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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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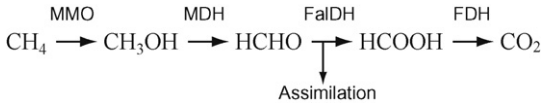

Scheme 1. Methane oxidation in the metabolic pathway of methanotrophic bacteria. MMO, methane monooxygenase; MDH, methanol dehydrogenase; FalDH, formaldehyde dehydrogenase; FDH, formate dehydrogenase.

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

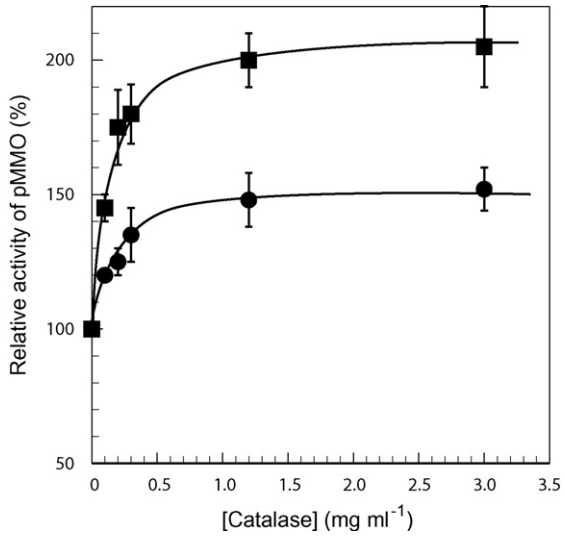

Fig. 1. Effect of catalase on pMMO activity in the membrane fractions (■) and purified enzyme (●). 100% of each activity is 3.4 and 2.4 nmol-propene oxide min^{-1} mg-protein^{-1} respectively.

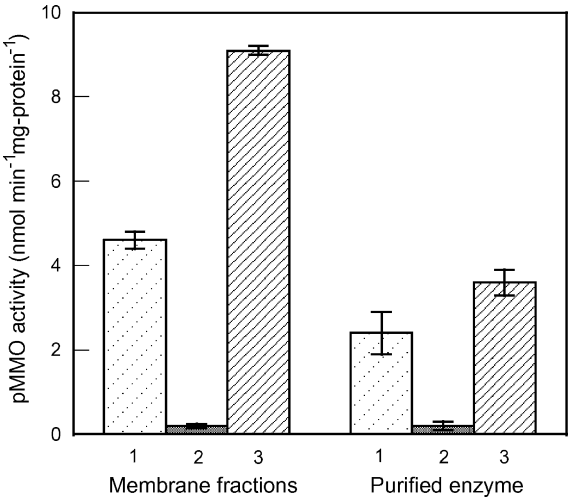

Fig. 3. Effect of catalase on duroquinol-driven pMMO activity in H_2O_2-treated membrane fractions and purified enzyme. The activity was measured prior to the H_2O_2 treatment of pMMO (1), after the treatment with H_2O_2 (1 mM) (2), and after the addition of catalase (final concentration: 1 mg ml^{-1}) to the H_2O_2-treated pMMO (3).

Ar. Next, 500 μl of the mixture was transferred anaerobically to a quartz EPR tube, followed by rapid freezing in liquid nitrogen. The concentration of EPR-detectable copper was obtained by double integration of the copper signal measured, and comparison to Cu(II)-EDTA as the standard.

3. Results

3.1. Inhibitory effect of H_2O_2 on purified pMMO

The effect of catalase, which catalyzes decomposition of H_2O_2, on pMMO activity is shown in Fig. 1. pMMO activity increased when catalase was added, implying the presence of H_2O_2 in our pMMO sample and that H_2O_2inhibits pMMO activity.

