The MtsA Subunit of the Methylthiol:Coenzyme M Methyltransferase of Methanosarcina barkeri Catalyses Both Half-reactions of Corrinoid-dependent Dimethylsulfide: Coenzyme M Methyl Transfer*

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Methanogenesis from dimethylsulfide requires the intermediate methylation of coenzyme M. This reaction is catalyzed by a methylthiol:coenzyme M methyltransferase composed of two polypeptides, MtsA (a methylcobalamin: coenzyme M methyltransferase) and MtsB (homologous to a class of corrinoid proteins involved in methanogenesis). Recombinant MtsA was purified and found to be a homodimer that bound one zinc atom per polypeptide, but no corrinoid cofactor. MtsA is an active methylcobalamin: coenzyme M methyltransferase, but also methylates cob(I)alamin with dimethylsulfide, yielding equimolar methylcobalamin and methanethiol in an endergonic reaction with a $K_{eq}$ of $5 \times 10^{-4}$. MtsA and cob(I)alamin mediate dimethylsulfide:coenzyme M methyl transfer in the complete absence of MtsB. Dimethylsulfide inhibited methylcobalamin:coenzyme methyl transfer by MtsA. Inhibition by dimethylsulfide was mixed with respect to methylcobalamin, but competitive with coenzyme M. MtbA, a MtsA homolog participating in coenzyme M methylation with methanethiol, was not inhibited by dimethylsulfide and did not catalyze detectable dimethylsulfide:cob(I)alamin methyl transfer. These results are most consistent with a model for the native methylthiol:coenzyme M methyltransferase in which MtsA mediates the methylation of corrinoid bound to MtsB with dimethylsulfide and subsequently demethylates MtsB-bound corrinoid with coenzyme M, possibly employing elements of the same methyltransferase active site for both reactions.

Methanosarcina species and related genera comprise a branch of methanogenic euryarchaeota capable of methylo trophic methanogenesis from compounds such as methanol, trimethylamine (TMA), dimethylamine (DMA), and monomethylamine (MMA) (1, 2). During methylotrophic methanogenesis, substrate methyl groups are reduced to methane with reducing power derived from the simultaneous oxidation of methyl groups to carbon dioxide (1, 2).

In recent years, it has been found that methylated thiols such as dimethylsulfide (DMS) and methylmercaptopropionate (MMPA) are also methylo trophic methanogenic precursors (3–7). These compounds arise in nature from processes such as the breakdown of methionine (8), reduction of dimethyl sulfide (9), anaerobic degradation of methoxylated aromatics (10, 11), photosynthesis by some anaerobic photrophs (12), and demethylation of the osmolyte dimethylsulfoniopropionate (13, 14). Since emission of DMS over the open ocean is thought to influence cloud formation, the metabolism of these compounds has wide-ranging implications (15).

Methanosarcina species such as Methanosarcina acetivorans and Methanosarcina siciliae will couple growth to methane production from the methylated thiols DMS and MMPA (3, 4, 16, 17). In contrast, Methanosarcina barkeri was long thought not to utilize methyli thio ls as methane precursors (18). However, it was found that this species differentially expressed enzymes to produce methane from DMS or MMPA depending upon its growth substrate (19, 20). During growth on acetate, but not methanol, cells of M. barkeri MS will convert either methylated thiol to methane. Cell-free extracts of these cells will catalyze methylation of coenzyme M (CoM) with either DMS or MMPA. Methyl-CoM is the precursor of methane with all known methanogenic substrates, and its reduction is the major site of energy conversation in methanogens (1, 2).

A single enzyme was identified and purified from acetate grown cells of M. barkeri (21) that carries out CoM methylation by either DMS or MMPA (19). This methylthiol:CoM methyltransferase is a 480-kDa corrinoid protein that is comprised of equimolar amounts of 40- and 30-kDa subunits termed, respectively, MtsA and MtsB. One corrinoid cofactor is bound per MtsA:MtsB pair, and six or seven MtsA:MtsB pairs comprise the 480-kDa protein (21). The corrinoid cofactor in the Co(I) state is methylated by DMS, while the methylated Co(III) corrinoid can in turn be demethylated by CoM, regenerating the Co(I) corrinoid cofactor (19). These results indicate that protein-bound corrinoid serves as an intermediate in the methylation of CoM with DMS or MMPA.

The methylation of CoM with methylated thiols such as DMS by a two-subunit protein (19) stands in contrast with the three component systems effecting CoM methylation with methanol (22–24), monomethylamine (25), dimethylamine (26), or trimethylamine (27) (Fig. 1, panel A). In the latter CoM methylation pathways, a corrinoid-binding protein sequentially interacts with two methyltransferases. Substrate:corrinoid methyltrans-
ferases initiate the demethylation of the methanogenic substrate and methylation of a cognate corrinoid protein that specifically interacts with that methyltransferase (25, 26, 28). Each cognate corrinoid protein is then demethylated by a methylcorrinoid:CoM methyltransferase. In the case of the methylamines, the methylcorrinoid:CoM methyltransferase is a single protein, MtbA (29). A different yet homologous protein, MtaA, methylates CoM with the methanol cognate corrinoid protein, MtcA (24).

