METHYLTRANSFERASES AND CORRINOID BINDING PROTEINS INVOLVED IN METHYLAMINE DEPENDENT METHANOGENESIS IN METHANOSARCINA BARKERI

DISSERTATION

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By

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ABSTRACT

*Methanosarcina barkeri* is a methanogen capable of producing methane from a wide range of substrates including carbon dioxide with molecular hydrogen, acetate, methylated sulfur compounds, methanol, and the methylamines. In all known pathways of methanogenesis, the penultimate step involves the methylation of 2-mercaptoethanesulfonate (CoM). At the onset of this investigation, the pathways of CoM methylation from CO₂/H₂, acetate, and methanol were largely understood, however, methylamine dependent CoM methylation was still uncharacterized. Therefore, this study was undertaken to identify the role of the methylcorrinoid:CoM methyltransferase, MtbA, in methylamine dependent CoM methylation, isolate and characterize the enzymes involved in the initial steps of CoM methylation from trimethylamine (TMA) and dimethylamine (DMA), and to reconstitute the pathways of CoM methylation from TMA and DMA *in vitro*.

An immunochemical approach was employed as a test for the functional activities of the methylcorrinoid:CoM methyltransferases, MtbA and MtaA, in the metabolic pathways of methane formation from monomethylamine (MMA), DMA, and TMA. Specific removal and subsequent addition of MtbA from extracts of TMA grown cells demonstrated
the involvement of MtbA in the pathways of CoM methylation from TMA, DMA, and MMA. It was shown that MtbA functions as the exclusive methylcorrinoid:CoM methyltransferase in the MMA:CoM and DMA:CoM methyl transfer pathways. However, both MtbA and MtaA were shown to function in the TMA:CoM methyl transfer pathway.

Reconstitution of the TMA:CoM methyl transfer pathway was achieved with three purified polypeptides: MttB, MttC, and MtbA. Two of these polypeptides copurified as the MttBC complex which stimulated TMA:CoM methyl transfer activity in extracts. MttB is a 280 kDa protein composed of 52 kDa subunits and MttC is a 26 kDa protein which can exist as a monomer or in complex with MttB. The 26 kDa MttC was shown to bind 1.1 mol corrinoid per mol polypeptide whereas MttB bound no apparent prosthetic group. It was found that either methylcorrinoid:CoM methyltransferase could interact with MttC to carry out TMA:CoM methyl transfer although MttC showed a greater affinity for MtbA than for MtaA.

DMA dependent CoM methylation was reconstituted in vitro for the first time using only highly purified proteins. These proteins included the previously unidentified corrinoid protein, MtbC, which copurified with MtbA, and a DMA:corrinoid methyltransferase MtbBl. MtbC was shown to bind 1 mol corrinoid per mol polypeptide whereas MtbBl bound no apparent prosthetic group. A molar ratio of MtbC to MtbBl of 1 was found to be optimal for activity. Other than the ability of MtbBl to methylate free cob(I)alamin with DMA at a low rate, MtbC and MtbBl showed a high degree of specificity for one another and for DMA as the substrate.
This work is dedicated to my parents

Don and Donna Ferguson
ACKNOWLEDGMENTS

I would like to thank my fiancée Tsuneo Hill for her love, support, and advice over the last few years of my graduate career, for which I will be eternally grateful. I also thank the members of my committee Dr. John Reeve, Dr. Tina Henkin, and Dr. Neil Baker for all of their advice and helpful suggestions on this project. I would like to extend a special thanks to Dr. Kathy Kendrick who was kind enough to spend the time and effort required to serve on my committee during her period of illness. I am thankful for the opportunity to spend my graduate career among the members of the Krzycki laboratory, past and present, whose advice, generosity, and friendship made graduate school much more enjoyable. Finally, I would like to thank my adviser Dr. Joe Krzycki for his invaluable help and advice and for helping to mold me into the scientist that I am today.
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<tr>
<td>Ab</td>
<td>antibody</td>
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<tr>
<td>AdoMet</td>
<td>S-adenosyl-L-methionine</td>
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<tr>
<td>CO</td>
<td>carbon monoxide</td>
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<td>CODH/ACS</td>
<td>carbon monoxide dehydrogenase/acetyl CoA synthase</td>
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<td>CoB</td>
<td>coenzyme B or mercaptoheptanoylthreonine phosphate</td>
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<td>CFeSP</td>
<td>corrinoid/iron-sulfur protein</td>
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<td>CoM</td>
<td>coenzyme M or 2-mercaptoethanesulfonate</td>
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<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
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<td>DMA</td>
<td>dimethylamine</td>
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<td>DMAMT</td>
<td>dimethylamine methyltransferase</td>
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<td>DMS</td>
<td>dimethylsulfide</td>
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<td>DTNB</td>
<td>5,5'-dithiobis(2-nitrobenzoic acid)</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<td>EPR</td>
<td>electron paramagnetic resonance</td>
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<td>H₄folate</td>
<td>tetrahydrofolate</td>
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<td>H₄SPt</td>
<td>tetrahydrosarcinapterin</td>
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<td>HEPES</td>
<td>N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]</td>
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<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<td>HS-HTP</td>
<td>mercaptoheptanoylthreonine phosphate</td>
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<td>IgG</td>
<td>immunoglobulin G</td>
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<tr>
<td>MAP</td>
<td>methyltransferase activating protein</td>
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<td>MCR</td>
<td>methyl coenzyme M reductase</td>
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<tr>
<td>MeOH</td>
<td>methanol</td>
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<tr>
<td>MES</td>
<td>2-[N-morpholino]ethanesulfonic acid</td>
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<td>MFR</td>
<td>methanofuran</td>
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MMA  monomethylamine
MMPA methylmercaptpropionate
MOPS 3-[N-morpholino]propanesulfonic acid
MS  methionine synthase
MSH methane thiol
MT1 methyltransferase I
MT2-A amine specific methyltransferase II
MT2-M methanol specific methyltransferase II
FAGE polyacrylamide gel electrophoresis
PCR polymerase chain reaction
PVDF polyvinylidene fluoride membrane
RAM reductive activation, methylamines
SDS sodium dodecyl sulfate
THF tetrahydrofolate
THMP tetrahydromethanopterin
THSP tetrahydrochloropterin
TMA trimethylamine
Tris tris(hydroxymethyl)aminomethane
UV ultraviolet light

Gene designations of enzymes involved in methylo trophic methanogenesis

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<td>methanol specific methylcorrinoid: CoM methyltransferase</td>
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<td><em>mtbB1</em></td>
<td>DMA:corrinoid methyltransferase version I (linked to <em>mtt</em>/<em>mtb</em> operon)</td>
<td>Paul et al., 2000</td>
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<td>putative MMA permease</td>
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CHAPTER 1

GENERAL INTRODUCTION

The Methanogens

Dating back to the time of Alessandro Volta's early experiments with "combustible air," scientists have been interested in the nature of biological methane production. In recent years we have witnessed the discovery of many novel cofactors (Figure 1) and enzymes and the dawn of genetic manipulation in methanogens. Over fifty methanogenic strains have been identified, all of which are phylogenetically distinct from the Bacteria and Eukarya (Boone et al., 1993). This observation, based on 16S ribosomal RNA sequences, prompted the reclassification of the methanogens and other phylogenetically similar organisms into a separate domain known as the Archaea (Woese et al., 1978; Woese et al., 1990; Balch et al., 1977; Fox et al., 1977). The Archaea include both mesophilic and thermophilic species of methanogens as well as halophiles (Woese et al., 1978; Woese et al., 1990). Methanogens are found in a wide range of anaerobic environments including
Figure 1.

Structures of the coenzymes and cofactors involved in methanogenesis (from White and Zhou, 1993).
Figure 1
freshwater and marine sediments, landfills, anaerobic sludge digestors, and the intestinal tracts of animals (Zinder, 1993). In these anaerobic environments, methanogens play a key role in the carbon cycle by converting organic carbon from the end-products of fermentative and acetogenic metabolism to methane. Although some methanogens are acetotrophic or methylotrophic, the catabolic pathway shared by most methanogens is the reduction of carbon dioxide to methane (Zinder, 1993).

Reduction of carbon dioxide to methane using molecular hydrogen

Most methanogens possess the capability to reduce carbon dioxide (CO₂) to methane (CH₄) with molecular hydrogen (H₂) or formate (Figure 2). The metabolic pathway by which this occurs has been well studied using both biochemical and molecular biological methods. This pathway involves multiple step-wise reductions of the carbon from CO₂ which is bound to one of three novel methanogenic cofactors at various steps in the pathway until the methyl group bound to coenzyme M (CoM) is finally reduced to methane (DiMarco et al., 1990). These stepwise reductions are generally facilitated by the splitting of H₂ by hydrogenase enzymes to liberate two electrons which can be used to reduce coenzyme F₄₂₀, by the coenzyme F₄₂₀-reducing (NiFe) hydrogenase, or an unknown electron acceptor, by the coenzyme F₄₂₀-nonreducing (NiFe) hydrogenase (DiMarco et al., 1990; Ferry, 1999). Although the physiological electron acceptor of the coenzyme F₄₂₀-nonreducing hydrogenase is unknown, it is believed to be involved in the initial reduction of CO₂ to the formyl-methanofuran state, due to the inability of reduced coenzyme F₄₂₀ to function at this step (Karrasch et al., 1989).
Figure 2.

Schematic of the pathway of methanogenesis from CO₂ and H₂. The sites of energy conservation are shown at step 6 and linked to step 8 which lead to ATP synthesis at steps 10 and 11 (from Deppenmeier et al., 1996).
Figure 2
Energy conservation during methanogenesis from \( \text{CO}_2 \) and \( \text{H}_2 \) occurs at two steps in the pathway: the transfer of the methyl group from \( N^5 \)-methyltetrahydromethanopterin (methyl-THMP) to CoM and the reduction of the heterodisulfide CoM-S-S-coenzyme B to HS-CoM and HS-CoB. Both of these steps are catalyzed by membrane bound enzyme complexes (Blaut et al., 1993). The former step is catalyzed by the \( N^5 \)-methyltetrahydromethanopterin:CoM methyltransferase and will be discussed below. The latter step is catalyzed by heterodisulfide reductase and has been implicated in the generation of a transmembrane proton gradient involved in ATP synthesis (Blaut et al., 1993; Blaut et al., 1986).

**Methanosarcina barkeri**

*M. barkeri* is a member of the order *Methanosarcinales*, family *Methanosarcinaceae*, and is the type species of the genus *Methanosarcina* (Boone et al., 1993). Nearly all of the methylotrophic methanogens are found within the family *Methanosarcinaceae*. *M. barkeri* is one of the most studied of the methylotrophic methanogens. Like most methanogens, it is capable of methanogenesis from \( \text{CO}_2 \) and \( \text{H}_2 \). However, one of the most interesting aspects of this organism is its ability to carry out methanogenesis from acetate and nearly all of the known methylotrophic methanogenic substrates including methanol (MeOH), monomethylamine (MMA), dimethylamine (DMA), trimethylamine (TMA), dimethylsulfide (DMS), methane thiol (MSH), and methylmercaptopropionate (MMPA) (Tallant and Krzycki, 1996). The only known methylotrophic methanogenic substrate which *M. barkeri* appears unable to catabolize is tetramethylammonium. Nearly all of the
research performed on the catabolism of methylotrophic substrates in methanogens, with the exception of tetramethylammonium (Tanaka, 1994; Asakawa et al., 1998), has been done using M. barkeri.

**Methanogenesis from methylotrophic substrates**

Most of the work done previously on the biochemistry of methanogenesis has focused on CO₂ and acetate metabolism. Until recently, little focus has been placed on the catabolism of methylotrophic substrates such as methylated thiols and methylamines. The initial steps of methanol-dependent methanogenesis were studied originally by Vogels and collaborators and also recently by Thauer and collaborators. Much of the work done on the biochemistry of methylamine-dependent methanogenesis and all of the work done on the enzymes involved in methylated thiol-dependent CoM methylation has been performed in our laboratory. The processes by which methanogens convert methylotrophic substrates to methyl-CoM is discussed below and in the following chapters. The final step of methylotrophic methanogenesis is identical to all known pathways of methanogenesis. Following methylation of CoM, a two electron reduction of the methyl group to methane is catalyzed by methyl-CoM reductase with electrons donated by coenzyme B (Ferry, 1999). The products of this reaction are methane and the heterodisulfide CoM-S-S-CoB. Free and reduced CoM and CoB are generated by heterodisulfide reductase with electrons derived from H₂ (Ferry, 1999).
Methanogenesis from methanol

Methanogenesis from methanol is one pathway in which all of the enzymes responsible for the transfer of a methyl group from growth substrate to CoM have been isolated and characterized. The first enzyme isolated was a 37 kDa enzyme which contained no apparent prosthetic group and was capable of methylating CoM using methylcobalamin as the methyl donor (van der Meijden et al., 1983). This enzyme, originally termed methyltransferase II or MT2 and later called MT2-M due to its role in methanol catabolism, is now referred to by its gene designation, MtaA. MtaA is now known to bind 1 mol zinc per mol enzyme (LeClerc and Grahame, 1996). The presence of MtaA indicated that a corrinoid protein was also involved in the pathway. A corrinoid binding protein was isolated which in the presence of MtaA and catalytic amounts of methanol-grown cell extract was able to catalyze the ATP-dependent methylation of CoM from methanol (van der Meijden et al., 1984). This enzyme, originally termed methyltransferase I or MT1, has now been renamed MtaBC. MtaBC consists of two distinct subunits in an α2β configuration (van der Meijden et al., 1984; Sauer, et al., 1997). The α subunit, MtaB, a 51 kDa polypeptide which has been shown to bind 1 mol zinc per mol polypeptide, is believed to activate methanol to allow for the methanol:corrinoid methyl transfer reaction to take place (van der Meijden, et al., 1984; Sauer and Thauer, 1997). The β subunit, MtaC, is a 27 kDa polypeptide which binds a corrinoid. The corrinoid bound to MtaC is presumed to be 5-hydroxybenzimidazolyl cobamide (Figure 3) (van der Meijden et al., 1984) based on bulk corrinoid isolations and
Figure 3.

Structure of the predominant corrinoid in methanogens, 5-hydroxybenzimidazolyl cobamide (from Keltjens and Vogels, 1993). The corrinoid is shown in the base-on methylcob(III)amide state in which a methyl group is bound at the upper axial ligand site and the 5-hydroxybenzimidazole is serving as the lower axial ligand. When the corrinoid is bound to proteins, a histidine residue of the protein may serve as the lower ligand and the 5-hydroxybenzimidazole tail serves to anchor the corrinoid in the protein.
identifications (Pol et al., 1982), as is presumed to be the case for all methanogen corrinoid binding proteins (Ferry, 1999). In order for the methanol:CoM methyl transfer to take place, the central cobalt atom in the corrinoid must be in the catalytically active Co(I) state (van der Meijden et al., 1984; Sauer et al., 1997). The corrinoid in the Co(I) state is a highly reactive nucleophile which is able to demethylate methanol leaving the corrinoid in the methyl-Co(III) state. The methyl group is then removed by MtaA and the Co(I) state is regenerated (van der Meijden et al., 1984; Sauer et al., 1997). The zinc in MtaB is believed to act as a Lewis acid to allow for the nucleophilic attack on the methyl group by the Co(I) form of the corrinoid of MtaC (Sauer and Thauer, 1997; Ferry, 1999). However, if the corrinoid is exposed to an oxidant such as O₂, the corrinoid can be inactivated to the Co(II) state. In this case, a cellular reductive activation system is required to regenerate the Co(I) state. A methyltransferase activating protein (MAP), with no apparent prosthetic group, has been described which appears to be able to use ATP and reducing equivalents from H₂ passed through hydrogenase and ferredoxin to reduce the corrinoid from Co(II) to Co(I) (Daas et al., 1993; Daas et al., 1996a; Daas et al., 1996b; Wassenaar et al., 1996). However, this reaction has not been demonstrated. Only methylation of the corrinoid on MtaC from methanol in the presence of the activation fractions and ATP has been demonstrated which may indicate a requirement for methanol in the activation reaction.

The native MtaBC enzyme exists in a tight complex and thus far it has not been possible to separate the native polypeptides for further study without the use of detergents. Therefore, mtaB and mtaC have been cloned, sequenced, and expressed as recombinant proteins in E. coli (Sauer et al., 1997;
Sauer and Thauer, 1998b). EPR and site directed mutagenesis studies of rMtaC have suggested that the His136 residue is the lower axial ligand of the corrinoid, analogous to His759 of methionine synthase (discussed below), due to the inability of mutants substituted with Lys or Gly at this site to bind cobalamin and form the MtaC holoprotein (Sauer and Thauer, 1998b). However, it has not been conclusively demonstrated that His136 is the active site of the enzyme; His136 may simply be important in proper folding of the protein. Recombinant MtaB has also been studied and was found to bind 1 mol zinc per mol polypeptide (Sauer and Thauer, 1997). An ATP binding motif was also discovered and ATP was found to stimulate methanol-dependent methylation of the corrinoid of MtaC by MtaB 3-fold (Sauer et al., 1997). It has been demonstrated that MtaB can also methylate free cob(II)alamin with methanol (Sauer and Thauer, 1999). This activity can proceed in the presence or absence of MtaA but is stimulated by the presence of MtaA. Interestingly, an MtaAB complex able to bind free cobalamin appears to form in the presence of methanol which leads to a stimulation of MtaA activity (Sauer and Thauer, 1999).

**Oxidation of methanol to CO₂ during methanol dependent methanogenesis**

Methylo trophic methanogenesis is typically a disproportionation reaction which requires the oxidation of one methyl group from substrate per three methyl groups reduced to methane (Keltjens and Vogels, 1993). This leads to an overall stoichiometry of methanogenesis from methanol of:

$$4\text{CH}_3\text{OH} \rightarrow 3\text{CH}_4 + \text{CO}_2 + 2\text{H}_2\text{O} \quad (\Delta\text{G}^\circ = -106 \text{ kJ/mol CH}_4)$$

(Keltjens and Vogels, 1993)
The oxidation of 1 methyl group generates the 6 reducing equivalents necessary to reduce 3 methyl groups to methane. The oxidation of this methyl group appears to occur via a reversal of the pathway used to reduce CO₂ to methane using molecular hydrogen (Figure 4) (Keltjens and Vogels, 1993; Ferry, 1999; Müller, et al., 1988). In order for this to occur, a methyl group from the substrate must first be transferred to tetrahydrosarcinapterin (THSP), the pterin derivative found in Methanosarcina species (Müller et al., 1988). There is some controversy as to how this occurs and there is evidence to support two different pathways. One theory suggests that the methyl group from substrate must first be transferred to CoM through the reductive pathway discussed above and then transferred to THSP by a reverse reaction of the membrane-bound N⁵-methyltetrahydromethanopterin:CoM methyltransferase (MtrA-H) in an energy consuming process. This is supported by experiments in which methanol-dependent methanogenesis without H₂ required a transmembrane Na⁺ gradient (inside high) but in the presence of H₂ no gradient was required (Müller et al., 1988; Müller et al., 1993). Another theory involves the direct methylation of THSP from methanol by an as yet unidentified enzyme(s). This reaction would be energetically favorable and would eliminate the need for the energy requiring step catalyzed by MtrA-H. Evidence for the direct methylation of THSP with methanol has recently been obtained by Thauer and collaborators but has not yet been published (R.K. Thauer, personal communication).
Pathway of methanol oxidation to generate reducing equivalents for the reductive pathway (from Deppenmeier et al., 1996). It is currently unknown whether methylation of \( \text{H}_4\text{SPt} \) (THSP) from methanol occurs directly through step (a) or indirectly through steps (b) and (c). Oxidation of 1 methyl group generates the 6 reducing equivalents necessary to reduce 3 methyl groups to methane.
Figure 4
Methanogenesis from methylated thiols

Until recently, it was believed that utilization of methylated thiols such as DMS by methanogens was limited to some of the halophilic species and *M. barkeri* was not believed to possess this activity (Keltjens and Vogels, 1993; Oremland *et al.*, 1989). Early studies in our laboratory led to the discovery of a 480-kDa corrinoid binding protein in extracts of acetate-grown cells (Cao and Krzycki, 1991; Kremer *et al.*, 1993). The protein consists of two subunits of 40 kDa and 30 kDa in an $\alpha_6\beta_6$ configuration (Kremer *et al.*, 1993). The 30 kDa $\beta$ subunit, MtsB, was identified as the corrinoid binding subunit based on sequence similarities to known corrinoid binding proteins such as methionine synthase and methylmalonyl-CoA mutase (Pau and Krzycki, 1996). The physiological function of this enzyme was unknown; however, it was isolated in the methylated form and was able to transfer its methyl group to CoM thereby suggesting a role for this enzyme in methanogenesis (Kremer *et al.*, 1993). This activity was explored further and it was found that the 40 kDa $\alpha$ subunit, MtsA, possessed the methylcorrinoid:CoM methyltransferase activity (Tallant and Krzycki, 1996). Sequence analysis of MtsA revealed similarity to uroporphyrinogen decarboxylases and the homologous methylcorrinoid:CoM methyltransferases, MtaA and MtbA (Pau and Krzycki, 1996). It was later discovered that cells grown on acetate could convert DMS or MMPA to methane (Tallant and Krzycki, 1997). These activities in extracts eluted from a gel permeation column in the range of 470 kDa. The purified 480 kDa MtsAB complex could stimulate DMS- and MMPA-dependent methanogenesis from acetate-grown cell extracts 2.5-fold. The purified methylated form of the MtsAB complex was then tested for the ability to
methylate CoM from DMS and MMPA and was found to possess these activities (Tallant and Krzycki, 1997). MtsA is believed to serve two functions in these reactions: transfer of the methyl group from DMS or MMPA to the corrinoid of MtsB and also transfer of the methyl group from MtsB to CoM (T.C. Tallant, personal communication).

**Methanogenesis from methylamines**

Methylamines are important precursors to methane production in marine environments and appear to be a way in which methanogens can coexist and compete with sulfate reducing bacteria (King, 1984). The primary source of methylamines in marine environments appears to be from the breakdown of trimethylamine-N-oxide and glycine betaine (Zinder, 1993; King, 1984). Both of these compounds are commonly employed by fish, plants, and bacteria as osmoprotectants. Early studies of methylamine-dependent methanogenesis indicated that, like methanol, the methyl group from substrate is transferred intact to CoM (Walther et al., 1981). Studies by Naumann et al. (1984) also suggested that CoM methylation from TMA, DMA, and MMA are catalyzed by separate and inducible enzyme systems. These studies led our laboratory to conduct further studies on the enzyme systems responsible for methylamine-dependent CoM methylation.

Unlike in methanol grown cells, the predominant methylcorrinoid:CoM methyltransferase in methylamine grown cells is not MtaA but instead a homologous enzyme named MtbA (Grahame, 1989). MtaA and MtbA are 37% identical at the amino acid level (LeClerc and Grahame, 1996; Harms and Thauer, 1996). MtbA was discovered during the
purification of MtbA from acetate-grown cells after a second peak of methylcorrinoid:CoM methyltransferase activity was detected from a hydroxyapatite column. MtbA was originally suspected to be involved in acetate metabolism (Grahame, 1989). A study by Yeliseev et al. (1993) demonstrated a correlation between the presence of MtbA in cells grown on TMA and H₂/CO₂ and the ability of these cells to utilize TMA. Prior to work done in our laboratory, however, no physiological function of MtbA had been assigned.

The first demonstration of a physiological role of MtbA came from work done in our laboratory on MMA catabolism (Burke and Krzycki, 1995). A 29 kDa corrinoid protein of unknown function was discovered in our laboratory from acetate grown cells (Cao and Krzycki, 1991). This protein was determined to have the ability to stimulate MMA dependent methanogenesis from TMA grown cells. The MMA:CoM methyl transfer activity was purified from TMA-grown cell extracts and this fraction was found to contain two polypeptides. N-terminal sequencing identified these polypeptides as the 29 kDa corrinoid protein (MtmC) discovered previously and one of the methylcorrinoid:CoM methyltransferases, MtbA (Burke and Krzycki, 1995). Each of these polypeptides could independently stimulate MMA:CoM methyl transfer activity in extracts but were insufficient to catalyze the reaction in a resolved system (Burke and Krzycki, 1995). In order to reconstitute the MMA:CoM methyl transfer pathway in a resolved system, a third component, the MMA:corrinoid methyltransferase, MtmB, was isolated (Burke and Krzycki, 1997) and will be discussed in greater detail in Chapter 4. Work done
on the enzymes involved in the TMA:CoM and DMA:CoM methyl transfer pathways will be discussed in detail in Chapters 3 and 4, respectively.

