THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 14, Issue of April 3, pp. 7957–7966, 1998

Printed in U.S.A.

The Particulate Methane Monooxygenase from *Methylococcus capsulatus* (Bath) Is a Novel Copper-containing Three-subunit Enzyme

ISOLATION AND CHARACTERIZATION*

(Received for publication, July 7, 1997, and in revised form, December 22, 1997)

Hiep-Hoa T. Nguyen‡, Sean J. Elliott, John Hon-Kay Yip, and Sunney I. Chan§

From the Arthur Amos Noyes Laboratory of Chemical Physics, California Institute of Technology, Pasadena, California 91125

The particulate methane monooxygenase (pMMO) is known to be very difficult to study mainly due to its unusual activity instability *in vitro*. By cultivating *Methylococcus capsulatus* (Bath) under methane stress conditions and high copper levels in the growth medium, membranes highly enriched in the pMMO with exceptionally stable activity can be isolated from these cells. Purified and active pMMO can be subsequently obtained from these membrane preparations using protocols in which an excess of reductants and anaerobic conditions were maintained during membrane solubilization by dodecyl β-D-maltoside and purification by chromatography. The pMMO was found to be the major constituent in these membranes, constituting 60–80% of total membrane proteins. The dominant species of the pMMO was found to consist of three subunits, α, β, and γ, with an apparent molecular mass of 45, 26, and 23 kDa, respectively. A second species of the pMMO, a proteolytically processed version of the enzyme, was found to be composed of three subunits, α′, β, and γ, with an apparent molecular mass of 35, 26, and 23 kDa, respectively. The α and α′ subunits from these two forms of the pMMO contain identical N-terminal sequences. The γ subunit, however, exhibits variation in its N-terminal sequence. The pMMO is a copper-containing protein only and shows a requirement for Cu(I) ions. Approximately 12–15 Cu ions per 94-kDa monomeric unit were observed. The pMMO is sensitive to dioxygen tension. On the basis of dioxygen sensitivity, three kinetically distinct forms of the enzyme can be distinguished. A slow but air-stable form, which is converted into a "pulsed" state upon direct exposure to atmospheric oxygen pressure, is considered as type I pMMO. This form was the subject of our pMMO isolation effort. Other forms (types II and III) are deactivated to various extents upon exposure to atmospheric dioxygen pressure. Under inactivating conditions, these unstable forms release protons to the buffer (~10 H^+/94-kDa monomeric unit) and eventually become completely inactive.

The enzyme methane monooxygenase, found in methanotrophic bacteria, catalyzes the conversion of methane to methanol using dioxygen as a co-substrate at ambient temperatures and pressures (1, 2). This system has attracted considerable attention, since it provides an ideal natural model to study methane activation and functionalization, a subject of significant current interest (3). Two distinct species of methane monooxygenase (MMO)[1] are known to exist at different cellular locations, a cytoplasmic (soluble) MMO and a membrane-bound (particulate) MMO (4). The soluble MMO (sMMO) is a complex three-component system consisting of a hydroxylase, a reductase, and a small regulatory protein (4). The sMMO has been investigated extensively by several research groups (5–21). The x-ray crystal structure of the sMMO hydroxylase isolated from *Methylococcus capsulatus* (Bath) has been solved (22, 23). The hydroxylase active site contains a non-heme binuclear iron cluster. In contrast, the particulate methane monooxygenase (pMMO) appears to be a copper protein (24–29). This enzyme is much less well characterized mainly due to its unusual activity instability.

Despite the lability of the enzyme activity *in vitro*, the pMMO appears to be expressed in all methanotrophs (1, 2, 4). So far, the sMMO has been detected in only the following strains and species: *M. capsulatus, Methylosinus trichosporium, Methylosinus sporium, Methylocystis* sp. M and *Methylomonas methanica* 68–1 (6, 30–34). In strains capable of expressing either the sMMO or pMMO, the sMMO is expressed under copper stress only (low copper/biomass ratio) (35–39). Otherwise, the pMMO is expressed. Copper ions not only regulate the expression of the pMMO but have been found to be crucial for pMMO activity. The expression of the pMMO is accompanied by the formation of an extensive network of intracytoplasmic membranes, where the membrane-bound pMMO resides (35–39). An increase in carbon to biomass conversion efficiency is also observed. Three new polypeptides with apparent molecular masses of 45, 35, and 26 kDa were observed in the membrane fractions when *M. capsulatus* (Bath) switched from expressing the sMMO to the pMMO (35–39).

Recent progress in our laboratory indicates that the pMMO is a novel copper-containing enzyme. Metal/protein ratio data analysis clearly suggests that the pMMO is a multiple copper-containing enzyme (24–26, 28, 29, 40). Activity was found to be proportional to the level of membrane-bound copper ions (24, 27–29). The pMMO-associated copper ions appear to be orga-

* This work was supported by NIGMS, National Institutes of Health, Grant GM 22432 (to S. I. C.). Unrestricted financial support was also received from the George Grant Hoag Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡Recipient of a W. R. Grace fellowship and a National Research Service Predoctoral Award.

§ To whom correspondence should be addressed: Noyes Laboratory of Chemical Physics 127-72, California Institute of Technology, Pasadena, CA 91125. Tel.: 626-395-6508; Fax: 626-578-0471; E-mail: chans@cco.caltech.edu.

[1] The abbreviations used are: MMO, methane monooxygenase; sMMO, soluble methane monooxygenase; pMMO, particulate methane monooxygenase; pMMOH, pMMO hydroxylase; AMO, ammonia monooxygenase; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PAGE, polyacrylamide gel electrophoresis.

nized into trinuclear cluster units with rather defined magnetic and redox properties (24–26, 28, 29, 40). The as-isolated pMMO-enriched membranes often contain a mixture of Cu(I) and Cu(II) ions in various proportions, depending on the handling of the samples (25, 26, 28, 29, 40). Hence, the functional form of the enzyme has been suggested to be the reduced or partially reduced form. The chemistry catalyzed by this enzyme is also highly specific. pMMO-catalyzed hydroxylation of cryptically chiral ethanes has implicated a reaction mechanism proceeding with complete retention of alkane substrate configuration (41, 42). This extraordinary chemistry currently has no precedent in known model and biological systems. Accordingly, insights regarding the copper-containing active site of the pMMO can provide a new direction in the design of biominetic catalysts for methane activation and functionalization.

The pMMO has been known to be very difficult to study. As noted earlier, one of the main obstacles in studying the pMMO is the unusual instability of the activity of the enzyme. Activity is frequently lost upon cell lysis, detergent solubilization, and freeze-thaw cycles. In several cultures, no activity was observed in cell-free extracts, or activity quickly disappeared within 6 h after cell lysis (24–26, 28, 29, 40). Enzymatic activity is also known to be very sensitive to exogenous ligands as well as the choice of buffer. This highly unusual instability has hampered efforts in characterizing the pMMO. The addition of copper ions is known to enhance enzymatic activity under certain conditions (cells grown at low copper levels), but the effect of copper ions in the extension of pMMO activity is not known. No reagent is currently known to reactivate the enzyme once the protein becomes inactive. As a result, a highly active, stable, and purified preparation of the enzyme has been slow in forthcoming.

Past efforts in isolating the pMMO have resulted in significant confusion regarding the nature of the enzyme. An early report of pMMO isolation from *M. trichosporium* OB3b indicates that this system can utilize ascorbate in addition to NADH as electron donors and was found to consist of three components: a 47-kDa polypeptide containing various amounts of copper, a smaller 9.4-kDa subunit, and a 13-kDa CO-binding cytochrome *c* (43). In later studies, the aforementioned ascorbate-linked activity was not observed, and attempts to solubilize the enzyme resulted in complete deactivation of the protein. Solubilization of pMMO from *M. capsulatus* (Bath) using a nonionic detergent was attempted, and upon detergent removal and lipid vesicle reconstitution, partial activity was observed (44). According to the authors, any attempts to purify the enzyme further resulted in complete loss of activity. A few reports including a recent work (45, 46) appear to support the notion that the active site of the pMMO may contain iron despite the overwhelming evidence accumulated to date suggesting that copper is the element responsible for catalysis within the enzyme active site. Thus, to advance the field, the presence of iron and copper in the pMMO must be resolved.

This paper summarizes our efforts to isolate and purify the pMMO from *M. capsulatus* (Bath) for biochemical and biophysical characterization. Toward the development of suitable protocols for pMMO isolation, we have embarked on an extensive investigation of factors contributing to enzymatic activity stability, including various methods of bacterial cultivation and membrane isolation, and various schemes of enzyme stabilization and purification. We find that the details of the bacterial cultivation and isolation methods significantly affect the quality of the membranes and the protein isolated from them. Methods of bacterial cultivation and pMMO isolation were optimized such that active and purified preparations of the enzyme could be recovered. Active membrane fractions, highly enriched in pMMO and exhibiting exceptionally stable activity, were subsequently isolated using various procedures, assayed for activity, solubilized with detergents, and fractionated using available methods of protein purification. For such preparations, activity can be maintained in the membrane-bound forms for an extended period of time, a minimum of 3–4 days and up to 10 days at 4 °C with stable or enhanced activity (stable with respect to repeated freeze-thaw cycles and prolonged storage at −80 °C). Aside from describing these procedures in this report, we will discuss several other critical issues relating to the nature of the pMMO, particularly whether or not the pMMO is a copper-containing enzyme only, the subunit composition of the enzyme, and whether or not there is more than one form of the protein as suggested by recent genetic data.

