Grant: DiSpirito, A.A. and Antholine, W.E. Membrane-Associated Methane Monooxygenases from Type X and Type I Methanotrophs, Department of Energy. Project Number: DE-FG02-00ER15446

Research Summary:

Introduction

Methanotrophs are a widely distributed group of Gram-negative bacteria with representative genera in the α - (Type II methanotrophs) and γ - (Type I methanotrophs) subdivisions of the *Proteobacteria* and in the *Verrucomicrobia* (thermoacidophilic methanotrophs) phylum. Despite the diversity of methanotrophs the general pathway by which these cells oxidize CH_4 to CO_2 is identical, with methanol, formaldehyde and formate as intermediates (Fig. 1).

For the conversion of CH_4 to CH_3OH , two forms of methane monooxygenase (MMO) have been found. One form the membrane-associated or particulate MMO (pMMO) is found in most known methanotrophs and is located in the cytoplasmic membrane. Another form, the soluble MMO (sMMO) is found in some methanotrophs and is located in the cytoplasm. The two MMOs are genetically and structurally distinct enzymes. In methanotrophs that have both forms of MMO, copper is known to regulate the expression of the genes encoding both MMOs as well the activity of these enzymes. The pMMO was the focus of this study.

Fig. 1. Proposed pathway of methane oxidation in cells cultured under high and low copper conditions. Proteins showing positive or negative Cu-regulation are shown in blue and red, respectively. Abbreviations: Cyt, cytochrome; d-FalDH, dye-linked formaldehyde dehydrogenase; FDH, formate dehydrogenase; MDH, methanol dehydrogenase; N-FalDH, NAD(P)-linked formaldehyde; PQQ, pyrroloquinoline quinone; pMMO, membrane-associated methane monooxygenase; Q, ubiquinone; RuMP, ribulose monophosphate; sMMO, soluble MMO.

Membrane-associated or particulate methane monooxygenase (pMMO).

Methanotrophs cultured at higher copper to biomass ratios **exclusively** express the pMMO. In contrast to the sMMO little is known about the molecular properties of the pMMO, mainly due to the low specific activity of most enzyme preparations. All laboratories studying the pMMO agree that the pMMO is a copper-containing enzyme, composed of three polypeptides with approximate molecular masses of 45,000 (α -subunit, *pmoB*), 26,000 (β -subunit, *pmoA*) and 23,000 (γ -subunit, *pmoC*) Da, with a $(\alpha\beta\gamma)_3$ subunit structure. However, researchers in the field disagree on the number, type, and function of metal centers associated with the pMMO as well as the nature of the physiological electron donor. Based on metal composition and physiological reductant four different models for the pMMO have been proposed.

S. Chan's group (Taiwan University) has proposed that the pMMO contains 15 Cu atoms per $\alpha\beta\gamma$, with 12 Cu atoms arranged into trinuclear catalytic (C-clusters) and trinuclear electron transfer (E-clusters) clusters, in addition to the mononuclear and dinuclear copper sites identified in the crystal structure from *Mc. capsulatus* Bath of this enzyme. This model proposes that the E-clusters accept electrons from NADH and transfer them to the C-clusters.

The model proposed by Rosenzweig and coworkers is based on the crystal structures of the enzyme that showed the pMMO contains two metal sites in *Methylosinus trichosporium* OB3b, or three in *Mc capsulatus* Bath. Namely, a (tentative) dinuclear Cu site, a mononuclear Cu center (present in the *Mc. capsulatus* Bath structure, but absent in the *Ms. trichosporium* OB3b structure) and a third metal binding site that is occupied by Zn in the *Mc. capsulatus* Bath structure and by Cu in the *Ms. trichosporium* OB3b structure (Fig. 2). In this model the putative

dinuclear Cu center was suggested as the site of methane oxidation, although the authors do not rule out the possibility that the third site occupied by either Zn or Cu in the crystal structures could be the locus of methane oxidation. **It should be noted that the enzyme preparations used for obtaining the crystal structure for *Mc. capsulatus* Bath was inactive, and the preparation used for *Ms. trichosporium* OB3b showed less than 0.01 % of the physiological rate. In addition, the presence of different metals in the third binding site suggests that this is a labile metal binding site.** In a side remark, Rosenzweig and Lieberman point out that the third site could accommodate a diiron center.