The reductive cellular activation systems involved in methanogenesis from methylamines have not been extensively studied thus far. Work done using MAP from methanol grown cells has suggested that MAP, which was reported to contain no prosthetic group, can function to activate the corrinoid of the TMA corrinoid protein and the DMA corrinoid protein. However, in our laboratory we have observed that extracts of methanol grown cells were unable to activate the corrinoid proteins of the TMA:CoM and DMA:CoM methyl transfer pathways (Ferguson and Krzycki, 1997; Chapter 4). Work done in our laboratory has led to the purification of a redox active protein capable of activating MtmC in the MMA:CoM methyl transfer reaction in an ATP-dependent resolved system. UV-visible spectra of this protein, RAM, are consistent with the presence of an iron-sulfur cluster which could indicate that RAM and MAP are in fact different proteins (T.K. Hill, personal communication). It has recently been shown that RAM can function in the activation of DMA and TMA specific corrinoid proteins as well (T.K. Hill, personal communication).

Tetramethylammonium-dependent methanogenesis has not been shown to occur in M. barkeri, however, the activity and the enzymes responsible for the activity have recently been identified in Methanococcoides strain NaT1 (Tanaka, 1994; Asakawa et al., 1998). Three proteins were isolated which were required for tetramethylammonium:CoM methyl transfer activity with apparent molecular masses of 50, 40 and 22 kDa (Asakawa et al., 1998). The 22 kDa protein, MtqC, had the typical characteristics of a corrinoid
protein including a UV-visible spectrum characteristic of cob(III)alamin. The N-terminus of the 40 kDa protein, MtqA, revealed sequence similarities to all of the known methylcorrinoid:CoM methyltransferases. The 50 kDa polypeptide, MtqB, is proposed to be the tetramethylammonium:corrinoid methyltransferase although its N-terminus showed no sequence similarity to the previously isolated methanol or methylamine:corrinoid methyltransferases (Asakawa et al., 1998). However, this is not surprising due to the fact that the previously isolated methyltransferases show no similarities to one another with the exception of size and the presence of an in-frame UAG codon in the methylamine:corrinoid methyltransferases (Sauer et al., 1997; Burke et al., 1998; Paul et al., 2000). It is unknown whether the tetramethylammonium:corrinoid methyltransferase also contains an in-frame UAG codon. The activity of the system was dependent on Ti(III)-citrate and ATP (Asakawa et al., 1998). The requirement for ATP may indicate the presence of a contaminating activation protein in one or more of the fractions. Data in our laboratory have suggested that trace amounts of an activation protein could be sufficient to activate the corrinoid protein (T.K. Hill, personal communication).

**Corrinoid-dependent methyltransferase systems**

Corrinoid-dependent methyltransferase systems have been implicated in a variety of metabolic processes including methionine biosynthesis, acetyl-CoA biosynthesis and cleavage, and a range of methanogenic reactions (Banerjee, 1997). In the case of methionine biosynthesis, mutations in the human methionine synthase enzyme have been shown to have
ramifications on health. These mutations, which map to the B12 and adenosyl methionine binding regions of the enzyme, have been implicated in the accumulation of homocysteine which leads to the condition known as hyperhomocysteinemia (Banerjee, 1997). One advantage of corrinoid-dependent methyltransferase reactions over the more common S-adenosyl methionine-dependent (AdoMet) methyltransferase reactions is in their energy requirement. AdoMet-dependent reactions require the hydrolysis of 1 ATP per turnover to regenerate AdoMet as opposed to corrinoid-dependent reactions which may only require ATP if the corrinoid becomes inactivated. This is particularly useful when corrinoid-dependent reactions are utilized in catabolic pathways in which one of the desired end products of the pathway is ATP. However, corrinoid-dependent methyltransferase reactions by nature require reduced environments which may be inopportunity in some instances. Discussed below are some of the best studied enzymes and corrinoid binding proteins involved in corrinoid-dependent methyltransferase systems.

Cobalamin-dependent methionine synthase (MetH)

One extensively studied corrinoid containing enzyme is the 136 kDa monomeric methionine synthase enzyme (MetH) from E. coli. MetH catalyzes the transfer of a methyl group from methyltetrahydrofolate (CH3-THF) to enzyme bound cobb(I)alamin and then from methylcobalamin to homocysteine to generate methionine and THF (Banerjee et al., 1989; Amaratunga et al., 1996; Jarrett et al., 1996). This reaction proceeds via a heterolytic cleavage of the carbon-cobalt bond on methylcobalamin so that cobb(I)alamin is regenerated with each cycle (Amaratunga et al., 1996; Jarrett et
However, if the corrinoid becomes oxidized to Co(II), MetH is able to use S-adenosyl-methionine and an electron from flavodoxin to regenerate the enzyme-bound corrinoid to the methylcob(III)alamin form (Figure 5) (Banerjee et al., 1990; Amaratunga et al., 1996; Jarrett et al., 1998a). The enzyme has been shown to be a modular protein with four distinct regions. The region of the protein containing residues 2-353 catalyzes the methyl transfer from methylcobalamin to homocysteine. Residues 354-649 are involved in the binding and activation of CH₃-THF. The region containing residues 650-896 is responsible for cobalamin binding and residues 897-1227 are responsible for S-adenosyl-methionine-dependent activation of the enzyme (Goulding et al., 1997).

The 27 kDa cobalamin-binding domain of MetH has been actively expressed in E. coli and purified to homogeneity (Drennan et al., 1994). The X-ray structure of the methylcobalamin form of this enzyme fragment has been determined at 3 Å resolution (Drennan et al., 1994). The structure revealed that the lower axial ligand of bound methylcobalamin is the Ne of His759 rather than a nitrogen from the dimethylbenzimidazole as it is in free methylcobalamin (Drennan et al., 1994). The dimethylbenzimidazole nucleotide tail was shown to be anchored inside the protein. The His759 residue appears to be hydrogen bonded to two other residues, Asp757 and Ser810, when coordinated to the cobalt atom to form a ligand triad (Drennan et al., 1994). These three residues are contained within the α/β domain of the fragment which interacts with the lower face of the corrinoid. There is also an α-helical domain of the fragment which interacts with the upper face of
Figure 5.

Schematic of the catalytic and reactivation cycles of cobalamin-dependent methionine synthase (MS) (from Drummond et al., 1993). The cob(I)alamin form of the enzyme is able to demethylate methyltetrahydrofolate (CH$_3$-H$_4$Folate) generating the methylcob(III)alamin form of the enzyme and H$_4$Folate. The methylcob(III)alamin form of the enzyme is demethylated by homocysteine (Hcy) generating methionine (Met) and regenerating cob(I)alamin. When cob(I)alamin enzyme becomes oxidized to the Co(II) state, the enzyme is reactivated by a one electron reduction by flavodoxin (not shown) and methylated by adenosyl methylionine (AdoMet).
Figure 5
the corrinoid. This helical domain is postulated to be a mechanism for shielding of the methyl ligand thus forming a methyl cap region (Drennan et al., 1994). Conformational changes in the protein during methylation or demethylation of the corrinoid could momentarily displace this cap so that the reaction could take place. Although portions of the methyl cap appear to be conserved, it is unclear whether this methyl cap structure exists in the methanogenic enzymes studied thus far, however, the ligand triad does appear to be conserved (Burke et al., 1998; Paul and Krzycki, 1996; Sauer et al., 1997; Paul et al., 2000).

Sequence analysis of the metH gene as well as genes encoding corrinoid proteins involved in methanogenesis have shown the putative cobalamin binding region to be highly conserved (Burke et al., 1998; Paul and Krzycki, 1996; Sauer et al., 1997; Paul et al., 2000). The sequences of mtaC encoding the corrinoid protein of MtaBC, mtsB encoding the corrinoid protein of the methyl thiol:CoM methyltransferase, and mtmC, mibC, and mtC encoding the methylamine specific corrinoid proteins all show a high degree of conservation throughout this region (Burke, et al., 1998; Paul and Krzycki, 1996; Sauer, et al., 1997; Paul et al., 2000). MtmC, Mtbc, and MttC contain the exact residues of the proposed ligand triad of MetH (Burke et al., 1998; Paul et al., 2000). MtsB and MtaC contain the His and Asp residues of the ligand triad and each contain a conservative Thr substitution in place of Ser (Paul and Krzycki, 1996; Sauer et al., 1997). Site-directed mutagenesis of each of the residues in the ligand triad of MetH had negative effects on the methyltransferase activity of the 27 kDa cobalamin-binding enzyme fragment.
Mutations in the Asp and Ser residues decreased the activity and a mutation in the His residue had a deleterious effect on the activity (Jarrett et al., 1996). These results seem to indicate that the ligand triad is important in catalysis for MetH. Similar studies on MtaC have suggested that the His136 residue is important in methanol:CoM methyl transfer activity (Sauer and Thauer, 1998).

Recent studies of cob(I)alamin formation and substrate binding to MetH have indicated that changes in protonation affect the reactivity of the enzyme (Jarrett et al., 1997). It was demonstrated that proton uptake occurs at a rate equal to that of cob(I)alamin formation. Also, binding of homocysteine to the cob(II)alamin form of MetH results in the release of protons believed to be derived from the thiol of homocysteine (Jarrett et al., 1997). The uptake of a proton on the enzyme is believed to occur within the ligand triad based on comparative studies of the wild-type enzyme and the Asp757Glu mutant. The protonation of the enzyme is believed to stabilize the 4-coordinate cob(I)alamin form of the enzyme and may be involved in a conformational change in the enzyme (Jarrett et al., 1997).

Zinc has also been implicated in the mechanism of methyl transfer from methylcob(III)alamin to homocysteine (Goulding and Matthews, 1997). Zinc appears to be required for homocysteine activation. The wild-type enzyme binds 0.9 equivalents of zinc; however, Cys310Ser and Cys311Ser mutants bind only 0.05 equivalents of zinc and are unable to catalyze the methylation of homocysteine. The zinc appears to be involved in the deprotonation of the thiol of homocysteine (Goulding and Matthews, 1997).
The enzyme must have a mechanism by which cob(I)alamin interacts preferentially with either AdoMet during reactivation or methyl-THF during the catalytic cycle (Jarrett et al., 1998b). This mechanism appears to be related to the ability of the enzyme to exist in two different conformations. In the primary turnover conformation, the enzyme reacts with methyl-THF and homocysteine but not flavodoxin and AdoMet. In the reactivation conformation, the enzyme reacts with flavodoxin and AdoMet but not methyl-THF and homocysteine. In the cob(II)alamin form of the enzyme there is interconversion between these two conformational states (Jarrett et al., 1998b). The reduction of cob(II)alamin to cob(I)alamin is carried out by the strong reductant flavodoxin hydroquinone, however, very little cob(I)alamin can be observed in this reaction (Jarrett et al., 1998a). The small amount of cob(I)alamin produced is believed to be trapped by immediate methylation by AdoMet. Methylation by AdoMet has been shown to produce the 5-coordinate methylcob(III)alamin which slowly decays to the 5-coordinate base-on state. Therefore, the rapid electron transfer from flavodoxin to cob(II)alamin and methyl transfer from AdoMet to cob(I)alamin are believed to be masked by the relatively slow rate-limiting conformational changes required for these reactions to take place (Jarrett et al., 1998a). It was once believed that a conformational change in the enzyme leading to base-off 4-coordinate cob(II)alamin would raise the midpoint potential of the Co(II)/Co(I) couple allowing flavodoxin to reduce the cobalamin to the Co(I) state. However, direct measurement of the redox potential required to reduce base-off cob(II)alamin revealed that the base-off and base-on require essentially the same redox potential (Hoover et al., 1997).
\textit{N}^5\text{-methyltetrahydromethanopterin}:CoM methyltransferase (MtrA-H)

The membrane-bound CH$_3$-THMP:CoM methyltransferase complex, MtrA-H, has been isolated and characterized from \textit{Methanobacterium thermoautotrophicum} strains Marburg and \textit{\Delta H}, \textit{Methanosarcina barkeri} and \textit{Methanosarcina mazei} G\ö1 (Gärtner et al., 1993; Gärtner et al., 1994; Lienard et al., 1996). This enzyme complex is responsible for the methylated pterin-dependent CoM methylation reaction during growth on acetate and H$_2$/CO$_2$. The complex has been shown to contain 8 different subunits (Harms et al., 1995). The genes encoding these subunits are arranged in an operon and cotranscribed (Harms et al., 1995; Lienard and Gottschalk, 1998). The MtrA subunit has been shown to bind the corrinoid in the complex (Harms and Thauer, 1996). The consensus corrinoid binding region from MetH appears to be conserved in MtrA and site directed mutagenesis studies of different His residues in MtrA have suggested that His84 is the active site histidine analogous to His759 in MetH (Sauer and Thauer, 1998; Lienard and Gottschalk, 1998). The MtrH subunit has been shown to catalyze the transfer of the methyl group from CH$_3$-THMP to the bound corrinoid of MtrA (Hippler and Thauer, 1999). The precise functions of subunits B-G have not been demonstrated although it has been suggested that MtrD could be involved in the sodium translocation reaction (Lienard and Gottschalk, 1998).

In order for MtrA-H to carry out the methyltransferase reaction, the cobalt atom in the corrinoid must be in the Co(I) state (Kengen et al., 1988). Activation of the complex \textit{in vitro} requires Ti(III)-citrate and addition of ATP also provides a two-fold stimulation of activity. It has been suggested that MtrA-H is responsible for the methylation of THSP from methyl-CoM during
the oxidative pathway of methylotrophic methanogenesis (Müller et al., 1988), although this is still unclear.

One of the most interesting features of this enzyme complex is its role in energy conservation during growth on acetate or H₂/CO₂. The activity of the MtrA-H complex in the exergonic methyl transfer reaction appears to be linked to the endergonic extrusion of sodium ions (Thauer, 1998). The methyltransferase reaction appears to be dependent on sodium ions. The CH₃-THMP:CoM reaction catalyzed by the purified MtrA-H complex was stimulated 4-5 fold by sodium ions with half-maximal rates obtained with 50 μM Na⁺ concentration. The purified complex is a primary sodium pump and, when reconstituted in ether lipid liposomes, is capable of translocating 1.7 mol Na⁺/mol methyl groups transferred. This allows the MtrA-H complex to achieve a transmembrane sodium gradient of approximately 1 Na⁺ₘₐₙ:4 Na⁺ₜₐₙ in whole cells of *M. barkeri*. Therefore, this enzyme clearly plays a significant role in the generation of a transmembrane gradient necessary for energy conservation (Müller et al., 1987a) and ATP synthesis via a Na⁺ translocating F₁F₀ ATP synthase (Becher and Muller, 1994). This process generates 1 ATP per 4 methyl groups transferred.

**Carbon monoxide dehydrogenase/Acetyl-CoA synthase complex (CODH/ACS)**

The CO dehydrogenase/acetyl-CoA synthase complex has been studied from several organisms, however, due to the nature of this study, only the CODH/ACS complex from methanogens will be discussed here in detail. In methanogens, the CODH/ACS complex is one of the key enzymes of the
acetate catabolism pathway and is responsible for the cleavage of acetyl-CoA to generate a methyl group and a CO group (Terlesky et al., 1986; Abbanat and Ferry, 1990). The methyl group is used to methylate THSP via the bound corrinoid which leads to methane production whereas the CO group is oxidized to generate CO₂ and reducing equivalents. The complex consists of five subunits (α,β,γ,δ,ε) encoded in the cdh operon which are cotranscribed and subject to repression and derepression due to growth substrate (Ferry, 1999). The complex has been shown to bind a corrinoid prosthetic group, Ni, and three Fe₄S₄ clusters (A,B, and C). Cluster B is a conventional Fe₄S₄ cluster, however, clusters A and C both also contain a Ni atom within the cluster bridged to an Fe atom by an unknown linker atom. Cluster A is believed to be the active site of acetyl-CoA cleavage and synthesis. It has been proposed that the Ni atom of cluster A binds acetyl-CoA at the carbonyl group and breaks the C-S bond releasing CoA. The C-C bond is then broken leaving the methyl group and the CO group each bound to the Ni atom independently. The methyl group is then transferred to the corrinoid and the CO group is released. It is believed that CO is then oxidized to CO₂ (CO dehydrogenase activity) at cluster C. The methyl group bound to the corrinoid is then transferred to THSP and used to methylate CoM (Ferry, 1999).

The enzyme complex can be disaggregated by either cationic detergents or partial proteolysis (Abbanat and Ferry, 1991; Grahame and DeMoll, 1996). The stability of the complex can also vary due to strain differences. For example, the CODH originally isolated from M. barkeri MS contained only the α and ε subunits (Krzycki and Zeikus, 1984). The 200 kDa α/ε fraction
binds the Ni/Fe-S cluster C and is the site of CO dehydrogenase activity (Grahame and DeMoll, 1996). The reducing equivalents generated by the oxidation of CO are thought to be transferred to the membrane bound heterodisulfide reductase by way of ferredoxin and cytochrome b (Heiden et al., 1993). The 100 kDa γ/δ fraction constitutes the corrinoid/Fe-S portion of the enzyme and is responsible for the transfer of the methyl group from the corrinoid to THSP. The β subunit is responsible for the acetyltransferase activity (Grahame and DeMoll, 1996). This reaction appears to be redox and pH dependent in which increases in pH necessitate lower redox potentials. This suggests the possibility that a proton is taken up by the enzyme during reduction to the active form (Bhaskar et al., 1998).

Reducing equivalents necessary for acetyl-CoA synthesis in vivo appear to originate from H₂ and one of two hydrogenases which are found in association with the complex (Grahame and DeMoll, 1995). One hydrogenase has the ability to reduce ferredoxin but not F₄₂₀ and the other has the ability to reduce F₄₂₀ but not ferredoxin. Only the ferredoxin-reducing hydrogenase has the ability to reconstitute acetyl-CoA synthesis in vitro. Ferredoxin is essential for reconstitution of acetyl-CoA synthesis (Grahame and DeMoll, 1995) and may be the electron acceptor of the CO dehydrogenase reaction. The midpoint potential of the Co(II)/Co(I) redox couple has been measured at -426 mV and it appears as if redox potentials within the cell generated by hydrogenases and the CO dehydrogenase reaction are sufficient to maintain the corrinoid of CODH/ACS primarily in the Co(I) state (Grahame, 1993).
Corrinoid/iron-sulfur protein (CFeSP) from *Clostridium thermoaceticum*

The corrinoid/iron-sulfur protein (CFeSP) from *C. thermoaceticum* is involved in two key steps of the Wood-Ljungdahl pathway of acetyl-CoA biosynthesis (Zhao *et al.*, 1995). The first reaction involves the transfer of a methyl group from methyl-THF to the bound cob(I)amide of the CFeSP, yielding methylcob(III)alamin and THF, catalyzed by the methyltetrahydrofolate:corrinoid/iron-sulfur protein methyltransferase (MeTr). The second reaction involves the transfer of the methyl group from methylated CFeSP to the CODH/ACS complex (Zhao *et al.*, 1995; Menon and Ragsdale, 1998). The CFeSP appears to serve an analogous function to the corrinoid/Fe-S component of the CODH/ACS complex in methanogens. The CFeSP is an 88 kDa protein composed of a 33 kDa corrinoid binding subunit and a 55 kDa Fe₄S₄ cluster binding subunit (Menon and Ragsdale, 1998).

Originally, the function of the Fe₄S₄ cluster was unknown and it was postulated that the methyl transfer reaction may be a redox reaction (Menon and Ragsdale, 1998). However, the methyl transfer reaction is now believed to be an S_N2 reaction analogous to reactions carried out by MethH and methanogenic enzymes. The Fe₄S₄ cluster is now known to catalyze the reduction of the central cobalt of the corrinoid to the active Co(I) state (Menon and Ragsdale, 1998). The midpoint potential of the Co(I)/Co(II) redox couple of the corrinoid on CFeSP is -504 mV whereas the midpoint potential of the 2+/1+ couple of the Fe₄S₄ cluster is -523 mV (Menon and Ragsdale, 1998). The reducing equivalents necessary for the reduction are believed to be derived from the oxidation of CO by CODH/ACS and the presence of ferredoxin also increases the rate of electron transfer (Menon and Ragsdale, 1998).
It is interesting to note that in this protein, the corrinoid binding subunit and the activation subunit exist in a tight complex. The activation subunit is analogous to the MAP and RAM proteins described above which do not exist in a complex with their respective corrinoid proteins and have generally proven difficult to isolate. The analogy between the activation subunit of CFeSP and RAM is especially strong due to the apparent presence of an iron-sulfur cluster in RAM. Studies of CFeSP will likely prove beneficial in expanding our knowledge of the mechanism of RAM during MtmC activation.

Studies on MeTr could also prove relevant in the study of methylotrophic methanogenesis. The reaction carried out by MeTr is nearly identical to the reaction carried out by residues 354-649 of MetH and highly analogous to that of the Mrtr subunit of the MtrA-H complex in methanogens. The reaction is also analogous to the methanol and methylamine:corrinoid methyltransferases in that it binds the substrate and allows for nucleophilic attack on the methyl group by the co(l)rrinoid of the corrinoid protein. It has been determined that two protonation events are important in the CH$_3$-THF:CFeSP methyltransferase reaction. First, protonation of MeTr itself allows for a rate-limiting conformational change in the enzyme (Zhao and Ragsdale, 1995). Second, NMR studies have determined that CH$_3$-THF becomes protonated on the pterin ring very rapidly upon binding to MeTr making the methyl group more electrophilic which helps facilitate the nucleophilic attack by co(l)rrinoid (Seravalli et al., 1999). It is unknown whether a conformational change takes place in the methanol or methylamine:corrinoid methyltransferases and, if so, whether it is dependent
on protonation of the enzyme. It has been postulated that methanol must first be protonated in order to serve as a methyl donor and Zn in MtaB is believed to play a role in this process (Sauer and Thauer, 1997). However, methylamines are already protonated at physiological pH, therefore this step would be unnecessary for the methylamine:corrinoid methyltransferases. Nonetheless, detailed studies of the mechanism of MeTr could aid in the understanding of the mechanisms of the methylamine:corrinoid methyltransferases.

Goals of these studies

During the time at which this project began, little was known about the enzymes involved in methanogenesis from methylated amines. The studies of these pathways and enzymes were still at the level of crude extracts. Two of the enzymes involved in these pathways, MtmC and MtbA, had been isolated although no physiological function had been assigned at that time. Several corrinoid binding proteins and enzymes involved in corrinoid-dependent methyltransferase reactions had been identified. However, these proteins were often components of multi-enzyme complexes such as MetH and CODH/ACS making the partial reactions difficult to study without disruption of the complex through the use of detergents or proteolytic enzymes. Much of the work done on the methanol:CoM methyl transfer pathway was done using partially purified enzyme fractions and the pathway had never been reconstituted in a completely purified system. None of the genes encoding the enzymes involved in methylotrophic methanogenesis had been cloned and sequenced. Therefore, the degree of similarity between
the enzymes of the different pathways had never been assessed. We therefore embarked on a study consisting of three primary goals: 1) identification of the physiological role of the methylcorrinoid:CoM methyltransferase, MtbA, in the catabolism of methylotrophic substrates; 2) purification and characterization of the enzymes involved in TMA:CoM methyl transfer; and 3) identification of the role of a corrinoid binding protein which co-purified with MtbA from TMA-grown cell extracts.
CHAPTER 2

SPECIFIC ROLES OF THE HOMOLOGOUS METHYLCORRINOID: COENZYME M METHYLTRANSFERASES IN METABOLISM OF METHYLAMINES IN METHANOSARCINA BARKERI

INTRODUCTION

*Methanosarcina barkeri* is a methanogenic archaeal species which is capable of growth on a relatively wide range of substrates (Zinder, 1993). Although *Methanosarcina* spp. generally share in common with most methanogens the ability to use hydrogen for reduction of CO$_2$ to methane, these species have been most useful in the study of methylotrophic methanogenesis. Many of the catabolic substrates of *M. barkeri* are compounds from which methyl groups are converted essentially intact to methane (Ferry, 1992). These compounds include acetate, methanol, trimethylamine (TMA), dimethylamine (DMA) and monomethylamine (MMA). In many microbial ecosystems, these compounds serve as the
primary precursors of methane. In numerous freshwater environments acetate is the predominant source of methane. Methylated substrates other than acetate often serve as the major source of methane in brackish and salt waters (King, 1984). In marine sediments active in sulfate reduction, mono-, di-, and trimethylamines serve as methane precursor substrates rather than carbon dioxide or acetate (King, 1984).

