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ORIGINAL PAPER

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Intracellular localization of the particulate methane monooxygenase and methanol dehydrogenase in *Methylomicrobium album* BG8

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Abstract The methanotrophic bacterium *Methylomicrobium album* BG8 uses methane as a sole source of carbon and energy. This bacterium forms an extensive intracytoplasmic membrane. The first enzymes of the methane oxidation pathway are the membrane-bound particulate methane monooxygenase and the periplasmic methanol dehydrogenase. Immunoelectron microscopy with specific antibodies was used to localize these enzymes to the intracytoplasmic membrane.

Keywords *Methylomicrobium album* BG8 · Methane monooxygenase · Methanol dehydrogenase · MMO · MDH · Immunoelectron microscopy · pMMO

Abbreviations *BSA* Bovine serum albumin · *ICM* Intracytoplasmic membrane · *MMO* Methane monooxygenase · *pMMO* Particulate methane monooxygenase · *MDH* Methanol dehydrogenase · *sMMO* Soluble methane monooxygenase · *TBST* Tris-buffered saline with Tween

Introduction

Methanotrophic bacteria are gram-negative obligate aerobes that use methane as sole source of carbon and energy. Methanotrophs are included among the methylotrophs which grow on one-carbon compounds. The first two enzymes in the pathway of methane oxidation are methane monooxygenase (MMO), which oxidizes methane to methanol, and methanol dehydrogenase (MDH), which oxidizes methanol to formaldehyde. The latter has been localized to the periplasm in biochemical and immunoelectron microscopic studies of methylotrophs (Alefounder and Ferguson 1981; Jones et al. 1982; Kasprzak and Steenkamp 1983; Fassel et al. 1992). MMO exists in distinct soluble (sMMO) and membrane-bound particulate (pMMO) forms (Murrell et al. 2000a, b). All known methanotrophs, with one possible exception (Dedysh et al. 2000), contain the pMMO enzyme. Some methanotrophs can make either MMO enzyme, depending upon growth conditions (Prior and Dalton 1985). In these organisms the switch between sMMO and pMMO is affected by copper availability in the growth medium (Murrell et al. 2000a, b).

Methylomicrobium album BG8 is a well-studied methanotroph strain originally isolated by Whittenbury et al. (1970). *M. album* BG8 has an extensive intracytoplasmic membrane (ICM) that forms stacks of membranes throughout the cell (Brantner et al. 1997; Collins et al. 1991). *M. album* BG8 contains only pMMO (Stanley et al. 1983; Stainthorpe et al. 1990), which is a copper protein (Zahn and DiSpirito 1996; Yuan et al. 1997, 1998, 1999). Approximately 50% of the total membrane protein in *M. album* BG8 is contributed by pMMO (Collins et al. 1991). Both MMO activity and ICM content in these cells increase with increasing copper concentration in the growth medium (Brantner et al. 1997). Moreover, addition of copper to copper-limited cells results in ICM formation (Brantner et al. 1997). There is also a correlation of pMMO activity with ICM formation in mutants of *Methylosinus trichosporium* OB3b (Phelps et al. 1992). The correlation between copper content of the medium and ICM formation suggests a role for pMMO in ICM formation. In *M. album* BG8, increased copper in the medium may increase the amount of pMMO in the membranes due to an effect on synthesis and/or assembly; assembly of pMMO in the membrane may in turn stimulate the proliferation of ICM. This would be consistent with the suggested role of major membrane proteins in ICM formation in phototrophic bacteria (Hessner et al. 1991; Cheng et al. 2000). A prediction of this hypothesis is that pMMO must

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be located in the ICM. While this is suggested by our previous studies, it has not been directly demonstrated. A recent review (Murrell et al. 2000a) identified the intracellular location of pMMO to be an important unanswered question. The present work uses immunoelectron microscopy to localize the pMMO to the ICM of *M. album* BG8. In addition, the MDH was shown to be associated with the ICM.

