Reconstitution of Dimethylamine:Coenzyme M Methyl Transfer with a Discrete Corrinoid Protein and Two Methyltransferases Purified from *Methanosarcina barkeri*

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Methyl transfer from dimethylamine to coenzyme M was reconstituted *in vitro* for the first time using only highly purified proteins. These proteins isolated from *Methanosarcina barkeri* included the previously unidentified corrinoid protein MtbC, which copurified with MtbA, the methylcorrinoid:coenzyme M methyltransferase specific for methanogenesis from methylamines. MtbC binds 1.0 mol of corrinoid cofactor/mol of 24-kDa polypeptide and stimulated dimethylamine:coenzyme M methyl transfer 3.4-fold in a cell extract. Purified MtbC and MtbA were used to assay and purify a dimethylamine:corrinoid methyltransferase, MtbB1. MtbB1 is a 230-kDa protein composed of 51-kDa subunits that do not possess a corrinoid prosthetic group. Purified MtbB1, MtbC, and MtbA were the sole protein requirements for *in vitro* dimethylamine:coenzyme M methyl transfer. An MtbB1:MtbC ratio of 1 was optimal for coenzyme M methylation with dimethylamine. MtbB1 methylated either corrinoid bound to MtbC or free cob(I)alamin with dimethylamine, indicating MtbB1 carries an active site for dimethylamine demethylation and corrinoid methylation. Experiments in which different proteins of the resolved monomethylamine:coenzyme M methyl transfer reaction replaced proteins involved in dimethylamine:coenzyme M methyl transfer indicated high specificity of MtbB1 and MtbC in dimethylamine: coenzyme M methyl transfer activity. These results indicate MtbB1 demethylates dimethylamine and specifically methylates the corrinoid prosthetic group of MtbC, which is subsequently demethylated by MtbA to methylate coenzyme M during methanogenesis from dimethylamine.

*Methanosarcina barkeri* is a methanogenic archaean 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 (1, 2).

The methylamines are important methylotrophic substrates in marine environments where they arise from the anaerobic breakdown of choline derivatives, betaine, and trimethylamine oxide (3). 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 source (4, 5).

The final step of CoM methylation with each methylamine is catalyzed by the same polypeptide. This is the 37-kDa methylcorrinoid:CoM methyltransferase, termed MtbA, that was originally identified in cells grown on acetate (6) but was found in highest abundance in cells grown on TMA (7). MtbA was shown to be involved in the MMA:CoM methyl transfer reaction due to its stimulation of this activity in cell extracts (8). Removal of MtbA from cell extracts with immobilized antibodies 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 (9). MtbA does not contain a corrinoid prosthetic group, and since little corrinoid exists in *M. barkeri* that is not protein-bound (10), these results indicated that corrinoid-binding proteins must be involved in CoM methylation from each methylamine.

This prediction was confirmed with the reconstitution of MMA-dependent CoM methylation reaction (11) with highly purified proteins (Fig. 1A; also see Fig. 9). A 50-kDa polypeptide, MtbM, which lacks a detectable prosthetic group, was required for the methylation with MMA of MtbC, a 29-kDa corrinoid-binding protein. The two independently isolated proteins can form a complex and are most active in CoM methylation at a MtbM:MtbC ratio of 2. Methyl-MtbM 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 methyle viologen. The latter two components are required for reductive activation of MtbM to the active Co(I) form, since MtbM is isolated in a mixture of the Co(II) and Co(III) redox states. Use of this reductant and redox mediator circumvents a requirement for a physiological ATP-dependent reductive activation system that is not yet completely resolved (12, 13), allowing *in vitro* MMA:CoM methyl transfer with the aforementioned three polypeptides.

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1 The abbreviations used are: CoM, coenzyme M; MMA, monomethylamine; DMA, dimethylamine; TMA, trimethylamine; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; MOPS, 3-(N-morpholino)propanesulfonic acid; DTNB, 5,5'-dithio(2-nitrobenzoic acid); MAP, methyltransferase activation protein.