MATERIALS AND METHODS

Growth of Methanotrophs and Membrane Isolation—M. capsulatus (Bath) used in the studies were maintained on Petri plates containing the nitrate mineral salts medium with added $CuSO_4$ (20 μM) and solidified with 1.7% agar. Cultures were maintained under an atmosphere of 20% methane in air and streaked onto fresh plates every 4–6 weeks (47). Chemostat cultures (9–10 liters) were grown according to the following procedure. The organisms were first transferred from Petri plates to 250-ml flasks and subsequently to 2-liter Erlenmeyer flasks, containing 40 and 300 ml, respectively, of the nitrate mineral salts medium with added $CuSO_4$ (10 μM), a 20% methane in air atmosphere, and continual shaking. The organisms were allowed to grow for 48 h in these small scale cultures. The 300-ml cultures were used to seed a fermentor containing 9 liters of the above described medium with added 20 μM $CuSO_4$ and 20 μM CuEDTA. The methane feeding rate was controlled such that methane is growth-limiting (feeding rate ~0.01–0.012 $feet^3$/h·liter). The methane/air ratio was 1:4. A cell density of >10 g/liter of culture can be obtained at a higher methane feeding rate. However, the employed methane feeding rate, termed as methane stress condition (semistarvation growth condition), results in less biomass; typically only ~5–6 g of wet cells/liter would be obtained. However, this condition was found to stimulate the overproduction of the intracytoplasmic membranes, which also contain exceptionally high levels of the pMMO (see below). Furthermore, it also stimulates copper uptake (high copper/protein ratio), resulting in exceptionally high pMMO specific activity. Approximately 24 h after inoculation and 6 h prior to cell harvest, additional $CuSO_4$ (or CuEDTA) was added to bring the total added copper concentration to 50 and 60 μM, respectively. Six h and 3 h prior to cell harvest, the methane feeding rate was increased incrementally to 0.03–0.04 $feet^3$/h·liter to relieve the starvation condition (partly to increase cell density). Without this step, the activity was not stable although the membranes contained unusually high levels of the pMMO. *M. capsulatus* (Bath) was grown at 42 °C. The pH must be maintained at 6.8–7.4 during growth. Cells were harvested in late log phase (typically 48–52 h after inoculation) by centrifugation at 27,000 × *g* for 15 min and washed twice with 50 mM Pipes (pH 7.2). Washed cells were suspended in lysis buffer containing 50 mM Pipes, 4 mM ascorbate, 50 μg of catalase/ml of buffer, pH ~7.2 (typically 60 g of wet cells and buffer to a volume of ~75 ml of cell suspension). Cu(II) ions (100 μM $CuSO_4$) can also be added to this buffer to improve the enzyme stability further. However, the addition of copper often caused the ascorbate-containing buffer to lose its effectiveness rather quickly and would complicate metal content analysis of the purified protein, so it was routinely omitted.

Cell suspensions (~0.8–1.0 g of cells/ml) were passed three times through a French pressure cell at 20,000 p.s.i. to separate the cytosolic and membrane fractions. Less dense cell suspensions (<0.5 g of cells/ml) often result in low activity or completely inactive cell-free extract and membranes. It appeared that the dense cell suspensions used here (i) kept the dioxygen tension low, hence minimizing copper oxidation and (ii) resulted in highly viscous lysate, which helped to protect the integrity of the membrane-bound pMMO during the isolation process. Unlysed cells and cell debris were removed by centrifugation at 27,000 × *g* for 40 min. The supernatant was then ultracentrifuged at 220,000 × *g* for 90 min to pellet the membrane fraction. The clear supernatant obtained after ultracentrifugation was used as the cytosolic fraction. The pelleted membranes often show distinct layers. The minor bottom layer containing bluish and black materials and the thin, white top layer were discarded. Only the middle layer, or the *translu-*

cent intracytoplasmic membranes, constituting the bulk of the membrane fractions, were collected. These membranes can be separated further on the basis of their texture into "soft" and "hard" membranes, albeit with difficulty. The difference between these two types of membranes is not great although the hard membranes appear to have higher intact pMMO content. The translucent membranes were washed by suspending them in washing buffer containing 50 mM Pipes, 5 mM ascorbate, 25 μg of catalase/ml (pH 7.2) using a Dounce homogenizer, repelleted by ultracentrifugation, and resuspended in washing buffer of 2–3 times the volume of the original cell suspension. This process was repeated a few more times until the supernatant was virtually free of soluble proteins. Finally, the pelleted membranes were suspended in storage buffer (low ionic strength storage buffer, 20–25 mM Pipes, 5 mM ascorbate, 25 μg of catalase/ml of buffer, pH ~7.25; or high ionic strength storage buffer, 75–100 mM Pipes, 50 mM imidazole, 5 mM ascorbate, 25 μg of catalase/ml of buffer, pH ~7.25) in a volume equal to the original cell suspension volume. Sucrose (200 mM) can also be added to the above buffers to improve stability further. The membrane suspensions then can be kept at 4 °C or frozen at liquid nitrogen temperature and stored at −80 °C for future use. It should be noted that activity was found to quickly decrease if the membranes were too dilute (*i.e.* the storage buffer volume was several times higher than that of the original cell suspension).

Membrane Solubilization—The membrane suspension was first degassed by several vacuum/argon cycles. The membranes in storage buffer (in either low or high ionic strength buffer) were then treated with either solid or 20% (w/v) stock solution of dodecyl β-D-maltoside (to a final concentration of 3–5% (w/v) or ~2 mg of detergent/mg of protein). The mixture was mixed rigorously and incubated on ice for 30 min to 1 h and then centrifuged at 37,000 × *g* for 45 min to remove unsolubilized materials. The clear supernatant was taken as the solubilized membranes, and used for subsequent steps.

Rapid Isolation Procedure Using L-Lysine-Agarose Affinity Chromatography and Removal of Positively Charged and Iron-containing Proteins—The L-lysine-agarose column (Sigma) (20 × 2 cm) was equilibrated with buffer containing 25 mM Pipes, 5 mM ascorbate, with or without 200 μM $CuSO_4$ and 0.05% (w/v) dodecyl β-D-maltoside, pH ~7.25. Dithionite (5 mM) can be used in lieu of ascorbate; however, strict anaerobic protocol must be followed. The solubilized membranes (~2 ml; ~40–60 mg of total protein) were applied to the column, and 0.5-ml effluent fractions were collected, employing an elution buffer of 20–25 mM Pipes, 5 mM ascorbate, 0.05% (w/v) dodecyl β-D-maltoside, pH 7.2, but with no sucrose or imidazole added. The elution rate from the column was typically 0.5–1.0 ml/min. Three to four fractions can be obtained. The flow-through fraction contains large pieces of the solubilized membranes and most of the positively charged proteins. A fast moving fraction contains several proteins (heme-containing proteins) but also pMMO (purity of ~70% or higher). Next, a slow moving fraction constitutes the bulk of the solubilized membranes and contains mostly the three-subunit form of the pMMO (purity of ~90% or higher). Finally, a minor binding fraction can be eluted out of the column using buffer containing 50 mM Pipes, 100 mM NaCl, pH ~7.25. This binding fraction consists of mostly heme-containing proteins but also some residual pMMO.

Large Scale Isolation Procedure Using Anion Exchange Chromatography and Removal of Positively Charged and Iron-containing Proteins—Large scale isolation of the enzyme can also be obtained with a variation of the above procedure using DEAE-Sepharose Fast Flow (Amersham Pharmacia Biotech). A DEAE-Sepharose Fast Flow column was equilibrated with buffer containing 100 mM Pipes, 50 mM imidazole, 5 mM ascorbate, 200 μM $CuSO_4$, 0.05% (w/v) dodecyl β-D-maltoside buffer at pH ~7.25. Sucrose (200 mM) can also be included in this buffer, but its effectiveness is not great. 5–7 ml of solubilized membranes (concentration 20–30 mg/ml) in high ionic strength storage buffer were then applied to the column. When an anaerobic protocol was used, the column was first degassed, dithionite (5 mM) was added to the equilibrating buffer to remove dissolved dioxygen, and the manipulations were performed in an anaerobic chamber. The column was then washed with one column volume of the equilibrating buffer. DEAE-Sepharose FF column fractionates the solubilized membranes into four fractions. There are two flow-through fractions, a fast moving fraction (~10 ml) containing a mixture of two forms of pMMO (see below) and a slow moving fraction (~10–20 ml) containing positively charged proteins (proteins of high pI), a truncated form of pMMO, and other impurities. The bound proteins are eluted out using the above high ionic strength buffer combining with a NaCl (or NH_4Cl) gradient from 0 to 200 mM. They are separated into two fractions. The fraction eluted out at <100 mM NaCl (~200 ml) contained mostly the pMMO as judged from the SDS-PAGE assay (purity >90%). The second fraction (50 ml or less) eluted out of the column at higher salt concentration (>100 mM NaCl) with a characteristic low pI and low molecular mass contaminant (~22 kDa) as well as other minor impurities. The isolated proteins were concentrated using Amicon ultrafiltration membranes (M_r cut-off 50,000 or 100,000).