The inhibitory effect of H_2O_2 on pMMO activity was measured by the addition of H_2O_2 to pMMO. Fig. 2 shows the dependence

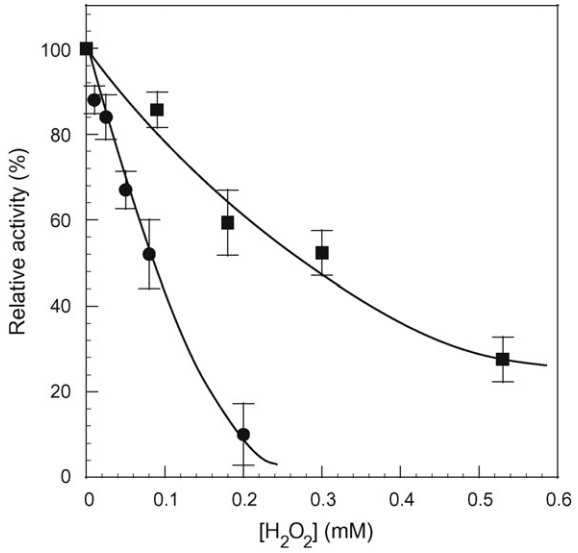

Fig. 2. Inhibitory effect of H_2O_2 on pMMO activity in the membrane fractions (■) and purified enzyme (●). 100% of the activity is 3.4 and 2.6 nmol-propene oxide min^{-1} mg-protein^{-1} respectively.

of pMMO activity on H_2O_2 concentration. The pMMO activity decreased as H_2O_2 concentration increased, indicating that H_2O_2 inhibits pMMO activity. The pMMO activity inhibited by H_2O_2 was restored by the addition of catalase (Fig. 3).

In some copper-containing enzymes, such as dopamine β-monooxygenase and amine oxidase, the inhibitory effect of H_2O_2 on enzyme activity is time dependent. This is caused by modification of the tyrosine residue near the copper site of the enzymes via the reaction of H_2O_2 with the copper site, which prevents the catalytic cycle of these enzymes [17,18]. Structural analysis has revealed that pMMO does not have tyrosine residues near the copper binding sites [10], suggesting that the mechanism of H_2O_2 inhibition of pMMO differed from these enzymes. In fact, in the present study time dependence of pMMO inhibition by H_2O_2 was not observed; even after a 30 min incubation of pMMO with H_2O_2, pMMO activity was recovered completely by the addition of catalase (data not shown). The inhibition of pMMO by H_2O_2 is reversible and clearly distinct from the case of dopamine β-monooxygenase and amine oxidase.

pMMO contains copper ions that are involved in the activity of pMMO, and therefore the coordination and/or electron state of the copper ions may be affected by H_2O_2. The effect of H_2O_2 on the copper ions in pMMO was measured by EPR spectroscopy. A type 2 copper signal ($g_{//} = 2.23$, $|A_{//}| = 18.8$ mT, $g_{\perp} = 2.06$) with a super-hyperfine structure at $g_{\perp}$ was observed in purified pMMO (Fig. 4a) [21] EPR spectrum quantitation and metal content analysis revealed that about 80% of copper in the purified pMMO is EPR-detectable. No change in the type 2 copper signal was observed when H_2O_2 was added to pMMO anaerobically (data not shown).

Although duroquinol is an electron donor for pMMO, the type 2 copper (24 μmol g-protein^{-1}) in pMMO inhibited by H_2O_2 was not markedly reduced with duroquinol (Fig. 4b). In contrast, in the presence of catalase, about 50% of the type 2 copper (13 μmol g-protein^{-1}) was reduced with duroquinol (Fig. 4c). These results indicate that H_2O_2 prevents the reduction of type 2 copper with duroquinol, most likely by re-oxidation by H_2O_2 of the copper that had once been reduced by duroquinol. The correlation between the inhibition of pMMO activity and the prevention of type 2 copper reduction suggests that the prevention of reduction of type 2 copper in pMMO causes the inhibition of pMMO by H_2O_2.