Detailed studies have been carried out on the metabolic pathways by which methane is produced from methanol, acetate, and CO₂. However, pathways of methanogenesis from methylated amines are still not well characterized. All methanogenic substrates ultimately serve to methylate 2-mercaptopethanesulfonate (CoM), thereby forming the final metabolic intermediate in methanogenesis 2-(methylthio)ethanesulfonate (methyl-CoM). In all methanogens, methyl-CoM is converted to methane by the enzyme methyl-CoM reductase (MCR). Since MCR is the only enzyme known to form significant amounts of methane in methanogens, the problem of understanding methane formation from the various growth substrates requires knowledge of the mechanisms by which each of these methylated substrates is converted to methyl-CoM.

Methanol utilization was one of the earlier studied pathways of methanogenesis (Blaylock and Stadtman, 1964; Blaylock, 1968). Conversion of chiral [²H,³H]methanol to methyl-CoM in extracts of M. barkeri was shown to proceed with overall retention of stereochemical configuration, consistent with a pathway that contains an even number of S_N2 displacement reactions (Zydowsky et al., 1987). Two different enzymes that catalyze separate methyl group transfer reactions in the pathway have been identified. The first
enzyme, originally designated methyltransferase I (MT1), consists of two subunits with molecular weights of 51,000 (MtaB) and 28,000 (MtaC) (van der Meijden et al., 1984). The enzyme contains a non-covalently bound 5-hydroxybenzimidazolylcobamide prosthetic group which becomes methylated by interaction with methanol. The purified enzyme has been obtained with the bound corrinoid cofactor either in the Co(II) or Co(III) oxidation state. However, reduction to the Co(I) level is required prior to reaction with methanol. Reductive activation of the enzyme is a process which is not well characterized, apparently involving an ATP-dependent step, and the participation of hydrogenase, ferredoxin, and an uncharacterized protein termed the methyltransferase activating protein (MAP) (van der Meijden et al., 1984). Apparently, reaction of the active form of the MtaBC complex with methanol results in the automethylation of the enzyme, forming MtaC-bound methylcob(III)amide. Methyl-CoM is then produced by the second enzyme in the pathway, a methylcorrinoid:CoM methyltransferase originally termed methyltransferase II (MtaA), which catalyzes transfer of the methyl group from the MtaC-bound methylcob(III)amide to CoM. MtaA is a monomeric protein of approximately 37 kDa that does not contain a prosthetic group distinguishable by UV-visible spectroscopy. A convenient assay for methylcorrinoid:CoM activity has been developed based on the finding that MtaA also catalyzes methyl group transfer to CoM from free methylcobalamin.

During the course of investigations on the pathway of acetate conversion to methane it was discovered that extracts of acetate-grown *M. barkeri* contained two different homologous forms of the
methylcorrinoid:CoM methyltransferase (Grahame, 1989). These homologues had nearly identical molecular masses as judged by SDS polyacrylamide gel electrophoresis, but exhibited different electrophoretic mobilities under non-denaturing conditions, indicating that they differ in overall charge. The two methyltransferases are also distinguishable based upon their relative affinities for hydroxylapatite, and based on their different reactivities with polyclonal antibodies directed against each of the two proteins. One of the homologues was predominant in acetate-grown cells, and was termed MtbA. The other form accounted for nearly all of the methylcorrinoid:CoM activity in cells grown on methanol, and was designated MtaA. It is likely that MtaA was the major form of the enzyme originally described as being involved in the pathway of methanol conversion to methyl-CoM. Discovery of a different form of the methyltransferase in cells grown on acetate, MtbA, suggested at first that this form might be specifically involved in metabolism of acetate, however, a specific metabolic function of MtbA was not demonstrated. Later it was found that MtbA was also the major form of the enzyme present in cells grown on TMA or H2/C02. Since methanogens grown autotrophically contain low levels of methylcorrinoid:CoM methyltransferase activity, it was hypothesized that MtbA functioned in methanogenesis from TMA (Yeliseev et al., 1993).

Recently two proteins, a 29 kDa protein that contains a corrinoid cofactor and MtbA, were implicated in methane formation from MMA (Burke and Krzycki, 1995). The results strongly supported the hypothesis that MtbA functions in the pathway of methane formation from
monomethylamine. Both MMA and DMA are produced during methanogenesis from TMA by either cell cultures (Hippe et al., 1979) or extracts (Naumann et al., 1984), and subsequently serve as methanogenic substrates. However, it was unknown if MtbA might function in methanogenesis from the other methylamines as well. Furthermore, it was unknown to what extent different pathways might exhibit specific requirements for either one of the methyltransferase homologues. In this study, an immunochemical approach was employed in order to test for specificity of the involvement of individual methylcorrinoid:CoM methyltransferase homologues in methyl-CoM formation from monomethylamine, dimethylamine, and trimethylamine. Resolution and reconstitution of the individual homologues in extracts active in conversion of the different substrates is performed. Direct evidence was obtained demonstrating that MtbA was capable of functioning in the transformation of all three methylated amine substrates, whereas, MtaA acts only in the conversion of trimethylamine and methanol.

MATERIALS AND METHODS

General reagents

CNBr-activated Sepharose 4B, and GammaBind™ G Sepharose (a preparation of recombinant streptococcal Protein G covalently immobilized on Sepharose 4B) were obtained from Pharmacia LKB Biotechnology Corp. Ovine antibodies specific for MtbA and MtaA were raised in two separate
animals as described previously (Grahame, 1989) (performed by Dr. David Grahame). The γ-globulin fraction from the immune serum of each animal was prepared by ammonium sulfate precipitation, as described earlier (Grahame, 1989), and was maintained frozen at -70°C (performed by Dr. David Grahame).

Cell culture and preparation of cell-free extracts

*Methanosarcina barkeri* was grown at 37°C on 80 mM trimethylamine hydrochloride (Burke and Krzycki, 1995). The cells were harvested anaerobically, washed three times with 50 mM MOPS buffer, pH 7.0, and stored at -70°C until use. For extract preparation the cells were doused with liquid nitrogen and broken up in a commercial blender. The cells were then suspended in MOPS buffer (1 g [wet weight] per ml of buffer) under hydrogen and passed through a French pressure cell at 20,000 lb/in². Cell debris and membrane fragments were then removed by centrifugation at 150,000 × g (Cao and Krzycki, 1991). Supernatants were then decanted and stored under hydrogen at -70°C until use. Protein concentration was estimated by the method of Bradford (1976).

Enzyme assays

Two methods of analysis of methylcorrinoid:CoM methyltransferase activity were used. In one method, the demethylation of 1 mM methylcobalamin in the presence of 4 mM CoM was measured by use of the cyanide derivatization method described by Grahame (1989) in which 50 μl aliquots of reaction mixture were removed at appropriate time intervals and
placed into 950 µl of aerobic 10 mM potassium cyanide in 50 mM Tris-HCl buffer at pH 7.2. The absorbance due to the formation of dicyanocobalamin was measured at 367 nm. One unit (U) of enzyme is defined as the amount required for methyl group transfer at 1 µmol/min. In the other method, relative amounts of activity were measured spectrophotometrically based on the increase in absorbance at 620 nm due to the demethylation of methylcobalamin and the appearance of cob(I)alamin and cob(II)alamin. Reactions were carried out in 1 ml cuvettes with a 1 cm path length containing 50 mM Tris-HCl, pH 8.0, 4 mM CoM and 0.5 mM methylcobalamin. The initial rate of increase in absorbance was proportional to the amount of enzyme added to the solution.

Assays of methyl-CoM formation from CoM and methylamine substrates in extracts were performed by measurement of the substrate-dependent loss of the free thiol group of CoM over time by use of Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). All assays were carried out in anaerobic sealed 2 ml vials under an atmosphere of H₂. The reaction mixtures contained 8 mM CoM, 10 mM ATP, 20 mM MgCl₂, 3.2 mM sodium 2-bromoethanesulfonate (BES), 0.4 mM Ti(III)-citrate, and 16 mM methyl donor substrate (TMA, DMA, or MMA). The total volume of the assay was 125 µl made up of 45 µl of cell-free extract of TMA-grown cells, 55 µl of 50 mM MOPS pH 7.0 containing 1mM Ti(III)-citrate, 20 µl of a solution containing ATP, MgCl₂, CoM, and BES, and 5 µl of mono-, di- or trimethylamine substrate in water. The vials were loaded with extract samples to be analyzed and sealed inside an anaerobic chamber (Coy Laboratory Products, Inc. Grass Lake, MI). The vials were removed from the chamber, chilled on ice, and all
other reagents were added under an atmosphere of 100% H₂, maintained by use of a gassing manifold. The reactions were initiated by transfer of the vials from ice to a shaking water bath at 37°C. Samples (3 μl) were removed at specific time points and mixed in wells of a microtiter plate containing 250 μl of 0.5 mM DTNB in 150 mM Tris-HCl pH 8.0. Absorbance at 410 nm was measured by use of an MR700 ELISA plate reader (Dynatech Laboratories, Inc). Calculations of thiol concentration were based on the value of 13.6 mM⁻¹cm⁻¹ as the molar absorptivity of the 2-nitro-5-thiobenzoate dianion at 412 nm (Habeeb, 1972).

Affinity-purified anti-MtaA and anti-MtbA antibodies immobilized on protein G sepharose (performed by Dr. David Grahame)

Affinity purification of antibodies against MtbA was carried out by Dr. David Grahame by a modification of the methods described by Kincaid (1988). A column containing MtbA linked to Sepharose 4B was constructed by reaction of 2.5 mg of the pure MtbA with 1 g of CNBr-activated Sepharose 4B prepared for coupling in pH 8.3 sodium bicarbonate solution according to the procedure recommended by the manufacturer. Affinity purification from approximately 1700 mg total IgG was carried out in three separate runs, and yielded a total of approximately 10 mg of affinity purified antibody. The IgG fraction was applied at a concentration of approximately 71 mg/ml, and the column was subsequently washed with 50 ml of 0.1 M Tris-HCl, 0.5 M NaCl, pH 8.0. Thorough washing of the column was confirmed by monitoring of the effluent absorbance at 280 nm. Elution of the bound antibodies was then carried out with 0.1 M sodium acetate, pH 3.5, in 10% glycerol. Fractions were
promptly neutralized with an equal volume of 0.8 M Tris-HCl, pH 8.0. The unbound IgG was reapplied in subsequent runs in order to remove residual antibody reactivity, as judged by the eventual loss of ability of the unbound IgG to function in Western blot analyses. Affinity purified antibodies against MtaA were prepared in a similar manner, by use of an immobilized antigen column constructed from 1 g of CNBr-activated Sepharose and 5.7 mg of MtaA.

Prior to immobilization on GammaBind G Sepharose, affinity purified antibody preparations were concentrated by ultrafiltration, and adjusted to contain 50 mM MOPS, 100 mM NaCl, pH 7.2. Affinity-purified anti-MtbA antibody-GammaBind G-Sepharose gels were prepared containing 14 mg of affinity purified antibody per ml of gel. The control IgG-GammaBind G Sepharose matrix (1.0 ml) contained 16 mg of non-specific IgG, obtained during affinity purification as the IgG fraction that did not bind to the MtbA matrix.

**Immunosorptive depletion of methylcorrinoid:CoM methyltransferase homologues from cell-free extracts**

The strategy used to bring about efficient removal from the extract of either one or both of the homologous methyltransferases was designed to minimize dilution of the extract preparations. The buffer soluble cell extracts were incubated with IgG-protein G Sepharose gel preparations for 25 min at approximately 25°C with gentle mixing under strictly anaerobic conditions inside a Coy anaerobic chamber containing approximately 3% H2 in N2. The suspension was then centrifuged for 2-3 min and the supernatant was
analyzed for total methylcorrinoid:CoM methyltransferase activity, and for specific activity of methyl-CoM formation in assays of the overall conversion of a particular methanogenic substrate (either methanol, MMA, DMA, or TMA). Measurement of the remaining methylcorrinoid:CoM methyltransferase activity was performed routinely in order to assess both the time required, and the amount of immunosorbent gel necessary to obtain the maximal extent of removal of methylcorrinoid:CoM methyltransferase from each of the different cell extracts. The time course of depletion showed that removal of methylcorrinoid:CoM activity reached apparent completion after approximately 15-20 min. The minimum amount of antibody-linked gel required to accomplish the maximal extent of methylcorrinoid:CoM methyltransferase removal was found to vary depending upon the amount of methylcorrinoid:CoM activity present in the extract. Maximal depletion of methylcorrinoid:CoM activity from the trimethylamine-grown cell extract was achieved by use of a volume of gel approximately equal to that of the extract.

Control incubation experiments were carried out in which the specific activity of methanogenic substrate conversion to methyl-CoM was measured following incubation of the extract with samples of the control gel (non-specific IgG bound to protein G Sepharose). The effect of incubation on the overall methyl-CoM formation activities was also measured in the absence of gel, with or without dilution with 50 mM MOPS buffer, pH 7. Dilution of the extracts by up to 50% with 50 mM MOPS buffer alone was found to have no effect on specific activity of the extracts in conversion of any of the methanogenic substrates tested. Similarly, no decrease in specific
activity was observed in mixtures that contained samples of the control gel incubated under conditions that were otherwise identical to those which contained the specific antibody-linked gel preparations.

**Gel electrophoresis and Western blotting**

Gel electrophoresis was performed after Laemmli (1970). The samples were prepared for Western blotting by electrophoresis in a 12% acrylamide-SDS gel. The polypeptides were then electroblotted overnight at 14V onto a nitrocellulose membrane in a BioRad Trans-blot cell containing 20 mM Tris, 150 mM glycine, and 20% methanol (pH 8.0) at 4°C. The membrane was then soaked in a BLOTTO solution containing 10 mM Tris, 0.9% NaCl, and 5 g dry milk per 100 ml for 2 h at room temperature while shaking. A 1:2000 dilution of primary antibody (Ab), as indicated in text, was added to the BLOTTO solution and the membrane was incubated for an additional 2 h while shaking. The membrane was then rinsed in 5 changes of 10 mM Tris, 0.9% NaCl, pH 7.4 over the course of 30 min. A fresh BLOTTO solution containing a 1:2000 dilution of peroxidase-conjugated rabbit anti-sheep Ab (Sigma) was applied to the membrane followed by a 2 h incubation while shaking. The membrane was then rinsed in the 10 mM Tris solution as described above. The peroxidase reaction was developed by incubating the membrane in 100 ml of a 2.9 mM 4-chloro naphthol, 17% ethanol solution.
RESULTS

Functional analysis of the methylcorrinoid:CoM methyltransferase homologues in methylamine metabolism

In order to establish conditions that would permit direct measurements of the function of the methylcorrinoid:CoM methyltransferase homologues in pathways of methyl-CoM formation, extracts were depleted of either one or both of the homologues by incubation with immobilized, affinity purified anti-MtaA or anti-MtbA antibody preparations. Immunosorptive depletion of the methylcorrinoid:CoM methyltransferase from extracts of TMA-grown cells was performed by use of an anti-MtbA isozyme matrix, as described under Materials and Methods. The procedure resulted in removal of 95% (± 2%, n=6) of the methylcorrinoid:CoM methyltransferase specific activity initially present in the extract (Table 1). In contrast, much less methylcorrinoid:CoM methyltransferase activity was removed by incubation with a control matrix containing non-specific IgG which had not bound to the matrix during affinity purification.

Samples of the depleted extract were used, both directly, and after addition of specified amounts of purified MtbA and MtaA, for analysis of methyl-CoM formation from TMA, DMA, and MMA. The reaction mixtures that remained after the assays were completed were frozen and thereafter subjected to SDS PAGE and Western blot analyses. Results of one such analysis are shown in Figure 6. A band on a gel stained for protein was observed that was present in the crude extract [Figure 6, panel A, lane 1] and migrated at the position of purified MtbA [Figure 6, panel A, lane 5]. The
<table>
<thead>
<tr>
<th>Extract Treatment</th>
<th>% CoM methylase activity</th>
<th>Substrate dependent rates of methylCoM formation$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>TMA 405</td>
</tr>
<tr>
<td>non-immune IgG</td>
<td>92</td>
<td>449</td>
</tr>
<tr>
<td>anti-MtbA IgG</td>
<td>4.9</td>
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<td>387</td>
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<tr>
<td>+MtbA</td>
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</tr>
<tr>
<td>anti-MtbA IgG</td>
<td>96.2</td>
<td>282</td>
</tr>
<tr>
<td>+MtaA</td>
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Table 1. Effect of immunodepletion of MtbA on methylamine dependent methylation of CoM in cell extracts of *M. barkeri*. Immune or non-immune IgG coupled to Protein A-Sepharose 4B was used to remove MtbA from each extract. Immune IgG was affinity purified with MtbA bound to a CNBr-activated Sepharose 4B matrix. Non-immune IgG was the fraction of IgG not retained by the affinity column when the anti-MtbA IgG was purified. Immunodepleted extract was then tested before and after supplementation with exogenous MtaA or MtbA to the levels indicated.

$^a$ nmol CoM methylated/min·mg extract.

$^b$ methylcorrinoid:CoM methyltransferase activity.
Figure 6.

SDS-PAGE and Western blot analyses of the specific immunosorption of MtbA from extracts of TMA-grown cells. (A) A 12% polyacrylamide-SDS gel. Lane 1 contains untreated cell extract. Lanes 2 and 4 contain extract samples treated with immobilized anti-MtbA antibodies which were tested for MMA:CoM and TMA:CoM methyl transfer activity. Lane 3 contains treated extract to which purified MtbA was added and tested for MMA:CoM methyl transfer activity. Lane 5 contains purified MtbA, lane 6 contains purified MtaA, and lane 7 contains molecular mass standards. Molecular masses are indicated in kDa. (B) A western blot using anti-MtbA antibodies. (C) A western blot using anti-MtaA antibodies. The lanes for the western blots shown in parts B and C are identical to those in part A with the exception that lane 6 is blank and lane 7 contains purified MtaA.
Figure 6
intensity of this band was significantly diminished after treatment with the anti-MtbA matrix, as shown in Figure 6, panel A, lanes 2 and 4. MtbA was specifically revealed on Western blots developed with antibodies raised against MtbA, as shown in Figure 6 (panel B, lanes 1-5). Anti-MtbA antibodies did not react with MtaA, as shown in Figure 6 (panel B, lane 6), verifying the specificity of the anti-MtbA antibody preparation. Anti-MtaA antibodies showed little cross reaction with MtbA (lanes 5 and 7 in Figure 6, panel C). Moreover, MtbA was almost completely eliminated from the extract after treatment with immobilized antibody, whereas the level of MtaA was unchanged (Figure 6, panels B and C).

Methyl-CoM formation from TMA, DMA, and MMA was assayed in antibody-treated and untreated extracts as described under Materials and Methods. Treatment of the TMA-grown cell extract with anti-MtbA matrix resulted in a large decrease in the specific activity of CoM methylation from all three methylamine substrates, as shown in Table 1. Loss of activity correlated with the observed decrease in methylcorrinoid:CoM methyltransferase specific activity. Methyl-CoM formation from MMA was found to be highly dependent on the presence of MtbA (Figure 7, panel A). Removal of MtbA led to a 97% decrease in the rate of CoM methylation (Table 1). Addition of purified MtbA to the depleted extract resulted in reconstitution of MMA:CoM methyl transfer activity. However, activity was not recovered when purified MtaA was added indicating that requirement for the MtbA isozyme was specific. Similar results were obtained when MtbA depleted extracts were tested for DMA:CoM methyl transfer activity (Table 1). Elimination of MtbA from the extract also resulted in approximately 97%
Figure 7.

MMA-dependent (A), DMA-dependent (B), and TMA-dependent (C) coenzyme M methylation by TMA-grown cell-free extracts treated with immobilized IgG preparations. The assays were performed as described under Materials and Methods with untreated extract (○), extracts treated with non-specific antibodies (□), anti-MtbA antibody matrix treated extracts (■), and anti-MtbA matrix treated extracts supplemented with either 2.3 units of purified MtbA/mg extract protein (●) or 2.1 units of purified MtaA/mg extract protein (▲).
Figure 7
Figure 7 (cont.)

Coenzyme M (mM) vs. Time (min) for the TMA:CoM pathway.

C
loss of DMA-dependent CoM methylation specific activity (Figure 7, panel B). Addition of exogenous, purified MtaA to the extract did not reconstitute the activity. However, addition of pure Mtba resulted in complete restoration of DMA:CoM methyl transfer activity (Table 1).

Whereas both MMA- and DMA-dependent CoM methylation pathways exhibited a specific requirement for Mtba, it was found that both homologues were able to participate in methylation of CoM by TMA (Table 1 and Figure 7, panel C). In contrast to the virtually complete elimination of methyl-CoM formation from MMA and DMA, extracts depleted of Mtba continued to carry out TMA-dependent methylation of CoM at about 20% of the specific activity present in undepleted extracts. As noted also for the other amine substrates, TMA-dependent CoM methylation activity was restored by addition of exogenous Mtba (Table 1 and Figure 7, panel C). However, unlike the DMA-and MMA-dependent reactions, a substantial amount (approx. 61%) of TMA:CoM methyl transfer activity was restored by addition of MtaA.

In control experiments, the same extracts were treated with a matrix containing non-specific IgG, prepared as described under Materials and Methods. Treatment of extracts with similar volumes and amounts of IgG as used in the above experiments resulted in no decrease in the specific activities of the MMA- DMA- or TMA-dependent methylation of CoM (Table 1 and Figure 7).

**Titration of DMA:CoM methyl transfer activity by adjustment of the level of Mtba in extracts**

The effect of varying the amounts of the Mtba isozyme on the rate of
DMA conversion to CH$_3$-CoM was tested in two ways. In one experiment the extract was incubated with decreasing amounts of the immobilized anti-MtbA antibody to produce samples containing different levels of residual methylcorrinoid:CoM activity. In the other experiment, 95% of initial methylcorrinoid:CoM activity was removed, and then increasing amounts of either MtaA or MtbA were added back to the extract. The results of both experiments are shown in Figure 8. A strong correlation was found between the amount of methylcorrinoid:CoM activity remaining in the extract, and the rate of DMA-dependent methylation of CoM. The response curve (Figure 8) indicated that saturation with MtbA occurs at levels somewhat higher than originally present in the extract. The curve extrapolated to zero activity as MtbA approached zero, indicating that dependence on MtbA was absolute. No increase in DMA:CoM methyl transfer activity was produced in extracts depleted of MtbA by addition of MtaA to 250% of the initial methylcorrinoid:CoM specific activity again indicating specificity for MtbA.

**DISCUSSION**

Herein the first direct evidence is given of MtbA involvement in the formation of methyl-CoM from either DMA or TMA. These results also confirm biochemical data indicating that MtbA is involved in the MMA:CoM methyl transfer pathway (Burke and Krzycki, 1995). MtbA is a versatile enzyme since it is capable of participating in the methylation of CoM by all three of the methylamines tested. MtbA appears to be the amine-specific
Figure 8.

Titration of DMA:CoM transmethylation activity by adjustment of the amount of MtbA in cell extracts. The effect of varying amounts of MtbA activity on the DMA:CoM methyl transfer system was tested by adjusting the amount of MtbA activity in two ways. Extracts were incubated with decreasing amounts of immobilized anti-MtbA antibody (□). In a second experiment, MtbA activity was removed to 5% of initial values and MtbA activity was added to the extract in the form of increasing amounts of the purified enzyme (△).
Figure 8
homologue of the methylcorrinoid:CoM methyltransferase. In contrast, MtaA appears able to participate in both the TMA-and methanol-dependent pathways of methanogenesis.