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The DMA-CoM methyl transfer pathway has 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. (21, 22) and designated DMAMT (see Fig. 1B). Purified preparations of homodimeric DMAMT contained 0.45 mol of corrinoid/mol of 50-kDa DMAMT polypeptide. DMAMT and MtbA alone were insufficient for DMA-CoM methyl transfer and required addition of methyltransferase activation protein (MAP), a protein involved in ATP-dependent repressive activation of the corrinoid, as well as a fraction from a primary DEAE separation of cell extract that contained hydrogenase activity (22). A mechanism was proposed in which DMAMT methylated with DMA its own bound corrinoid prosthetic group, which was then demethylated by MtbA and CoM (Fig. 1B). This scheme stands in contrast with the resolved pathway for MMA-CoM methyl transfer (Fig. 1A) and the analogous pathways for TMA and methanol-dependent CoM methylation in that a small discrete corrinoid-binding protein is not required.

In this paper, 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 methyl transfer reaction.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures and Preparation of Extracts—**M. barkeri MS was cultured on 80 mM TMA (8, 23) as described previously. Cell extracts were also prepared as described previously (24), except where indicated below for MtbC purification. Briefly, cell suspensions were lysed anaerobically at 20,000 pounds/square inch with a French pressure cell prior to ultracentrifugation at 150,000 × g. The supernatant was stored at -70 °C in hydrogen-flushed stoppered serum vials until used.

**Materials—**Gases were purchased from Linde Specialty Gases (Columbus, OH) and passed through catalyst R3-11 (Chemical Dynamics Corp., South Plainfield, NJ) to remove O2. Column chromatography media were manufactured by Amersham Pharmacia Biotech unless otherwise indicated. MOPS, CoM, TMA, DMA, MMA, methyl viologen, hydroxocobalamin, methylcobalamin, ATP, and 5,5'-dithio-2-nitrobenzoic acid, or DTNB, were purchased from Sigma. Titanium (III) chloride (10% in aqueous solution) was purchased from Aldrich. Reagents for electrophoresis were purchased from Bio-Rad.