If desired, the binding fraction can be fractionated further using QEA-Sephadex A-50, albeit with a significant reduction in recovered activity. QEA-Sephadex A-50 column was equilibrated with buffer containing 50 mM Pipes, 50 mM NaCl, 50 mM imidazole, 200 mM sucrose, 5 mM ascorbate, 200 μM $CuSO_4$, and 0.03% (w/v) dodecyl β-D-maltoside, pH ~7.2. The binding fraction was then applied to the column. The pMMO can be eluted out using a NaCl (or NH_4Cl) gradient. The pMMO (a light green band) is normally eluted out at around 200 mM NaCl.

Lipids and Membrane-bound Quinone Isolation—Membrane suspensions (protein concentration >20–30 mg/ml) were mixed with a methanol/chloroform (1:3, v/v) mixture. The wet membrane suspension/extraction solution mixture (typically 1:5 to 1:10, v/v) was shaken rigorously and decanted. The process was repeated at least three times to ensure complete extraction. The extracts were combined, dried over anhydrous $MgSO_4$, and decanted. After solvent removal, the crude yellowish lipids were dried and stored under vacuum for later use.

Membrane-bound quinones were extracted using an ethanol/*n*-hexane mixture (2:5, v/v). The extracts were combined, dried over $MgSO_4$, and decanted. After solvent removal, the isolated quinones were reduced by sodium borohydride. The resulting quinols, obtained as precipitates, were washed with a minimum amount of water and then with ethanol, dried, and stored under vacuum for later use.

Protein Reconstitution—A 2–3-ml volume of the buffer containing 10–20 mg/ml of the isolated lipids was sonicated for 10–15 min to disperse the lipids and mixed with 1 ml of purified protein in detergent-containing buffer (protein concentration 20–30 mg/ml). The resulting mixture can be sonicated briefly for a few seconds to assure dispersion. As soon as the detergent-containing protein solution was added, the solution became clear. The mixture was then loaded into dialysis tubing (M_r cut-off 50,000 or 100,000). The tube was dialyzed against a buffer containing 50 mM Pipes, 10 mM ascorbate, 200 μM $CuSO_4$, 100 mM $(NH_4)_2SO_4$ for 12 h with continuous stirring. The reconstituted protein was assayed immediately for activity and stored either at 4 °C or −80 °C for later use. The excess detergent can also be removed using BioBeads SM-2. A volume of ~2–3 ml of the purified protein (~30–50 mg/ml) was passed through a column (1 × 5 cm) of Bio Beads, and the eluate was concentrated using Amicon ultrafiltration membranes and mixed immediately with a sonicated lipid suspension (10–20 mg/ml) as described above (lipid/protein ratio 1:1 or 2:1, v/v). This mixture can be sonicated briefly for a few seconds to ensure dispersion. The reconstituted protein was then assayed for activity immediately. The BioBeads method did not prove to be a useful approach to prepare lipid-reconstituted protein, since the pMMO tended to precipitate out of the buffer as soon as the detergent was removed.

MMO Activity Assay—The MMO activity of samples was measured by alkane substrate (propane, butane) oxidation or propene epoxidation assays. For membrane fractions, solubilized membranes, purified protein, and reconstituted pMMO, the reductant of choice was NADH. Dithionite (3–5 mM) was also found to be capable of supporting turnover; however, it must be used in conjunction with a strong buffer (100 mM Pipes) and must be assayed using alkane substrates. Duroquinol and membrane-originated quinols were tested as potential sources of reducing equivalents. Duroquinone obtained from Sigma and quinones isolated from the membranes were reduced by sodium borohydride and purified as described above.

Each of the reductants was added to the membrane suspensions to give a final concentration of 5 mM in a total volume of ~1.0 ml. The assay was performed at 45 °C, and at ~5–7-min intervals, a 1-μl aliquot of the solution was removed and injected directly onto a gas chromatograph for chemical analysis. Oxidation products were identified and quantified by GC using a flame ionization detector. The activity of the pMMO was determined from the limiting initial slope of product(s) *versus* time plot. Specific activity was then obtained by dividing the activity by the total amount of protein in the sample as determined by the Lowry method.

SDS-PAGE, Protein Blotting, and N Terminus Sequencing—Each pool of protein obtained during purification as well as the concentrated purified protein was analyzed using SDS-PAGE (12.5–15%) according to Laemmli (48). It is essential not to heat the protein in dissociating buffer before loading, since this step results in substantial degradation and cross-linking. The polypeptide bands were visualized by staining with Coomassie Blue.

The purified protein were first subjected to SDS-PAGE (12.5%) using the protocols described above. The proteins were then blotted into Immobilon-P membranes using the TransBlot apparatus (Bio-Rad) with a modified procedure in which the SDS concentration in the Tris/glycine transfer buffer was at least 0.2% (49, 50). Upon staining the Immobilon-P membranes with Coomassie Blue to visualize the polypeptides, the bands corresponding to each subunit were excised, and the N terminus sequence was determined using the Edman degradation method.

Metal Assay—Metal ion analysis (copper, iron, zinc, cobalt, manganese, and nickel) was performed by inductively coupled plasma-mass spectroscopy. The copper concentration of the samples was determined relative to standard solutions of $Cu(NO_3)_2$, ranging in concentration from 7.3 to 155 μM in 0.1 N HNO_3 (Aldrich). A solution of 0.1 N HNO_3 in distilled water was used as a copper-free control. Samples of purified pMMO were used as obtained or digested at 45 °C using ultrapure metal-free sulfuric acid obtained from Aldrich. The protein solution was diluted with ultrapure water containing 0.1 N HNO_3 to the appropriate concentration prior to analysis. The values reported are the averages of three separate determinations. The same samples were used for iron analysis, but the standards were prepared by diluting an iron atomic absorption standard purchased from Sigma with 0.1 N HNO_3.

UV-visible and EPR Spectroscopy—EPR spectra were recorded on a Varian E-line Century X-band spectrometer. In the EPR experiments, sample temperature was maintained at 77 K with a liquid nitrogen Dewar or at 4.2–100 K with an ESR-900 Oxford Instruments (Oxford, United Kingdom) liquid helium cryostat. The EPR samples were prepared by sealing 200 μl of protein solution under an atmosphere of argon in quartz EPR tubes at a total protein concentration of ~50 mg/ml in 20 mM Pipes (pH 7.2). UV-visible spectra were taken using a Hewlett-Packard HP 3502A UV-visible spectrometer.

RESULTS AND DISCUSSION

The Overproduction of Highly Enriched pMMO-containing Membranes—The cultivation of methanotrophs is often plagued by a foreign organism contamination, possibly another methylotroph, which thrives on the byproducts of methanotroph metabolism. As a result, samples of methanotroph chemostat cultures were routinely withdrawn and scrutinized under a microscope every 12 h to ensure the culture purity. Only absolutely pure cultures as ascertained by microscopy were subjected to membrane isolation and further experiments. At low methane feeding rate conditions as described, the release of methane metabolism by-products was minimized, and bacterial contamination was completely eliminated. In addition to eliminating bacterial contaminants, maintaining a low methane feeding rate also stimulates the overproduction of the intracytoplasmic membranes and the pMMO. We routinely obtain a minimum of 60% or more cellular mass in the form of these membranes using growth conditions as described above. In these membranes, the pMMO indeed constitutes the bulk of the membrane-bound proteins (Fig. 1).

In the case of *M. capsulatus* (Bath), the addition of a substantial level of iron (>2 μM FeEDTA) often results in the concomitant expression of sMMO in some bacterial population, as evident by sMMO activity being detected in the soluble fraction. Furthermore, sMMO associates itself rather tenaciously to the membrane fractions and distorts the activity or oxidation product distribution when the membranes are assayed using other hydrocarbon substrates. It can be also copurified with the pMMO, hence giving a false impression of recovered activity. Therefore, care must be exercised to wash the membranes thoroughly to remove cytosolic materials as much as possible.

To obtain pMMO preparations with high and stable activity, we have observed that it is essential to maintain relatively high copper concentrations consistently throughout growth. High levels of copper, however, must be coupled with low methane feeding rates. High methane feeding rates give substantially higher biomass, but the membrane yield is low. Furthermore, low and unstable pMMO activity is frequently observed in these membranes, which also contain significantly less copper. Iron is also found to be another important element for growth, but copper is the only ion found to be consistently crucial for pMMO activity. These organisms have unusual tolerance for high copper concentrations. The presumed toxicity of copper ions was found to be due to the fact that that high $CuSO_4$ concentrations (>50 μM) added to the nitrate mineral salts medium buffered at pH ~7 cause a significant precipitation of nutrients in the form of highly insoluble complexes. This precipitation depletes the pool of essential nutrients, resulting in poor growth. The addition of CuEDTA to the growth medium, along with $CuSO_4$, to buffer the free Cu(II) concentration alleviates the depressed growth, as long as the pH is maintained around 7 for the duration of the growth. This protocol allows us to obtain high quality pMMO preparations routinely without supplementing the medium with any other elements (iron included).