The methylation of CoM by DMA or MMA exhibited specificity for MtbA. MtaA was unable to replace MtbA in either pathway following depletion of MtbA from extracts. The overall rate of CoM methylation by DMA may also be adjusted solely by variation of the amount of MtbA present in the extract, suggesting a possible means for regulation of the pathway. In contrast, the TMA-dependent pathway did not display strict dependence on the MtbA isozyme. Removal of MtbA markedly restricted the TMA:CoM methyl transfer pathway, however a significant percentage of the initial specific activity remained following immunodepletion. Although our measurements of the TMA:CoM methyl transfer pathway would include some activity from the DMA and MMA generated from the demethylation of TMA, it may be concluded that the TMA pathway displays the ability to use either enzyme, since both the DMA and MMA pathways showed a strict dependence on MtbA.

The involvement of a methylcorrinoid:CoM methyltransferase in methyl-CoM formation from MMA, DMA, or TMA implies that one or more corrinoid proteins are also involved in these pathways. As mentioned earlier methylcorrinoid:CoM activity may be measured by use of methylcobalamin as a methyl donor substrate. However, there is little corrinoid in Methanosarcina that is not bound to protein (Pol, 1982). Both methylcorrinoid:CoM methyltransferase homologues have Km values of approximately 13 mM for free methylcobalamin (D. A. Grahame, personal
communication), suggesting that in vivo both of the homologous enzymes would recognize primarily determinants found on the protein rather than on the corrinoid coenzyme. It seems likely that the specificity of protein-protein interactions may be responsible for the specificity shown here for functional activity of the two homologues in pathways of methanol, MMA and DMA conversion.

It seems certain that proteins with corrinoid prosthetic groups are involved in the methylation of CoM by each of the methylamines, possibly in a manner analogous to the pathway of methanol conversion to methane. Perhaps the simplest hypothesis is that, like the methanol-dependent MtaBC corrinoid enzyme, these methylamine MtaBC-type enzymes automethylate the enzyme bound corrinoid by reaction with a specific methylamine substrate. The corrinoid cofactor would then be demethylated by the requisite methylcorrinoid:CoM methyltransferase homologue, catalyzing transfer of the methyl group to CoM. Available data at the time of this study indicated that the corrinoid proteins of the TMA and MMA pathways were separate enzymes (Ferguson and Krzycki, 1997; Burke and Krzycki, 1995). Each pathway of methanogenesis from the different methylamines is separately inducible by its substrate, and cells grown on MMA do not convert TMA to methane at a high rate (Naumann et al., 1984). The difference in the methylcorrinoid:CoM methyltransferase homologue requirement for methyl-CoM formation from TMA versus DMA and MMA also suggests that the TMA pathway must diverge from that of DMA and MMA utilization. Our laboratory isolated a 29 kDa corrinoid protein involved in the MMA:CoM methyl transfer pathway (Burke and Krzycki, 1995). This protein did not
appear to function in the utilization of TMA. Another corrinoid protein has recently been isolated which appears to be involved in the TMA:CoM methyl transfer pathway (Ferguson and Krzycki, 1997). Both corrinoid proteins may serve as MtaBC-type enzymes in the utilization of their respective substrates. At the time of this study it was unknown whether or not the DMA pathway would have a separate corrinoid containing enzyme, since it shares the same methylcorrinoid:CoM methyltransferase homologue with the MMA pathway. Conceivably, both DMA and MMA pathways would involve different corrinoid binding enzymes that contain similar structural determinants recognized by MtbA.

The utilization of the same methylcorrinoid:CoM methyltransferase homologue in both the DMA and MMA pathways would be advantageous to the organism, since it would limit the energetic cost of transcribing and translating two different genes with products that catalyze a similar reaction. However, the ability of the TMA pathway to utilize either homologue is not as easily rationalized. One might speculate that since trimethylamine may be the form in which methylamines are most often encountered in the environment, that cells which had primarily been using methanol as growth substrate might have only to induce the protein possessing TMA-dependent, MtaBC-like activity. This corrinoid protein could then use either methylcorrinoid:CoM methyltransferase homologue, leading to formation of MMA and DMA. These substrates might serve in turn to induce the amine-specific methylcorrinoid:CoM methyltransferase, MtbA.
CHAPTER 3

RECONSTITUTION OF TRIMETHYLAMINE DEPENDENT COENZYME M METHYLATION WITH THE TRIMETHYLAMINE CORRINOID PROTEIN (MttC) AND THE HOMOLOGUES OF THE METHYLCORRINOID:COENZYME M METHYLTRANSFERASES FROM METHANOSARCINA BARKERI

INTRODUCTION

Carbon dioxide is reduced to methane by the vast majority of methanogens in culture. Methanosarcina barkeri is one of the few species which is also capable of methylotrophic methanogenesis from substrates such as methanol, acetate, methylated thiols, and methylamines (Boone et al., 1993). These compounds can be significant methane precursors in freshwater or marine ecosystems (Zinder, 1993).

Methylotrophic substrates destined to become methane are first used to methylate the thiol of coenzyme M (CoM). Methyl-CoM is then reduced to

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methane by methylreductase, a step which is linked to the major site of energy conservation in methanogens (Deppenmeier et al., 1990; Deppenmeier et al., 1999). Methanol, trimethylamine, and acetate are converted to methane with retention of most of the original hydrogen atoms of the methyl group (Lovley et al., 1984; Walther et al., 1981; Zydowsky et al., 1987).

CoM is methylated by acetate through the intermediate methyl-tetrahydrosarcinapterin (methyl-THSP) (Ferry, 1992; Grahame, 1991; Krzycki and Zeikus, 1984; Terlesky et al., 1986). In contrast, methanol is used to directly methylate CoM as demonstrated by Vogels and collaborators (Daas et al., 1996a; Daas et al., 1996b; Keltjens and Vogels, 1993; van der Meijden et al., 1983; van der Meijden et al., 1984) and summarized below:

(1) \[ H^+ + CH_3OH + MtaBC-[Co(I)] \rightarrow MtaBC-[Co-CH_3] + H_2O \]

(2) \[ HSCoM + MtaBC-[Co-CH_3] \rightarrow MtaBC-[Co(I)] + CH_3-S-CoM + H^+ \]

MtaBC is a 122 kDa corrinoid protein with subunits of 51 kDa (MtaB) and 28 kDa (MtaC). The corrinoid prosthetic group is methylated by methanol when in the Co(I) redox state. Methylated MtaBC serves as substrate for CoM methylation by the methylcorrinoid:CoM methyltransferase (MtaA) (Leclerc and Grahame, 1996; Sauer et al., 1997), a 37 kDa zinc binding polypeptide. Hydrogenase, ferredoxin, and a methyltransferase activating protein (MAP) also appear to be required to supply an ATP dependent activation system to reduce the central cobalt ion of the MtaC-bound corrinoid to the Co(I) state (Daas et al., 1996a; Daas et al., 1996b).
The pathways of methanogenesis from trimethylamine (TMA),
dimethylamine (DMA), and monomethylamine (MMA) are still being
elucidated. A homologue of the methylcorrinoid:CoM methyltransferase has
been isolated (Grahame, 1989) and found to participate in CoM methylation
from the methylamines (Burke and Krzycki, 1995; Ferguson et al., 1996;
Chapter 2) This enzyme is known as MtbA. MtbA shares 50% sequence
similarity to MtaA (involved in methanol metabolism) (Harms and Thauer,
1996; Leclerc and Grahame, 1996). Involvement of MtbA in trimethylamine
metabolism was first postulated from the abundance of MtbA in cells grown
on TMA (Yeliseev et al., 1993). Involvement of MtbA in MMA metabolism
was directly demonstrated by the stimulation of the rate of MMA:CoM
methyl transfer by purified MtbA, but not MtaA, in cell extracts (Burke and
Krzycki, 1995). Cell extracts depleted of MtbA by affinity columns can
methylate CoM with DMA or MMA only after addition of purified MtbA, but
not MtaA, to the depleted extract (Ferguson et al., 1996; Chapter 2). The
involvement of MtbA in DMA and MMA metabolism implies the
involvement of corrinoid proteins in these pathways as well. The 29 kDa
monomeric MMA corrinoid protein (MtmC) interacting with MtbA during
methanogenesis from MMA has been purified (Burke and Krzycki, 1995; Cao

While corrinoid proteins mediating CoM methylation from DMA or
MMA have a specific requirement for MtbA, methane formation from TMA
appears to be supported by either methylcorrinoid:CoM methyltransferase
homologue. Immunodepletion of MtbA from extracts did not completely
eliminate TMA:CoM methyltransferase activity, and supplementation of the
immunodepleted extract with either homologue reconstituted activity (Fergusen et al., 1996; Chapter 2). This result also indicated that the methylation of CoM by TMA requires a corrinoid protein. Thus far, corrinoid proteins like MtmC or MtaC have shown a strict requirement for one or the other methylcorrinoid:CoM methyltransferase homologue. However, the ability of the TMA:CoM methyl transfer pathway to utilize two different homologues is consistent with either the involvement of multiple corrinoid proteins in TMA metabolism, or that one corrinoid protein involved in the TMA pathway could interact with either homologue.

These ambiguities are resolved here by the further elucidation of the pathway of TMA:CoM transmethylation. A corrinoid protein was isolated which, when combined with either methylcorrinoid:CoM methyltransferase homologue, mediated the methylation of CoM specifically with TMA.

MATERIALS AND METHODS

Cell cultures and preparation of extracts

*M. barkeri* MS (DSM 800) was cultured in a phosphate-buffered medium supplemented with 80 mM TMA or methanol in 15 L carboys as described in Chapter 2. Cells were lysed anaerobically at 20,000 p.s.i. in a French pressure cell prior to ultracentrifugation at 150,000 x g (Cao and Krzycki, 1991). The supernatant was stored at -70°C in hydrogen flushed stoppered serum vials until use.
Materials

Gases were purchased from Linde Specialty Gases (Columbus, OH) and passed through catalyst R3-11 (Chemical Dynamics Corp., South Plainfield, New Jersey) to remove O_2. Column chromatography media were purchased from Pharmacia LKB Inc. (Pleasant Hill, Calif.). TMA, DMA, MMA, 3-(N-Morpholino) propanesulfonic acid (MOPS), CoM, methyl viologen, BES, ATP, and 5,5’-dithio-bis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co. (St. Louis, MO). Titanium (III) chloride (10% in aqueous solution) was purchased from Aldrich (Milwaukee, WI). Reagents for electrophoresis were purchased from Bio-Rad (Hercules, CA).

Assay of trimethylamine-methyl transfer activity employed during purification

The TMA:CoM methyl transfer activity (MttBC activity) was detected by stimulation of the rate of TMA dependent CoM methylation in cell extracts monitored by the loss of the CoM thiol group with DTNB (Ellman, 1958), essentially as described in Chapter 2 except that the BES concentration was 3.2 mM and 16 mM TMA was used. Other modifications are described in the text of this chapter.

Purification of proteins involved in TMA:CoM methyl transfer

All chromatography steps were carried out in an anaerobic chamber containing 97% N_2 and 3% H_2 (Coy Laboratory Products, Inc., Grass Lake, MI). Oxygen was removed by circulating the chamber atmosphere through palladium catalysts. In a representative purification of the TMA:CoM methyl
transfer activity (see Table 2) 115 ml of cell extract from TMA grown cells was applied to a DEAE-Sephacel column measuring 5 x 15 cm. The column contents were then eluted with a 1.2 L linear gradient of 100 mM to 450 mM NaCl in 50 mM HEPES buffer, pH 8.0, at a rate of 2 ml/min. The peak eluted after 512 ml of the gradient had been applied in a volume of 149 ml. The active fractions were pooled, adjusted to pH 6.5, and loaded onto a Q-Sepharose column measuring 2.5 x 19 cm. The column was then eluted at 1 ml/min with 400 ml of a 200 mM to 600 mM NaCl linear gradient in 50 mM MOPS, pH 6.5. The peak of activity eluted after 193 ml had been applied to the column in 51 ml. The fractions were pooled, concentrated to 7 ml using an Amicon YM-10 membrane, and loaded onto a Sephacryl S-300 column measuring 2.5 x 80 cm. Elution at 1 ml/min was carried out with 75 mM NaCl in 50 mM MOPS buffer, pH 7.0. The peak of activity eluted after 224 ml in a volume 31 ml. The active fractions were collected and loaded onto a Mono-Q 10/10 column (Pharmacia) using a Pharmacia FPLC system kept in the anaerobic chamber, and eluted with a 200 ml gradient of 250 mM to 300 mM NaCl in 50 mM Tris-HCl, pH 8.75, at a rate of 4 ml/min. The peak of activity eluted after 60 ml in a volume of 27 ml. The active fractions were collected and loaded onto a Mono-Q 5/5 column. A 40 ml gradient of 250 mM to 350 mM NaCl in 50 mM Tris-HCl pH 8.75 was run over a volume of 40 ml at a rate of 1 ml/min. The peak of activity eluted after 11 ml in a volume of 7.5 ml.

Purification of MtbA and MtaA was performed using DEAE-Sephacel, hydroxylapatite, Sephacryl S-100, Q-Sepharose, and phenyl-Sepharose (Burke and Krzycki, 1995). The activity was detected using a methylcorrinoid:CoM
methyl transfer assay (Grahame, 1989) described in Chapter 2. The identity of
the homologues was confirmed using an affinity purified MtbA specific
antibody (Ferguson et al., 1996; Chapter 2), which was the kind gift of Dr.
David Grahame.

**Measurement of TMA:CoM methyl transfer with purified proteins**

Reactions were conducted in stoppered 2 ml serum vials under an
atmosphere of H₂. The reaction mixtures contained final concentrations of 2
mM TMA, 2 mM CoM, 0.5 mM methyl viologen, 1.5 mM Ti(III) citrate in 50
mM MOPS buffer, pH 7.0, in a total volume of 125 μl. The amounts of MtbA,
MtaA or MttBC added are indicated in the text. The 93 mM Ti(III) citrate
solution was prepared as described by Seefeldt and Ensign (1994) in which
2.225 ml of 500 mM sodium citrate and 2.25 ml of saturated Tris-NaOH were
added to 0.5 ml of Ti(III) chloride inside an anaerobic chamber. The 93 mM
stock solution had a final pH of 8.8. Methyl viologen added to the reaction
mixture was pre-reduced to the neutral species. Reduction to the blue
monovalent cationic form of methyl viologen was achieved with addition of
a slight molar excess of Ti(III) citrate to methyl viologen, then further reduced
by addition of 2 μl 93 mM Ti(III) citrate to 5 μl of 12.5 mM methyl viologen.
The reactions were initiated by transferring the vials from ice to a shaking
water bath at 37°C. Samples (10 μl) were removed at specific time points and
mixed in wells of a microtiter plate containing 90 μl of 0.5 mM Ellman's
reagent in 150 mM Tris-HCl, pH 8.0. Absorbance at 410 nm was measured by
use of an MR700 enzyme-linked immunosorbent assay plate reader (Dynatech

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Laboratories, Inc.). Calculations of thiol concentration were based on standard curves constructed using known amounts of CoM.

In experiments where methylamine concentrations were followed during the course of the reaction, 12.5 μl samples of reaction mixture were removed and added to 2.5 μl 2 M HCl. The samples were then centrifuged for 5 minutes at 10,000 x g. An aliquot (10 μl) of each supernatant was mixed with 16 μl of 4% KOH. Methylamines were then analysed by gas chromatography using a column operated at 110°C containing 60/80 Carbowax 20M, and 0.8% KOH (Supelco Inc., Bellefonte, PA) with a nitrogen flow rate of 20 ml/min (Burke and Krzycki, 1995). Retention times of methylamines on this system were 2.3 min (DMA), and 2.9 min (TMA).

**Separation of the polypeptides in the MttBC protein**

A Superdex-200 (16/60) size exclusion column (Pharmacia LKB Inc., Pleasant Hill, CA) was equilibrated in 50 mM Tris-HCl, pH 8.0 containing 0.5% SDS. The protein solution was diluted 1:1 in 100 mM Tris-HCl, pH 8.0 containing 2% SDS and 5% β-mercaptoethanol and loaded onto the column in a volume of 1 ml which was eluted with equilibration buffer at 0.5 ml/min. The A₂₈₀ peaks which eluted were pooled and concentrated using Centricon YM-10 membranes for analysis by SDS gel electrophoresis, UV-visible spectra, or corrinoid content.

**Other analytical techniques**

Corrinoid concentrations were determined using the dicyano derivatization method (Grahame, 1991). In this procedure, protein solutions
were mixed with an equal volume of 100 mM 2-(N-
cyclohexylamino)ethanesulfonate, 2% SDS, pH 10. The samples were then
heated to 60°C for 30 min, centrifuged, and spectra were recorded. A second
spectrum was then recorded after the addition of cyanide (20 mM final
concentration) and a 20 min incubation. The observed change in absorbance
at 368 nm was then used to calculate the corrinoid concentration using a
difference extinction coefficient of 1.7 mM⁻¹ cm⁻¹ (Grahame, 1991). Protein
concentrations were determined by the method of Lowry et al. (1954) using
bovine serum albumin as standard. Ultraviolet-visible spectra were obtained
using a Beckman DU-70 spectrophotometer equipped for use with 50 µl
microcuvettes or a Hewlett Packard model 8453 photodiode array
spectrophotometer. SDS gel electrophoresis was performed after Laemmli
(1970) using a Mini-slab electrophoresis system (Idea Scientific Co.,
Minneapolis, MN). Molecular size markers (Pharmacia LKB Inc., Pleasant
Hill, CA) were phosphorylase b (94 kDa), bovine serum albumin (67 kDa),
ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa),
α-lactalbumin (14.4 kDa). Non-denaturing gradient polyacrylamide gel
electrophoresis was performed with the same buffer system, but in the
absence of sodium dodecyl sulfate (SDS) and β-mercaptoethanol.

N-terminal sequencing was performed as described in Kremer et al.
(1993). The samples were prepared by electrophoresis of 25 µg of the MttBC
complex in a 12% acrylamide-SDS gel. The polypeptides were then
electroblotted overnight at 14V onto a PVDF protein sequencing membrane
(0.2 µm pore size) (Bio-Rad Laboratories) in a BioRad Trans-blot cell
containing 20 mM Tris, 150 mM glycine, and 20% methanol (pH 8.0) at 4°C.

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The membrane was then stained with ponceau S and the polypeptides were cut from the membrane and dried. The polypeptides were then submitted to individual quantitative sequence analysis on a model 470A protein sequencer equipped with an on-line 120A phenylthiohydantoin analyzer and a 900A control data analysis module (Applied Biosystems Inc., Foster City, CA).

To determine the native sizes of the proteins in the MttBC fraction, a Sepharose CL-6B column (1.6 x 84 cm) was adapted to a Bio-Rad medium pressure liquid chromatography system (BioLogic) inside a Coy anaerobic chamber and equilibrated with 100 mM NaCl in 50 mM MOPS pH 7.0. An aliquot (460 µg) of MttBC was loaded onto the column in a volume of 250 µl and eluted at a rate of 0.5 ml/min. Molecular mass standards used to generate the standard curve were blue dextran (2,000,000), thyroglobulin (669,000), apoferritin (443,000), β-amylase (200,000), alcohol dehydrogenase (150,000), bovine serum albumin (66,000), carbonic anhydrase (29,000), cytochrome c (12,400), and ferricyanide (324) purchased from Sigma Chemical Co.

RESULTS

Isolation of polypeptides involved in the TMA:CoM methyl transfer pathway

It was anticipated that several proteins might participate in the methylation of CoM with TMA. Therefore, individual components in the multi-component reaction were detected by stimulation of TMA dependent CoM methylation in a cell free extract. This assay was a variation of that used previously to detect the corrinoid protein involved in MMA dependent CoM
methylation (Burke and Krzycki, 1995). An extract with low TMA:CoM methyl transfer activity was obtained by diluting 1:32 (v/v) an extract of TMA grown cells with an extract of cells grown on methanol. The trace amount of TMA grown cell extract provided a low rate of TMA:CoM methyl transfer from 2 to 10 nmol/min/mg total protein. The methanol grown cell extract lacked detectable TMA:CoM methyl transfer activity and maintained a low redox potential via hydrogenases and the hydrogen gas phase. With this assay, a single peak of activity was detected following initial chromatography of a TMA grown cell extract on DEAE-Sephasel under anaerobic conditions. The pooled activity was further purified by low pressure chromatography using a strong cation exchanger and gel permeation, and finally by two passes on Mono-Q columns using medium pressure chromatography (Table 2). The final fraction, termed the TMA methyl transfer activity (MttBC fraction), was used in the subsequent experiments described here. The dependence of the rate of CoM methylation using TMA as the methyl donor on the MttBC fraction is illustrated in Figure 9. In this particular experiment, a seven fold stimulation over the background rate of TMA dependent CoM methylation was observed with the highest amount of Mono-Q2 fraction tested.

Composition of the MttBC fraction

SDS polyacrylamide gel electrophoresis the MttBC fraction revealed two polypeptides with apparent molecular masses of 52 and 26 kDa (Figure 10A). Densiometric analysis indicated that these polypeptides were present at a molar ratio of 3.6:1, respectively. Two independent purifications of MttBC
Table 2. Purification of the MttBC activity from an extract of TMA grown cells. Activity is expressed as μmol CoM methylated/min·mg protein of the purification fraction that was dependent on the addition of TMA to the assay. Details of this purification are presented in Materials and Methods.
Figure 9.

Stimulation by the isolated MttBC fraction of TMA:CoM methyl transfer in cell extract. Increasing amounts of the MttBC fraction from the final Mono-Q purification step were assayed as described in Materials and Methods. The reaction was supplemented with the indicated amounts of the MttBC protein in 25 μl MOPS buffer, pH 7.0. The activity corresponds to the TMA dependent loss of the free thiol of coenzyme M, and is not corrected for the background activity observed in the extract.
Figure 10.
SDS gel electrophoresis of the protein components of the reconstituted TMA:CoM methyl transfer reaction. (A) Coomassie stained 12% acrylamide gel of the MttBC fraction from the final Mono-Q purification step. Lanes were loaded with the following samples: 1, 33 μg of protein from a methanol grown cell extract; 2, 32 μg of protein from a TMA grown cell extract; and 3, 5 μg protein of the TMA:CoM methyl transfer activity. The relative positions of standard proteins with the indicated masses in kilodaltons are indicated on the right. (B) Coomassie stained 12% acrylamide gel of MtaA and MtbA preparations used in these experiments. The lanes were loaded with: 1, molecular mass standards; 2, MtbA (3 μg); and, 3, MtaA (3 μg).
Figure 10
were performed, and both resulted in a MttBC fraction containing only these two polypeptides. The MttBC polypeptides co-migrated with prominent bands observed following electrophoresis of cell extracts of TMA grown cells. Cell extracts of methanol grown cells, which do not convert TMA to methane, did not appear to have prominent polypeptides of these molecular weights.

The temperature at which MttBC protein was heated for 5 min in sample buffer prior to SDS PAGE was found to influence the electrophoretic migration of the smaller polypeptide. Samples heated at or higher than 80°C yielded only the form of the polypeptide with an apparent molecular mass of 26 kDa. However, when samples were heated at 40°C, very little 26 kDa band was observed, and primarily a form with an apparent molecular weight of 21 kDa was observed. When MttBC samples were heated at temperatures between 40 and 60°C, a mixture of both the 26 and 21 kDa bands were observed (Figure 11).

Non-denaturing gel electrophoresis of the MttBC fraction under anaerobic conditions yielded a fast migrating band and a slow migrating band. Two dimensional gels using non-denaturing gel electrophoresis in the first dimension followed by an SDS gel in the second dimension revealed that the slower migrating band was composed of both 52 and 26 kDa polypeptides. The faster migrating band consisted only of the 26 kDa polypeptide. Approximately 30% of the 26 kDa polypeptide migrated with the 52 kDa polypeptide during non-denaturing PAGE.

The association of the 26 kDa polypeptide and the 52 kDa polypeptide was further demonstrated by chromatography of the MttBC fraction on a
Figure 11.

Different migration of MttC in SDS-PAGE upon incubation at different temperatures prior to electrophoresis. The lanes labeled 40-100 each contain 4 µg of partially purified MttC which were heated to the temperatures indicated (in °C) for 5 min in SDS sample buffer prior to loading into the gel. The molecular mass standards were loaded into the lane labeled stds and their respective masses are indicated to the left.
Figure 11
Sepharose 6B column under anaerobic conditions. Two well separated and symmetrical protein peaks eluted from the column with apparent molecular masses of 280 kDa and 33 kDa. The larger peak consisted primarily of the 52 kDa polypeptide, while the second peak consisted only of the 26 kDa polypeptide. However, some 26 kDa polypeptide also eluted with the 280 kDa peak. The molar ratio of 52 kDa to 26 kDa polypeptide in the 280 kDa peak was 20:1. This indicates that 20% of the 26 kDa polypeptide remained associated with the 52 kDa polypeptide during gel permeation, the remainder eluted as a monomeric protein.