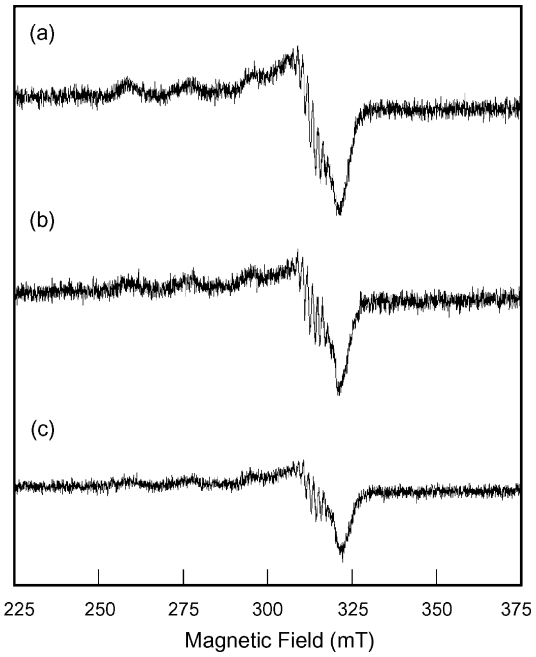

Fig. 4. Effect of catalase on the redox behavior of type 2 copper in the purified pMMO measured by X-band EPR. (a) pMMO inhibited by H_2O_2 (0.2 mM); (b) addition of duroquinol (60 μM) to sample (a) anaerobically; and (c) addition of duroquinol (60 μM) anaerobically in the presence of catalase (1 mg ml^{-1}) (c). The spectra were recorded under the following conditions: temperature, 77 K; modulation width, 0.5 mT; gain, 400; power, 1 mW; frequency, 9.007 GHz.

3.2. H_2O_2 formation by pMMO with an electron donor

The increase in pMMO activity by catalase (Fig. 1) suggests the inhibition of pMMO by H_2O_2, which was confirmed by the results shown above, and also suggests that our pMMO sample contained H_2O_2. One possibility of the source of H_2O_2 in our sample is the pMMO itself. As suggested previously, in the case of sMMO [12] over-reduction of the enzyme causes H_2O_2 formation by the reaction as shown in reaction 1, which is the reaction without MMO substrate, methane (reaction 2).

$$O_2 + 2H^+ + 2e^- \rightarrow H_2O_2 \quad (1)$$

$$CH_4 + O_2 + 2H^+ + 2e^- \rightarrow CH_3OH + H_2O \quad (2)$$

The mixture for the pMMO assay contains duroquinol, an electron donor for pMMO, which may induce the formation of H_2O_2 by pMMO under aerobic conditions. Duroquinol-induced H_2O_2 formation by pMMO under aerobic conditions was measured fluorometrically (Table 1). In this experiment H_2O_2 was detected in the reaction mixture of the pMMO assay, whereas H_2O_2 was not detected in the mixture without duroquinol or pMMO. These results indicate that duroquinol induces H_2O_2 formation by pMMO. In the presence of pMMO substrate, methane, the H_2O_2 formation was diminished, which is likely to be caused by the consumption of electrons by methane oxidation (reaction 2), not by H_2O_2 formation (reaction 1).

Table 1
H_2O_2 generation by pMMO in the presence of duroquinol under aerobic conditions.

Sample	H_2O_2 (μM)
Purified pMMO + duroquinol	3.7 ± 0.1
Purified pMMO + duroquinol + CH_4	2.1 ± 0.2

4. Discussion

We have demonstrated for the first time the inhibitory effect of H_2O_2 on pMMO activity and on reduction of type 2 copper in pMMO with duroquinol. The formation of H_2O_2 by pMMO with duroquinol was also observed. These results strongly suggest that reversible H_2O_2 inhibition of pMMO activity is generated during the assay of pMMO activity. Addition of catalase to the reaction mixture is effective to measure the pMMO activity without the inhibitory effect of H_2O_2.

In addition, we found that pMMO activity increased upon addition of catalase despite H_2O_2 not being added to the pMMO sample (Fig. 1), indicating that H_2O_2 is also generated during the isolation steps of pMMO. The pMMO sample during the isolation steps, particularly at the beginning steps, still contained some reductant from the bacteria, and the reaction mixture of the pMMO assay also contained duroquinol. Via these reductants H_2O_2 was generated by pMMO under aerobic conditions and thus accumulated in the isolated pMMO. The H_2O_2 accumulation in pMMO preparation is one of the reasons for the importance of anaerobic conditions for the isolation of pMMO with high activity. In fact, we purified pMMO in the presence of catalase, resulting that the purified pMMO had quite higher activity (8–9 nmol min^{-1} mg^{-1}) than aerobically purified pMMO (<0.4 nmol min^{-1} mg^{-1}). This result indicates that preventing H_2O_2 accumulation during isolation is effective for obtaining pMMO with high activity.