The sequence of genes encoding the polypeptides effecting CoM methylation with methylamines (26, 30–32) and methanol (24, 32) have revealed similarities to the two subunits of the methylthiol:CoM methyltransferase (33) (see Fig. 1 and accompanying legend). MtsA is 50% similar over its entire length to the methylcorrinoid:CoM methyltransferases of the methylene (MtbA) and methanol (MtaA) pathways. Consistent with this, the methylthiol:CoM methyltransferase has a methylocobalamin:CoM methyltransferase activity, which is significantly enhanced in the presence of SDS (34). Activity staining of SDS-PAGE gels revealed that MtsA catalyzes methylocobalamin:CoM methyl transfer, while MtsB did not display this activity (34).

The primary structure of MtsB itself is on the average 50% similar to the entire lengths of each of the cognate corrinoid proteins of the methanol- and methylene-dependent CoM methylation pathways (Fig. 1). The methylamine cognate corrinoid proteins each bind one corrinoid per polypeptide (21, 26, 35), and these proteins as a group are homologous to the cobalamin binding domains of methionine synthase, as well as coenzyme B12-dependent enzymes (30, 33, 36). MtsB contains all expected signature residues for binding corrinoid (33, 37) and is therefore the likely corrinoid binding subunit of methylthiol:CoM methyltransferase. Thus, a clear route for the demethylation of methylated MtsB corrinoid protein and methylation of CoM can be proposed, in that MtsA mediates the demethylation of MtsB and methylation of CoM (Fig. 1, panel B). It is striking, however, that MtsA:MtsB complex is capable of demethylating DMS without a third polypeptide initiating methyl transfer from DMS, such as seen in the otherwise completely analogous pathways for CoM methylation from methanol or the methylamines, such as MMA (Fig. 1, panel A). This leaves the manner by which the methylthiol:CoM methyltransferase effects demethylation of DMS an open question. Unfortunately, efforts to physically separate the two subunits using denaturants and chromatography to search for the sites of the relevant subreactions have not yielded active preparations of either subunit.2

To resolve this problem, we expressed MtsA as a recombinant protein, and tested its ability to interact with CoM, DMS, and free cobalamin. We find that MtsA acts both to demethylate DMS and to methylate CoM using non–protein-bound cobalamin as an intermediate methyl carrier. Thus, this subunit carries out both CoM methylation and DMS demethylation and can account for both corrinoid-dependent methyltransferase activities of the methylthiol:CoM methyltransferase. This appears to not be a universal property of methylcorrinoid:CoM methylases, since a representative homologous CoM methylase, MtbA, does not interact with DMS in any detectable manner.

**Experimental Procedures**

**Chemicals and Reagents—** N2 was purchased from Linde Specialty Gases (Columbus, OH) and passed through catalyst R3-11 (Chemical Dynamics Corp., South Plainfield, NJ) to remove O2 prior to use. MOPS, 2-mercaptoethanesulfonate (CoM), dithiothreitol (DTT), hydroxycobalamin, methylcobalamin, isopropyl-1-thio-β-D-galactopyranoside (IPTG), EDTA, and Tris were purchased from Sigma. Sodium dodecyl sulfate (SDS), acrylamide, 2-mercaptoethanol, bromothymol blue, and Coomasie R-250 were purchased from Bio-Rad. Titanium(III) chloride was purchased from Roche Molecular Biochemicals.

2 T. C. Tallant, unpublished results.
were grown in Luria-Bertani broth (40) with 34 mM 
E. coli of MtsA in hydrochloride, 50 mM DTT, and 20 mM 2-mercaptoethanol. MtsA was eluted at a 
50–500 mM NaCl gradient in the same buffer. MtsA eluted at 
parts anaerobic 1M KCl, 10 mM DTT, 10 mM 2-mercaptoethanol, 100 mM 
solution before solubilization in anoxic 6M guanidine 
and 100 mM NaCl. Solubilized inclusion bodies were then washed with 0.5, 1, and 2 M urea and again 
3 g for 15 min at 4 °C. The inclusion bodies were then 
grown in duplicate. The apparent 
control of a T7 promoter. Further details are found under “Experimental Procedures.”

FIG. 2. Construction of pET-40ex for heterologous expression of MtsA in E. coli. A NdeI restriction site was introduced overlapping the 
start codon of mtsA by PCR and the PCR fragment introduced into 
pGEM-T to generate pGEM-4. A complete mtsA gene was reconstructed from the 
HindIII/PstI and PstI/SalI fragments from pET500 and 
pGEM-4, clones generated during the sequencing of mtsA and mtsB, and 
pGEM-4 to generate pGEM-4HS. MtsA was excised from pGEM-4HS 
with NdeI and SalI and inserted into pET-17b digested with NdeI/ 
EcoRV to generate pET-40ex with transcription of mtsA under the 
control of a T7 promoter. Further details are found under “Experimental Procedures.”

otherwise present in the pET-17b vector. The mtsA insert and flanking vector regions were sequenced. The mtsA gene in the insert was found to be identical to the previously characterized mtsA sequence (GenBank™ U63637) and correctly oriented in the expression vector.