**Reconstitution of the TMA:CoM methyl transfer pathway with purified proteins**

The MttBC fraction was combined with another known participant in this reaction, MtbA (Figures 10A and 10B illustrate the purity of these preparations). In the presence of Ti(III) citrate and methyl viologen, these three proteins were found to mediate the TMA:CoM methyl transfer reaction (Figure 12). Three separate preparations of the MttBC fraction were made whose specific activities varied from 600 nmol/min/mg MttBC protein to 150 nmol/min/mg MttBC protein when 40 μg MttBC fraction was assayed with 40 μg MtbA. Controls in which either MtbA or the MttBC fraction was deleted from the reaction mixture did not methylate CoM with TMA. The reaction was strictly dependent on TMA (see Figure 14). TMA concentrations higher than 2 mM did not increase the observed reaction rate (Figure 13). DMA and MMA did not serve as substrates for CoM methylation by the MttBC proteins and MtbA (Figure 14).
Figure 12.

Minimal requirements for TMA:CoM methyl transfer in vitro include the purified MttBC fraction, purified MtbA, Ti(III) citrate, and methyl viologen. The reactions initially contained 2 mM CoM and TMA, 1.5 mM Ti(III) citrate and 0.5 mM methyl viologen unless otherwise indicated, and 40 µg of MtbA and 40 µg of MttBC unless otherwise indicated. Time courses are presented for the complete reaction (o), as well as the complete reaction minus MttBC (□), minus MtbA (△), minus Ti(III) citrate (■), or minus methyl viologen (▲).
Figure 12
Figure 13.

TMA:CoM methyl transfer activity of the MttBC fraction at increasing concentrations of TMA. The effect of substrate concentration on TMA:CoM methyl transfer activity using the purified MttBC complex. The reactions contained 2 mM CoM, 1.5 mM Ti(III) citrate, 0.5 mM methyl viologen, 40 μg of MtbA and 40 μg of MttBC in a total volume of 125 μl. The concentration of the TMA was varied from 0 to 3 mM.
Figure 13
Figure 14.
Substrate specificity of the MttBC complex. The purified MttBC complex and MtbA were tested for their ability to transfer methyl groups from TMA, DMA, and MMA to CoM. The reactions contained 2 mM CoM, 1.5 mM Ti(III) citrate, 0.5 mM methyl viologen, 40 µg of MtbA, 40 µg of MttBC, and 2 mM substrate as indicated in a total volume of 125 µl.
Figure 14
TMA dependent CoM methylation was strictly dependent on addition of Ti(III) citrate and methyl viologen to the assay mixture. Either agent alone was insufficient (Figure 12). Best results were obtained when Ti(III) citrate was present at 3 fold excess over methyl viologen and when these agents were incubated together prior to the addition of protein. Methyl viologen was immediately reduced to the blue monovalent cation, but was then further reduced by the Ti(III) citrate to the colorless neutral species as evidenced by the loss of the methyl viologen absorbance peak at 605 nm. Ti(III) citrate preparations which were not freshly prepared were not capable of reducing methyl viologen to the neutral species, and were inactive in promoting the TMA:CoM methyl transfer reaction. MttBC that was exposed to oxygen for 6 h at 4°C, then made anaerobic by flush and evacuation, could still be activated by Ti(III) citrate and methyl viologen with little (4%) loss of original activity.

**Substrates and products of the TMA:CoM methyl transfer reaction**

The methylation of CoM by the MttBC polypeptides and MtbA was typically monitored by the loss of the free thiol of CoM using Ellman's reagent (Ellman, 1958). Since this indirectly measures the demethylation of TMA and the subsequent methylation of CoM, the reaction rate was also monitored by consumption of TMA and production of DMA (Figure 15). The rate of TMA utilization was very similar to that of consumption of the free thiol of CoM (350 nmol/min/mg protein MttBC fraction). DMA was produced during the entire course of the reaction at a rate very near to that of TMA and CoM disappearance. After 20 minutes, 520 nmol of TMA and 470 nmol of CoM had been consumed, and 520 nmol of DMA had been produced.
Figure 15.

Substrate and product stoichiometry of the TMA:CoM methyl transfer reaction mediated by the MttBC fraction and MtbA. The reaction initially contained 2 mM CoM and TMA, 1.5 mM Ti(III) citrate, 0.5 mM methyl viologen, 80 μg of MtbA and 80 μg of MttBC. Samples (12.5 μl) were removed from the 250 μl reaction mixture at the indicated timepoints for measurement of the free thiol of CoM and methylamines.
No MMA was detected during the course of the reaction. These results indicate that the demethylation of 1 mol of TMA yields an equimolar amount of both DMA and methyl-CoM. The production of DMA was dependent on the addition of CoM to the reaction mixture.

The 26 kDa polypeptide is the trimethylamine specific corrinoid protein

The requirement for a methylcorrinoid:CoM methyltransferase such as MtbA in the reconstituted TMA:CoM methyl transfer reaction indicated that one or both of the polypeptides in the MttBC fraction was a corrinoid protein. The anaerobically purified MttBC fraction had an orangish-red color, also indicating a corrinoid cofactor was present. This was confirmed by the UV-visible spectrum of the protein (Figure 16B). The "as-isolated" scan of the Mono-Q2 fraction had absorbance peaks characteristic of protein bound corrinoid. The maxima at 480 nm and 532 nm are indicative that the corrinoid was present in a mixture of Co(II) and Co(III) redox states. The corrinoid cofactor was extracted and quantified by the dicyano method (Grahame, 1991). The molar ratio was determined to be 0.92 nmol corrinoid:1 nmol 26 kDa polypeptide:4 nmol 52 kDa polypeptide.

This ratio indicated that the 26 kDa polypeptide might bind the corrinoid cofactor present in the MttBC fraction. The two polypeptides form a loosely associated complex, however, and were not completely separated by gel permeation chromatography except in the presence of a denaturant. Chromatography of the MttBC fraction was performed using a medium pressure gel permeation column eluted with buffer containing 0.5% SDS under aerobic conditions. The two polypeptides were cleanly separated and
Figure 16.

The 26 kDa polypeptide binds the corrinoid cofactor present in the MttBC fraction. The two polypeptides of the MttBC fraction were separated on a gel permeation column eluted with a buffer containing SDS. (A) SDS 10% polyacrylamide gel of MttBC before and after gel permeation in presence of SDS. The lanes were loaded with: 1, molecular size standards, masses in kDa are indicated on the left; 2, MttBC fraction (3.5 μg); 3, 26 kDa polypeptide (2 μg); and 4, 52 kDa polypeptide (2 μg). (B) UV-Visible spectra of MttBC (2.3 mg/ml) fraction in 50 mM MOPS, pH 7.0. The anaerobic sample was removed, and scanned immediately in an aerobic cuvette. Inset is the same sample on an expanded scale. (C) UV-Visible spectrum of the 52 kDa polypeptide of the MttBC fraction (0.27 mg/ml). The spectrum was taken under aerobic conditions in 50 mM Tris HCl, pH 8.0 and 0.5% SDS. (D) UV-visible spectrum of the 26 kDa polypeptide (0.49 mg/ml) in 0.5% SDS in 50 mM Tris HCl, pH 8.0.
Figure 16 (cont.)

B

MttBC

Absorbance

0.15

0.25

0.35

250 350 450 550 650

Wavelength (nm)

C

52 kDa

Absorbance

30

20

10

250 350 450 550 650

Wavelength (nm)

D

26 kDa

Absorbance

0.6

0.4

0.2

250 350 450 550 650

Wavelength (nm)
eluted as symmetrical peaks at 52.9 ml and 70.5 ml. The latter peak was found to contain entirely the 26 kDa polypeptide, while the former peak contained entirely the 52 kDa polypeptide (Figure 16A). Each peak was pooled, concentrated, and the UV-Vis spectra collected. The 52 kDa polypeptide had a featureless spectrum except for the peak of major absorbance centered at 276 nm (Figure 16C). In contrast, the 26 kDa polypeptide clearly retained the corrinoid cofactor with prominent peaks at 351, 532, and a shoulder at 570 nm which are characteristic of protein bound Co(III) corrinoid (Figure 16D). The amount of corrinoid was quantitated by extraction and conversion to the dicyano form and 1.1 nmol of corrinoid was found per nmol of 26 kDa polypeptide. Similar treatment of the 52 kDa polypeptide yielded no detectable corrinoid.

The identity of the 26 kDa polypeptide as the TMA specific corrinoid protein was further confirmed by Edman degradation sequencing of the N-termini of the MttBC polypeptides. The N-terminal sequence of the 52 kDa polypeptide was determined as MAKNNAVAGFNLGVELNL. The N-terminus of the 26 kDa polypeptide was determined as MANKEEIAKAKAITEITDFDD. The N-terminus of the 52 kDa MttB polypeptide showed no significant homology to any known proteins. However, the N-terminus of the 26 kDa MttC polypeptide showed significant homology (30% identity) to MtmC, the MMA specific corrinoid protein (Burke et al., 1998; Paul et al., 2000).
Relative efficacy of methylcorrinoid:CoM methyltransferase homologues in TMA dependent CoM methylation

The MttBC polypeptides were found to be capable of coupling TMA demethylation to CoM methylation with either purified methylcorrinoid:CoM methyltransferase homologue (Figure 17). The concentration of each homologue was varied while the amount of the MttBC fraction was held constant at 35 µg of protein (approximately 0.15 nmol 26 kDa polypeptide). The concentration of both homologues was increased until they were no longer the rate limiting component in the reaction. At saturation, MtbA supported a maximal rate of 170 nmol/min/mg protein of MttBC fraction. MtaA supported a rate of 88 nmol/min/mg protein. The reaction displayed a marked preference for MtbA. The amount of MtbA required to reach half maximal rate was 0.35 nmol, or a molar ratio of MtbA to the corrinoid protein of 2.3. The amount of MtaA required to reach saturation was 1.5 nmol, corresponding to a molar ratio of MtaA to the corrinoid protein of 10.

DISCUSSION

The involvement of methylcorrinoid:CoM methyltransferases in TMA conversion to CoM (Ferguson et al., 1996; Chapter 2) entailed the participation of a corrinoid binding protein in the pathway, since little free corrinoid is present in M. barkeri (Dangel et al., 1987). The isolation of that corrinoid protein is described here for the first time. The isolated MttBC fraction
Figure 17.

Relative activity of different methylcorrinoid:CoM methyltransferases with MttBC in the transfer of methyl groups from TMA to CoM. The amount of MttBC was held constant at 35 μg protein, while the amount of either MtbA (○) or MiaA (□) was varied over the indicated range. The molecular mass of both isozymes was taken as 37 kDa, and the total assay volume was 125 μl.
Figure 17
consists of a 26 kDa corrinoid binding polypeptide weakly associated with a 52 kDa polypeptide. The 26 kDa polypeptide is therefore designated MttC and the 52 kDa polypeptide is designated MttB, consistent with the nomenclature in the field. The MttBC fraction containing these two polypeptides, along with either methylcorrinoid:CoM methyltransferase homologue, could mediate CoM methylation by TMA.

With the isolation of MttC and MtmC (Burke and Krzycki, 1995), two different corrinoid proteins involved in the methylamine pathways have been purified. The N-termini of the polypeptides in the MttBC fraction have been obtained, and they are both different from the N-terminus of MtmC. MttC does not stimulate MMA dependent CoM methylation in cell free extracts, nor does the reconstituted TMA:CoM methyl transfer activity utilize MMA or DMA. MtmC specifically stimulates MMA, but not TMA, dependent CoM methylation in cell extracts. The participation of MtbA in the methylation of CoM from TMA, DMA, and MMA had raised the possibility that a single corrinoid protein might exist which participated in all three pathways. The activities of MtmC and MttC show that at least two methylamine pathways are mediated by separate corrinoid proteins. The independent corrinoid protein involved in the DMA:CoM methyl transfer pathway will be described in the next chapter.

The involvement of a corrinoid protein in TMA:CoM methyl transfer indicates a mechanism similar to methanol:CoM methyl transfer, that is, MttC carries the corrinoid cofactor which is methylated by TMA and demethylated by MtbA or MtaA for the subsequent methylation of CoM. However, the role of MttB in the TMA:CoM methyl transfer reaction is not as
clear. We did not observe activity without MttB in the MttBC fraction. MttB
eluted as a 280 kDa protein and MttC as a monomer, but some MttC was
always present in low amounts with MttB. The co-purification of the two
polypeptides, and their weak association, indicates that MttB may also be
involved in the TMA pathway. The direct involvement of both polypeptides
in TMA dependent CoM methylation would be consistent with the dimeric
nature of MtaBC, which interacts with MtaA during the methanol dependent
methylation of CoM (van der Meijden et al., 1983). A simple hypothesis is
that MttB acts to methylate MttC with TMA prior to CoM methylation by
MtbA or MtaA. This is supported by the fact that the genes encoding MttB
and MttC are located immediately adjacent to one another in the genome and
are cotranscribed in the mtt/mtb operon (Paul et al., 2000). The mtt/mtb
operon was cloned using primers designed from the N-termini of the MttB
and MttC proteins described in this chapter.

In cell free extract, the TMA:CoM methyl transfer reaction requires the
presence of hydrogen and ATP for reductive activation of the transmethylases
mediating the reaction (Naumann et al., 1984). The reconstituted TMA:CoM
methylation required Ti(III) citrate and methyl viologen. This low potential
reducing system replaced the cellular ATP dependent reductive activation
system and allowed the reaction to be mediated by MttBC and MtbA or MtaA.
Corrinoid dependent methyltransferases are active in the extremely
nucleophilic Co(I) state, which is converted to the methylated Co(III) state
upon interaction with a methyl donor (Banerjee et al., 1990a; Banerjee et al.,
1990b). Ti(III) citrate can result in the direct reduction of even free cobalamin
to Co(I) (Gärtnert et al., 1994). However, Ti(III) citrate was unable to activate
the MttBC fraction without methyl viologen as a mediator. Methyl viologen was further inactive unless it was reduced completely to the colorless neutral species. The requirement for the neutral species of methyl viologen indicates that a polar agent may be excluded from the site of reduction on MttC.

This is the first demonstration of CoM methylation by a methylamine in a purified system. However, the rate at which MttBC and MtbA catalyze CoM methylation was lower than expected, since cell extracts mediate the reaction at 400 nmol/min/mg protein (Chapter 2). Assuming that the MttBC polypeptides constitute 5% of soluble protein in the cell (which is a typical value for the major catabolic enzymes in methanogenesis), only 2 to 5% of the expected activity was achieved using the resolved system. Isolated enzymes of methanogenesis often have lower than expected specific activities. For example, specific activities of 800 nmol/min/mg MtaBC protein were reported for methanol:CoM methyl transfer (van der Meijsen et al., 1983). The MttBC fraction also has higher specific activity when assayed in the presence of cell extract (see Table 2). This may reflect the presence of components in the cell extract which stimulate activity of the MttBC proteins, such as the cellular reductive activation system. In addition, MtbA must associate with MttC during TMA dependent CoM methylation, and higher concentrations of protein might increase the association of the proteins significantly.

It was initially surprising to find that either MtbA or MtaA supported TMA:CoM methyl transfer activity in the MtbA depleted cell extract, since MtaBC and MtmC show a marked ability to use only one of the homologues. Our current results show that these results can be explained by the ability of
one corrinoid protein, MttC, to interact with either MtbA or MtaA. MttC must possess determinants which allow it to interact with either homologue. This flexibility could fulfill a useful physiological function. Cells growing primarily on methanol contain little MtbA and primarily MtaA. If MttBC can function with either methylcorrinoid:CoM methyltransferase, induction of only these components of the pathway could assist the transition from growth primarily on methanol to growth on TMA.

Although either methylcorrinoid:CoM methyltransferase can interact with MttC, it is clear that, on a molar basis, the affinity of the corrinoid protein is greater for MtbA than for MtaA. The strict requirement of the MMA and DMA pathways of CoM methylation for MtbA, as well the higher affinity of the TMA pathway for MtbA, further emphasizes that this enzyme can be considered the amine specific methylcorrinoid:CoM methyltransferase.
CHAPTER 4

RECONSTITUTION OF DIMETHYLAMINE:COENZYME M METHYL TRANSFER WITH A DISCRETE CORRINOID PROTEIN AND TWO METHYLTRANSFERASES PURIFIED FROM METHANOSARCINA BARKERI

INTRODUCTION

* Methanosarcina barkeri* is a methanogenic archaeon capable of methanogenesis from a wide range of substrates. Aside from H₂:CO₂ and acetate, the majority are methylotrophic substrates such as methylated thiols, methanol and methylamines. Methanogenesis from a methylotrophic substrate requires methyl group abstraction and subsequent methylation of the thiol of 2-mercaptoethanesulfonic acid (Coenzyme M or CoM). Methyl-CoM is then converted to methane with reducing equivalents obtained from the concomitant oxidation of the methylotrophic substrate to carbon dioxide (Ferry, 1999; Thauer, 1998).
The methylamines (TMA, DMA, and MMA) are important methylo trophic substrates in marine environments where they arise from the anaerobic breakdown of cholines, betaine, and TMAO (King, 1984). During the breakdown of trimethylamine (TMA) methyl groups are sequentially removed from the substrate with the intermediate production of dimethylamine (DMA) and monomethylamine (MMA) which are subsequently consumed for the production of methane. DMA or MMA can also serve as sole carbon and energy sources (Naumann et al., 1984; Hippe et al., 1979).

The actual methylation of CoM with each methylamine is catalyzed by the same polypeptide. This is a 37-kDa methylcorrinoid:CoM methyltransferase termed MtbA that was originally identified in cells grown on acetate (Grahame, 1989), but found in highest abundance in cells grown on TMA (Yeliseev et al., 1993). MtbA was shown to be involved in the MMA:CoM methyl transfer reaction due to its stimulation of this activity in cell extracts (Burke and Krzycki, 1995). Removal of MtbA with affinity columns from cell extracts greatly diminished CoM methylation with TMA, DMA, or MMA, but each activity could be fully restored by supplementing the MtbA depleted extract with purified MtbA (Ferguson et al., 1996; Chapter 2). MtbA does not bind a corrinoid prosthetic group, and since little corrinoid exists in M. barkeri that is not protein bound (Dangel et al., 1987), these results indicated that corrinoid binding proteins must be involved in CoM methylation from each methylamine.

This prediction was confirmed with the reconstitution of the MMA dependent CoM methylation reaction (Burke and Krzycki, 1997) with highly
purified proteins (Figure 18, panel A; see also Figure 33). A 50-kDa polypeptide, MtmB, which lacks a detectable prosthetic group, was required for the methylation with MMA of MtmC, a 29 kDa corrinoid binding protein. The two independently isolated proteins can form a complex, and are most active in CoM methylation at a MtmB:MtmC ratio of 2. Methyl-MtmC serves as substrate for CoM methylation as catalyzed by MtbA. These three protein components are sufficient for MMA:CoM methyl transfer in vitro in the presence of Ti(III) citrate and methyl viologen. The latter two components are required for reductive activation of MtmC to the active Co(I) form, since MtmC is isolated in a mixture of the Co(II) or Co(III) redox states. Use of this reductant and redox mediator circumvent a requirement for a physiological ATP dependent reductive activation system that is not yet completely resolved (Daas et al., 1996a; Daas et al., 1996b; Wassenaar et al., 1996), allowing in vitro MMA:CoM methyl transfer with the aforementioned three polypeptides.

TMA:CoM methylation has also been resolved to three purified polypeptides (Ferguson and Krzycki, 1997; Chapter 3). In this case, the 52-kDa MttB and the 26-kDa MttC polypeptides are required to catalyze TMA specific CoM methylation in the presence of MtbA. MttB and MttC do not separate during purification. Dissociation of MttB and MttC using gel permeation in the presence of SDS demonstrated that each moi of MttC bound 1 mol corrinoid, while no corrinoid was bound by MttB.

Methanol dependent methylation of CoM also requires a minimum of three polypeptides. Methanol was the first methylotrophic substrate of *M. barkeri* for which the pathway of coenzyme M (CoM) methylation was
Figure 18.

Proposed reaction schemes for in vitro CoM methylation from two different methylamines. (A) CoM methylation from MMA requires a small corrinoid protein, MtmC, and two methyltransferases (MtmB and MtbA). MtmB binds no corrinoid prosthetic group, but is required for the methylation of the corrinoid prosthetic group of MtmC. MtmC is active in the Co(I) state and inactive in the Co(II) state. Activity is maintained by the presence of Ti(III) citrate and methyl viologen in the assay. Methyl-MtmC is then used by MtbA to methylate CoM. (B) DMA dependent CoM methylation as proposed by Wassenaar et al. (1998b). A dimeric methyltransferase, DMAMT, binds one corrinoid group/dimer and automethylates the prosthetic group which is then demethylated by MtbA to methylate CoM. The corrinoid bound to DMAMT is kept in the active Co(I) state by components of a cellular ATP dependent reductive activation system added to the system as purified MAP as well as hydrogenase added as a primary DEAE fraction of cell extract. References supporting both models are cited in the text.
Figure 18
elucidated. MtaBC is a corrinoid containing enzyme composed of two tightly bound polypeptides, MtaB and MtaC (van der Meijden et al., 1984; Sauer et al., 1997). The 51-kDa MtaB subunit uses methanol to methylate the corrinoid prosthetic group of the 28-kDa MtaC subunit, as shown by the capability of recombinant MtaB to methylate free cob(I)alamin (Sauer and Thauer, 1999). Methyl-MtaC is then demethylated by MtaA, a methylcorrinoid:CoM methyltransferase (Sauer et al., 1997; van der Meijden et al., 1983) which is homologous to MtbA (Leclerc and Grahame, 1996; Harms and Thauer, 1996), to generate methyl-CoM. Reductive activation of MtaC can be accomplished with either Ti(III) citrate (Sauer et al., 1997) or the partially resolved ATP dependent corrinoid activation system (Daas et al., 1996). MtbC does not function in the methylation of CoM with MMA or DMA, but will function to a limited extent in the TMA dependent CoM methylation pathway (Ferguson and Krzycki, 1997; Chapter 3).

Thus, methanol and TMA dependent CoM methylation systems are analogous to the MMA:CoM methylation scheme shown in Figure 18, panel A. Each system has in common a requirement for a small corrinoid binding polypeptide, yet these are distinct corrinoid binding proteins specific for each pathway. As demonstrated for the methanol and MMA systems, methylation of the corrinoid protein with a methylotrophic substrate requires a separate protein such as MtbM or MtaB. The methylated corrinoid is then demethylated by a methylcorrinoid:CoM methyltransferase such as MtaA or MtbA.

The DMA:CoM methyl transfer pathway had not previously been reconstituted with highly purified proteins. However, in addition to the CoM
methylase MtbA, another protein was recently implicated in DMA:CoM methyl transfer by Wassenaar et al. (1998a; 1998b) and designated DMAMT (see Figure 18, panel B). Purified preparations of homodimeric DMAMT contain 0.45 mol corrinoid/mol 50-kDa DMAMT polypeptide. DMAMT and MtbA alone are insufficient for DMA:CoM methyl transfer and required addition of MAP, a protein involved in ATP dependent reductive activation of the corrinoid, as well as a fraction from a primary DEAE separation of cell extract that contained hydrogenase activity (Wassenaar et al., 1998b). A mechanism was proposed in which DMAMT methylated with DMA its own bound corrinoid prosthetic group, which was then demethylated by MtbA and CoM (Figure 18, panel B). This scheme stands in contrast to the resolved pathway for MMA:CoM methyl transfer (Figure 18, panel A) and the analogous pathways for TMA and methanol dependent CoMmethylation in that a small discrete corrinoid binding protein is not required since DMAMT automethylates its own corrinoid prosthetic group.