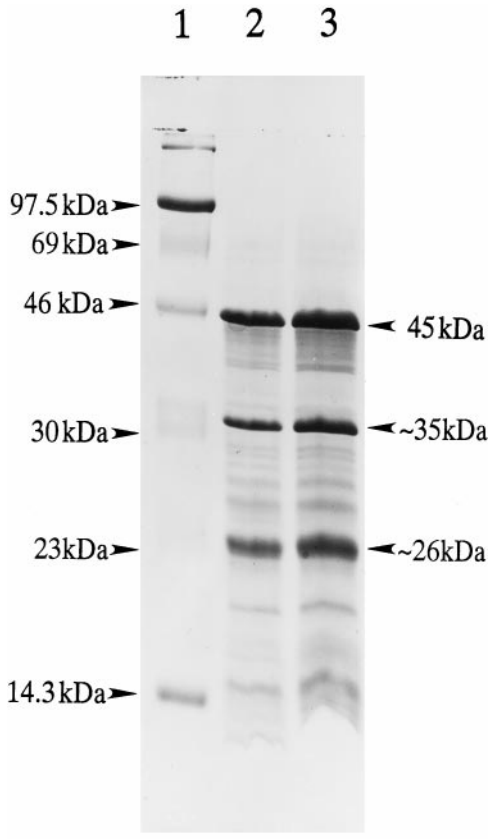

FIG. 1. **SDS-PAGE of pMMO-overexpressed membranes isolated from *M. capsulatus* (Bath).** *Lane 1*, standards. *Lanes 2* and *3*, electrophoretic profile of pMMO-overexpressed membranes isolated from *M. capsulatus* (Bath) grown under methane stress conditions. Note the absence of high molecular weight polypeptides in these preparations.

Methodology for pMMO Isolation—To develop a suitable protocol for successful isolation of the active enzyme, several unusual features of the pMMO had to be recognized (1, 2). The enzyme appears to require a lipid environment to function, since solubilization often results in >90% loss of activity (44). Previous attempts at isolating the protein indicated that once being removed from the lipid bilayer, the enzyme deactivates quickly. Our on-going characterization of the enzyme *in situ* indicates that the enzyme contains an exceptionally high level of Cu(I) ions, a unique feature for a monooxygenase (25, 26). As such, the loss of activity upon solubilization and during purification could be a result of overoxidation and subsequent loss of some of the more labile copper cofactors. In the protocols for

isolating the sMMO hydroxylase developed by Fox *et al.* (5), the reduced form of iron was introduced into the isolating buffer, resulting in preparations with a 10–20-fold increase in activity. Similar approaches may not work for the pMMO, a copper-containing protein, since an air-stable Cu(I) complex with a high dissociation constant is not readily available. Despite the fact that Cu(II) ions have been found to improve the pMMO activity for certain pMMO preparations, the bulk of the copper ions associated with the pMMO actually exists in the reduced Cu(I) form. As Cu(I) ions are extremely insoluble in aqueous solution, these copper sites are relatively inert. However, once oxidized in an aerobic environment, they can become quite labile, particularly for those copper cofactors that are relatively exposed.

In light of these considerations, we have elected to develop either an anaerobic procedure *or* an aerobic procedure that can be carried out under an environment where copper oxidation is minimized during solubilization and isolation. Accordingly, in our protein isolation and purification experiments, copper oxidation was minimized by using deoxygenated buffer, performing manipulations in an anaerobic chamber, and by adding dithionite (2–5 mM) to the buffers to remove dissolved oxygen. Indeed, our first successful attempt at recovering activity from purified pMMO was achieved using a protocol in which degassed buffer was used with an excess of ascorbate to reduce all the copper ions in the preparation.

Another unusual feature of the pMMO system is that the enzyme is overexpressed in the membranes. As such, the main purpose of the isolation procedure is not to enrich the pMMO several hundred- or thousand-fold, as is commonly done for many other proteins and enzymes, but the objective is to remove other contaminating proteins in the membranes, particularly heme proteins and other iron-containing proteins, as much as possible. Once purified, we relied on reconstitution experiments on these pMMO preparations to optimize the recovered activity.

Components of the pMMO System—Assuming that we can isolate intact pMMO with full metal content, it does not follow that we will observe optimal activity, since maximal activity may require the presence of other crucial components such as a pMMO reductase and possibly an activity-regulatory protein. The existence of a pMMO reductase is certain, since NADH, a reductant capable of supporting pMMO turnover *in vitro* is a two-electron donor, while each copper ion is a one-electron acceptor. This fact suggests the presence of a mediator. From the available literature, we can postulate possible types of reductase systems for the pMMO. A two-component pMMO system would consist of a pMMO hydroxylase and a reductase that accepts electrons from NADH and channels them directly to the hydroxylase, a scenario found to be the case for the sMMO. A three-component system might consist of a pMMO hydroxylase; a mediator component, which could be a species like cytochrome *b* or *c* or a quinone analog; and a NADH oxidoreductase, which accepts electrons from NADH and channels them to the mediator molecule. Depending on the nature of the mediator, this oxidoreductase could be a NADH/quinone oxidoreductase or a NADH/cytochrome oxidoreductase and could be either membrane-bound or membrane-soluble. Recent results of Shiemke *et al.* (51) suggest that the pMMO system might be a three-component quinone oxidoreductase/quinol/hydroxylase. Confirmation of this hypothesis can be readily made by assaying the enzyme in the form of membrane-bound, solubilized, or purified/reconstituted pMMO hydroxylase using the quinones isolated from the membranes, purified and reduced as described. However, a conclusive result has yet to be obtained. In any case, this scenario now seems rather unlikely, with the recent isolation and characterization of a flavin-containing NADH oxidoreductase that appears to be associated with the pMMO.[2]

TABLE I
Metal content and activity of representative purified pMMO hydroxylase preparations

Copper/Protein[a]	Iron/Protein	Activity[b]
μmol/mg protein	*μmol/mg protein*	*nmol/min · mg protein*
0.141[c] (15)	~0.00[d]	2.6[e] (9.66, 27%)
0.133[f] (12.4)	~0.00	5.1[e] (12.5, 41%)

[a] The number of copper atoms per protein molecule is shown in parentheses. It was determined assuming that the monomeric pMMO hydroxylase has a subunit composition of $\alpha\beta\gamma$ and a molecular mass of 94 kDa.
[b] The membrane activity and percentage of recovered activity are shown in parentheses.
[c] This preparation was prepared using the DEAE-Sepharose procedure.
[d] The residual level of iron in the purified pMMO is a copper/iron ratio of ~70–100:1.
[e] NADH was used as electron donor in the activity assay. Dithionite as reductant also yielded comparable results. The recovered activity was found to vary from preparation to preparation.
[f] This preparation was obtained using the L-lysine-agarose protocol.

Characterization of the Purified pMMO Hydroxylase Activity: Metal Content—The major protein isolated from the membranes from now on will be referred to as the pMMO hydroxylase (pMMOH). The isolated pMMOH upon detergent removal and lipid reconstitution exhibits a significant level of recovered activity (Table I). Although the recovered activity has yet to be as high as the level in whole cells or in our most active membrane preparations to date, the level of observed activity is significant enough for us to draw important and critical conclusions regarding the nature of the pMMO.

Although the membrane fractions contain certain levels of iron (the copper/iron ratio fluctuates greatly, ranging from 7:1 to 20:1 or higher), the purified pMMOH contains only copper ions (Table I). Since only copper ions are detected in these active preparations, one can conclude now that copper, not iron, is the metallic cofactor of the pMMOH and constitutes the active site(s) of the enzyme. Even in inactive and "purified" pMMOH preparations, copper was also the only metal ion detected in any significant and stoichiometric amounts. A recent report regarding the ammonia monooxygenase (AMO) isolated from *Paracoccus denitrificans* also indicates that only copper was found in the purified enzyme (52). Considering the sequence homology shared by the two enzymes and biochemical similarities between the pMMOH and the AMO (2, 40, 52–59), metal analysis of the AMO is consistent with our conclusion that the pMMOH contains copper only; in other words, it is a copper protein. The number of copper ions in active preparations is high (12–15 copper atoms/protein molecule). It has been suggested that the copper ions in pMMOH can be grouped into two distinct classes of copper on the basis of their preferential functions. These copper ions have been referred to by their roles in either catalysis (C-copper) *or* electron transfer (E-copper). In active and purified preparations of the pMMOH, a significant amount of copper is reduced (26). These Cu(I) ions (the E-copper ions) could serve as the source of endogenous reducing equivalents for turnover and hence probably were partially responsible for the observed recovered activity upon lipid reconstitution in the absence of added external reductant like NADH. The presence of Cu(I) ions in the pMMOH is consistent with other studies known to date regarding the biochemistry of the pMMO and AMO (2). Both pMMO and

[2] S. J. Elliott and S. I. Chan, unpublished data.

AMO are known to be very sensitive to copper chelators, particularly the ones highly specific to Cu(I) ions (for instance, allylthiourea) and are sensitive to light, a possible manifestation of Cu(I) photochemistry (2). Thus, it is understandable that the isolation of active AMO was achieved by using a protocol performed in the dark, which is consistent with the inactivation of Cu(I) ions in the enzyme by light (52). Growth inhibition by light was observed for certain methanotrophs that are known to express only the pMMO (1, 2).