Materials and methods

Cell growth, specimen preparation, and enzyme activity

M. album BG8 was grown as described previously (Brantner et al. 2000). Cells were prepared for immunoelectron microscopy essentially as previously described (Collins et al. 1991), with the exception that post-fixation with OsO_4 was omitted. Briefly, cells were washed with 5 mM potassium phosphate buffer, fixed for 1 h in 0.5% glutaraldehyde prepared in buffer, and dehydrated in a graded alcohol series at room temperature. Dehydrated cells were placed in ethanol:LR White (1:1, v/v) before infiltration with LR White (Electron Microscopy Sciences, Fort Washington, Penn., USA) and polymerized for 48 h at 50 °C. Ultrathin (70–90 nm) sections were post-stained with 1% aqueous uranyl acetate. For complementary ultrastructural experiments, concentrated cells were placed into specimen carriers that had been precoated by dipping in 3% (v/v) lecithin in chloroform and air drying (Kiss and Staehelin 1995). Specimens were frozen with a Balzers HPM 010 high-pressure freezing apparatus and stored in liquid N_2. Specimens were freeze-substituted in 2% (w/v) OsO_4 in anhydrous acetone using a Leica EM AFS freeze substitution apparatus (Leica Microsystems, Wetzlar, Germany) according to the following schedule: –100 °C for 2 h followed by increases in temperature of 10 °C per h for 2 h, –80 °C for 120 h, followed by increases in temperature of 10 °C per h for 6 h, –20 °C for 4 h, followed by increases in temperature of 10 °C per h for 1.6 h, –4 °C for 2 h, increased to 4 °C over 1 h, held at 4 °C for 1 h then at room temperature in a fume hood for 2 h. Cells were collected by centrifugation and resuspended in 100% ethanol. Two additional changes of 100% ethanol were made before the cells were embedded in LR White resin as above. MMO activity was assayed as previously described (Collins et al. 1991).

Antibody preparation

A polyclonal rabbit anti-pMMO was raised. The immunogen consisted of bands of approximately 22 kDa (Collins et al. 1991) excised from five lanes of SDS-polyacrylamide gels (Laemmli 1970). Immunogen was prepared by the method of Harlow and Lane (1988) and dispersed in Freund's complete adjuvant. The purification of pMMO and the recognition of three subunits (Zahn and DiSpirito 1996) suggest that the immunogen injected may have contained both of the smaller pMMO proteins, designated PmoA and PmoC.

A rabbit polyclonal antibody was prepared against *M. album* BG8 MDH (Fassel et al. 1988). Briefly, MDH was purified from the soluble fractions of the cell sonicate by ammonium sulfate precipitation, DEAE-cellulose chromatography, isoelectric focusing and hydroxyapatite chromatography. The purified MDH consisted of subunits of 10 and 60 kDa. For immunization, MDH was homogenized with Freund's complete adjuvent. This anti-MDH antibody has been used in other studies (Stephens et al. 1988). Serum samples were obtained from all rabbits before immunization.

Immunoblots

Cells were solubilized by treatment for 5 min at 100 °C and electrophoresed on SDS-polyacrylamide gels. The resolved proteins were transferred to Immobilon-P (Millipore, Bedford, Mass., USA) with a semi-dry blotter (Hoefer Scientific Instruments, San Francisco, Calif., USA). The transfer conditions were 0.8 mA/cm^2 of the gel for 3.5 h at 4 °C using a Hoefer PS 500X energy supply unit. Membranes were blocked by treatment with Blotto [10% non-fat dry milk (w/v), 155 mM NaCl, 0.02 M Tris buffer with 0.1% Tween, 0.001% merthiolate (v/v), pH 7.5] (Johnson et al. 1984). The blocked membranes were incubated in immune serum (1:1,000) or pre-immune serum in Tris-buffered saline with 0.1% Tween (TBST) overnight at 4 °C. Blots were washed in TBST and incubated in the secondary antibody [1:20,000 dilution of goat anti-rabbit IgG horseradish peroxidase conjugate (A-6154, Sigma, St. Louis, Mo., USA)] in TBST for 1 h with shaking. The immunoblots were developed using the chemiluminescence reaction of the Super Signal reagents (Pierce, Rockford, Ill., USA). A Hoefer scanning densitometer equipped with a GS-360 Hoefer Data System was used to quantify the amount of pMMO antigen present in the samples detected by immunoblot.