In this chapter, we describe the isolation of a small corrinoid protein, MtbC, from M. barkeri which stimulated DMA:CoM methyl transfer when added to cell extract. MtbC was used in an assay to isolate MtbB1, a DMA:corrinoid methyltransferase that does not have a corrinoid prosthetic group. Highly purified MtbB1, MtbC, and MtbA are sufficient to catalyze in vitro CoM methylation by DMA in a manner most analogous to the resolved MMA:CoM methylation reaction.
MATERIALS AND METHODS

Cell cultures and preparation of extracts

*M. barkeri* MS was cultured on 80 mM TMA (Burke and Krzycki, 1995; Krzycki, 1989) as described in Chapter 2. Cell extracts were also prepared as described previously in Chapter 2 (Cao and Krzycki, 1991), except where indicated below for MtbC purification. Briefly, cell suspensions were lysed anaerobically at 20,000 p.s.i. with a French pressure cell prior to ultracentrifugation at 150,000 x g. The supernatant was stored at -70°C in hydrogen flushed stoppered serum vials until use.

Materials

Gases were purchased from Linde Specialty Gases (Columbus, OH) and passed through catalyst R3-11 (Chemical Dynamics Corp., South Plainfield, New Jersey) to remove O₂. Column chromatography media were manufactured by Pharmacia Biotech (Uppsala, Sweden) unless otherwise indicated. 3-(N-Morpholino) propanesulfonic acid (MOPS), CoM, TMA, DMA, MMA, methyl viologen, hydroxocobalamin, methylcobalamin, ATP, and 5, 5'-dithio(2-nitrobenzoic acid), or DTNB, were purchased from Sigma Chemical Co. (St. Louis, MO). Titanium (III) chloride (10% in aqueous solution) was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). Reagents for electrophoresis were purchased from Bio-Rad Laboratories (Hercules, CA)
Purification of the corrinoid protein involved in DMA-CoM methyl transfer (Performed by Dr. Natalia Goriatova and Dr. David Grahame)

The 24-kDa corrinoid protein MtbC was purified from cultures in exponential growth phase. The purification was performed under aerobic conditions at room temperature. Cell extract was prepared by French press disruption of 60 g of cell paste resuspended in 150 ml of 0.1 M potassium phosphate buffer, pH 7.5, containing 10% glycerol, 4 mM EDTA, 0.02% sodium azide and 23 μg/ml DNase I, and centrifuged to remove insoluble components. MtbA was assayed by the methylcobalamin:CoM reaction described previously in Chapter 2 (Grahame, 1989). MtbC itself was followed by SDS-PAGE analyses of the fractions, and in later steps also by absorbance at 360 nm and 546 nm (absorption maxima for the bound corrinoid cofactor). The corrinoid protein co-purified with MtbA through the first two columns employed for purification. Cell extract was first applied at 5 ml/min to a 200 ml column of Q-Sepharose (5.0 cm diameter x 10.0 cm) that had been equilibrated with a solution of 0.10 M KCl in Buffer A (20 mM potassium phosphate, 1 mM EDTA and 0.02% sodium azide, pH 7.5). The column was then washed with two bed volumes of Buffer A, and elution then carried out with a linear gradient of 0.10 M to 0.25 M KCl in Buffer A. The bulk of both proteins, MtbA and the 24-kDa MtbC, was recovered in the fractions eluting between 0.10 M and 0.15 M KCl. A separate 25-kDa corrinoid protein eluted in earlier fractions and was largely removed at this step. The pooled fraction containing MtbA and MtbC were then concentrated by ultrafiltration in a stirred cell using a YM-30 membrane (Amicon, Inc., Beverly, MA) and then diluted to approximately 0.07 M KCl. The protein solution was then applied
to a 140 ml (1.5 x 20 cm) column of Q-Sepharose equilibrated and then eluted as described above. The resulting fraction of MtbA/MtbC was then concentrated and adjusted to 1.58 M (NH$_4$)$_2$SO$_4$ in a final volume of 3 ml. The fraction was then applied at 0.5 ml/min to a Pharmacia HR 16/10 phenyl-Superose column (Pharmacia Biotech, Uppsal, Sweden) equilibrated with 1.58 M (NH$_4$)$_2$SO$_4$ in Buffer A. Elution was carried out with a 30 ml gradient decreasing linearly in (NH$_4$)$_2$SO$_4$ from 1.58 M to 0 M and simultaneously increasing in 1,3-propanediol from 0% to a final concentration of 10%.

Continued application of 10% 1,3-propanediol in Buffer A resulted in the elution of two protein peaks, the first contained MtbA and the second consisted primarily of MtbC. The pooled MtbC fraction was dialysed against Buffer A that was adjusted to pH 6.5 then concentrated to 2 ml and applied to a Pharmacia Mono Q HR 16/10 column equilibrated with the same buffer. During application of a linear gradient of 0 M to 0.25 M KCl in Buffer A at pH 6.5, a peak of residual MtbA emerged, which was followed by a peak of MtbC at 150 mM KCl. The now homogeneous MtbC preparation was dialyzed against 20 mM HEPES, pH 7.5, concentrated by a Centricon 30 unit (Amicon), and stored as aliquots frozen in liquid nitrogen. The N-terminal amino acid sequence was determined by automated Edman degradation.

**Purification of the dimethylamine:corrinoid methyltransferase**

Purification of MtbB1 was performed in an anaerobic chamber containing 97% N$_2$ and 3% H$_2$ (Coy Laboratories, Grass Lake, MI) with buffers and column materials made anaerobic by repeated cycles of evacuation and flushing with N$_2$. Protein purification was initiated by applying 35 ml of cell-
free extract to a 25 x 2.5 cm DEAE-Sepharose (Sigma Chemical Co., St. Louis, MO) equilibrated in 50 mM Tris pH 8.0. A 500 ml gradient of 100 mM to 500 mM NaCl in 50 mM Tris-HCl, pH 8.0, was applied to the column at 2.2 ml/min. MtbB1 activity was assayed as described below and eluted in a volume of 85 ml after approximately 400 ml of the gradient had been applied. The MtbB1 active fraction was adjusted to pH 6.5 with 75 mM MOPS, pH 6.5, and 50 ml was loaded onto a Pharmacia Mono-Q HR 10/10 column. A 120 ml gradient of 50 mM to 500 mM NaCl in 50 mM MOPS, pH 6.5 was applied to the column at 2 ml/min. The remaining 35 ml of DEAE-Sepharose fraction was then subjected to the Mono-Q step. The MtbB1 activity from both Mono-Q runs eluted at approximately 320 mM NaCl in a total volume of 12 ml. The pooled active fractions were then loaded onto two UNO Q1 columns connected in series (BioRad, Hercules, CA) equilibrated with 50 mM Tris, pH 8.0. A 100 ml gradient of 150 mM to 350 mM NaCl in 50 mM Tris, pH 8.0, was then applied to the column at 1 ml/min. The MtbB1 activity eluted at approximately 245 mM NaCl in a volume of 13 ml. The pooled active UNO Q1 fractions were adjusted to 700 mM (NH₄)₂SO₄ and 6.5 ml were loaded onto a phenyl-Sepharose HP cartridge (Pharmacia Biotech, Uppsala Sweden) in 50 mM MOPS, pH 7.0, and a 39 ml gradient of 500 mM to 0 mM (NH₄)₂SO₄ was applied to the column. The phenyl-Sepharose column was repeated with the remaining 6.5 ml of the MtbB1 UNO Q1 fraction. The active MtbB1 fractions from both runs eluted at a concentration of approximately 180 mM (NH₄)₂SO₄ in a total volume of 20 ml. The pooled active fractions were then adjusted to pH 8.0 in 50 mM Tris and loaded onto a single UNO Q1 column. A gradient of 150 mM to 350 mM in 50 mM Tris pH 8.0 was applied to the column at 2
ml/min over a volume of 80 ml. The active MtB1 fractions eluted at a concentration of approximately 245 mM NaCl in a volume of 11 ml. The purified MtB1 was then concentrated using four Amicon Centricon 10 concentrators to a volume of 1.5 ml and adjusted to a volume of 3.2 ml with 50 mM MOPS adjusted to pH 7.0.

**Purification of the methyltransferases and corrinoid protein involved in MMA:CoM methyl transfer**

The monomethylamine methyltransferase, Mtmb, was purified essentially as described by Burke and Krzycki (1997). The purification of Mtmb was carried out anaerobically inside a Coy anaerobic chamber. An extract of MMA grown *M. barkeri* was loaded onto a column containing DE-52 (Whatman Inc., Fairfield, NJ) and eluted with a gradient of 50 mM to 500 mM NaCl in 50 mM Tris, pH 8.0. The Mtmb activity eluted at approximately 240 mM NaCl. The active fractions were collected and loaded onto a Mono-Q 10/10 column in 50 mM MES, pH 6.0, and eluted with a gradient of 50 mM to 500 mM NaCl. The activity eluted at approximately 190 mM NaCl. The active fractions were then collected and concentrated using Centricon YM-10 microconcentrators and loaded onto a Superdex-200 16/60 gel filtration column equilibrated with 50 mM MOPS, pH 6.5, 100 mM NaCl. The peak of activity eluted at 58 ml. The active fractions were then loaded onto a Mono-Q 10/10 column equilibrated in 50 mM MOPS, pH 6.5, 100 mM NaCl. A gradient of 100-400 mM NaCl was applied to the column and the activity eluted at approximately 210 mM NaCl. The active fractions were then stored at -70°C under an atmosphere of H₂ until use.
The methylcorrinoid:CoM methyltransferase, MtbA, and the monomethylamine specific corrinoid protein, MtmC, were purified as described previously in Chapter 3 where separation of MtbA and MtmC was not fully achieved until the final column. It was found that purification of each protein could be performed aerobically at room temperature with no loss of activity. Aerobically purified proteins were made anaerobic by repeated cycles of evacuation and flushing with H₂ prior to use in the enzyme assays described below.

**Dimethylamine dependent coenzyme M methylation assays**

All reactions were performed in anaerobic sealed 2 ml glass vials under an atmosphere of H₂ at 37°C in a shaking water bath. An extract stimulation assay that identified fractions promoting DMA:CoM methyl transfer in crude cell extract was used to locate activity peaks eluting from the primary DEAE-Sepharose fractionation of total soluble proteins. These reactions were carried out in hydrogen flushed vials containing 50 µl (850 µg) of MMA grown cell extract, 1.5 µl (39 µg) of TMA grown cell extract, 61.5 µl of column fraction, 1.5 mM Ti(III)-citrate, 10 mM ATP, 20 mM MgCl₂, 2 mM HS-CoM, 100 mM DMA, and 1 mM bromoethanesulfonate in a total volume of 125 µl. The latter compound inhibits methane formation from the methyl-CoM product. The remaining free thiol of unmethylated CoM was quantitated by removing 10 µl of reaction mixture and adding it to 90 µl of 0.5 mM DTNB reagent at fixed time points as previously described (Chapter 2; Burke and Krzycki, 1995). This method has previously been found to be an accurate measure of the DMA
dependent methylation of CoM in both whole and fractionated extracts of *M. barkeri* (Wassenaar et al., 1998a).

All other CoM methylation assays in subsequent experiments employed reactions supplemented with purified proteins. These reactions relied on activation of the methyltransferase reaction by Ti(III) citrate with methyl viologen as a redox mediator. Screening of columns following the initial DEAE column for purification of Mtbb1 was done with hydrogen flushed reaction vials containing 4 mM HS-CoM, 100 mM DMA, 15 µg of MtbbA, 15 µg of MtbbC, 4.5 mM Ti(III) citrate, 0.75 mM methyl viologen, and appropriate amounts of column fractions in a total volume of 125 µl. For other experiments, 30 µg purified Mtbb1 were added in place of the column fractions in reactions containing purified MtbbA and MtbbC as the only other protein components. Protein concentrations in the assay were varied for some experiments, as indicated in the text. At intervals during the reaction timecourse, 5 µl of the reaction mixture were removed and reacted with 95 µl of 0.5 mM DTNB to determine the amount of unmethylated CoM. A low level of CoM less (approximately 0.5-1 nmol/min) was observed in reactions lacking enzymes or DMA. This was not observed at 1.5 mM concentrations of Ti(III) citrate and was attributed to interference of higher concentrations of Ti(III) citrate with DTNB detection of free thiols. This low background rate was subtracted from all of the CoM methylation reactions reported here in which the Ti(III) citrate/methyl viologen reducing system was employed.
Corrinoid methylation reactions

The reactions contained 50 μg (10 μM) of either MtmB or Mtbb1 and 35 μg of either MtmC or Mtbc (12 μM or 15 μM respectively) as indicated, as well as 4 mM Ti(III) citrate, 0.75 mM methyl viologen, and 100 mM MMA or DMA in a total volume of 100 μl. The vials were incubated at 37°C for 20 min in a shaking water bath under dim red light. The corrinoid was then extracted under dim red light as described by Kremer et al. (1993). The protein sample (100 μl) was mixed with 100 μl of 95% ethanol and heated to 80°C for 10 min. The mixture was then placed in an ethanol-dry ice bath for 10 min prior to centrifugation at 10,000 x g for 10 min. The supernatant was then concentrated to dryness under vacuum with a rotary evaporator and resuspended in 110 μl of distilled deionized water. Methylcobalamin was quantitated by C-18 reverse phase HPLC (Burke and Krzycki, 1997). A Microsorb-MV C-18 column was equilibrated with 20% methanol in 25 mM sodium acetate, pH 6.0. Samples (100 μl) were eluted with a linear gradient of 20-100% methanol over 60 min at 1 ml/min. The amount of methylated corrinoid extracted for each reaction was calculated by comparison to a standard curve of methylcobalamin analyzed by the same HPLC method.

DMA dependent free cobalamin methylation by Mtbb1 was measured spectrally as follows. The reactions were performed in an H₂ flushed anaerobic cuvette with a 2 mm path length. H₂ rather than N₂ was used to minimize Ti(III) citrate oxidation by protons. The reactions contained 250 μg Mtbb1, 15 mM Ti(III)-citrate, 0.5 M DMA, and 2.5 mM hydroxocobalamin in 50 mM MOPS, pH 6.5, in a total volume of 0.6 ml. The spectrophotometer was blanked against the cuvette containing only the DMA, purified Mtbb1,
and buffer. The reactions were started by the addition of hydroxocobalamin and were performed at 23°C under dim red light. The conversion of cob(I)alamin to methylcob(III)alamin was quantitated at 540 nm using an extinction coefficient of 4.4 mM⁻¹ cm⁻¹ (Kreft and Schink, 1993). DMA did not methylate cob(I)alamin in the absence of MtbB1.

Cloning and sequencing of a fragment of DNA encoding a portion of a duplicate copy of MtbC and MtbB

Several products were generated by the polymerase chain reaction (PCR) using Taq polymerase (GIBCO BRL, Gaithersburg, MD), M. barkeri MS genomic DNA as a template, and the primers COB8 [5'-CACGACAT(ATC)GG(TCAG)AA(AG)AA(TC)AT-3'] and DMA4 [5'-CT(TC)TT(TCAG)CC(AG)TC(TCAG)CCCAT-3']. COB8 and DMA4 were constructed to be complimentary to the consensus corrinoid binding region and the 5' terminus of MtbB (based on the published N-terminus of DMAMT) respectively. The PCR reactions contained 10 µl 10X PCR buffer (GIBCO BRL), 200 µM dNTP mixture, either 2 or 4 mM MgCl₂ as indicated in text, either 20 or 50 pmol of primers as indicated in text, 300 ng template M. barkeri MS genomic DNA, and 5 U Taq polymerase (GIBCO BRL), in a total volume of 100 µl. The PCR reaction was initiated by a 5 min incubation at 96°C followed by 30 cycles of 94°C for 45 sec, 42°C for 1 min, and 72°C for 1.5 min. Following the cycles, the reactions were incubated at 72°C for 10 min and held at 4°C until used. The product containing a portion of the duplicate MtbC was identified by Southern analysis using the primer DMA5 [5'-GC(TCAG)AC(TCAG)GA(AG)TA(TC)GC-3'] end labeled with [γ-³²P] ATP.
(Amersham Corp., Arlington Heights, IL) using T4 polynucleotide kinase (GIBCO BRL) at 37°C for 45 min. This product was then purified from a 1% agarose gel and ligated with pGEM-T vector (Promega Corp., Madison, WI) and propagated in *E. coli* DH5α. To ensure that the proper clone was obtained, a colony lift was performed using a Nytran nylon membrane (Schleicher & Schuell, Keene, NJ) as recommended by the manufacturer and probed with 32P-labeled DMA5 primer as described above. The plasmid DNA was isolated using the QIAPrep Plasmid DNA purification system (Qiagen) as recommended by the manufacturer. DNA sequencing was carried out using the Dye Terminator Cycle Sequencing Reaction Mix (Perkin-Elmer Corp., Foster City, CA) supplemented to 5% (v/v) dimethylsulfoxide and sequencing reactions were analyzed on an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer). Sequence alignments were performed using CLUSTALW (Thompson *et al.*, 1994).

**Other analytical techniques**

Protein bound corrinoid concentrations were determined using a dicyano method derivitization method described in Chapter 2 (Grahame, 1991). Protein concentrations were determined by using the bicinchoninic acid protein assay (Smith *et al.*, 1985) with reagent purchased from Pierce Chemical Co. (Rockford, IL) using bovine serum albumin as standard. Plasma emission spectroscopy was performed at the University of Georgia Chemical Analysis Laboratory using a Jarrell-Ash 965 plasma emission spectrometer. Ultraviolet-visible spectra were obtained using a Hewlett Packard model 8453 photodiode array spectrophotometer. SDS gel
electrophoresis was performed after Laemmli (Laemmli, 1970) using a Mini-
slab electrophoresis system (Idea Scientific Co., Minneapolis, MN). Molecular
size markers (BioRad Laboratories, Hercules, CA) were rabbit skeletal muscle
myosin (200,000), E. coli β-galactosidase (116,250), phosphorylase B (97,400),
bovine serum albumin (66,200), hen egg white ovalbumin (45,000), bovine
carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), hen egg white
lysozyme (14,400), and bovine pancreas aprotinin (6,500). N-terminal
sequencing was performed as described in Chapter 3 using 15 µg of MtbB1 (300
pmol). HPLC size exclusion columns G4000SW_{XL} and G3000SW_{XL} (Supelco,
Bellefonte, PA) equilibrated with 100 mM NaCl in 50 mM MOPS, pH 7.0
connected in series and adapted to a Bio-Rad medium pressure liquid
chromatography system inside a Coy anaerobic chamber were used to
determine native protein masses. MtbB1 or MtbC (100 µg each) were injected
onto the columns in a volume of 250 µl and eluted at a rate of 0.75 ml/min.
The molecular mass standards used to generate the standard curve were blue
dextran (2,000,000), thyroglobulin (669,000), apoferritin (443,000), β-amylase
(200,000), alcohol dehydrogenase (150,000), bovine serum albumin (66,000),
carbonic anhydrase (29,000), and ferricyanide (324) purchased from Sigma
Chemical Co.
RESULTS

Purification of MtbC, a small corrinoid protein stimulating DMA:CoM methyl transfer (Performed in collaboration with Dr. Natalia Gorlatova)

During the isolation of the amine specific methylcorrinoid:CoM methyltransferase, MtbA, from cell extracts of TMA grown cells of M. barkeri, a previously uncharacterized protein termed MtbC was identified by Dr. Natalia Gorlatova in Dr. David Grahame’s laboratory. The two proteins copurified through several columns until the final hydrophobic interaction column yielded homogenous preparations of MtbC (Performed by Dr. Gorlatova). Purified MtbC migrated as a single 24-kDa protein band during SDS gel electrophoresis (Figure 19). The purified protein eluted with a molecular mass of 34 kDa from a HPLC silica gel permeation column. The UV-Vis spectra indicated MtbC is a corrinoid binding protein (Figure 20, panel A). The aerobic protein had an absorbance maximum at 360 nm, typical of protein bound corrinoid in the Co(III) state with water or a hydroxyl group as the beta axial ligand. The stoichiometry of cofactor bound to the protein was determined by SDS and heat denaturation of the protein following conversion of the cofactor to the dicyano derivative. Each mol of 24-kDa polypeptide bound 1.0 mol corrinoid. The first 10 residues were determined by Edman degradation by Dr. Gorlatova to be SXEELLQELAD, where X was a residue that could not be unambiguously assigned.

Extracts of cells grown on TMA possessed appreciable DMA:CoM methyl transfer activity (550 nmol/min/mg protein), but this activity was

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Figure 19.

SDS PAGE of proteins used to reconstitute the DMA:CoM methyl transfer or MMA:CoM methyl transfer reactions. Samples were loaded onto an 11% acrylamide gel and electrophoresed followed by staining with Coomassie dye. The samples for each lane are indicated at the top of the gel reading top to bottom: Stds, standard proteins of the indicated molecular masses in kDa; MMAX, extract of MMA grown cells (42 μg protein); TMAX, extract of TMA grown cells (42 μg protein); purified MtbC (4.0 μg protein); purified MtbB1 (4.5 μg protein); purified MtbA (4.0 μg protein); purified MtmC (4.0 μg protein); and purified MtmB (4.7 μg protein).
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![Image of gel electrophoresis with molecular weight markers and labeled bands.](image)

Figure 19
Figure 20.

UV-visible spectra of MtbC and MtbB1. (A) Spectrum of purified MtbC (0.4 mg/ml). (B) Spectrum of purified MtbB1 (0.4 mg/ml). The isolated proteins were in aerobic 50 mM MOPS, pH 7.0, and at the level of purity depicted in Figure 19.
Figure 20
much less in MMA grown cells (≤2.5 nmol/min/mg protein). There was a highly abundant 24-kDa protein in TMA-grown cell extract, which co-migrated with purified MtbC during SDS gel electrophoresis. No such prominent 24-kDa band was detectable in electrophoresed extract from MMA-grown cells (Figure 19). This was consistent with a possible role for MtbC in CoM methylation by DMA. CoM methylation with TMA, DMA, or MMA was therefore measured in an extract of TMA grown cells before and after addition to the extract of purified MtbC. CoM methylation from TMA, DMA, or MMA was measured at rates (in nmol/min/mg total protein) of 30.4, 38.5, or 24.2, respectively. Addition of 20 μg of purified MtbC to the same extract resulted in rates of CoM methylation from TMA, DMA, or MMA of 35.9, 130.3, or 31.3, respectively. The marked stimulation of DMA:CoM methyl transfer by the addition of MtbC indicated that MtbC plays a role in the initiation of methanogenesis from DMA.

Isolation of MtbB1 using purified MtbC and MtbA

A role for MtbC in DMA:CoM methyl transfer implies the existence of a DMA:MtbC methyltransferase. Extracts of TMA grown cells were anaerobically fractionated in order to identify such a methyltransferase and study its possible interaction with MtbC. The ability of protein fractions to stimulate the low rate of DMA:CoM methyl transfer in an extract of MMA grown cells was used to screen the initial DEAE-Sepharose column (Figure 21) for potential components of the DMA:CoM methyl transfer reaction. Two peaks of activity were detected. The first peak to elute (peak I) is most likely to be due to MtbC itself (see following section). From the second activity peak to
Figure 21.

Stimulation of DMA:CoM methyl transfer activity in extracts by two separate peaks from anion exchange chromatography. An extract of TMA grown cells was chromatographed on a DEAE-Sepharose column and the fractions collected assayed for their ability to stimulate DMA:CoM methyl transfer in extracts. The reactions contained 50 μl MMA grown cell extract, 1.5 μl TMA grown cell extract, 61.5 μl column fraction, 10 mM ATP, 20 mM MgCl₂, 100 mM DMA and 1 mM BES in a final volume of 125 μl. An initial concentration of 2 mM CoM was used in these reactions, which accounts for the flattened tops of the activity peaks. The solid line represents the absorbance at 280 nm and the open circles represent the DMA dependent CoM methylation activity assayed for individual fractions.
elute (peak II) a protein that acts as a DMA:corrinoid methyltransferase was isolated which was designated as MtbB1.