Since the number of copper ions per monomeric unit (94 kDa) is high, it is evident that the copper ions cannot all be ligated by multiple histidines, since there are only 11 histidine residues in the gene sequences of *pmoA* and *pmoB* published to date, and not all of these histidines are conserved and positioned at suitable locations for copper ligation (*e.g.* the conserved histidine residue at the N terminus). It also seems likely that many of these copper ions may be ligated to the nitrogen atoms in the peptide backbone or other side chain ligands, such as the carboxylates of glutamates and aspartates. These "hard" peptide-backbone nitrogen and carboxylate ligands of the pMMO-associated copper ions may explain several unique aspects of the copper chemistry exhibited by the enzyme. Since this type of coordination sphere confers rather weak binding to Cu(I) ions and since these E-copper ions are located in the exposed domains of the proteins,[2] they are readily disrupted during protein isolation and purification and oxidized to Cu(II). Once oxidized and dissociated, the enhanced solubility of the Cu(II) ions renders the inactivation process irreversible. This scenario explains in part the lability of the E-coppers as well as the rapid loss of the enzyme activity when they become oxidized.

The fact that our pMMO preparations contain only copper ions is in conflict with a recent report, which suggested that the pMMO may contain iron (45). The presence of iron in the latter pMMO preparation is clearly a result of unaccounted for iron-containing contaminants in the preparations as well as the experimental protocols employed in the study, where the organisms were cultivated under unusually high iron concentrations. Cultivating the organism in the presence of excess iron (60 μM) could have significant physiological consequences, ranging from concomitant sMMO expression and the expression of iron transport and storage proteins (bacterial ferritin) to increases in nonspecific iron binding, etc.

DiSpirito *et al.* (53) also reported the isolation of an aa_3-type oxidase and an aa_3-cytochrome *c* complex from the membranes isolated from *M. capsulatus* (Bath). This oxidase preparation contained at least 7–10 copper and 2–3 iron atoms/94-kDa monomer. An electrophoretic profile of this oxidase preparation, however, showed remarkable similarity to the pMMO polypeptides reported here. Considering the level of copper ions in oxidases isolated from other organisms (2–3 copper atoms/monomer), it is clear that this oxidase preparation was heavily contaminated by the pMMO. On the other hand, we have observed only ba_3-type oxidase so far in the membranes isolated from *M. capsulatus* (Bath). In light of the likelihood of bacterial contamination noted earlier, efforts to isolate oxidase from these membranes must ensure that the methanotroph culture used is pure. It should also be noted that attempts to isolate oxidase from these membranes are hampered by the low expression levels of the oxidase compared with those of the pMMO. Our results indicated that the oxidase level in the membranes is very low, at most constituting ~2–3% of total membrane proteins. The existence of an oxidase, however, points to the presence of electron transport chain complexes (complexes I, II, and III) in the membranes. These respiratory enzymes contain mostly heme-iron and iron-sulfur clusters. The existence of these multiple iron-containing proteins explains the presence of iron in the membranes detected previously (24, 27–29). On the basis of the observed level of oxidase expression, ~10–15% of total membrane proteins are probably respiratory enzymes. This expression level of these enzymes, which contain an average of 5–7 iron atoms/monomer of respiratory enzyme, affords an estimate of a membrane copper/iron ratio of 7–20:1, given that the pMMO expression level is ~60–80% of total membrane proteins and that the average level of copper incorporation into the pMMO is ~15 copper atoms/94 kDa. This is indeed the ratio observed (24–29). These results reinforce the conclusion that the pMMOH is a copper protein only.

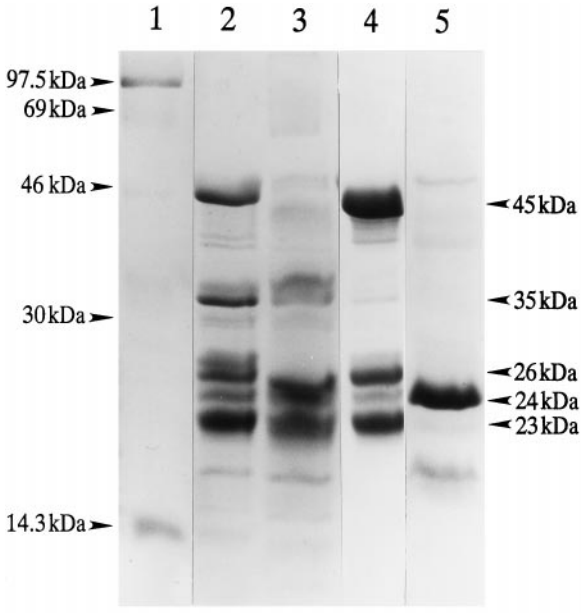

FIG. 2. **SDS-PAGE of fractions obtained from DEAE-Sepharose chromatography of solubilized membranes isolated from *M. capsulatus* (Bath).** *Lane 1*, standards. *Lane 2*, fraction 1 (flow-through, fast moving fraction). Note the prominent presence of all of the 45-, 35-, 26-, and 23-kDa polypeptides. The purity of the pMMO (both forms of the enzyme) in this fraction is estimated at >70%. *Lane 3*, fraction 2 (flow-through, slow moving fraction). Note the near absence of the 45-kDa band; however, the 35-kDa band is still present (albeit with smearing) as well as the ~26- and ~23-kDa bands. The presence of lightly stained high and low molecular weight polypeptides can also be seen. *Lane 4*, fraction 3 (weakly binding fraction eluted at <100 mM NaCl or NH_4Cl). This fraction is mostly the pMMO (purity >90%). The 26-kDa band exhibits some smearing. *Lane 5*, fraction 4 (strongly binding fraction eluted at >100 mM NaCl or NH_4Cl). This fraction contains mostly a very low pI, 24-kDa polypeptide which has an N-terminal sequence of AAQASLERNL. Other minor impurities can also be seen.

Subunit Composition of the pMMO Hydroxylase and pMMO Polymorphism—Purification of pMMOH from solubilized membranes and assaying of the polypeptide profile of the collected fractions demonstrates that three polypeptides are consistently co-purified together concomitant with recovered activity. This result suggests that at least one form of pMMO contains three subunits, since co-elution during purification is one of the criteria to determine the subunit association of an enzyme. SDS-PAGE analysis of the purified pMMOH fractions obtained from these experiments indicates an apparent molecular mass of 45, 26, and 23 kDa, for these subunits, named α, β, and γ, respectively (Fig. 2, *lane 3*). The assertion that the pMMOH contains a core of three different subunits is also supported by the observation that in several highly active and stable preparations, electrophoretic profiles of membranes (as well as purified pMMO) display *only* three major bands intensely, implying that these three subunits are all of the essential membrane-bound components needed for pMMO activity. The 35-kDa

TABLE II
The N-terminal sequence of the pMMO hydroxylase

Subunit	Molecular mass	Sequence
	kDa	
α	45	HGEKSQAAFMRTIHWYDLSWSKEKVKINGTV
α'	35	HGEKSQAAFMRTIHW[a]
β	26	SAVRSHAEAVQSRTIDWMALFVVFFVI
γ	23	AAAEAPLKDKKWLTFA[b]

[a] Approximately 90% of the 35-kDa band had the above sequence. This sequence was obtained using the pMMO fraction shown in Fig. 3, *lane 1*. The minor 10% contaminant of this 35-kDa polypeptide appeared to have the following N-terminal sequence: ARPLIDLALTL-DIPD.

[b] Polypeptides with the following N-terminal sequences were also observed: AAEAPLKDKKWLTFA and AEAPLKDKKWLTFA.

polypeptide observed previously in MMO expression switch-over experiments is absent in these preparations (35–39) (see below).

The 45-kDa subunit is one of the polypeptides observed previously in the electrophoretic profile of the membranes when the organism switches from sMMO to pMMO expression (35–39). In these experiments, when the organism switches from sMMO to pMMO expression, increasingly higher levels of the 45- and 26-kDa polypeptides were observed concomitantly with decreasing amounts of sMMO hydroxylase-associated polypeptides (35–39). We surmise that the E-clusters reside in the exposed domains of this subunit on the cytoplasmic side. The 26-kDa subunit is the acetylene-binding polypeptide, which almost certainly contain the C-clusters, namely, the active site(s) of the enzyme (24–29). This subunit contains an N-terminal sequence identical to the gene sequence already reported, except it started with a serine residue instead of a methionine (40, 54). The function of the 23-kDa subunit is not clear at the moment. Since previous studies did not detect its presence, it remains to be established that this subunit is conserved in all methanotrophs.

The N-terminal sequences of these pMMO subunits have been determined using Edman degradation, and the results are tabulated in Table II. Using oligonucleotide probes derived from these N terminus sequences, Lidstrom and co-workers (25, 40, 54) have identified two copies of the genes coding these polypeptides and have determined their gene sequences. The genes encoding these subunits have been named *pmoA* (26 kDa), *pmoB* (45 kDa), and *pmoC* (23 kDa). The closely arranged proximity of these pMMO genes is further evidence in strong support of a three-subunit pMMO hydroxylase.

The presence of two pMMO gene copies first suggested that two distinct forms of the enzyme may exist. Sequencing results, however, have indicated that the two pMMOH structural genes are almost identical, hence the existence of significantly different forms of the pMMO in terms of primary sequence is unlikely (25). Instead, the presence of almost identical multiple pMMO gene copies suggests a genetic mechanism for control of the level of enzyme expression; *i.e.* the level of pMMO expression can be altered by the rate of protein synthesis *via* a transcription mechanism in response to metabolic need. This conclusion implies that pMMO can be overexpressed, and it is found to be the case. As described above, the conditions for pMMO overexpression have been identified and exploited, allowing us to obtain large quantities of the membranes containing mostly pMMO.