Preparation of MtsA and MtsB— Cultures of E. coli with pET-40ex were grown in Luria-Bertani broth (40) with 34 μg/ml chloramphenicol and 100 μg/ml ampicillin to an optical density of 0.6 at 600 nm and induced with 0.4 mM IPTG at 37 °C with aeration. Samples were removed at 1-h intervals for analysis by polyacrylamide gel electrophoresis. Cultures were harvested 3 h after induction, washed with 50 mM MOPS, pH 7.0, and resuspended in 50 mM Tris, pH 8.0, with 2 mM EDTA. Cells were lysed by passage through a French pressure cell at 20,000 pounds per square inch.

Cell debris was removed from the extract by centrifugation at 1000 × 
g for 5 min at 4 °C, and then the inclusion bodies were pelleted by 
subsequent centrifugation at 27,000 × g for 15 min at 4 °C. The inclusion 
bodies were then washed with 0.5, 1, and 2 mM urea and again collected by centrifugation before solubilization in anoxic 6 M guanidine hydrochloride, 50 mM DTT, and 20 mM 2-mercaptoethanol. MtsA was refolded by rapid dilution of 1 part solubilized inclusion bodies into 59 parts anaerobic 1 mM KCl, 10 mM DTT, 10 mM 2-mercaptoethanol, 100 μM ZnCl2, in 0.5 mM Tris, pH 8.0. The solution was stirred for 20 h at 20–24 °C. All anoxic solutions were prepared by repeated evacuation and flushing with 
N2.

The refolded MtsA was concentrated by ultrafiltration, dialyzed against 50 mM MOPS, pH 6.5, and further purified by anion exchange chromatography using an Uno column (Bio-Rad) equilibrated with 100 mM MOPS, pH 6.5. Proteins binding to the column were eluted with a 50–500 mM NaCl gradient in the same buffer. MtsA eluted at ~130 mM NaCl.

MtbA, the methylcobalamin:CoM methyltransferase of methylamine catabolism, (formerly known as MT2-A) was purified from M. Barkeri as described previously (35).

Metal Analysis of MtsA—MtsA was dialyzed to equilibrium against 2 liters of 10 mM TES, pH 7.0, and 0.7 mg (2 ml) dialyzed MtsA submitted for metal analysis. MtsA and a sample of the dialysis buffer were analyzed for the content of 30 metals by plasma emission on a Thermo Jarrell-Ash 965 inductively coupled argon plasma spectrometer by the Chemical Analysis Laboratory (University of Georgia, Athens, GA). The metal content for MtsA was determined by subtraction of the metal content of the dialysis buffer from the metal content of the dialyzed protein sample.

**Gel Electrophoresis and Molecular Sieving**—Denaturing polyacrylamide gel electrophoresis (12% acrylamide) was performed with the buffer system of Laemmli (41) in the presence of 0.1% SDS, using a Mini-slab electrophoresis system (Idea Scientific Co., Minneapolis, MN). Samples for electrophoresis were prepared with 5% mercaptoethanol, 1% SDS, 10% glycerol, and 0.002% bromthymol blue and were heated to 80 °C for 15 min prior to electrophoresis. Molecular size markers (Bio-Rad) were β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and hen egg white lysozyme (14.4 kDa). Gels were stained with Coomassie R-250.

The molecular mass of renatured recombinant MtsA was estimated with a Biologic medium pressure chromatography unit (Bio-Rad) equipped with a Superose 6HR column (Amersham Pharmacia Biotech) and eluted with 100 mM NaCl, 50 mM MOPS, pH 7.0, at a flow rate of 0.4 ml/min. Molecular mass standards (Sigma) for the Superose 6HR column included β-amylose (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (31 kDa), and cytotoxic e (12.4 kDa).

**Methylcobalamin:CoM Methyltransferase Activity**—CoM methylation with methylcobalamin was measured by either the cyanide derivatization assay or by direct spectral assay. For the cyanide derivatization assay, samples (300 μl) were prepared in nitrogen-flushed vials at 4 °C containing 1–20 μg of MtsA or MtbA, 1 mM methylcobalamin, and 20 mM CoM in MOPS buffer, pH 7.0, unless otherwise stated. Concentrations of methylcobalamin and CoM were varied for determination of apparent 
Km values. Reactions were initiated by transfer of the vials to 37 °C, and methyltransferase activity and the reaction rates determined by periodic removal of aliquots that were subjected to the cyanide derivatization (42). In the cyanide derivatization procedure, hydroxycobalamin reacts with cyanide to generate dicyanocobalamin while methylcobalamin does not react with cyanide (42, 43). Rates were determined with a minimum of three linear time points. The apparent 
Km value reported in the paper were the average of two determinations that varied by no more than 6%, the only exception being the apparent 
Km value for CoM in the methylcobalamin:CoM methyltransferase reaction, which is an average of five separate determinations with S.D.

**DMS:CobDialamyl Methyl Transfer—**CobDialamyl methylation was measured by the increase in absorbance at 540 nm upon methylcobalamin demethylation (10) in the presence of 0.2 mM methylcobalamin in 
N2-flushed cuvets (1-cm pathlength) under dim red light at 22 °C. Samples (750 μl) were prepared with 5 μg of MtbA and 40 μM 
CoM in MOPS buffer, pH 7.0.