**Pathways for Methyltransferases and Corrinoid Proteins Involved in DMA-CoM and MMA-CoM Methyl Transfer—**The 24-kDa corrinoid protein MtbB 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 0.1M 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. MtbB was assayed by the methlycobalamin:CoM reaction described previously (6). MtbC itself was followed by SDS-PAGE analyses of the fractions and in later steps also by absorbance at 360 and 546 nm (absorption maxima for the bound corrinoid cofactor). The corrinoid protein copurified with MtbB 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 × 10 cm) that had been equilibrated with 50 mM 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 (6). MtbC itself was followed by SDS-PAGE analyses of the fractions and in later steps also by absorbance at 360 and 546 nm (absorption maxima for the bound corrinoid cofactor). The corrinoid protein copurified with MtbB 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 × 10 cm) that had been equilibrated with a solution of 0.10 mM 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 was then carried out with a linear gradient of 0.10 to 0.25 mM KCl in Buffer A. The bulk of both proteins, MtbB and the 24-kDa MtbC, was recovered in the fractions eluting between 0.10 and 0.15 mM KCl. A separate 25-kDa corrinoid protein eluted in earlier fractions and was largely removed at this step. The pooled fractions containing MtbB and MtbC were then concentrated by ultrafiltration in a stirred cell apparatus using an YM-30 membrane (Amicon, Inc., Beverly, MA) and then diluted to approximately 0.07 mM KCl. The protein solution was then applied to a 140-ml (1.5 × 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 (NH₄)₂SO₄ in a final volume of 3 ml. The fraction was then applied at 0.5 ml/min to a Amersham Pharmacia Biotech HR 16/10 phenyl-Sepharose column equilibrated with 1.58 M (NH₄)₂SO₄ in Buffer A. Elution was carried out with a 30-ml gradient decreasing linearly in (NH₄)₂SO₄ from 1.58 to 0 M and simultaneously containing 50 mM Tris and loaded onto a single UNO Q1 column. A 100-ml gradient of 150–350 mM NaCl in 50 mM Tris, pH 8.0, was then 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 eluted at approximately 320 mM NaCl in a total volume of 12 ml. The pooled active fractions from both Mono-Q runs eluted at approximately 320 m M NaCl in a total volume of 12 ml. The pooled active fractions were then loaded onto two UNO Q1 columns connected in series (Bio-Rad) equilibrated with 50 mM Tris, pH 8.0. A 10-ml gradient of 50–500 mM NaCl in 50 mM MOPS, pH 6.5, was applied to the column at 2 ml/min. The methylcorrinoid:CoM methyltransferase, MtbA, was also purified aerobically purified proteins were made anaerobic by repeated cycles of evacuation and flushing with N₂. Protein purification was initiated by applying 35 ml of cell-free extract to a 25 × 2.5-cm DEAE-Sepharose (Sigma) equilibrated in 50 mM Tris, pH 8.0. A 500-ml gradient of 150–350 mM NaCl in 50 mM Tris, pH 8.0, was applied to the column at 2.2 ml/min. MbB1 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 then eluted at pH 6.5 with 75 mM MOPS, pH 6.5, and 50 ml was loaded onto a Amersham Pharmacia Biotech Mono-Q HR 10/10 column. A 120-ml gradient of 50–500 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 eluted at a concentration of approximately 180 mM (NH₄)₂SO₄ in a total volume of 20 ml. The pooled active fractions were then loaded onto two UNO Q1 columns connected in series (Bio-Rad) equilibrated with 50 mM Tris, pH 8.0. A 100-ml gradient of 150–350 mM NaCl in 50 mM Tris, pH 8.0, was then applied to the column at 1 ml/min. The MbB1 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 (Amersham Pharmacia Biotech) in 50 mM MOPS, pH 7.0, and a 29-ml gradient of 500 to 0 mM (NH₄)₂SO₄ was applied to the column. The phenyl-Sepharose column was then used to isolate MbB1 or MtbB1 UNO Q1 fraction. The active MbB1 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 150–350 mM NaCl in 50 mM Tris, pH 8.0, was applied to the column at 2 ml/min. A 10-ml gradient of 50 to 0 mM (NH₄)₂SO₄ was applied to the column. The monocorrin-like protein, MtmC, was purified as described previously (11), essentially following the protocol of Yeliseyev et al. (7). It was found that both purifications 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 extracts was used to locate activity peaks eluting from DEAE-Sepharose fractionation of total soluble proteins. These reactions were carried out in hydrogen-flushed vials containing 50 μl (850 μg) of MGA-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 CoM, 100 mM DMA, and 1 mM bromothymol blue in a total volume of 125 μl. The reaction mixture was removed and reacted with 95 μl of 0.5 mM DTNB to determine the amount of unmethylated CoM. A low level of CoM loss (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 ascribed to interference of higher concentration with DTNB detection. The 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 MbB1 and 35 μg of either MtmC or MtbC (12 or 15 μM, respectively) as indicated, as well as 4 mM Ti(III) citrate, 0.75 mM methyl viologen, and 100 mM DMA 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 as described by Kremer et al. (25) and methylcobalamin quantitated by C-18 reverse phase HPLC (11). The amount of methylcobalamin extracted for each reaction was calculated by comparison to a standard curve of methylcobalamin analyzed by the same HPLC method.

Other Analytical Techniques—Protein-bound cobamide concentrations were determined using a dicyano derivatization method (27). Protein concentrations were determined by using the bicinchoninic acid protein assay (28) with reagent purchased from Pierce 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 following the procedure of Laemmli (29) using a Mini-slab electrophoresis system (Idea Scientific Co., Minneapolis, MN). Molecular size markers (Bio-Rad) were rabbit skeletal muscle myosin (200,000), Escherichia 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). HPLC size exclusion columns G4000SWXL and G3000SWXL (Supelco, Bellefonte, PA) equilibrated with 100 mM NaCl in 50 mM MOPS, pH 7.0, were used to size protein preparations. The samples 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-amylose (200,000), alcohol dehydrogenase (150,000), bovine serum albumin (66,000), carbonic anhydrase (29,000), and ferricyanide (324) purchased from Sigma.

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 MbB1 was done with crude cell extracts containing 4 mM CoM, 100 mM DMA, 15 μg of MtbA, 15 μg of MbB1, 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 of purified MbB1 were added in place of the column fractions in reactions containing purified MtbA and MtbC as the only other protein components. Protein concentrations in the assay were occasionally varied for some experiments, and this is indicated in the text. At intervals during the reaction time course, 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.