Having said that, many researchers have succeeded in the isolation of pMMO with high activity without the addition of catalase. Furthermore, pMMO from *Methylococcus capsulatus* (Bath) appears not to be oxygen sensitive [23]. This may be due to the expression of proteins that decompose H_2O_2 or inhibit H_2O_2 generation in methanotrophic bacteria. For instance, cytochrome c peroxidase and glutathione peroxidase, which have the ability to decompose H_2O_2, have been detected in *Mc. capsulatus* (Bath) [24,25]. In those studies cytochrome c peroxidase and glutathione peroxidase were probably present in the pMMO sample at the beginning of the isolation procedure, and may have protected pMMO during isolation by decomposing H_2O_2. Thus, optimization of culture conditions such that cytochrome c peroxidase and glutathione peroxidase are expressed efficiently in methanotrophic bacteria may yield high quality purified pMMO.

The finding that H_2O_2 is formed by pMMO in the presence of duroquinol raises the question of how H_2O_2 is formed from oxygen and duroquinol by pMMO. One possible site forming H_2O_2 is di-nuclear metal site in pMMO. According to X-ray crystallographic analysis and EXAFS studies, there is the site modeled as di-nuclear copper in pMMO [10,23,26]. Also, Mössbauer studies implied a di-nuclear iron cluster at the site occupied by zinc ion in the pMMO crystal [27]. Reduced form of these di-nuclear metal sites such as Cu^+–Cu^+ and Fe^{2+}–Fe^{2+} can donate two electrons to reduce molecular oxygen to form H_2O_2, thus might be H_2O_2 generation site. Alternatively, to interpret our results in this study, the heterogeneity should be considered. Our pMMO preparation seems to be heterogeneous regarding the number of copper ion per pMMO protein molecule. Our pMMO sample contains 2–3 copper ions per pMMO protomer (about 100 kDa), whereas pMMO crystal from *M. trichosporium* OB3b has 3 copper ions per protomer. This discrepancy is indicative that some pMMO proteins in our pMMO preparation are likely to lack some of the copper ions. These subpopulations may cause the H_2O_2 formation and the inhibitory effect on pMMO activity. Only the pMMO proteins lacking some copper ions may be associated with the production of H_2O_2 with duroquinol, though the pMMO that all copper sites are fully occupied with copper ions is not. For instance, the protein having only di-nuclear copper center produces hydrogen peroxide. Also, if the

mono-nuclear site occupied by one copper and the di-nuclear site occupied only one copper, hydrogen peroxide may be produced between these two sites. Close to both the two copper sites, there is a nonprotein patch of strong electron density that could accommodate at duroquinol. Therefore, both of the copper sites can be accept electrons from duroquinol, then produce H_2O_2.

The decrease in type 2 copper EPR signal of pMMO by the addition of duroquinol indicates that type 2 copper in pMMO undergoes redox chemistry. Based on the results of crystallographic, EPR and EXAFS analysis of pMMO reported by Resenzweig and coworkers [26,28], the decrease in the type 2 Cu^{2+} EPR signal of pMMO by the reduction with duroquinol can be explained as follows: First of all, there should be two Cu^{2+} exhibiting type 2 Cu^{2+} EPR signal in pMMO, which accept electrons from duroquinol. Probably one of the copper ions exhibiting type 2 copper signal is originated form the mono-nuclear site. If there is the companion redox partner accepting an electron from duroquinol in pMMO, it should be one of the copper ions at the di-nuclear site. From the EPR measurement of our pMMO sample, there was no signal originated from the delocalized copper center, $Cu^{+1.5}$–$Cu^{+1.5}$, in our pMMO sample. Therefore, in our as-isolated pMMO sample, there should be a completely localized or trapped valence copper center, Cu^{+}–Cu^{2+} at the di-nuclear site, which shows the type 2 copper signal. We do not have any evidences of this interpretation that there is the completely delocalized, or trapped valence copper center in pMMO. For making this point more clear, highly homogeneous pMMO with fully complement of copper ions should be required.

5. Conclusion

In conclusion, H_2O_2 is formed by pMMO in the presence of its electron donor, duroquinol, and inhibits pMMO via oxidation of the reduced form of type 2 copper in pMMO, which is involved in the enzyme activity. Anaerobic conditions are important for retaining the activity of isolated pMMO as these conditions suppress H_2O_2 formation by pMMO. Another way to protect pMMO from H_2O_2 during the isolation step and activity assay is the coexistence of proteins that decompose H_2O_2.

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