As first suggested by the N-terminal sequence of these polypeptides and later by the amino acid sequence deduced from the genes cloned to date, it is clear that pMMOH and AMO do not exhibit significant homology to any proteins sequenced to date (40, 54–59). This result establishes that pMMO is indeed a novel enzyme that together with AMO constitutes a new class of copper-containing proteins. These two enzymes share about ~40% homology on the gene portion already sequenced, significantly enough to indicate that these two enzymes are evolutionary related (54).

The above described results establish that the 45- and 26-kDa polypeptides and the newly elucidated 23-kDa polypeptide are parts of the pMMO hydroxylase complex. However, when the organism switches from sMMO to pMMO expression, a 35-kDa polypeptide has been observed as well, and its role has yet to be resolved. In several membrane preparations exhibiting high and stable activity, this polypeptide is also observed in significant levels, regardless of switch-over conditions, and it copurified with the three polypeptides described above (Fig. 2, *lanes 1* and *2*). This result suggests the formation of a tight complex among these polypeptides that does not dissociate under purification conditions. To our surprise, this 35-kDa polypeptide contains an N-terminal sequence identical to that of the large 45-kDa subunit. This result indicates that it is a product of the C terminus proteolytic cleavage of the 45-kDa subunit. However, since it is copurified or observed with two or three other subunits, it is likely that it is part of another form of the pMMO instead of a proteolytic degradation of the 45-kDa subunit. Two possible scenarios can account for this result. The first scenario is that there are three-subunit ($\alpha\beta\gamma$) and four-subunit forms of the pMMO ($\alpha_1\alpha_2\beta\gamma$), where α_1 and α_2 are the 45- and 35-kDa polypeptides, respectively. The other possibility is that there are two forms of the three-subunit pMMO. One form is composed of the 45-, 26-, and 23-kDa polypeptides ($\alpha\beta\gamma$), while the other consists of the 35-, 26-, and 23-kDa polypeptides ($\alpha'\beta\gamma$). The latter scenario is more consistent with our data, since we have obtained fractions containing mostly the 35-, 26-, and 23-kDa polypeptides with only a minor presence of the 45-kDa polypeptide (Fig. 3, *lane 1*). In this preparation, we obtained two distinct fractions with significantly different polypeptide compositions (Fig. 3, *lanes 1* and *2*), and the intensity of the bands in this preparation is consistent with a protein with a subunit composition of 35-, 26-, and 23-kDa polypeptides (Fig. 3, *lane 1*). As such, the 35-kDa polypeptide may be a post-translational modified version of the 45-kDa subunit. The process of proteolytic maturation via extensive peptidase action could lead to new folding structure and different catalytic properties. The presence of this 35-kDa polypeptide, however, has been observed only for the *M. capsulatus* (Bath) strain. Taken together, the nearly identical but multiple gene copies for each subunit, the variation in the N-terminal sequence, and the existence of a proteolytic processed version of the enzyme all point to a unique and rather unexpected sequence, composition, and structural polymorphism regarding the pMMO hydroxylase.

Spectroscopic Characterization of the Purified pMMO Hydroxylase—The UV-visible and EPR spectra of various purified pMMO preparations are shown in Figs. 4, 5, 6, and 7. The purified pMMOH also appears not to contain any other common biological cofactors as suggested by its UV-visible absorption spectrum (Fig. 4). The UV-visible spectrum of the purified pMMOH exhibits only the protein absorption (~280–300 nm) and a very weak band at 410 nm that can be attributed to a very slight cytochrome contaminant(s). Further efforts to remove the cytochrome contaminants resulted in complete deactivation of the enzyme and hence were not pursued further.

The purified pMMOH also exhibited an EPR spectrum similar to the spectrum of the overexpressed pMMO membranes reported previously (Fig. 5). This EPR spectrum is typical of Cu(II) ions in a quasi-square planar coordination environment. As noted earlier, most of the E-cluster coppers remain reduced

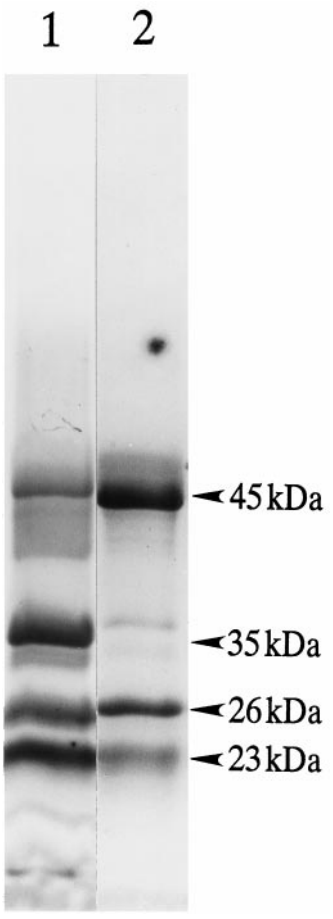

FIG. 3. **SDS-PAGE of two major fractions obtained from DEAE-Sepharose chromatography of unusual solubilized membranes isolated from *M. capsulatus* (Bath).** *Lane 1*, low ionic strength fraction (<100 mM), where the 35-kDa polypeptide is prominent. The 35-, 26-, and 23-kDa band intensities are nearly the same, whereas the 45-kDa band is virtually absent. *Lane 2*, high ionic strength fraction (>100 mM), where the 45-, 26-, and 23-kDa band intensities are nearly the same, whereas the 35-kDa band is almost completely absent.

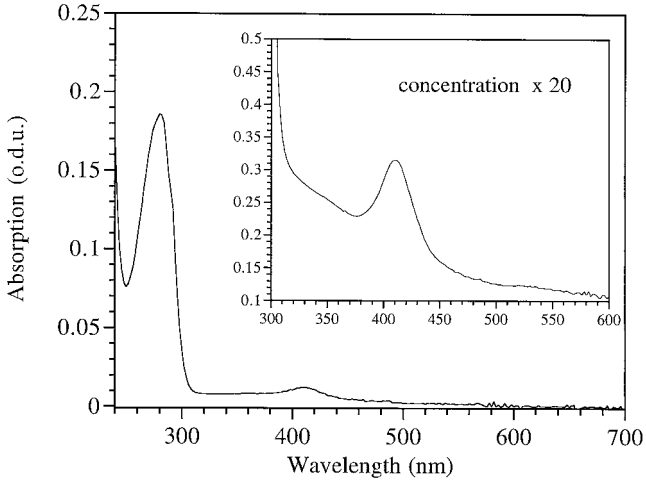

FIG. 4. **UV-visible spectrum of the purified pMMO that has the $\alpha\beta\gamma$ (45-, 26-, and 23-kDa) subunit composition.** Note the presence of a very weak absorption band at ~410 nm due to minor heme contamination. The concentration was 2.3 μM (*inset*, 40.6 μM) at pH 7.2 in 25 mM Pipes buffer and 0.05% dodecyl β-D-maltoside.

following the isolation and purification, and the EPR spectrum observed arises from the turnover of the C-cluster coppers by dioxygen.

The coppers of the purified pMMO can also be fully oxidized by ferricyanide treatment. Excess oxidant was removed by repeated washing. This preparation exhibited a quasi-isotropic EPR signature (Fig. 6), which has been attributed to trinuclear copper clusters (25, 26). The observed EPR intensity is consistent with the oxidation of all 15 copper ions arranged in the five trinuclear clusters. Interestingly, the ferricyanide-oxidized pMMO showed a broad optical absorption band at ~500 nm (Fig. 7). The origin of this absorption band will be examined in future investigations.

Factors Contributing to pMMO Activity Stability and Kinetically Distinct pMMO Forms—Maintaining high copper concentrations during growth has a marked effect on the isolated membranes, particularly higher and longer lasting activity *in vitro*. Equally important factors are cell growth/life cycle, pH, and levels of copper oxidation in solution (which are linked to dioxygen tension). Cell cycle is critical, since we obtain preparations with stable activity only with mature cells grown on Petri plates for more than 7 days and less than 4 weeks. The pH strongly affects the activity of the enzyme. Upon exposure of highly active membrane preparations to acidic pH (pH <7, or even a short exposure to 6.8), the membranes quickly lose all of the activity. While readjusting the pH to physiological ranges (7.2), no activity, or at best base-line activity, can be observed. Interestingly, certain membrane preparations become acidic rather quickly upon direct exposure to atmospheric oxygen, and once this phenomenon occurs, all activity is lost quickly. It has become clear to us that this is the underlying reason for the unusual instability of the pMMO activity: in all instances where activity is lost at any stage (cell lysis, membrane isolation, membrane solubilization, or the thawing of frozen membranes), a significant drop in pH is *always* observed.