Immunogold labeling procedure

Ultrathin sections of samples obtained using an RMC MT-7000 ultramicrotome (RMC, Tucson, Az., USA) were retrieved on 300-mesh nickel grids. The grids were incubated in a drop of 3% bovine serum albumin (BSA) buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mg/ml BSA, pH 8.2) for 1 min and blotted. Grids were incubated on a 30-µl drop of diluted primary antiserum for 1 h at room temperature on a Teflon-printed slide in a humid chamber. The grids were then transferred through four drops of 3% BSA buffer on a stir plate. This was followed by incubation for 1 h at room temperature on a 25-µl drop of secondary antibody conjugated to 10 nm colloidal gold particles (1:40 dilution of anti-rabbit immunoglobulin G; G-7402, Sigma). The grids were then rinsed by application of 25 drops of 3% BSA buffer and then in H_2O drops on a stir plate for 40 min. Pre-immune serum was used in place of the primary antibody as a control. Finally, post-staining was done with 1% aqueous uranyl acetate. An Hitachi H-600 TEM electron microscope operating at 75 kV was used to visualize the location of the bound gold particles.

Quantitation of gold particles

To determine the distribution of the gold particles on the sections, the image was displayed on a 15 inch monochromic TV screen system (Advanced Microscopy Techniques, Danvers, Mass., USA). A 7×8-inch frame was used for each count; there were two to four cells per frame and ten frames were scored. Two investigators counted gold particles on the cells and the background.

To determine the intracellular distribution of the gold particles, digital images were acquired by scanning electron micrographs with an AGFA Duo Scan scanner and analyzed with Optimas 6.2 software (Media Cybernetics, Silver Spring, Md., USA). Each cell was analyzed for the following parameters: total area of the cell, total area occupied by ICM, total gold particles within the cell boundary, and total gold particles associated with ICM. For this purpose, only regions of clearly defined ICM were designated as such.

Results and discussion

Specificity of anti-pMMO

An immunoblot probed with the anti-pMMO antibody indicated that the rabbit serum contained antibodies specific to a band of approximately 22 kDa in *M. album* BG8 cells that could be PmoA and/or PmoC (not shown). No bands were detected in blots incubated with the preimmune serum (not shown). To provide additional evidence that

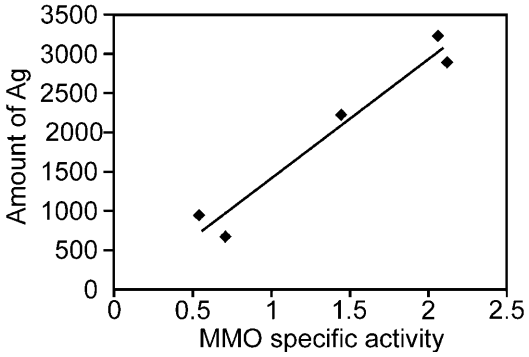

Fig. 1 Correlation of reactive antigen with particulate methane monooxygenase (pMMO) activity. Linear relationship between pMMO activity [nmol propylene oxide min^{-1}(mg wet weight)$^{-1}$] and amount of antigen (Ag) reported in arbitrary units in cells reactive with anti-pMMO antibody. The antigen was measured in immunoblots of gels loaded with equivalent amounts of cells normalized on the basis of OD; r^2=0.95

the antibody to the 22-kDa protein reacts with pMMO, the amount of reactive antigen was correlated with enzyme activity. For this purpose, cells with varying levels of pMMO were obtained by growth on medium with different levels of copper (Brantner et al. 1997). From these cultures, the amount of pMMO activity was measured using the propylene oxide assay and the amount of pMMO reactive antigen was measured by densitometric analysis of immunoblots prepared with these same cells. The pMMO reactive antigen and pMMO activity were found to be directly correlated, indicating the specificity of the antibody for pMMO (Fig. 1).