**DMS:CobDialamyl Methyl Transfer—**CobDialamyl methylation was measured by the increase in absorbance at 540 nm upon 
methylcobalamin demethylation (10). Methyl transfer was performed in 
N2-flushed cuvets (2-mm pathlength) under dim red light at 22 °C. Assays were performed with 0.18–1.2 mg/ml MtsA or MtbA, 0.5–2.5 mg cobDialamyl, and 20–200 mM soluble DMS. CobDialamyl was generated from an equal amount of 
hydroxycobalt(III)dialamyl and 6-fold greater concentration of titanium(III) citrate.

**Methanethiol (MTHS)** generated from the cobDialamyl-dependent 
demethylation of DMS was measured by gas chromatography using a 
model SA gas chromatograph (Shimadzu Scientific Instruments, Columbia, MD) equipped with a flame ionization detector and a 0.5-meter Porapak R column (Alltech Associates Inc., Deerfield, IL). The column, injector, and detector were at 130 °C. The carrier gas was nitrogen at a flow rate of 20 ml/min. It was determined that, under the conditions of the assay, 84% of total MSH in the closed vial was in the liquid phase of the reaction mixture at equilibrium.

**DMS:CobMethyl Transfer—**MtsA catalyzed CoM methylation with DMS in the presence of 1 mM cobDialamyl was measured by monomobominate derivatization of thiols and analysis of bimane derivatives by reverse phase HPLC as described previously (19) and based on the procedures of Fahey and co-workers (44, 48). Reactions were performed in 
N2-flushed vials under dim red light at 37 °C. Reactions contained 0.53 mg/ml MtsA or 1.2 mg/ml MtbA, 10 mM CoM, 100 mM DMS, and 1 mM cobDialamyl generated from the reduction of 1 mM hydroxycobalamin by 6 mM titanium(III) citrate. Samples were removed at 30-min intervals for monomobominate derivatization. Reactions were performed in duplicate.
Protein samples are as indicated across the top of the gel: with pET-40ex induced with IPTG and purified recombinant MtsA.

denaturing polyacrylamide (12%) electrophoretic gel of whole cell extracts of largely in an insoluble form.

denaturation of the oxidation product of cob(D)alamin. The inset is a Lineweaver-Burk plot of the same data.

RESULTS

Heterologous Expression of MtsA—Methylcobalamin:CoM methyltransferase activity was quantified in cells of E. coli BL21(DE3)pLysS transformed with pET-40ex using an assay based on cyanide derivatization of the hydroxycobalamin produced from oxidation of the cob(D)alamin generated by demethylation of methylcobalamin (42). The 30,000 X g supernatant of extracts made with pET-40x-transformed cells catalyzed CoM-dependent demethylation of 16 nmol of methylcobalamin min^-1 mg^-1 total protein with 1 mM methylcobalamin and 20 mM CoM. No methylcobalamin:CoM methyltransferase activity was detectable in extracts of cells transformed with pET-17b lacking the mtsA insert. Although no increase in soluble methyltransferase activity was observed following induction with IPTG, a notable increase in a prominent 41-kDa polypeptide, the expected size of MtsA, was found upon electrophoresis of SDS extracts of induced cells (Fig. 3). Microscopic examination of the induced cells revealed the presence of inclusion bodies, indicating that MtsA had been expressed largely in an insoluble form.

The inclusion bodies were harvested from cells 3 h after induction and solubilized using 6 mM guanidine hydrochloride, 50 mM DTT, and 20 mM 2-mercaptoethanol. The solubilized protein was diluted 60-fold with a variety of refolding buffers, pH 8.0, and methylcobalamin:CoM methyltransferase activity was measured at 1, 4, and 24 h after dilution. Optimal activity was achieved by protein refolding at room temperature for 24 h in refolding buffer consisting of 500 mM Tris, pH 8.0, 1 mM KCl, 10 mM DTT, 10 mM 2-mercaptoethanol, and 100 µM ZnCl₂. Refolding buffers containing 500 mM guanidine hydrochloride, 33 mM CHAPS, 0.5% Triton X-100, 5 mM SDS, or 20% glycerol did not produce active methyltransferase, nor did refolding at 4 °C in the optimal refolding buffer. Preparations of recombinant MtsA made without addition of zinc typically had 3-fold lower methylcobalamin:CoM methyltransferase activity than MtsA refolded in the presence of added zinc.

The solubilized and refolded MtsA was further purified by a subsequent anion exchange chromatography step. SDS-PAGE of the purified recombinant MtsA preparation revealed a single homogenous polypeptide with a molecular mass of 41 kDa (Fig. 3). Approximately 40 mg of purified MtsA was obtained from 1 liter of induced recombinant cells. The purified recombinant enzyme catalyzed methylcobalamin:CoM methyl transfer at 15.8 µmol min^-1 mg^-1 MtsA in the presence of 20 mM CoM and 1 mM methylcobalamin. For comparison, the methylcobalamin:

CoM methyltransferase activity of the purified methylamine specific CoM methylase, MtbA, was measured as 34.6 µmol of methyl groups transferred min^-1 mg^-1 MtbA when assayed under the same conditions. MtsA in association with MtsB (the native methylthiol:CoM methyltransferase) catalyzed methylcobalamin:CoM methyl transfer at 0.4 µmol min^-1 mg^-1 (34).