This proton release phenomenon is linked to dioxygen tension (hence to copper oxidation). In several preparations, when treating these membranes with pure dioxygen (even prolonged storage at 4 °C, thus allowing more reaction time), a drop in pH is observed, and all pMMO activity is lost. Adding reductants (ascorbate or NADH) or deoxygenating the membranes does extend the life of these preparations, suggesting a link between dioxygen tension and copper oxidation with the observed proton release and activity loss. At the moment, it appears that the reaction of dioxygen with pMMO in the absence of substrates triggers the release of internal protons in this form of the pMMO. The source of these protons is yet to be determined, although they may be associated with glutamate/aspartate side chains that are not ligated to copper ion(s), as would be the case when the protein is not loaded with its full complement of copper cofactors. On the basis of the buffer strength (20 mM Pipes, pK_a ~6.8), the magnitude of the pH drop (from 7.2 to 6.6), and the protein concentration (~50 mg/ml), the level of proton release is estimated to be ~10 H^+/94-kDa protein monomer.

On the basis of results obtained to date, three kinetically distinct pMMO forms can be distinguished. Type I pMMO is the stable form. This form exhibits moderate but stable specific activity *in vitro* and is also stable with respect to repeated freeze-thaw cycles and prolonged storage at −80 °C. Type I pMMO is isolated only from slow growing bacteria and has been described in great detail in this paper. As a result of its enhanced stability, it is the most suitable for isolation. Increases in dioxygen tension (treating the protein suspensions with pure dioxygen or aerobic storage at 4 °C) will result in the formation of the "pulsed" state of the enzyme, which exhibits severalfold increases in activity *in vitro*, and only a minimal amount of proton release is observed (1–2 H^+/94-kDa monomer). Type II pMMO is the highly unstable form that is pre-

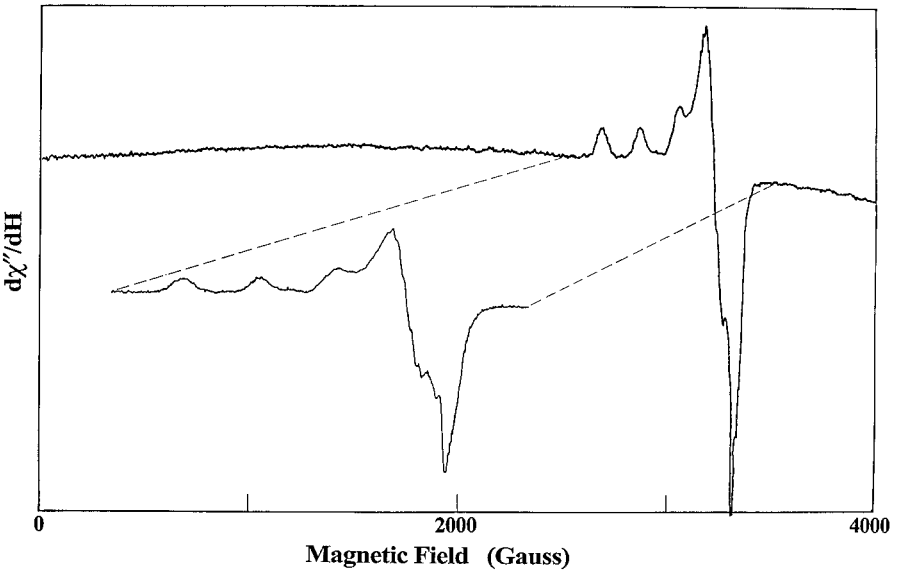

FIG. 5. **The X-band EPR spectrum of the purified pMMO as isolated.** The spectrum was recorded at the temperature of 8 K with microwave power of 0.1 milliwatt, modulation frequency of 100 kHz, modulation amplitude of 10 Gauss, and gain of 8×10^3. See "Materials and Methods" for protein concentration.

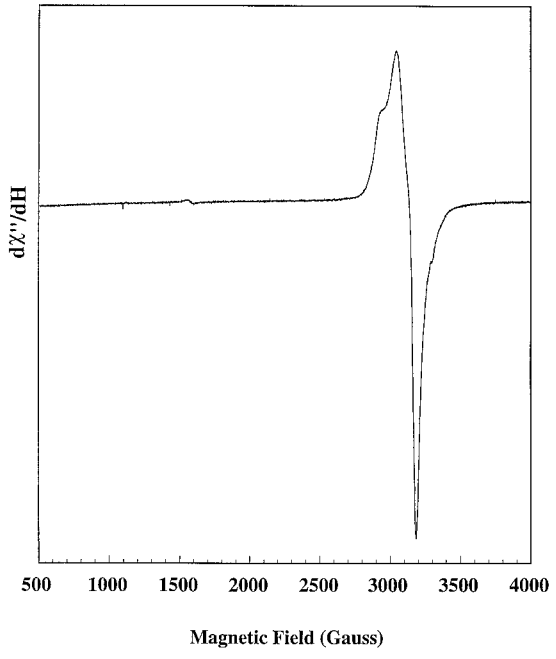

FIG. 6. **The X-band EPR spectrum of the fully oxidized purified pMMO obtained by ferricyanide treatment.** Excess ferricyanide was removed by repeated washing. The spectrum was recorded at 4 K with microwave power of 0.05 milliwatt, modulation frequency of 100 kHz, modulation amplitude of 10 Gauss, and gain of 4×10^3. See "Materials and Methods" for protein concentration.

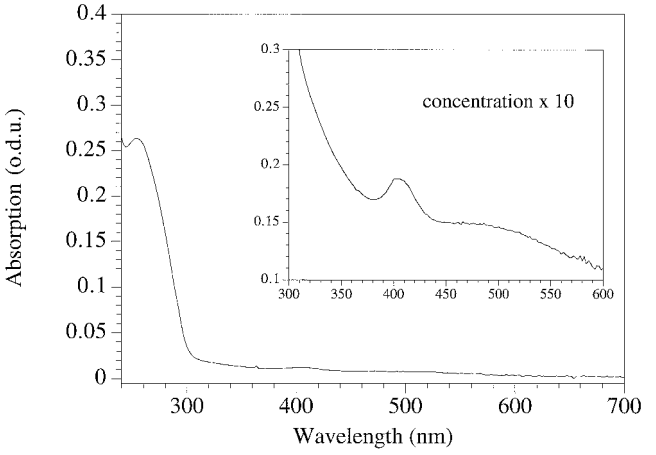

FIG. 7. **UV-visible spectrum of the fully oxidized purified pMMO that has the $\alpha\beta\gamma$ (45-, 26-, and 23-kDa) subunit composition.** Note the appearance of a broad absorption band at ~500 nm in addition to the ~400-nm band observed previously in the purified as-isolated pMMO. The concentration was 3.7 μM (*inset*, 37 μM) at pH 7.2 in 25 mM Pipes buffer and 0.05% dodecyl β-D-maltoside.

dominantly expressed by fast growing bacteria even under methane stress conditions (cell density often exceeds 8–10 g/liter of culture). Upon cell lysis and direct exposure to atmospheric oxygen pressure, it releases many protons (>10 H^+ released/94 kDa) and quickly loses all activity within 6 h after cell breakage. Activity assays using cell-free extracts in the first hour after cell lysis, however, indicate that this form exhibits extremely high specific activity, initially several times higher than type I pMMO. Anaerobic storage, and the addition of reductants like ascorbate to the buffer do prolong its activity. Type III pMMO is an intermediate between the two aforementioned forms and is most often obtained. This form is quite stable at first to direct exposure to atmospheric dioxygen tension, but the activity decays over several days and is unstable with respect to freezing and solubilization. Increased dioxygen tension does not lead to the formation of the pulsed state; instead, a significant drop in pH occurs (~5–7 H^+ released/94 kDa), and all activity is lost. Thawing frozen membranes of this type also leads to a substantial proton release and loss of activity.

The fact that the pMMO is sensitive to dioxygen and prone to inactivation as a result of dioxygen overexposure is surprising. However, ample evidence to indicate this dioxygen sensitivity can be found in the methanotroph literature. First and foremost, this characteristic reflects the environment of methanotrophs' natural habitat, namely the interface between anaerobic and aerobic environments, where there is sufficient methane but low dioxygen pressure. The shock of increased dioxygen pressure is enough to deactivate the enzyme (types II and III), which is consistent with the observation that methanotrophic growth is often slowed down or inhibited under normal atmospheric oxygen pressure (1, 2). The existence of the exception (type I pMMO), however, is a manifestation of an amazing biodiversity that allows the organisms not only to cope with but also to thrive under the many challenges of their environments.

Acknowledgments—We thank Prof. Mary E. Lidstrom and Drs. Andrei Chistoserdov, Ludmila Chistoserdova, and Roopa Ramamorthi

for helpful discussions and Dr. Peter Green for assistance with the inductively coupled plasma-mass spectroscopy analysis.