Immunolocalization of pMMO

Ultrathin sections of *M. album* BG8 were incubated with either anti-pMMO antibody or preimmune serum and then with secondary antibody conjugated to colloidal gold. The amount of bound gold particles for the pMMO immunolabeling was quantified for three dilutions of primary antibody. The greatest specificity was achieved with the most dilute serum (Table 1). The sample treated with the pre-immune serum showed only a very low level of label and a random distribution with approximately 50% of the particles on the background resin. Dilution is important when using whole sera as it improves the signal by reducing the concentration of cross-reacting antibodies that may be present in the sera (Bendayan 1995). Increasing the dilution of the immune serum decreases the non-specific binding thus increasing the signal-to-noise ratio.

Table 1 Quantification of immunogold particles on specimens treated with immune and pre-immune serum (number of particles in 10 fields as defined in Materials and methods)

Dilution	Pre-immune		Immune	
	Background	Cells	Background	Cells
Anti-pMMO				
1:2	102	236	26	571
1:10	497	547	97	1680
1:50	329	353	55	2765
Anti-MDH				
1:250	100	77	95	1440

The indirect immunogold labeling procedure used in this study localized the pMMO protein to the ICM in the cells (Fig. 2A). It should be noted that the preservation of the ICM ultrastructure is suboptimal because post-fixation with OsO_4 was omitted to minimize loss of antigenic sites. The majority of the gold particles were on the cells and followed the contours of the ICM. The subcellular location of the pMMO was evaluated quantitatively by measuring the area of the cell containing ICM and calculating the percent of the gold particles associated with the ICM in the section. ICM was found to occupy 38±10% of the cell area. This must be considered a minimum estimate of ICM content because only those portions of the cell in which the ICM stacks were perpendicular to the plane of the section (Fig. 2A, arrow) were classified as ICM. In 33 cells analyzed, 75±11% of the gold particles were on the ICM stacks. This is probably an underestimate of the total ICM-associated pMMO because gold particles bound to regions of apparent ICM not perpendicular to the plane (Fig. 2A, arrowhead) of the section were not included. Moreover, only those epitopes on the surface of the section would be available for antibody binding.

Immunolocalization of MDH

The observation that pMMO is located in the ICM would suggest that the next enzyme in the methane oxidation pathway, the periplasmic MDH, would also be associated with the ICM. The anti-MDH antibody prepared by Fassel et al. (1988) was characterized by immunoblot and shown to bind to *M. album* BG8 proteins of 60 and 10 kDa (not shown), representing the two subunits of MDH enzyme. Immunoblot analysis of the control pre-immune serum showed no bands (not shown).

This anti-MDH antibody was used to label ultrathin sections of *M. album* BG8 (Fig. 2C). The gold particles were associated with the ICM, with very little label appearing in other parts of the cell or on the background. Counts of gold particles on ultrathin sections in the TEM indicated that there were 15-fold more gold particles on the cells than in the background (Table 1). In 31 *M. album* BG8 cells, 82±9% of the gold label was associated with the ICM. As with pMMO, this is a minimum of the ICM-associated MDH since the area occupied by ICM is probably underestimated in the section. The use of pre-immune serum resulted in random labeling of the cell and the background (Fig. 2D).

This study provides direct evidence that both the pMMO and the MDH of *M. album* BG8 are associated with the ICM. This location of the MDH extends the find-