Characteristics of Recombinant MtsA—Methylcorrinoid:CoM methyltransferases are typically monomers in solution. However, the molecular mass of purified and refolded MtsA was determined by size exclusion chromatography as 77 kDa, indicating recombinant MtsA is a homodimer in solution. The purified protein was examined for metals by plasma emission spectroscopy. The only metal bound at stoichiometric levels was zinc, and the purified protein bound 1.06 nmol of zinc/nmol of MtsA polypeptide. Any cobalt bond to the enzyme was below the lower level of detection, which was 0.1 cobalt/polyepitope. The spectrum of MtsA was essentially featureless except for the peak at 279 nm, indicating that the enzyme as isolated from E. coli bound no corrinoid or other UV-visible detectable prosthetic group. An extinction coefficient of 86.9 mM^-1 cm^-1 at 279 nm was calculated for the homodimer of MtsA.

Kinetic analysis of the methylcobalamin:CoM methyltransferase reaction catalyzed by the recombinant enzyme was undertaken. The reaction did not appear to be first order with respect to methylcobalamin in the presence of 40 mM CoM and best fit a curve approaching saturation with this substrate (Fig. 4). However, complete saturation of the reaction rate was not possible due to the limited solubility of methylcobalamin (Fig. 4). The data allowed calculation of an apparent $K_m$ value of 5.5 mM methylcobalamin and an apparent $V_{max}$ of 103 µM of CoM methylated min^-1 mg^-1 MtsA. In contrast, a complete saturation of the reaction rate with respect to CoM was observed in the presence of 1 mM methylcobalamin, the enzyme displayed an apparent $K_m$ for CoM of 10.8 ± 0.6 mM and an apparent $V_{max}$ of 21 ± 2.2 µM of CoM methylated min^-1 mg^-1 MtsA Is a DMS:Cob(D)alamin Methyltransferase—Methylthiol:CoM methyltransferase-bound corrinoid has been implicated as a methylated intermediate in the methylation of CoM with DMS. As quantified above, recombinant MtsA catalyzes methylcobalamin:CoM methyl transfer activity, confirming the previously reported role of this subunit in the demethylation of corrinoid bound to MtsB and methylation of CoM. To determine if MtsA might also be involved in the methylation of MtsB-bound corrinoid with methylated thiols such as dimethylsulfide, MtsA was examined for its ability to catalyze methylation
Cob(I)alamin was generated by reduction of 1 mM hydroxycobalamin with 6 mM Ti(III) citrate, and DMS: cob(I)alamin methyl transfer measured as the increase in absorbance at 540 nm (10). MtsA catalyzed the methylation of 78 nmol of cob(I)alamin min⁻² mg⁻¹ MtsA with 100 mM DMS (Fig. 5, panel A). To confirm that cob(I)alamin was being methylated with DMS by MtsA, the formation of methanethiol was also monitored by gas chromatography (Fig. 5, panel B). Methanethiol and methylcobalamin were formed at nearly equivalent rates and at the same stoichiometry, indicating that DMS was converted to equimolar amounts of methanethiol and methylcobalamin by MtsA.

Saturation with increasing concentrations of DMS was observed for the initial rates of the DMS: cob(I)alamin methyltransferase reaction catalyzed by MtsA. In the presence of 1 mM cob(I)alamin, MtsA had an apparent $K_m$ for DMS of 33 mM, and an apparent $V_{max}$ of 98 nmol min⁻¹ mg⁻¹ MtsA (Fig. 5, panel C). Saturation of the rate of CoM methylation with 100 mM DMS was not observed with concentrations of cob(I)alamin from 0.5 to 2.5 mM. The rate of cob(I)alamin methylation increased linearly in response to increasing cob(I)alamin concentration (Fig. 5, panel D). Higher concentrations of cob(I)alamin could not be tested due to the limits of the spectrophotometric assay.

The DMS: cob(I)alamin reaction did not proceed to comple-
tion. Addition of fresh MtsA to reactions that had ceased did not result in further cob(I)alamin methylation; however, addition of more DMS to reactions that had ceased did result in further cob(I)alamin methylation (Fig. 5, panel E). This indicated that the reaction ceased because equilibrium had been reached. An equilibrium constant of \(5.4 \pm 0.4 \times 10^{-4}\) was calculated for the conversion of DMS and cob(I)alamin to methanethiol and methylcobalamin from a series of different reactions in which either DMS or methylcobalamin concentrations were varied.

The dependence on cob(I)alamin of DMS demethylation by MtsA was examined more closely using a high amount of MtsA in the absence of cob(I)alamin to sensitively detect DMS-dependent methanethiol formation. MtsA (19.5 nmol) incubated with 100 mM DMS for 30 min did not produce any detectable methanethiol. The lower limit of detection under these conditions was 200 pmol of methanethiol. To liberate any acid-labile methanethiol bound to the enzyme, 1 M HCl was subsequently added to the reaction mixture to denature MtsA. However, no methanethiol was detected after acidification. These results indicate that demethylation of DMS by MtsA obligatorily requires the presence of cob(I)alamin.

The methylamine-specific methylcobalamin:CoM methyltransferase, MtbA, did not catalyze any detectable methyl transfer from DMS to cob(I)alamin when 1.2 mg of MtsA/ml was incubated with 2.5 mM cob(I)alamin and 100 mM DMS and monitored for as long as 8 h. Assuming the same linear rate observed for MtsA mediated DMS: cob(I)alamin methyltransfer, this assay would have detected an activity as low as 70 pmol of cob(I)alamin methylated min\(^{-1}\) mg\(^{-1}\) protein. In contrast, with these same concentrations of reactants, MtsA catalyzed DMS-dependent cob(I)alamin methylation at a rate of 167 nmol min\(^{-1}\) mg\(^{-1}\) protein.