Peak II fractions combined with purified MtbC and MtbA carried out DMA:CoM methyl transfer in the presence of Ti(III) citrate and methyl viologen. This assay, rather than the extract stimulation assay, was used to purify MtbB1 to a single homogenous polypeptide that could carry out DMA:CoM methyl transfer in presence of purified MtbA, MtbC, and no other proteins. A single peak of activity was detected in each of the subsequent columns on which MtbB1 was purified (Table 3). The final preparation of MtbB1 revealed a single 51-kDa polypeptide upon SDS gel electrophoresis (Figure 19). The determined N-terminus of the polypeptide (MATEYALRMGDGKRVYLTKE) matched 14 of 16 residues from the DMAMT polypeptide isolated by Wassenaar et al. from M. barkeri Fusaro (Wassenaar et al., 1998a; Wassenaar et al., 1998b). In marked contrast to the report for DMAMT preparations, our preparations of homogenous MtbB1 polypeptide did not contain any detectable corrinoid prosthetic group when assayed by the dicyano method. The UV-Vis spectrum of MtbB1 did not have any of the characteristic peaks of a corrinoid protein (Figure 20, panel B). Analysis of the metal content of MtbB1 by plasma emission spectroscopy revealed no detectable cobalt in two different preparations of the homogenous enzyme. The lower limit of detection for cobalt using this procedure was 0.04 mol cobalt/mol polypeptide. No other metals were detected except 0.43 mol zinc/mol polypeptide which is comparable to the 0.3 mol zinc/mol MtmB polypeptide detected. Plasma emission also detected 3.4 mol boron/mol MtbB1 polypeptide from 2
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Table 3. Purification of MtbB1.

\(^a\) DMA:CoM methyl transfer activity was monitored by the DMA dependent disappearance of the free thiol of CoM. The first column was screened using a DMA:CoM stimulation assay, the MtbB1 activity was followed in the subsequent columns by supplementing fractions with MtbA and MtbC and activating methyl transfer using Ti(III) citrate/methyl viologen as described under Material and Methods of this chapter. In order to compare activities, all rates in the table were measured using the latter assay.

\(^b\) One unit is defined as 1 μmol CoM consumed per min.
preparations of purified MtbB1. The protein eluted as a 230-kDa protein during size exclusion chromatography, indicating a homotetrameric or possibly homopentameric configuration.

Minimum requirements for in vitro DMA:CoM methyl transfer

Previous work with DMA:CoM methyl transfer in vitro had been performed primarily using purified proteins added to crude extracts, or with extracts fractionated with only a single DEAE column (Ferguson et al., 1996; Chapter 2; Wassenaar et al., 1998a). Therefore, the minimal requirements for DMA:CoM methyl transfer could not be determined. However, as shown below, the isolation of MtbC and MtbB1 provided the final purified components required for a fully resolved in vitro DMA:CoM methyl transfer reaction.

In order to activate DMA:CoM methyl transfer, the presently unresolved cellular ATP and hydrogen dependent activation system was again replaced with Ti(III) citrate and methyl viologen. Purified MtbB1 and MtbA were found insufficient for DMA:CoM methyl transfer when incubated with DMA and Ti(III)citrate/methyl viologen. However, DMA:CoM methyl transfer occurred when MtbC was also added to these reactions. Activity was completely dependent on each protein (Figure 22). The preparations of all three proteins used in this and the following experiments were near homogeneous as illustrated in Figure 19. Preincubation of the proteins with 10 mM ATP and 20 mM MgCl2 had no effect on the rate of the reaction. A low molecular weight fraction, obtained as the filtrate from Amicon Centricon 3 membrane filtration unit treatment of a TMA-grown cell extract,
Figure 22.

Three polypeptides are required to reconstitute DMA:CoM methyl transfer activity in a purified system: MtbB1, MtbA, and MtbC. The minimum requirements for DMA:CoM methyl transfer were examined using the purified proteins analyzed by SDS-PAGE as depicted in Figure 19. The complete reaction contained 40 μg of MtbB1, 20 μg of MtbA, 20 μg of MtbC, 4.5 mM Ti(III) citrate, 0.75 mM methyl viologen, 100 mM DMA, and 4 mM CoM, in a final volume of 125 μl. Timecourses are presented for the complete reaction (●), as well as the complete reaction minus MtbB1 (■), minus MtbA (◇), or minus MtbC (▲).
also did not stimulate the rate of CoM methylation. No CoM methylation was detectable if MtbB1, MtbC, or MtbA were incubated with TMA or MMA as methyl donors. MtbC (typically present as 15 µg or 0.65 µM in the resolved reaction) could not be replaced by the addition of 20 µM non-protein bound cobalamin.

The resolved DMA:CoM methyl transfer reaction was used to putatively identify the nature of the protein responsible for the activity in peak I that eluted from the DEAE-Sepharose column depicted in Figure 21. The pooled peak I fractions, purified MtbB1, and purified MtbA carried out DMA:CoM methyl transfer in the presence of Ti(III) citrate and methyl viologen (Figure 23). However, peak I incubated with MtbC and MtbA could not carry out CoM methylation with DMA. These results indicated that the first peak of DMA:CoM methyl transfer stimulating activity to elute from the DEAE column is likely due to the presence of MtbC in this fraction, since this fraction replaced purified MtbC, but not MtbB1 (Figure 23).

The specificity of purified MtbB1 and MtbC for methylated substrate was examined using TMA, DMA, and MMA in the CoM methylation reaction. It was found that MtbB1 could not utilize either TMA or MMA in the CoM methylation reaction (Figure 24). Wassenaar et al. (1998a) reported that DMAMT displayed a high affinity for DMA in the CoM methylation reaction (apparent Km = 0.5 mM). We examined the kinetics of our system using an extract of TMA grown cells as well as purified proteins. Although preliminary, these data suggest an increase in the apparent Km for DMA when using purified MtbB1 (apparent Km approximately 8 mM) as compared to extracts (apparent Km approximately 2 mM) (Figure 25).
Figure 23.

Activity of the two stimulating peaks from anion exchange chromatography in a more resolved system. Peak I and peak II were combined with purified MtbB1 and MtbC to test for their abilities to replace the purified proteins and reconstitute DMA-CoM methyl transfer in the absence of the cellular activation system. The reactions contained 5 mM Ti(III) citrate, 0.75 mM methyl viologen, 100 mM DMA, and 15 μg MtbA. The reactions also contained 30 μg MtbB1, 15 μg MtbC, 50 μg peak I, and 50 μg peak II as indicated.
Figure 23
Figure 24.

Substrate specificity of enzymes of the DMA:CoM methyl transfer pathway. The reactions contained 4.5 mM Ti(III) citrate, 0.75 mM methyl viologen, 4 mM CoM, 30 µg MtβB1, 15 µg MtβC, 15 µg MtβA, and 100 mM methylated substrate as indicated, in a final volume of 125 µl.
Figure 24
Figure 25.

Estimations of apparent Km for DMA in DMA:CoM methyl transfer activity with an extract of TMA grown cells and the purified proteins of the DMA:CoM methyl transfer pathway. Panel A illustrates the DMA:CoM methyl transfer activity of an extract of TMA grown cells (0.5 mg/reaction) using increasing concentrations of DMA (0-10 mM). The inset in panel A shows the corresponding Lineweaver-Burke plot using the data from panel A. Panel B illustrates the DMA:CoM methyl transfer activity of the purified proteins of the DMA:CoM methyl transfer pathway (30 μg MtbB1) using increasing concentrations of DMA (0-100 mM). The inset in panel B shows the corresponding Lineweaver-Burke plot using the data from panel B.
Figure 25
Purified MtbB1 was titrated against a constant amount of purified MtbC (0.65 nmol) and purified MtbA (0.41 nmol) and DMA:CoM methyl transfer was measured (Figure 26). A linear increase in activity was seen with increasing MtbB1 until the reaction began to saturate with approximately 0.6 nmol MtbB1. This corresponds to an approximate molar ratio of 1:1 between MtbB1 and MtbC. Prior to saturation of the reaction with MtbB1, it can be calculated that methylation of CoM occurred at an average rate of 2.2 μmol/min/mg MtbB1.

**MtbB1 is a DMA: cob(I)alamin methyltransferase**

The requirement of a methylcorrinoid:CoM methyltransferase, a corrinoid protein, and MtbB1 for DMA:CoM methylation suggested that MtbB1 could function in demethylation of DMA and methylation of the corrinoid prosthetic group of MtbC. To test this hypothesis, the ability of MtbB1 to methylate free cobalamin with DMA was examined. Hydroxocobalamin was reduced to the Co(I) state with Ti(III) citrate in the presence of DMA, and MtbB1 dependent formation of methylcobalamin was monitored spectrally. Upon initiation of the reaction, a shift in the cob(I)alamin 553 nm absorbance peak occurred towards the methylcobalamin peak of 532 nm. An isobestic point at 578 nm for timepoints following T₀ indicated that the transformation of cob(I)alamin to methylcobalamin proceeded without a detectable intermediate (Figure 27, panel A). The spectrum taken at T₀ has contributions from hydroxocobalamin and cob(II)alamin since the reaction was began by introduction of hydroxocobalamin into the cuvette. The conversion of cob(I)alamin to
Figure 26.

Dependence of DMA:CoM methyl transfer activity on MtbB1 in reactions containing MtbA and MtbC. The rate of DMA dependent CoM consumption was measured in reaction vessels containing purified MtbA (0.41 nmol, 15 μg) and MtbC (0.65 nmol, 15 μg). Reactions were also supplemented with different amounts of MtbB1 as indicated. The reactions also contained 4.5 mM Ti(III) citrate, 0.75 mM methyl viologen, 4 mM CoM, and 100 mM DMA, in a final volume of 125 μl.
Figure 26
Figure 27.

DMA dependent methylation of free cobalamin by MtbB1. (A) UV-visible spectra of 2.5 mM cobalamin reduced with Ti(III) citrate during the course of a 2 hour incubation in the presence of DMA and MtbB1. Reaction conditions are as described in the Materials and Methods of this chapter. The spectra at T₀ and 120 min are indicated, the remaining spectra were taken 10 minute intervals. (B) The rate of cobalamin methylation determined using the extinction coefficient at 540 nm of 4.4 mM⁻¹cm⁻¹.
Figure 27
methylcobalamin can be followed at 540 nm, the isobestic point of Co(I)/Co(II) cobalamin (Kreft and Schink, 1993). MtbB1 methylated 2.5 mM cob(I)alamin at a rate of 14.4 nmol/min/mg MtbB1 (Figure 27, panel B). The formation of methylcobalamin from DMA and cob(I)alamin as catalyzed by MtbB1 was confirmed by HPLC analysis.

**Methylation of MtbC by MtbB1**

The methylation of free cobalamin with DMA by MtbB1 indicates that its role in the DMA:CoM reaction is in the methylation of the prosthetic group of MtbC. In order to test this directly, 35 μg of MtbC (1500 pmol corrinoid) and 1 nmol of MtbB1 were incubated in the presence of Ti(III) citrate, methyl viologen, and DMA but in the absence of MtbA and CoM (Table 4). The corrinoid prosthetic group of MtbC was then aerobically extracted with ethanol and analyzed by HPLC. No hydroxylated corrinoid peak was detected, and 1250 pmol of methylated corrinoid was recovered from MtbC. Methylation of MtbC was completely dependent on DMA.

**Specificity of MtbB1 and MtbC for CoM methylation by DMA**

The preceding results indicated that this resolved in vitro DMA:CoM methyl transfer system was analogous to the resolved three component MMA:CoM methyl transfer reaction (Burke and Krzycki, 1997). In addition to the methylcorrinoid:CoM methyltransferase MtbA, each of these systems require a methylamine specific methyltransferase and a corrinoid protein. In the following experiments we examined the ability of these protein
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Table 4. Methylation of corrinoid proteins by cognate and non-cognate methyltransferases.

<sup>a</sup> Amount of corrinoid attributed to Mtbc or MtmC based on a 1:1 ratio of corrinoid to protein.

<sup>b</sup> No other corrinoid peak was detectable during HPLC analysis of these fractions, recovery of total corrinoid averaged 80%.

<sup>c</sup> ND = below the limit of detection (20 pmol)
components to substitute for one another during CoM methylation from DMA or MMA.

MtMB and MtBB1 were incubated with either MtMC or MtBC in the presence of MtBA and CoM methylation from either DMA or MMA monitored (Figure 28). Only the combination of MtBB1 and MtBC supported CoM methylation with DMA, while only the combination of MtMB and MtMC supported CoM methylation with MMA. MtMC and MtBB1 incubated with MtBA did not result in detectable methylation of CoM with either DMA or MMA. Similarly, MtBC incubated with MtMB and MtBA resulted in no detectable CoM methylation with either MMA or DMA. These experiments demonstrate that MtMB and MtMC determine the specificity of CoM methylation via MtBA for MMA, while MtBB1 and MtBC are essential for CoM methylation by DMA. MtBB1/MtBC and MtMB/MtMC thus form specific cognate methyltransferase/corrinoid protein pairs initiating methanogenesis from DMA or MMA, respectively.

Specificity of MtBB1 and MtMB in methylation of their cognate corrinoid proteins

Neither MtBB1 nor MtMB carried out significant methylation of the non-cognate corrinoid protein with their specific methylamine substrates. Methylation of MtBC or MtMC was analyzed following extended incubation with either MtBB1 or MtMC in the presence of either DMA or MMA and Ti(III) citrate and methyl viologen (Table 4). Following incubation, the corrinoid prosthetic groups were aerobically extracted from the reaction mixture, and methyl-corrinoid quantitated by HPLC. In control reactions,
Figure 28.

Specificity of methylamine methyltransferases and their cognate corrinoid proteins for CoM methylation. The specificity of the DMA and MMA methyltransferases for their respective cognate corrinoid proteins and substrates was tested by mixing the reaction components required for CoM methylation by DMA or MMA. All reactions contained 15 μg MtbA, as well as the following proteins and substrates: MtbB1, MtbC, and DMA (Δ); MtbB1, MtmC, and DMA (○); MtbB1, MtmC, and MMA (□); MtmB, MtmC, and MMA (+); MtmB, MtbC, and DMA (●); MtmB, MtbC, and MMA (■); no enzyme (▲). All the reactions also contained 4.5 mM Ti(III)citrate, 0.75 mM methyl viologen, 4 mM CoM, and 100 mM methylated substrate, in a final volume of 125 μl. If present, 30 μg of MtbB1 or MtmB were added with either 15 μg of MtbC or MtmC.
Figure 28
MtbB methylated Mtc with MMA, as observed previously, and MtbB1 methylated MtbC with DMA as discussed above. No hydroxylated corrinoid was detected in either of these reactions.

In contrast, when MtbB was incubated with reduced MtbC in the presence of MMA only 1.8% of the initial corrinoid bound to MtbC was recovered as methylated corrinoid. No methylation of the corrinoid bound to Mtc by MtbB1 with DMA was detectable. The remainder of the corrinoid cofactor extracted from both reactions eluted as hydroxylated corrinoid during HPLC analysis.

Duplicate copy of mtbC lies immediately upstream of a duplicate copy of mtbB.

It has been determined that three copies of the mtbB gene exist in the genome of M. barkeri (Paul et al., 2000). It was suspected that these duplicate copies, mtbB2 and mtbB3, would also lie in close proximity in the genome to duplicate copies of mtbC although this had not yet been determined. Using primers complimentary to the published N-terminus of DMAMT (Wassenaar et al., 1998a) and the consensus corrinoid binding region (Paul et al., 2000), we generated a PCR product which encoded the C-terminus of a duplicate copy of mtbC and the N-terminus of either mtbB2 or mtbB3. Several products were generated in the reaction and by Southern analysis a fragment migrating as approximately 700 bp (Figure 29) was identified as the product containing the 5' region of a mtbB gene. Sequence analysis revealed that the fragment was only 280 bp in length and encoded the final 58 amino acids of a duplicate mtbC gene and the first 14 amino acids of either mtbB2 or mtbB3 (Figure 30). The portion of the duplicate mtbC was 82% identical at the nucleic acid level.
Figure 29.

A 1% agarose gel indicating the PCR products generated using the COB8 and DMA4 primers. The PCR reactions were performed as described in Materials and Methods of this chapter. The lanes contained the following: (L) 1 kb DNA ladder (GIBCO BRL), (1) 10 μl of a PCR reaction performed using 20 pmol of primers and 4 mM MgCl₂, (2) 10 μl of a reaction performed using 50 pmol of primers and 4 mM MgCl₂, (3) 10 μl of a reaction performed using 20 pmol of primers and 2 mM MgCl₂, (4) 10 μl of a reaction performed using 50 pmol of primers and 2 mM MgCl₂. The desired 280 bp product encoding the C-terminus of a MtbC copy and the N-terminus of a MtbB copy is indicated (▲).
Figure 30.

Nucleic acid sequence and the predicted amino sequence of the coding regions of the PCR product generated. The PCR product was generated using primers designed from the consensus corrinoid binding region and the N-terminus of DMAMT. A portion of the duplicate $mrbC$ gene ($mrbC-PP$) and the first 14 amino acids of a duplicate $mrbB$ gene ($mrbB-PP$) were amplified. The $mrbC-PP$ gene ends at residue 178 and the $mrbB-PP$ gene begins at residue 241 as indicated.
CCT TCA GGG CAG AGA GAT GTA ATT GAA CTC CTC AAG GAA
    P S G Q R D V I E L L K E

GAA GGG CTC AGA GGC AAA GTA AAG GTT ATG GTA GGC GGA
    E G L R G K V K V M V G G

GCC CCT GCA ACC CAG TCC TGG GCT GAC AAG ATA GGT GCA
    A P A T Q S W A D K I G A

GAC TGC TAT GCT GAA AAC GCA AGC GAA GCC GTG GCA AAA
    D C Y A E N A S E A V A K

GCA AAG GAA ATG CTG CTA TGA GCC TCA ACT CTC AAT AAA
    A K E M L L * mtbC-PP ends

ATC GAC ATA GTC TAA ACT AAG TTT GTC AAA AGG AGA ATT

AAA AAA ATG GCA ACT GAA TAC GCT TTG AGA ATG GGC GAC
    mtbB-PP M A T E Y A L R M G D

GGA AAG AGA
    G K R

Figure 30

156
(Figure 31) and 85% identical at the deduced amino acid level (Figure 32) to the mtbc contained in the mtt/mtb operon (Paul et al., 2000). The first 14 amino acids encoded by the duplicate mtbb gene (MATEYALRMDGDKR) were identical to the N-terminus of Mtbb1. This is not surprising due to the high degree of identity (>90%) between the three copies of Mtbb (Paul et al., 2000), however, this did not allow for identification of the mtbb copy.

DISCUSSION

This work is the first complete resolution of a pathway for the methylation of CoM with DMA using only highly purified proteins. Mtbb1, Mtbc, and Mtba are sufficient, and each necessary, for in vitro transfer of methyl groups from DMA to CoM. Our data support a model for the interaction of these proteins in the methylation of CoM with DMA that is analogous to the reactions for CoM methylation with TMA, MMA, and methanol (Figure 33). In each of these pathways, a small corrinoid protein specific for that pathway interacts with two methyltransferases to initiate CoM methylation from the methylotrophic substrate. One methyltransferase methylates the corrinoid protein with the methanogenic precursor, while the second methyltransferase catalyzes the demethylation of the corrinoid protein and methylation of CoM.

Several lines of evidence support this type of DMA:CoM methylation reaction in M. berkeri. Mtbc is a small corrinoid protein that specifically
Figure 31.

Comparison of the nucleic acid sequences of *mtbC* and the duplicate *mtbC* partial sequence denoted as *mtbC-PP* (PCR product). The alignment was generated using CLUSTALW.
Figure 31
Figure 32.

Alignment showing the homology between the deduced amino acid sequences of MtbC and the duplicate MtbC (MtbC-PP). The alignment was generated using CLUSTALW.
<table>
<thead>
<tr>
<th>Mtbc-PP</th>
<th>Mtbc</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A A D A M T A G V A A L K D L M P E G A S G S K L G V I V N G T V</td>
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<tr>
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</tr>
<tr>
<td>166</td>
<td>L K E E G L R D K V K V M V G G A P A T Q A W A D K I G A D C Y A</td>
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<tr>
<td>44</td>
<td>E N A S E A V A K A K E M E L</td>
</tr>
<tr>
<td>199</td>
<td>E N A T E A V A K A K E L L A</td>
</tr>
</tbody>
</table>

Figure 32
Figure 33.

Proposed models of CoM methylation during methanogenesis from methylotrophic substrates. The TMA, MMA, and methanol dependent CoM methylation systems are analogous to the MtbB1, MtbC, and MtbA mediated methylation of CoM with DMA. This figure is not meant to represent higher order structures of the proteins involved, but is designed to indicate protein components with analogous roles. Each pathway has a polypeptide of approximately 50 kDa that demethylates the growth substrate and methylates its cognate corrinoid protein. MtaB and MtbB1 have now been shown to methylate free cobalamin with the indicated substrates, while MtmB is required for methylation of the corrinoid bound to MtmC. MttB forms a tight complex with MttC and therefore the postulated role as a TMA:corrinoid methyltransferase has not been demonstrated. Each methylated cognate corrinoid protein is demethylated by a methylcorrinoid:CoM methyltransferase for the methylation of CoM. The corrinoid proteins are homologous proteins (approximately 50% sequence similarity), as are the CoM methylases (37% sequence identity). However, the methyltransferases initiating the CoM methylation reactions are not homologous. References supporting these models are cited in the text.
Figure 33
stimulated CoM methylation from DMA when added to cell extracts. Furthermore, purified MtbC was used as a component of the DMA:CoM methyl transfer assay employed to isolate MtbB1, a DMA:corrinoid methyltransferase. MtbB1 is the first protein reported to catalyze the direct methylation of non-protein bound cob(I)alamin with DMA. This indicates that MtbB1 possesses the active site used in DMA dependent methylation of the corrinoid prosthetic group of MtbC. Purified MtbB1, MtbC, and MtbA can methylate CoM with DMA, but not other methylamines. These results are fully consistent with a model in which MtbB1 specifically methylates the corrinoid prosthetic group of MtbC with DMA, and methyl-MtbC is subsequently demethylated by the methylcorrinoid:CoM methylase, MtbA.

Wassenaar et al. (1998a) have postulated the existence of a different pathway of DMA:CoM methylation in M. barkeri (Figure 18, panel B). In their work, they proposed that a 50-kDa DMA methyltransferase, DMAMT, binds corrinoid and automethylates its prosthetic group which is then demethylated by MtbA to methylate CoM (Wassenaar et al., 1998a; Wassenaar et al., 1998b). Their proposed pathway does not include a small corrinoid protein such as MtbC, rather, DMAMT binds 0.45 mol corrinoid/mol polypeptide, and is suggested to be a fusion of a corrinoid binding protein and a DMA:corrinoid methyltransferase. The full relationship of DMAMT to the DMA methyltransferase isolated here (MtbB1) is unknown, but the N-termini are 88% identical. The key difference between the two proteins is that our homogeneous preparations of MtbB1 do not bind detectable corrinoid, and
clearly methylate with DMA a discrete corrinoid polypeptide, MtbC. The sequence of DMAMT has not yet been obtained. We have obtained the full sequence of MtbB1, and it has no detectable homology with any corrinoid binding protein (Genbank accession number AF102623) (Paul et al., 2000). The specific activity of DMA demethylation measured for purified MtbB1 is comparable to that reported for DMAMT. The native structure of the two proteins is also different. DMAMT is a dimer, while MtbB1 is a tetramer or pentamer.

If both DMAMT and MtbB1 represent completely homologous gene products, then it seems clear that DMAMT bound corrinoid is not essential for activity, and a small corrinoid protein, MtbC, supplies the corrinoid cofactor for the DMA:CoM methyltransferase reaction. However, it is possible that both pathways are present in methanogens. Recently, it was demonstrated that chloromethane utilization by an aerobic methylotroph requires a protein that is a fusion of a methylotrophic corrinoid protein and a MtbA homolog (Vannelli et al., 1999). This observation indicates that the DMA methyltransferase-corrinoid protein fusion proposed by Wassenaar et al. might be feasible. However, since DMAMT required unresolved protein fractions to carry out in vitro DMA:CoM methyl transfer, it is difficult to determine if a small corrinoid protein is functioning in the DMAMT dependent methylation of CoM. Further resolution of the DMAMT dependent DMA:CoM methyl transfer system, and the sequencing of the gene encoding DMAMT, should establish whether a pathway of DMA:CoM methyl transfer exists other than that illustrated in Figure 33.
Recently, the genes encoding two polypeptides required to initiate TMA:CoM methylation, MttB and MttC, (see Figure 33) were cloned and sequenced (Genbank Af102623) (Paul et al., 2000). Two open reading frames were identified near these genes which initially could not be assigned to gene products. However, the N-termini of Mtbb1 and MtbbC obtained in this study indicate that each of these ORFs encode proteins involved in the DMA dependent methylation of CoM. Interestingly, the genes mttB, mtiC, mtbC, and mttB are encoded on a single transcript found in TMA grown cells (Paul et al., 2000). The co-transcription of the genes encoding MtbbC and Mtbb1 is consistent with the function of these two independently isolated proteins in the DMA:CoM methyl transfer reaction. It is of further interest that both the mtbb1 and mtbbC genes are co-transcribed with genes for initiating TMA catabolism. Since M. barkeri generates DMA as an intermediate of methanogenesis from TMA, co-transcription of both TMA and DMA methyltransferase genes would be an efficient means of coordinating expression of these methyltransferases.