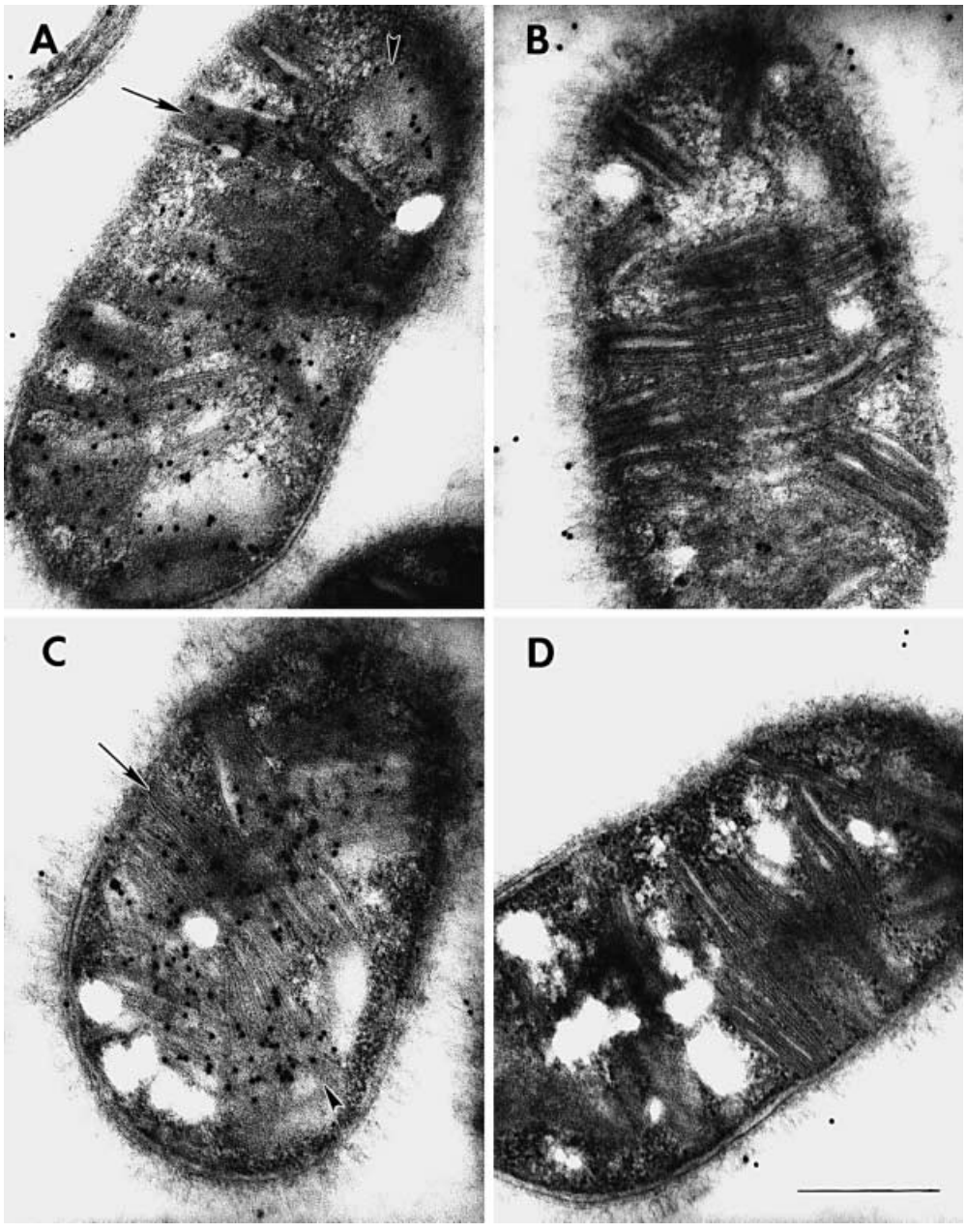

Fig. 2A–D Immunolocalization of pMMO and methanol dehydrogenase (MDH) in *Methylomicrobium album* BG8 cells. *M. album* BG8 labeled with **A** anti-pMMO antibody and **B** preimmune serum; **C** anti-MDH antibody and **D** preimmune serum. *Bar* 0.5 μm. *Arrows* Typical ICM; *arrowheads* oblique planes through apparent ICM; such areas were not scored as ICM for the quantitative analysis. The white areas are holes that formed when sections were placed in the electron beam. This is apparently due to inadequate fixation of the granules when osmium is not used for post-fixation. This has been observed with phosphate granules in cyanobacteria (Jensen 1985)