**DMS:CoM Methyl Transfer Is Catalyzed by MtsA and Cob(I)-alamin**—The above results demonstrated that MtsA alone can carry out both DMS: cob(I)alamin methyl transfer, as well as methylcobalamin:CoM methyl transfer. This indicated that MtsA possessed the active sites for both half reactions of the methylation of CoM with DMS; therefore, it was tested if DMS: CoM methyl transfer could be catalyzed solely by recombinant MtsA with the corrinoid cofactor bound to the native methionyl-CoM methyltransferase replaced by free cob(I)alamin.

MtsA was incubated in the presence of 1 mM cob(I)alamin (generated with 6 mM Ti(III)citrate and hydroxycobalamin), 100 mM DMS, and 10 mM CoM. Methyl transfer from DMS to CoM was assayed by monobromobimane derivatization of the unmethylated thiol of CoM and subsequent HPLC analysis. This assay has been previously demonstrated to accurately measure DMS-dependent CoM methylation, and due to the presence of the strongly reducing Ti(III)citrate is not subject to interference from CoM oxidation to the dithiol derivative (19). Under these conditions, MtsA catalyzed DMS:CoM methyl transfer (Fig. 6) at a rate of 18.7 nmol of CoM methylated min\(^{-1}\) mg\(^{-1}\) MtsA. In separate reactions performed under the same conditions, the evolution of methanethiol was followed, and found to proceed at nearly the same linear rate as CoM methylation (17.8 nmol of methanethiol liberated min\(^{-1}\) mg\(^{-1}\)). This rate of methythiol production was lower than measured in the absence of CoM, and this may be ascribed to the competition of CoM and DMS for the same active site, as described below.

The methylamine-specific CoM methylase, MtbA, did not catalyze DMS:CoM methyl transfer at a detectable rate under the same conditions used to assay methyl transfer by MtsA (Fig. 6). MtbA (1.2 mg/ml) was monitored for this activity for as long as 8 h. Assuming the same linear rate of reaction observed with the DMS:CoM methyltransferase reaction catalyzed by MtsA and cob(I)alamin, this would have been sufficient to detect 0.4 nmol of CoM methylated min\(^{-1}\) mg\(^{-1}\) protein. These results demonstrated that MtsA, but not MtbA, had determinants that allow the transfer of methyl groups from DMS to CoM with cob(I)alamin as a free intermediate methyl carrier.

**DMS Is a Competitive Inhibitor of MtsA-catalyzed CoM Methylation**—The ability of MtsA to carry out both DMS demethylation and CoM methylation with cobalamin intermediates raised the question as to whether one or two active sites might exist on MtsA for DMS demethylation and CoM methylation. MtsA-catalyzed methylcobalamin:CoM methyl transfer is 160-fold faster compared with DMS: cob(I)alamin methyl transfer; therefore, it was decided to study the effect of DMS on the former reaction, since this would limit the effects of product removal on kinetic analysis.

A plot of the reciprocals of the initial rates of methylcobalamin:CoM methyltransfer versus different concentrations of CoM in the presence of different amounts of DMS revealed a pattern of DMS inhibition of CoM methylation that was most consistent with DMS acting as a competitive inhibitor of the methylcobalamin:CoM methyltransferase reaction (Fig. 7, panel A). A replot of the slopes of the lines of the reciprocal plots versus the concentration of DMS allowed determination of an apparent \(K_{i}\) value for DMS as 14.2 mM.

The above results suggested that DMS inhibited MtsA CoM methylation with methylcobalamin due to competition with CoM at the same active site. As a further test of this model, the mode of inhibition of DMS relative to the methylcobalamin was also examined. A double-reciprocal plot of the rate of the methylcobalamin:CoM methyltransfer reaction versus methylcobalamin in the presence of different fixed concentrations of DMS revealed a pattern most consistent with mixed inhibition of methylcobalamin demethylation by DMS (Fig. 7, panel B).
methylcobalamin:CoM methyl transfer catalyzed by MtbA. The dicyano derivatization assay in both experiments.

Methylcobalamin, which compared favorably to 35 mM methylcobalamin, 2.3 mM Ti(III)citrate, 10.5 μg/ml MtsA, and from 0 to 80 mM DMS. B, mixed inhibition with respect to methylcobalamin. Methylocobalamin concentrations were varied from 1.07 to 7.47 mM in the presence of 20 mM CoM, 2.3 mM Ti(III)citrate, 12 μg/ml MtsA, and 0–80 mM DMS. Rates of methylocobalamin:CoM methyl transfer activity were measured by the dicyano derivatization assay in both experiments.

had an apparent $K_m$ of 38 μM in the presence of 200 μM methylcobalamin, which compared favorably to 35 μM CoM in the presence of 50 μM methylcobalamin reported previously by LeClerc and Grahame (32). Therefore, DMS was tested as a competitive inhibitor of the methylcobalamin:CoM methyl transfer reaction by MtbA with initial concentrations of 40 μM CoM and 200 μM methylcobalamin. The specific activity of purified MtbA was 8.9 μmol min$^{-1}$mg$^{-1}$ MtbA, and did not significantly change in the presence and absence of 20 or 100 mM DMS, indicating that DMS is not an effective inhibitor of methylcobalamin:CoM methyl transfer catalyzed by MtbA.

