modifies a tyrosine residue located near the copper site of the enzyme via a reaction with the copper [17,18]. This modification of the proteins causes inactivation of the enzymes, and thus may also affect pMMO activity. H_2O_2 also damages unsaturated fatty acids in a process of lipid peroxidation, which alters membrane structure and fluidity [19]. According to these observations, H_2O_2 may affect pMMO structure and its activity.

In the present study, the inhibitory effect of H_2O_2 on pMMO activity and H_2O_2 formation by pMMO with an electron donor for pMMO are investigated. The inhibition mechanism of pMMO with H_2O_2 is discussed on the basis of the redox behavior of type 2 copper in pMMO as measured by electron paramagnetic resonance (EPR) spectroscopy.

2. Materials and methods

2.1. Materials

All the chemicals were of the highest grade available and used without purification. 2-6[(4′-hydroxy) phenoxy-3*H*-xanthen-3-on-9-yl] benzoic acid (hydroxyphenyl fluorescein: HPF) was obtained from Daiichi Pure Chemicals Co. Ltd. (Tokyo, Japan). Catalase (EC 1.11.1.6) and horseradish peroxidase (HRP, EC 1.11.1.7, 100 units/mg) were purchased from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan).

2.2. Preparation of membrane fractions from M. trichosporium OB3b

Culture of the methanotrophic bacteria *Methylosinus trichosporium* OB3b was performed as described previously [20]. The bacteria were grown in NMS medium containing 10 μM $CuSO_4$. Isolation of the bacterial membranes was performed as reported previously [21] and described here briefly. All isolation steps were carried out at 4 °C unless otherwise mentioned. The buffer used for the isolation was deoxygenated by purging with nitrogen gas. Next, 30 g-wet cells of frozen bacteria were thawed at room temperature, and suspended in 15 ml of 25 mM MOPS buffer, pH 7.0 (Buffer A). The bacteria were broken on ice by sonication under N_2 stream. Just prior to breakage of the bacterial cells, 300 μM of $CuSO_4$, 10 μg ml^{-1} of DNase I, and 4 mM of $MgCl_2$ were added to the bacterial suspension. Then 1 mM of benzamidine in distilled water was added to the suspension while the cells were sonicated under N_2 flow. Debris and unbroken cells were removed by centrifugation at 27,720 × *g* for 10 min. The membranes in the supernatant were collected by centrifugation at 143,000 × *g* for 90 min. The pellet was resuspended in Buffer A containing 1 M KCl using a homogenizer, and the suspension was centrifuged under the same conditions to wash the collected membranes. The salt-washed membrane pellet was then suspended in Buffer A. The membrane fractions were used immediately for the activity assay, or stored at −80 °C.

2.3. Purification of pMMO

Purification of pMMO from the membrane fractions was performed as reported previously [21]. All steps were carried out at 4 °C unless otherwise indicated. The membrane fractions (ca. 10 mg ml^{-1}) suspended in Buffer A were degassed by bubbling with N_2 gas gently for 20 min, followed by incubation for 45 min with 2% (w/v) *n*-dodecyl-β, D-maltoside under N_2. After the incubation, the suspension was centrifuged for 90 min at 203,000 × *g*. The solubilized supernatant was then applied to a POROS 20 HQ column (1 × 10 cm) equilibrated with Buffer A containing 0.1% (w/v) Brij 58. The adsorbed protein was washed with Buffer A containing 0.1% (w/v) Brij 58, then eluted using a concentration gradient of KCl from 0 to 1 M. pMMO was eluted around 0.15 M. SDS-PAGE analysis of purified enzyme showed only three bands attributed to three subunits of pMMO [21]. The metal analysis by inductively coupled plasma atomic emission spectroscopy indicated that purified pMMO contains 2–3 coppers and no iron per pMMO protomer.

2.4. Methane monooxygenase assay

MMO activity was measured as described previously [20]. MMO activity was assayed using propene epoxidation in Buffer A containing duroquinol as the reductant. A reaction vial (3 ml) sealed with a Teflon-sealed septum contained 300 μl of reaction mixture containing pMMO sample (membrane fractions or purified pMMO) (2 mg-protein ml^{-1}) and duroquinol (5 mM) in Buffer A. The reaction was initiated by the injection of 0.3 ml of propene into the reaction vial using a gas-tight syringe at 30 °C. The amount of produced propylene epoxide was measured using a gas chromatograph equipped with a flame ionized detector. All the measurements were performed at least three times using the same sample.

2.5. Detection of H_2O_2 generation

H_2O_2 production during the pMMO assay was measured based on oxidation of the fluorogenic indicator hydroxyphenyl fluorescein (HPF) in the presence of horseradish peroxidase (HRP) [22]. The reaction mixture containing purified pMMO (2 mg ml^{-1}) and duroquinol (5 mM) in Buffer A was incubated for 1 min at 30 °C, and an aliquot was sampled from the reaction vial. The sample was immediately diluted 5% with Buffer A followed by the addition of both HPF and HRP. The final concentrations of HPF and HRP were 0.1 unit ml^{-1} and 10 μM, respectively. Fluorescence emission spectra (excited at 490 nm) were recorded at 515 nm. Standard curves were obtained by the addition of known amounts of H_2O_2 to the assay medium in the presence of both HPF and HRP. As the control, the generation of H_2O_2 was also measured in the absence of the pMMO samples.

2.6. EPR measurement

EPR spectra of type 2 copper in pMMO were recorded at 77 K on a JEOL RE1X ESR spectrometer. EPR samples were prepared as follows. Reduction of pMMO with duroquinol was carried out anaerobically at room temperature. H_2O_2 (200 μM) or catalase (1 mg ml^{-1}) was added to purified pMMO (1 mg ml^{-1}) in a 3 ml reaction vial sealed with a rubber septum and then the vial degassed under vacuum followed by flushing of Ar into the vial several times. The sample was transferred to a vial containing duroquinol (final concentration 60 μM) under Ar and the reaction continued for 30 min at room temperature with stirring under