Fig. 2. Crystal structures and membrane orientation of the $\alpha\beta\gamma$ monomer of pMMO from *Mc. capsulatus* Bath and *Ms. trichosporium* OB3b. Membrane orientation was predicted using the methodology of Lomize, et al., using structural information from Rosenzweig laboratory

A model proposed by Dalton and coworkers suggests that the hydroxylase component of the pMMO (pMMO-H) contains 2 – 3 Cu and 1 – 2 Fe atoms per $\alpha\beta\gamma$ subunit. This group also proposed that pMMO-H is assembled with the methanol dehydrogenase (MDH) in a super complex (pMMO-C) in which the MDH serves as the reductase for pMMO-H (63).

Summary of results from this grant

In 1996 we reported a model for this enzyme, which was controversial at the time. In this model, the pMMO was a complex composed of three polypeptides (pmoA, pmoB, and pmoC), i.e. the hydroxylase (pMMO-H) that contains two copper and two iron atoms. The presence of iron in pMMO-H was initially very controversial, but results funded from this grant demonstrated that the diiron center is the catalytic center of the enzyme. Active preparations of the pMMO also contains 5 to 8 copies of a novel copper containing chromopeptide which we eventually designated as methanobactin,

which is described below. The central feature of our model, developed with funding from this grant, is that pMMO contains two Cu atoms involved in electron transfer from the respiratory chain to a diiron center very similar to that observed in sMMO. Results generated with funds from this grant was also used to show that methanobactin is redox active molecule with anti-oxidant properties and provide strong evidence for its role as the electron donor to pMMO-H.

Methanobactin. During the initial characterization of pMMO, we identified a small, 1,218 Da copper binding chromopeptide that co-purified with the pMMO (Fig. 3). Originally called the copper-binding cofactor/compound (cbc), this molecule has been renamed methanobactin based on its antibiotic properties, and is the first example of a new class of copper-binding agents called chalkophore (Greek for copper handling). With funding from this grant and in collaboration with Dr. Graham's group at the University of Kansas, the crystal structure of methanobactin was determined to a resolution of 1.2 Å. Although the structure was consistent with the molecular mass determinations, it was difficult to reconcile the chemical composition of the hydroxyimidazolate and thiocarbonyl groups with possible biosynthetic pathways. To confirm or correct the structure we examined the chemical structure by NMR and mass spectroscopy in conjunction with a Dr. Hartsel and Gallagher's groups at U. Wisconsin-Eau Claire and revised the structure, which now reconciles the chemical analysis via NMR, X-ray, and mass spectrometry data with possible biosynthetic pathways.

Fig. 3 Proposed structure for mb from *Ms. trichosporium* OB3b; 1-(N-[mercapto-[5-oxo-(3-methylbutanoyl)oxazol-4-ylidene]methyl]-Gly¹-Ser²-Cys³-Tyr⁴-2-(pyrrolidin-2-yl-(mercapto-[5-oxazol-4-ylidene]methyl)-Ser⁵-Cys⁶-Met⁷). The model is shown is the $(M-2H^{+}+^{63}Cu)^{-1}$ charged species. The backbone is traced in blue (.

Since the initial identification, we also identified methanobactin in the extracellular fraction of methanotrophs where it appears to be the extracellular component in a copper acquisition system similar to the siderophore based iron uptake systems in prokaryotes. Funds from this grant allowed us to examine this potential physiological role we have examined the metal binding properties of mb. These studies have shown mb to be a dynamic molecule in solution, which appears to initially bind Cu(II) as a multimer, probably a tetramer, via the sulfur and nitrogen of one of the mercapto-[5-oxo-oxazol-4-ylidene]methyl group designated as A in figure 3 and Tyr. This initial binding is followed by a reduction to Cu(I) then by coordination to the second mercapto-[5-oxo-oxazol-4-ylidene]methyl group. Studies on the metal binding as well as on the solution and thermodynamic properties of mb from *Ms. trichosporium* OB3b suggest mb functions as a copper siderophore or chalkophore in addition to its apparent role in as an electron donor to pMMO-H.