**DISCUSSION**

The methylation of CoM with methanol or methylamines requires three different polypeptides (24–27). CoM methylation with DMS or MMPA is different, requiring an enzyme composed of two tightly complexed polypeptides. The results presented here resolve this apparent anomaly in the pathways for initiation of methanogenesis from methylocrotophic substrates. MtsA, the CoM methylase subunit of the methyliodide:CoM methyltransferase, is capable of both subreactions necessary for the methylation of CoM by DMS.

Our current results indicate methylation and demethylation of enzyme-bound corrinoid in the native methyliodide:CoM methyltransferase is mediated by MtsA. Our previous work has shown that the corrinoid cofactor bound to the native methyl-

thiol:CoM methyltransferase can serve as an intermediate methyl carrier in DMS:CoM methyl transfer (19, 34). DMS will methyle at the enzyme-bound (I) corrinoid, and CoM will demethylate the methylated enzyme-bound corrinoid. Recombinant MtsA itself does not possess tightly bound corrinoid; however, this protein does react with non–protein-bound corrinoid. MtsA carries out the methylation of cob(I)alamin with DMS, and the methylation of CoM with methylcobalamin. In the presence of DMS, CoM, and cob(I)alamin, MtsA will catalyze the DMS:CoM methyl transfer reaction. Taken together, our current and previous observations support a model of methylthiol:CoM methyltransferase action in which MtsA methylates enzyme-bound corrinoid with DMS, and subsequently demethylates that same bound corrinoid with CoM during DMS:CoM methyl transfer.

One corrinoid is bound per MtsA:MtsB pair, and it is most likely that the cofactor is bound to MtsB. MtsB is highly similar to the entire length of methylotrophic corrinoid proteins such as MttC, MtbC, or MtmC (30, 33). These latter proteins bind one corrinoid cofactor per polypeptide (21, 26, 27, 35). MtsA thus appears to have the essential methyltransferase activities for DMS:CoM methyl transfer with corrinoid as an intermediate methyl carrier, with MtsB serving to direct close interaction of its corrinoid cofactor with the methyltransferase active site of MtsA. In this, MtsB is similar to other corrinoid proteins involved in CoM methylation with methylotrophic substrates such as monomethylamine (25), dimethylamine (28), or methanol (28), which as yet have no detectable enzymatic capabilities and interact specifically with certain methyltransferases.

The role of MtsB in presenting the corrinoid cofactor to MtsA is quite dramatically illustrated by the 100-fold faster rate of DMS demethylation by the intact MtsA:MtsB complex (19) relative to the rates observed here with only MtsA.

The methylation and demethylation of corrinoid during DMS:CoM methyl transfer may require only a single active site, since in essence DMS demethylation by Co(I) corrinoid is functionally analogous to the reversal of methylcorrinoid:CoM methyl transfer. Our current data demonstrate that DMS is a competitive inhibitor with respect to CoM of the methylcobalamin:CoM methyltransferase reaction mediated by MtsA. Although it remains possible that DMS binds to a separate site on MtsA and deforms the CoM active site, the most straightforward interpretation of competitive inhibition is that the demethylation of DMS and methylation of CoM share at least some of the same active site determinants.

The relatively high apparent $K_m$ value found here for recombinant MtsA may reflect modifications of the active site to accommodate both CoM and DMS. MtbA and MtsA have apparent $K_m$ values for CoM reported in the range of 20–40 μM (32), whereas MtsA has an apparent $K_m$ value for CoM of ~10 mM. The native methylthiol:CoM methyltransferase also has a high apparent $K_m$ for CoM relative to MtbA and MtsA, and was measured as 1.4 mM using the methyliodide:CoM methyltransferase reaction catalyzed by the intact enzyme (19). The apparent $K_m$ value for CoM of MtsA in the MtsB:MtsA complex using the methyl iodide:CoM methyltransferase assay is lower than the apparent $K_m$ for CoM of recombinant MtsA using the methylcobalamin:CoM methyltransferase reaction. This may be due to measurement of the latter $K_m$ value with non–protein-bound methylcobalamin at a value below the $K_m$ for the free cofactor, while the former $K_m$ value is derived using an assay that relies upon methylation of enzyme-bound corrinoid by methyl iodide.

Recombinant MtsA catalyzes methylcobalamin:CoM methyl transfer approximately 40-fold faster than MtsA in the native methylthiol:CoM methyltransferase when measured with the same CoM and methylcobalamin concentrations. It is relatively
rare that a recombinant enzyme is more active than the native enzyme. The higher activity of recombinant MtsA relative to MtsA in the native intact complex may be due to the interaction of the corrinoid-binding site of native MtsA with MtsB-bound corrinoid. The corrinoid-binding site of MtsA would be accessible by free methylcobalamin upon dissociation from MtsB. In evidence of this, exposure of native methylthiol:CoM methyltransferase to SDS leads to a 9.5-fold higher rate of methylcobalamin:CoM methyl transfer. Treatment with the same concentration of SDS led to dissociation of the MtsA subunits from the MtsB subunits during SDS-PAGE (34).