The gene mtbC, encoding MtbbC, is approximately 50% similar to the predicted protein sequences from the cloned genes of the methylotrophic corrinoid proteins of TMA (Mtbc) (Paul et al., 2000), MMA metabolism (MtmC) (Burke et al., 1998), and methanol metabolism (MtaC) (Sauer et al., 1997). Each of these corrinoid proteins is in turn homologous to the cobalamin binding domain of corrinoid dependent methionine synthase and mutases (Sauer et al., 1997; Burke et al., 1998; Drennan et al., 1994; Paul and Krzycki, 1996). The homology of the corrinoid protein isolated here, MtbbC, to these other small discrete corrinoid proteins involved in methanogenesis
from methylotrophic substrates is consistent with our results demonstrating a role for MtbC in a CoM methylation pathway, that is, DMA:CoM methyl transfer.

The finding of a duplicate mibC adjacent to one of the two duplicate mibB genes is interesting given the arrangement of mibC and mibB1 in the mtt/mtb operon (Paul et al., 2000). In the mtt/mtb operon, although mibC and mibB1 are cotranscribed, the fact that mibC and mibB1 are not immediately adjacent to one another left a possibility that MtbC is not strictly used in DMA:CoM methyl transfer but rather is an isozyme of MttC. The close proximity of this duplicate mibC to an mibB gene along with the biochemical data presented in this chapter provide strong evidence that MtbC is an essential component of the DMA:CoM methyl transfer pathway and is not involved in TMA:CoM methyl transfer.

Another concern is the fact that the N-terminus of MtbB1 is identical to the N-terminus encoded by the mibB on the PCR product. This raises some concerns as to the identity of the MtbB enzyme used in the studies described above. However, the transcription data presented by Paul et al. (2000) indicated that mibB1 appears to be the primary mibB transcript expressed during growth on TMA. Given the fact that the MtbB used in these experiments was isolated from TMA grown cells, thus far we can conclude that this enzyme is the MtbB1 encoded in the mtt/mtb operon. A more detailed analysis of the expression of the three mibB genes during growth on different substrates and at different points in the growth cycle could conclusively demonstrate the identity of the predominant MtbB in TMA grown cells.
MtnB, MtbB1, and MttB are each implicated in analogous reactions, the methylation of corrinoid with methylamines. However, now that proteins and genes for all three methyltransferases have been identified, it is somewhat surprising to observe that the methylamine methyltransferases are not homologous (Burke et al., 1998; Paul et al., 2000). This may explain the presence of the different, yet homologous, methylotrophic corrinoid proteins of methylamine dependent methanogenesis. Although each corrinoid protein serves as a methyl donor for the same CoM methylase, MtbA, the differences in primary sequence must be necessary in order to optimize interaction of a particular corrinoid protein with one of the non-homologous methylamine:corrinoid methyltransferases. These differences appear to lead to highly specific interactions between a methyltransferase and its cognate corrinoid protein, and the dedication of a particular corrinoid protein to a particular pathway. Presumably, this specificity occurs with all three of the small corrinoid proteins identified as involved in methylamine metabolism. Unfortunately, this cannot be easily tested with the proteins initiating CoM methylation with TMA, since MttB and MttC form a complex that is difficult to separate. However, our results with the resolved MMA:CoM and DMA:CoM methyl transfer systems do illustrate this specificity. There is little detectable interaction between the MMA methyltransferase and the DMA corrinoid protein, or, between the DMA methyltransferase and the DMA corrinoid protein. Neither corrinoid protein will detectably support the non-cognate methyltransferase in CoM methylation. This lack of activity in CoM methylation coincides with the inability of either methyltransferase to rapidly
methyliate the corrinoid prosthetic group of the non-cognate corrinoid protein.

Mtbc greatly enhances the interaction of MtbB1 with corrinoid. During the course of this study, the observed rates of DMA dependent methylation of CoM with the reconstituted system varied from 0.8 to 2.2 μmol/min/mg MtbB1. MtbB1 methylation of the corrinoid bound to Mtbc is therefore much faster than observed for the methylation of free cobalamin, since 20 μM cobalamin did not replace Mtbc in the resolved DMA:CoM reaction. Indeed, even with 2.5 mM cobalamin, the rate of MtbB1 dependent methylation of corrinoid with DMA was slow relative to the rates observed for MtbB1, Mtbc, and MtbA mediated methylation of CoM with DMA. These results further illustrate that the corrinoid binding protein, Mtbc, must greatly and specifically enhance the proper interaction of corrinoid with the DMA:corrinoid methyltransferase, MtbB1, during the initial reactions leading to methane formation from DMA.

It is somewhat surprising to observe that the apparent Km for DMA of the purified MtbB1 is greater than that observed in extracts (Figure 25). There are several possible explanations as to why this true. The assays with purified MtbB1 were performed using the Ti(III) citrate/methyl viologen artificial reducing system which may be rather inefficient. The presence of the natural activation system (RAM and ATP) may not only increase the availability of corrinoid in the system but may also trigger a conformational change in either MtbB1, Mtbc, or both which could lead to higher affinity for substrate. Similar observations have been made using the activation system of the methyl-CoM reductase enzyme system (Thauer, 1998). Another possible
explanation is the relatively low protein concentration in the reaction mixtures as compared to extracts. Higher protein concentrations could lead to an increased interaction of the proteins of the pathway, which could again lead to an apparent increase in the affinity for substrate. A third explanation is possible damage to MtbB1 caused by the purification process and multiple freeze-thaw procedures. For example, plasma emission spectroscopy revealed the presence of 0.43 mol zinc/mol polypeptide and 3.4 mol boron/mol polypeptide. It may be possible that either of these elements are somehow involved in the binding and activation of DMA on MtbB1. These values may represent suboptimal ratios of the element to the polypeptide leading to decreased binding of the substrate.
CHAPTER 5

GENERAL CONCLUSIONS

Summary

Early studies on the mechanisms of methylotrophic methanogenesis by Gottschalk and coworkers indicated that, like with methanol, methyl groups are transferred intact to methane (Walther et al., 1981). It was also shown that the overall stoichiometry of the reaction is the same in methanogenesis from methylamines as it is from methanol, in that one methyl group is oxidized to generate the necessary reducing equivalents to reduce three methyl groups to methane (Hippe et al., 1979). However, prior to the start of this project, little was known about the enzymes responsible for methylation of CoM with methylamines. Work done by Burke and Krzycki (1995) in our laboratory demonstrated for the first time the involvement of MtB and MtM in methanogenesis from MMA. However, it was suggested previously that separate inducible enzyme systems existed for the breakdown of each of the methylamines (Naumann et al., 1984). Therefore, we undertook a project
with the goals of determining the role of the previously identified MtbA as well as identifying and initially characterizing the enzymes responsible for the conversion of TMA and DMA to methyl-CoM.

1) Identification of the role of the methylcorrinoid:CoM methyltransferase MtbA in methanogenesis from methylamines

Using a method which involved the specific removal of MtbA and subsequent addition of purified MtbA from TMA grown cell extracts of *M. barkeri*, it was determined that MtbA plays a key role in methanogenesis from TMA, DMA, and MMA. Removal of MtbA from extracts tested for their ability to methylate CoM from MMA or DMA resulted in essentially complete depletion of DMA:CoM or MMA:CoM methyl transfer activity. Subsequent addition of purified MtbA to these depleted extracts resulted in complete restoration of the DMA and MMA dependent CoM methylation activities. Addition of purified MtaA, the methylcorrinoid:CoM methyltransferase involved in methanol dependent CoM methylation, to the depleted extracts had no effect on DMA:CoM or MMA:CoM methyl transfer pathways. Removal of MtbA from extracts tested for their ability to methylate CoM with TMA only depleted approximately 80% of the TMA:CoM methyl transfer activity. Subsequent addition of either MtbA or MtaA could restore TMA:CoM methyl transfer activity. A direct correlation between the amount of MtbA activity in the extract and the amount of DMA:CoM methyl transfer activity in the extract was seen. These results indicated that MtbA is used exclusively in the DMA:CoM and MMA:CoM methyl transfer pathways and that MtbA is used in the TMA:CoM pathway although MtaA may substitute
in the pathway as well. The involvement of MtbA in the methylamine conversion pathways also indicated that at least one corrinoid protein was involved in each of these pathways.

2) Reconstitution of the TMA:CoM methyl transfer pathway using MtbA and the newly isolated MttBC complex

Methylation of CoM with TMA was determined to require three polypeptides: a 52 kDa polypeptide termed MttB, a 26 kDa polypeptide termed MttC, and the 37 kDa methylcorrinoid:CoM methyltransferase MtbA. The purified MttBC fraction stimulated the rate of methyl-CoM formation in extracts seven fold, up to 1.7 µmol/min/mg MttBC protein. The MttBC polypeptides had molecular masses of 52 kDa and 26 kDa. Gel permeation of the MttBC fraction demonstrated that the 52 kDa polypeptide eluted with an apparent molecular weight of 280 kDa. The 26 kDa protein eluted primarily as a monomer, but some 26 kDa polypeptide also eluted with the 280 kDa peak, indicating that the two proteins weakly associate. The two polypeptides could be completely separated using gel permeation in the presence of SDS. The corrinoid remained associated with the 26 kDa polypeptide at a molar ratio of 1.1 corrinoid/26 kDa polypeptide. This polypeptide was therefore designated as the trimethylamine corrinoid protein, or MttC. The MttBC polypeptides, when supplemented with purified MtbA, could effect the demethylation of trimethylamine with the subsequent methylation of coenzyme M and production of DMA at specific activities up to 600 nmol/min/mg MttBC protein. Neither DMA or MMA could serve as substrates and the activity required Ti(III) citrate and methyl viologen. MttBC could interact with either
homologue of the methylcorrinoid:CoM methyltransferase, but had the highest affinity for MtbA. These results suggested that MttC is uniquely involved in TMA dependent methanogenesis, that this corrinoid protein is methylated by substrate and demethylated by either methylcorrinoid:CoM methyltransferase homologue, and that the predominant homologue found in TMA grown cells, MtbA, is the favored participant in the TMA:CoM methyl transfer reaction.

3) Reconstitution of the DMA:CoM methyl transfer pathway using only purified MtbB1, MtbA, and the discrete corrinoid protein MtbC

Methyl transfer from DMA to CoM was reconstituted in vitro for the first time using only highly purified proteins. These proteins included the previously unidentified MtbC which copurified with MtbA. MtbC binds 1 mol corrinoid/mol 24 kDa polypeptide and stimulated DMA:CoM methyl transfer 3.4 fold in extracts. Purified MtbA and MtbC were used to assay and purify a DMA:corrinoid methyltransferase, MtbB1. MtbB1 is a 230 kDa protein composed of 51 kDa subunits that do not bind a corrinoid prosthetic group. Purified MtbB1, MtbA, and MtbC were the sole protein requirements for in vitro DMA:CoM methyl transfer. A MtbB1:MtbC ratio of 1 was optimal for CoM methylation with DMA. MtbB1 methylated either corrinoid bound to MtbC or free cob(I)alamin with DMA, indicating that MtbB1 carries an active site for DMA demethylation and corrinoid methylation. Specific activities for this system have been measured ranging from 0.5 to 2.2 μmol/min/mg MtbB1. Experiments in which different proteins of the resolved MMA:CoM methyl transfer reaction replaced proteins involved in
DMA:CoM methyl transfer indicated high specificity for MtbB1 and MtbC for DMA:CoM methyl transfer activity. These results indicate MtbB1 demethylates DMA and specifically methylates the corrinoid prosthetic group of MtbC, which is subsequently demethylated by MtbA to methylate CoM during methanogenesis from DMA.

**Discussion and future work**

Initially, the study of the enzymes responsible for methylotrophic methanogenesis was thought to be a highly specialized field with relevance to other systems coming primarily from the homology of methanogen corrinoid proteins to other known corrinoid proteins such as MetH. However, systems from the Bacteria domain have recently come to light with enzymes highly analogous and, in some instances, homologous to the methanogenic enzymes described in this study.

Work done by Diekert and coworkers has shown the existence of an O-demethylase system in the methylotrophic homoacetogen *Acetobacterium dehalogens* utilizing two methyltransferases and a corrinoid protein in a manner highly analogous to the methylamine:CoM methyl transfer systems in *Methanosarcina* species (Kaufmann et al., 1997; Kaufmann et al., 1998). The initial enzyme in the system, OdmB, is a vanillate:corrinoid protein methyltransferase which demethylates the phenyl methyl ether, vanillate, and methylates the corrinoid protein, OdmA. OdmA is then demethylated by a methylcorrinoid protein:THF methyltransferase. OdmB appears to be a functional analogue of the methanol: and methylamine:corrinoid methyltransferases and OdmA is certainly analogous to the methanogen
corrinoid proteins. OdmA is homologous to MtmC (55% similarity) and MtaC (54% similarity) as well the cobalamin binding region of MetH (60% similarity) (Kaufmann et al., 1998). The reaction catalyzed by the methylcorrinoid protein:tetrahydrofolate methyltransferase is actually most analogous to the reverse reaction catalyzed by the MtrH subunit of the methyl-THMP:CoM methyltransferase. However, the enzyme is still quite analogous to the methylcorrinoid:CoM methyltransferases, MtaA and MtbA.

Another analogous system has been found in the aerobic methylotrophic Methylobacterium sp. strain CM4, an α-proteobacterium (Vanelli et al., 1999). As mentioned in Chapter 4, this system contains an enzyme which represents a fusion of a substrate:corrinoid methyltransferase and a corrinoid protein. This protein, CmuA, catalyzes the demethylation of chloromethane and the automethylation of its bound corrinoid. CmuA is then demethylated by a methylcorrinoid:THF methyltransferase (Vanelli et al., 1999) analogous to the second methyltransferase described above. Once again, this system invokes the general mechanism utilized by methanogens in methylotrophic methanogenesis.

This existence of these two enzyme systems illustrates the effectiveness of the model shown in Figure 33, in which a discrete corrinoid protein interacts with two separate methyltransferases to catalyze the transfer of a methyl group from substrate to a coenzyme. The fact that the Odm and Cmu enzyme systems are found within the Bacteria domain indicates that this model is not limited to the Archaea and may indicate that this model is more widespread than originally anticipated.
This method of methylotrophic metabolism is interesting from an evolutionary point of view as well. It is likely that the known corrinoid proteins share a common ancestry due to their high degree of sequence homology (Paul et al., 2000) and the second methyltransferases tend to share sequence homology with functionally analogous enzymes as well (Harms et al., 1995; LeClerc and Grahame, 1996; Harms and Thauer, 1996). However, the substrate:corrinoid methyltransferases sequenced thus far share no sequence homology (Paul et al., 2000) and yet interact with homologous corrinoid proteins with high specificity. This may be due to highly conserved secondary and tertiary structures in the substrate:corrinoid methyltransferases.

The crystal structure is not yet known for these enzymes although preliminary work is underway in our laboratory to determine the structure of MtmB. The relative ease of isolation of MtbB1 suggests that it is a prospective candidate for crystallization as well. Initial purifications of MtmB were performed anaerobically and, under these conditions, MtmB and MtmC readily separate during the purification process and do not appear to form a tight complex (Burke and Krzycki, 1997). It has been observed, however, that aerobically purified MtmB and MtmC appear to form a tight complex (T.K. Hill, personal communication). Crystals have been obtained in a solution containing the MtmBC complex (T.K. Hill and M. Chan, personal communication), although it is currently unknown whether these crystals contain both proteins. If the structure of the MtmBC complex can be solved, this information will be very useful in determining the secondary and tertiary structure of MtmB to be used in future comparative studies with other substrate:corrinoid methyltransferases. It would also be interesting to
determine how MtmB and MtmC interact, at least when MtmC is in the hydroxycob(III)alamin state.

The fact that the substrate:corrinoid methyltransferases of the different systems are not homologous raises the question of whether their respective reaction mechanisms are also unique. This is especially true in the case of the methylamine:corrinoid methyltransferases. It has been shown that the genes encoding these enzymes each contain an in-frame and read-through amber (UAG) codon (Burke et al., 1998; Paul et al., 2000). The fact that MttB and MtbB1 are approximately 50 kDa polypeptides as isolated (Chapter 2 and Chapter 3, respectively) indicates that these amber codons are read-through and do not function as stop codons. The structure of the amino acid encoded by the amber codon is currently unknown although preliminary evidence has suggested that a lysine, or possibly a modified lysine, residue may be encoded at that site (C.M. James, personal communication). An additional copy of mtmb and two additional copies of mtbb have been discovered in the genome as well which each contain the in-frame amber codon (Burke et al., 1998; G. Srinivasan, unpublished results; Paul et al., 2000). The fact that all these genes contain the amber codon, which is not found in mtSA (Paul and Krzycki, 1996) or in mtAB (Sauer and Thauer, 1997), could indicate a mechanism shared by these enzymes for binding and possibly demethylation of methylamines specifically.

In order to test these possibilities, it will first be necessary to determine the structure of the residue encoded by the amber codon. Work is currently underway using mass spectroscopy to determine the mass of the residue. It may also be possible to perform NMR on a small fragment of MtmB
containing the residue of interest to further confirm the identity of the residue (C.M. James, personal communication). If the structure of the MtmbC complex can be obtained we would be able to determine if the residue encoded by the amber codon is positioned in such a way as to allow access to the corrinoid face. If so, this would be one indication that this residue is involved in catalysis. However, given the fact that the TMA and DMA methyltransferases are not homologous to Mtmb, it will be necessary to solve the structure of either MttaB or MttaB1 to determine if these proteins adopt a conformation similar to Mtmb before drawing any general conclusions regarding the role of the residue.

The possibility still remains, however, that the amber codon is not involved in catalysis but instead represents a means of regulation for these enzymes. If the residue encoded by the amber codon is in fact lysine, this may be a mechanism by which the cell responds to the levels of nitrogen in the environment. If nitrogen levels are low in the environment the cells may induce the expression of these genes as a means of scavenging for nitrogen since one of the end products of the methylamine catabolic pathways is ammonia. Therefore, induction might occur during periods of high methylamine availability or low ammonia availability or both and repression might then occur during periods of low methylamine availability or high ammonia availability or both. This is somewhat difficult to imagine however since this implies the increased read-through of a codon encoding a nitrogenous amino acid during periods of low nitrogen availability. There is evidence to suggest that mtmb and mtmc are expressed in methanol grown cells during periods of low ammonia availability (Burke, 1997). This may
provide a competitive advantage for *Methanosarcina* spp. in marine environments, where methylamines are plentiful (Zinder, 1993), due to the organism’s ability to utilize methylamines as a nitrogen source as well as a carbon source.

Although our ability to reconstitute the methylamine:CoM methyl transfer pathways using purified proteins has greatly increased our understanding of methylotrophic methanogenesis, the initial kinetic studies conducted thus far show inconsistencies between the resolved systems and extracts. It was originally suspected that incorporation of the natural activation system (RAM and ATP) into the assay mixtures would greatly increase the specific activities of the pathways. This has now been shown not to be the case, with specific activity only increasing approximately 20% in most cases (T.K. Hill, personal communication). However, in the cases of DMA:CoM (Figure 25) and MMA:CoM (T.K. Hill, personal communication) methyl transfer, there appears to be a higher Km for substrate when using purified proteins as compared to cell-free extract. It may be possible to decrease the apparent Km for substrate in the reconstituted pathways by replacing the Ti(III) citrate/methyl viologen artificial reducing system with RAM and ATP. Evidence for this has been shown by Wassenaar et al. (1998a; 1998b) using DMAMT in which they incorporated MAP fractions into their DMA:CoM assays and found an apparent Km for DMA of 0.5 mM. This phenomenon, if it is shown to exist in our systems, may be due to changes in the conformation of these enzyme systems. For example, interactions between the methylamine:corrinoid methyltransferases and their cognate
corrinoid proteins may become more favorable in the presence of RAM leading to an apparent increase in affinity for substrate.

It may be possible that the enzymes within a given methylamine:CoM methyl transfer pathway naturally exist in large complexes within the cell. It may also be possible that, when cells are grown on TMA, large complexes exist containing the enzymes of the TMA, DMA, and MMA catabolic pathways thereby allowing for much greater efficiency in the breakdown of these substrates. For example, when a molecule of TMA enters the complex, the DMA produced could be immediately utilized by the enzymes of the DMA:CoM methyl transfer pathway which would then produce MMA which would also be immediately utilized. A similar complex could also form when grown on DMA which does not include MttB and MttC. It would be interesting to determine if these complexes do exist within the cell and, if so, what are the composition and stoichiometry of these complexes.

As mentioned previously, work done in our laboratory has demonstrated the existence of multiple copies of the *mtm* and *mtb* genes. It is interesting to note that genes encoding putative permeases have been found within the *mtm*, *mtt/mtb*, and *mtb* operons (Burke *et al.*, 1998; Paul *et al.*, 2000). This could help explain the existence of the multiple copies of the genes encoding methylamine:CoM methyl transfer enzymes. Perhaps the methyltransferases encoded in the *mtm*, and *mtb* operons are uniquely suited to interact with their cognate permeases when the cells are grown on MMA or DMA respectively. This would also explain why there is only a single copy of the TMA pathway genes since the cells would only encounter TMA externally as opposed to DMA and MMA which can be produced
internally as catabolic end products. If these large complexes do exist then it may also be possible that these complexes interact with these permeases at the cell surface to funnel growth substrate directly into the catabolic enzyme complex.

By eluting extracts of TMA grown cells from a size exclusion chromatography column, it may be possible to detect these large molecular weight complexes by measuring the ability of fractions eluting from the column to convert TMA, DMA, and MMA to methyl-CoM. Antibodies raised against the proteins of the different pathways could also be used to detect these complexes. Either of these methods could quantitatively determine the composition of these putative complexes. It may be difficult to determine if these complexes contained or interacted with permeases due to the fact that the permeases are integral membrane proteins and would likely be separated from the complex upon lysis of the cells. However, truncated forms of these permeases, which do not contain the membrane spanning regions, could be engineered to examine the interactions of these proteins with the large complexes.

Significance of these studies

The data presented here significantly increased the current understanding of methanogenesis from TMA and DMA. Prior to this work, the enzymes involved in CoM methylation from DMA and TMA were unknown and no general model for CoM methylation from methylotrophic substrates during methanogenesis could be constructed. This work has identified the proteins involved in the TMA:CoM methyl transfer pathway
which led to the cloning and sequencing of the genes encoding these proteins. This work also led to the identification of a gene of previously unknown function, mtbC, involved in DMA catabolism and established the existence of a pathway for CoM methylation with DMA unique from the pathway previously described. This work led to the discovery that the in-frame amber codon present in the gene encoding the MMA:corrinoid methyltransferase is also present in the DMA:corrinoid and TMA:corrinoid methyltransferase genes as well. The work described here also demonstrated that MtbB1 and Mt/B are produced as full length products indicating that the amber codon is read-through and does not function as a stop codon. The isolation and characterization of the enzymes involved in methanogenesis from the methylamines, much of which is described in these studies, has allowed for the development of a general model for initiation of methyl transfer during methylotrophic growth. This mechanism has now proven applicable to some cases of bacterial one carbon metabolism as well. Therefore, these studies have elucidated a mechanism by which a variety of organisms may initiate catabolism of methylotrophic or one carbon substrates.
LIST OF REFERENCES


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Zhao S. and S. W. Ragsdale. 1996. A conformational change in the methyltransferase from Clostridium thermoaceticum facilitates the methyl transfer from (6S)-methyltetrahydrofolate to the corrinoid/iron-sulfur protein in the Acetyl-CoA pathway. Biochemistry. 35:2476-2481


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