Although mb preferentially binds copper, we have also shown mb will bind and reduce a variety of metals and may prove useful in the production of gold, platinum or palladium nanoparticles. In collaboration with Dr. Semrau's at the U. Michigan and

McEllistrem at the U. Wisconsin-Eau Claire we are examining methods to control nanoparticles size and shape.

As a consequence of our studies on the copper binding properties of methanobactin was a collaboration with Dr. Karl H. Summer from the Institute of Toxicology in the German Research Center for Environmental Health, Neuherberg Germany we are examining the potential use of methanobactin in treatment of Morbus Wilson and related diseases. Morbus Wilson disease is a progressive autonomic recessive disorder characterized by disorder of transport and excessive accumulation of copper in liver, central nervous system, and in other organs. Dr. Summer's research focuses on mechanistic aspects of Morbus Wilson disease and is testing the effectiveness of methanobactin as a decoppering agent in his experimental model, the Long-Evans Cinnamon (LEC) rat mutant, which shows similar clinical and biochemical features to individuals with Wilson disease. His initial results showed after five daily doses to LED rats, their glutamic oxaloacetic transaminase (GOT) and bilirubin values returned to normal and two days after the last dose, liver copper values were low. The potential long-term side effects needs to be examined, but results are very promising.

As with the diiron center of pMMO-H, the existence of methanobactin was initially viewed with skepticism, but few now question its existence and a number of laboratories have started examining the physiological and environmental importance of methanobactin as evidenced in a recent review by Balasubramanian and Rosenzweig "Copper methanobactin: a molecule whose time has come" in Current Opinion in Chemical Biology (online: www.sciencedirect.com).

Other C-1 enzymes and respiratory components. One unexpected consequence of the objective to determine the physiological reductant of the pMMO was the identification and characterization of a novel membrane-associated formaldehyde dehydrogenase, a new class of high molecular mass multi-heme c-type cytochromes, a new class of c'-type cytochromes and the identification of the phylogenetic link between c'-type cytochromes and the hydroxylamine oxidizing enzyme cytochrome P-460 found in both methane and ammonia oxidizing bacteria. Other researchers in the field have recently shown an outer membrane location for the high molecular mass c-type cytochromes and suggest they may be involved in a respiration-linked metal reduction processes possibly mediated by methanobactin.

Oxidation of halogenated hydrocarbons by the membrane-associated methane monooxygenase. Halogenated hydrocarbons such as trichloroethylene (TCE) are suspected carcinogens and are the most common ground water pollutants in the USA. The aerobic biotransformation of TCE is the preferred method of bioremediation since this process does not produce toxic intermediates. Since methane is non-toxic and methanotrophs are ubiquitous in the environment, these bacteria have been extensively examined for the bioremediation of halogenated hydrocarbons. With funding from this grant, we demonstrated that the (pMMO) can mineralize halogenated compounds such as TCE and determined the mechanism of TCE oxidation. Lastly, recent results have shown that the pMMO is the probable enzyme system oxidizing halogenated hydrocarbons in field situations.

Publications funded by this grant

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Invited Speaker:

- Gordon Research Conference on The Molecular Basis of Microbial One-Carbon Metabolism, Connecticut College, CN (2000)
- University of Wisconsin-Eau Claire. Department of Chemistry (2004)
- Montana State University. Department of Microbiology (2005)
- Gordon Research Conference on The Molecular Basis of Microbial One-Carbon Metabolism, Oxford UK (2006)
- University of Wisconsin-Eau Claire. Department of Chemistry (2007)
- Gordon Research Conference on The Molecular Basis of Microbial One-Carbon Metabolism, Bates College Lewiston, Maine (2008)