MtsA shares ~50% similarity over nearly its entire length to the CoM methylases involved in CoM methylation by methanol (MtaA) and methylamine (MtbA) (33), and, as one would predict, they share some similarities. Like MtaA and MtbA, MtsA catalyzes an active methylcobalamin:CoM methyl transfer reaction. The sequence of all three proteins (32, 33) possess the proposed binding motif (V/I)LHICG, where zinc may coordinate to the His and Cys residues, as well as to a second Cys residue found 75 residues C-terminal to the first Cys residue (32, 46). MtsA, like MtbA and MtaA, binds 1 mol of zinc/mol of polypeptide. Zinc has been implicated in deprotonation of CoM in preparation for that coenzyme’s methylation (32, 47) by MtaA. Zinc did seem necessary for folding and/or catalysis of MtsA, since deletion of exogenous zinc from the MtsA refolding buffer led to a significant drop in methylcobalamin:CoM methyltransferase activity.

Key differences are also found between MtsA and the other CoM methylation enzymes. These differences appear to be those that would adapt a member of this family of homologous CoM methylases to the functions required for DMS:CoM methyl transfer.

One of the major distinctions between MtsA and the homologous CoM methylases is that MtaA and MtbA, the methylcobalamin:CoM methyltransferases from methylamine and methanol catabolism, respectively (23, 32), do not form stable complexes with their cognate corrinoid proteins. In contrast, the methylthiol:CoM methyltransferase is part of a very stable 480-kDa protein complex composed of MtsA and the corrinoid protein homolog, MtsB, in equimolar stoichiometry (21). This unique arrangement of a corrinoid protein homolog tightly bound to a CoM methylase homolog was at the outset puzzling. However, our current results indicate why such a complex is achieved and is feasible for the methylthiol:CoM methyltransferase. Unlike the other CoM methylases such as MtaA or MtbA, recombinant MtsA is a homodimer. This may help in achieving the higher-order structure of the methylthiol:CoM methyltransferase. The complex itself is feasible since only the single active site of MtsA both methylates and demethylates the corrinoid bound to MtsB. In contrast, the corrinoid proteins of methanol- or methylamine-dependent CoM methylation must interact with two separate methyltransferases during CoM methylation from the methylamines or methanol (see Fig. 1, panel A). In these cases, it may be necessary to allow conformational changes that allow the corrinoid to sequentially interact with one polypeptide active site after another. Such a conformational change would not be required to expose the corrinoid of MtsB to methylation and demethylation by the active site of MtsA.

The energetics of the individual subreactions of DMS:CoM methyl transfer may provide a rationale for why formation of the MtsA:MtsB complex would be not only be feasible, but favorable. The methylation of the thiolate of CoM with methylcobalamin is an exergonic reaction, with \( \Delta G^\circ \) of -18.25 kJ reaction (48). The methylation of cobalt with a methylated thiol would be predicted to be endergonic, and this is indeed what is revealed by the measured equilibrium constant for the DMS: cob(I)alamin methyltransferase reaction mediated by MtsA, which corresponds to a free energy of +17.9 kJ/mol. The thermodynamically unfavorable DMS-dependent methylation of Co(I) corrinoid would be enhanced though constant product removal via the exergonic demethylation of methylcorrinoid by CoM in a stable complex of the MtsA active site with MtsB-bound corrinoid. The overall methylthiol:CoM methyl transfer reaction has \( \Delta G^\circ \) of -0.35 kJ reaction, but this reaction would further displaced toward CoM methylation by the subsequent exergonic reduction of methyl-CoM to methane.

The largest discernable difference between MtsA and the CoM methylase of methylamine:CoM methyl transfer, MtbA, is the lack of interaction between DMS and MtbA. MtsA will demethylate DMS and methylate cob(I)alamin, whereas such an activity is undetectable in MtbA. Indeed, inhibition of MtbA-catalyzed methylcobalamin:CoM methyl transfer by DMS is not detectable, even at CoM concentrations near the apparent \( K_m \). In sharp contrast, DMS is a potent competitive inhibitor with respect to CoM of MtsA-catalyzed methylcobalamin:CoM methyl transfer.

Recently it was demonstrated that metabolism of chloromethane by an aerobic methylotrophic bacteria required a 60-kDa protein designated CmuA (49). The sequence of CmuA reveals an apparent fusion of two proteins. The C-terminal region is most similar to MtmC, the corrinoid protein of MMA:CoM methyl transfer. The N-terminal region of the protein is most similar to MtbA, the CoM methylase of MMA:CoM methyl transfer (49). The methylation of the corrin-binding domain appears to be mediated by the MtbA homolog. This enzyme thus provides the first example of a CoM methylase homolog, which functions in corrinoid methylation and growth substrate demethylation, rather than demethylation of corrinoid and methylation of a cellular intermediate. The results presented here illustrate that members of the CoM methylase family of methyltransferases within methanogens themselves can function physiologically in either the methylation or demethylation of corrinoid. MtsA thus provides an interesting example of the how relative modifications of members of an existing protein family have resulted in the extended catalytic diversity of methanogenic Archaea.

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Corrinoid-dependent DMS:Coenzyme M Methyl Transfer


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