ings of our preliminary report (Fassel et al. 1988) and is consistent with the association of this enzyme with the ICM in another methanotrophic bacterium (Fassel et al. 1992). Because MDH is located in the periplasm of methylotrophs (Alefounder and Ferguson 1981; Jones et al. 1982; Fassel et al. 1992), its association with the ICM, as shown in the present study, implies that the enzyme is located in the intra-ICM space and that this is continuous with the periplasm. Continuity between the periplasm and the intra-ICM space is consistent with continuity between the cytoplasmic membrane and the ICM, as suggested by earlier work (De Boer and Hazeu 1972). This continuity can be observed in fortuitous cross-sections, especially in cells with less fully developed ICM (Fig. 3). Moreover, these micrographs show the presence of ICM in cells frozen prior to chemical fixation. This is consistent with our previous freeze-etch studies of *M. album* BG8 (Fassel et al. 1990) and clearly indicates that the ICM observed in conventionally prepared cells is not an artifact of chemical fixation.

The immunolocalization of the copper protein pMMO to the ICM of *M. album* BG8 is consistent with our observations that the structure and abundance of the ICM is dependent on copper in the medium (Collins et al. 1991; Brantner et al.1997). The demonstration of the location of pMMO in the ICM is further consistent with the possible role of this major membrane protein in the development and proliferation of the ICM.

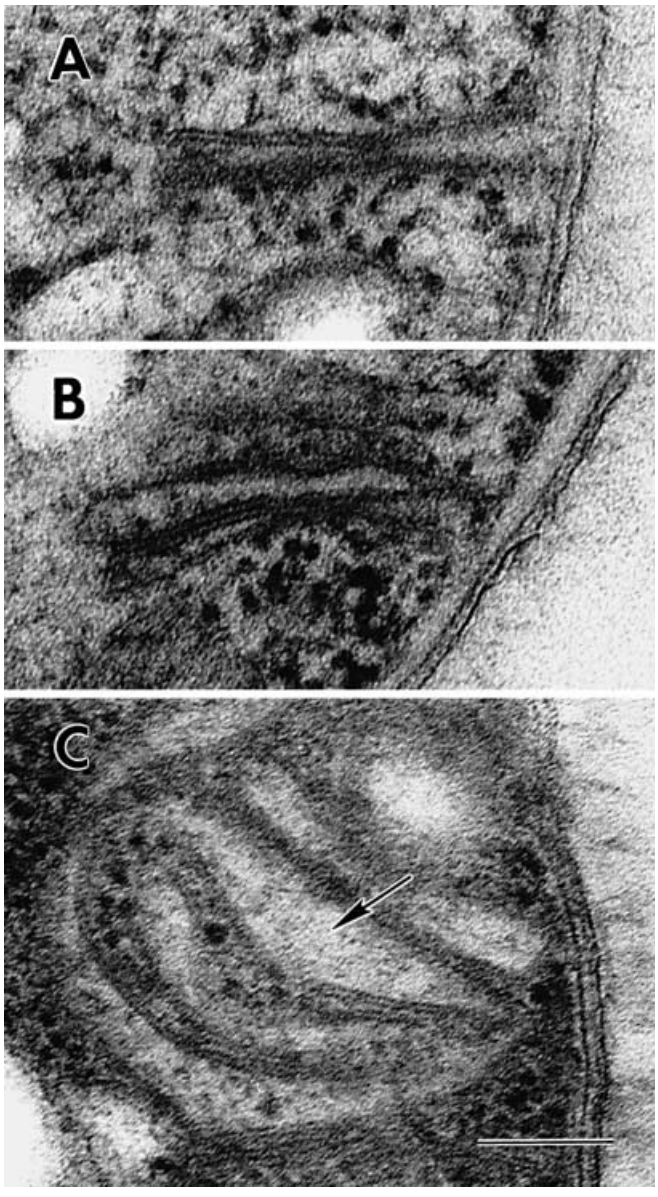

Fig. 3A–C Electron micrographs of *M. album* BG8 showing regions of continuity between the ICM and the cytoplasmic membrane. Cells were frozen under high pressure and freeze-substituted with OsO_4 **A, B** Regions of apparent continuity between the ICM and the cytoplasmic membrane. Note that the intra-ICM space (*arrow*) is distinguishable from the cytoplasm in that it lacks ribosomes; this is most apparent in **C**. *Bar* 0.1 μm

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