REFERENCES

1. Anthony, C. (1982) *The Biochemistry of Methylotrophs*, pp. 296–379, Academic Press, London
2. Bédard, C., and Knowles, R. (1989) *Microbiol. Rev.* **53,** 68–84
3. Crabtree, R. H. (1995) *Chem. Rev.* **95,** 987–1007
4. Lipscomb, J. D. (1994) *Annu. Rev. Microbiol.* **48,** 371–399
5. Fox, B. G., Froland, W. A., Jollie, D. R., and Lipscomb, J. D. (1990) *Methods Enzymol.* **188,** 191–202
6. Pilkington, S. J., and Dalton, H. (1990) *Methods Enzymol.* **188,** 181–190
7. Hendrich, M. P., Münck, E., Fox, B. G., and Lipscomb, J. D. (1990) *J. Am. Chem. Soc.* **112,** 5861–5865
8. Liu, Y., Nesheim, J. C., Lee, S. K., and Lipscomb, J. D. (1995) *J. Biol. Chem.* **270,** 24662–24665
9. Paulsen, K. E., Liu, Y., Fox, B. G., Lipscomb, J. D., Münck, E., and Stankovich, M. T. (1994) *Biochemistry* **33,** 713–722
10. Pulver, S., Froland, W. A., Fox, B. G., Lipscomb, J. D., and Solomon, E. I. (1993) *J. Am. Chem. Soc.* **115,** 12409–12422
11. Lee, S. K., Nesheim, J. C., and Lipscomb, J. D. (1993) *J. Biol. Chem.* **268,** 21569–21577
12. Fox, B. G., Hendrich, M. P., Surerus, K. K., Andersson, K. K., Froland, W. A., Lipscomb, J. D., and Münck, E. (1993) *J. Am. Chem. Soc.* **115,** 3688–3701
13. Froland, W. A., Andersson, K. K., Lee, S.-K., Liu, Y., and Lipscomb, J. D. (1992) *J. Biol. Chem.* **267,** 17588–17597
14. Green, J., and Dalton, H. (1988) *J. Biol. Chem.* **263,** 17561–17565
15. Green, J., and Dalton, H. (1985) *J. Biol. Chem.* **260,** 15795–15801
16. Lund, J., and Dalton, H. (1985) *Eur. J. Biochem.* **147,** 291–296
17. DeRose, V. J., Liu, K. E., Lippard, S. J., and Hoffman, B. M. (1996) *J. Am. Chem. Soc.* **117,** 121–134
18. Liu, K. E., Johnson, C. C., Newcomb, M., and Lippard, S. J. (1993) *J. Am. Chem. Soc.* **115,** 939–947
19. Liu, K. E., Valentine, A. M., Wang, D. L., Huynh, B. H., Edmondson, D. E., Salifoglou, A., and Lippard, S. J. (1995) *J. Am. Chem. Soc.* **117,** 10174–10185
20. Dewitt, J. G., Bentsen, J. G., Rosenzweig, A. C., Hedman, B., Green, J., Pilkington, S., Papaefthymiou, G. C., Dalton, H., Hodgson, K. O., and Lippard, S. J. (1991) *J. Am. Chem. Soc.* **113,** 9219–9235
21. Dewitt, J. G., Rosenzweig, A. C., Salifoglou, A., Hedman, B., Lippard, S. J., and Hodgson, K. O. (1995) *Inorg. Chem.* **34,** 2505–2515
22. Rosenzweig, A. C., Frederick, C. A., Lippard, S. J., and Nordlund, P. (1993) *Nature* **366,** 537–543
23. Rosenzweig, A. C., Nordlund, P., Takahara, P. M., Frederick, C. A., and Lippard, S. J. (1995) *Chem. Biol.* **2,** 409–418
24. Nguyen, H.-H. T., Shiemke, A. K., Jacobs, S. J., Hales, B. J., Lidstrom, M. E., and Chan, S. I. (1994) *J. Biol. Chem.* **269,** 14995–15005
25. Nguyen, H.-H. N. T., Zhu, M., Elliott, S. J., Nakagawa, K. H., Hedman, B., Costello, A. M., Peeples, T. L., Wilkinson, B., Morimoto, H., Williams, P. G., Floss, H. G., Lidstrom, M. E., Hodgson, K. O., and Chan, S. I. (1996) in *Microbial Growth on C_1 Compounds* (Lidstrom, M. E., and Tabita, F. R., eds) pp. 150–158, Kluwer Academic Publishers, Norwell, MA
26. Nguyen, H.-H. T., Nakagawa, K. H., Hedman, B., Elliott, S. J., Lidstrom, M. E., Hodgson, K. O., and Chan, S. I. (1996) *J. Am. Chem. Soc.* **118,** 12766–12776
27. Semrau, J. D., Zolandz, D., Lidstrom, M. E., and Chan, S. I. (1995) *J. Inorg. Biochem* **58,** 235–244
28. Chan, S. I., Nguyen, H.-H. T., Shiemke, A. K., and Lidstrom, M. E. (1993) in *Bioinorganic Chemistry of Copper* (Karlin, K. D., and Tyeklar, Z., eds) pp. 184–195, Chapman and Hall, New York
29. Chan, S. I., Nguyen, H.-H. T., Shiemke, A. K., and Lidstrom, M. E. (1993) in *Microbial Growth on C1 Compounds* (Murrell, J. C. and Kelly, D. P., eds) pp. 93–107, Intercept, Andover, Hampshire, United Kingdom
30. Stainthorpe, A. C., Salmond, G. P. C., Dalton, H., and Murrell, J. C. (1990) *FEMS Microbiol. Lett.* **70,** 103–108
31. Stainthorpe, A. C., Lees, V., Salmond, G. P. C., Dalton, H., and Murrell, J. C. (1990) *Gene* (*Amst.*) **91,** 27–34
32. Nakajima, T., Uchiyama, H., Yagi, O., and Nakahara, T. (1992) *Biosci. Biotech. Biochem.* **56,** 736–740
33. Prince, R. C., George, G. N., Savas, J. C., Crammer, S. P., and Patel, R. N. (1988) *Biochim. Biophys. Acta* **952,** 220–229
34. Patel, R. N., Hou, C. T., Laskin, A. J., and Pelix, A. (1982) *Appl. Environ. Microbiol.* **44,** 1130–1137
35. Cornish, A., MacDonald, J., Burrows, K. J., King, T. S., Scott, D., and Higgins, I. J. (1985) *Biotechnol. Lett.* **5,** 319–324
36. Prior, S. D., and Dalton, H. (1985) *J. Gen. Microbiol.* **131,** 155–163
37. Stanley, S. H., Prior, S. D., Leak, D. J., and Dalton, H. (1983) *Biotechnol. Lett.* **5,** 487–492
38. Scott, D., Brannan, J., and Higgins, I. J. (1981) *J. Gen. Microbiol.* **125,** 63–72
39. Dalton, H., Prior, S. D., Leak, D. J., and Stanley, S. J. H. (1984) in *Microbial Growth on C1 Compounds* (Crawford, R. L. and Hanson, R. S., eds) pp. 75–82., Intercept, Andover, Hampshire, United Kingdom
40. Semrau, J. D., Chistoserdov, A., Lebron, J., Costello, A., Davagnino, J., Kenna, E., Holmes, A. J., Finch, R., Murrell, J. C., and Lidstrom, M. E. (1995) *J. Bacteriol.* **177,** 3071–3079
41. Priestly, N. D., Floss, H. G., Froland, W. A., Lipscomb, J. D., Williams, P. G., and Morimoto, H. (1992) *J. Am. Chem. Soc.* **114,** 7561–7562
42. Wilkinson, B., Zhu, M., Priestley, N. D., Nguyen, H.-H. T., Morimoto, H., Williams, P. G., Chan, S. I., and Floss, H. G. (1996) *J. Am. Chem. Soc.* **118,** 921–922
43. Tonge, G. M., Harrison, D. E. F., and Higgins, I. J. (1977) *Biochem. J.* **161,** 333–344
44. Drummond, D., Smith, D., and Dalton, H. (1989) *Eur. J. Biochem.* **1982,** 667–671
45. Zahn, J. A., and DiSpirito, A. A. (1996) *J. Bacteriol.* **178,** 1018–1029
46. Akent'eva, N. F., and Gvozdev, R. I. (1988) *Biochemistry (Engl. Transl. Biokhimiya)* **53,** 91–96
47. Whittenbury, R., and Dalton, H. (1981) in *The Prokaryotes* (Starr, M. P., Truper, H. G., Balows, A. and Schlegel, H. G., eds) pp. 894–902, Springer-Verlag New York, Inc., New York
48. Laemmli, U. K. (1970) *Nature* **227,** 668–685
49. Schagger, H., and von Jagow, G. (1987) *Anal. Biochem.* **116,** 368–378
50. Gershoni, J. M., and Palade, G. E. (1982) *Anal. Biochem.* **124,** 396–405
51. Shiemke, A. K., Cook, S. A., Miley, T., and Singleton, P. (1995) *Arch. Biochem. Biophys.* **321,** 421–428
52. Moir, J. W. B., Crossman, L. C., Spiro, S., and Richardson, D. J. (1996) *FEBS Lett.* **287,** 71–74
53. DiSpirito, A. A., Shiemke, A. K., Jordon, S. W., Zahn, J. A., and Krema, C. L. (1994) *Arch. Microbiol.* **161,** 258–265
54. Holmes, A. J., Costello, A. M., Lidstrom, M. E., and Murrell, J. C. (1995) *FEMS Microbiol.* **132,** 203–208
55. Klotz, M. G., and Norton, J. M. (1995) *Gene* (*Amst.*) **163,** 159–160
56. Rothawe, J. H., Beboer, W., and Liesack, W. (1995) *FEMS Microbiol.* **133,** 131–135
57. Norton, J. M., Low, J. M., and Martin, G. (1996) *FEMS Microbiol.* **139,** 181–188
58. McTavish, H., Fuchs, J. A., and Hooper, A. B. (1993) *J. Bacteriol.* **175,** 2436–2444
59. Bergmann, D. J., and Hooper, A. B. (1994) *Biochem. Biophys. Res. Commun.* **204,** 759–762-