The methyltransferases of methanogenesis from dimethylamine were used to determine native protein masses. MbB1 or MbC (100 μg each) was 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-amylose (200,000), alcohol dehydrogenase (150,000), bovine serum albumin (66,000), carbonic anhydrase (29,000), and ferricyanide (324) purchased from Sigma.

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Methyltransferases of Methanogenesis from Dimethylamine

RESULTS

Purification of MtbC, a Small Corrinoid Protein Involved in DMA:CoM Methyl Transfer—During the isolation of the amine-specific methylcorrinoid:CoM methyltransferase, MtbA, from cell extracts of TMA grown cells from M. barkeri, a previously uncharacterized protein was identified and termed MtbC. The two proteins copurified through several columns until a hydrophobic interaction column separated MtbC from MtbA. Purified MtbC migrated as a single 24-kDa protein band during SDS-gel electrophoresis (Fig. 2). The purified protein eluted with an apparent molecular mass of 34 kDa from an HPLC silica gel permeation column. The UV-visible spectra indicated MtbC is a corrinoid-binding protein (Fig. 3A). The isolated aerobic protein had an absorbance maximum at 360 nm, typical of protein-bound corrinoid in the Co(III) state with water or a phobic interaction column separated MtbC from MtbA. Purified MtbC migrated as a single 24-kDa protein band during SDS-gel electrophoresis. The as isolated protein was a residue that could not be unambiguously assigned.

Extracts of cells grown on TMA possess appreciable DMA:CoM methyl transfer activity (550 nmol/min/mg protein), but this activity is much less in MMA-grown cells (<2.5 nmol/min/mg protein). There is a highly abundant 24-kDa polypeptide bound corrinoid in the Co(III) state with water or a hydroxyl group as the β-axial ligand. The stoichiometry of cofactor bound to the protein was determined spectrophotometrically following conversion of the cofactor to the dicyano derivative by SDS and heat denaturation of the protein in the presence of KCN. Each mol of 24-kDa polypeptide bound 1.0 mol of corrinoid. The N-terminal amino acid residues were determined by Edman degradation to be SXEELLQELADAIS, where X was a residue that could not be unambiguously assigned.

No other metals were detected except 0.43 mol of zinc/mol of polypeptide. The marked stimulation of DMA:CoM methyl transfer by the addition of MbC was consistent with a role for MbC in the initiation of methanogenesis from DMA.

Isolation of MtbB1 Using Purified MbC and MbA—A role for MbC in DMA:CoM methyl transfer implies the existence of a DMA:MbC methyltransferase. Extracts of TMA-grown cells were anaerobically fractionated in order to identify such a methyltransferase and study its possible interaction with MbC. 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 (Fig. 4) 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 MbC itself (see below). A protein that acts as a DMA:corrinoid methyltransferase was isolated from the second activity peak to elute (peak II) which was designated as MtbB1.

Peak II fractions combined with purified MbC and MbA 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 homogeneous polypeptide that could carry out DMA:CoM methyl transfer in the presence of purified MbA, MbC, and no other proteins. A single peak of activity was detected in each of the subsequent columns on which MtbB1 was purified (Table I). The final preparation of MtbB1 revealed a single 51-kDa polypeptide upon SDS-gel electrophoresis (Fig. 2). The determined N terminus of the polypeptide (MATEYALRMGD-GKRVYLTKE) matched 14 of 16 residues from the DMAMT (21, 22) of M. barkeri Fusaro. In marked contrast to the report for DMAMT preparations, our preparations of homogeneous MtbB1 polypeptide did not contain any detectable corrinoid prosthetic group when assayed by the dicyano method. The UV-visible spectrum of MtbB1 did not have any of the characteristic peaks of a corrinoid protein (Fig. 3B). Analysis of the metal content of MtbB1 by plasma emission spectroscopy revealed no detectable cobalt in two different preparations of the homogeneous enzyme. The lower limit of detection for cobalt using this procedure was 0.04 mol of cobalt/mol of polypeptide. No other metals were detected except 0.43 mol of zinc/mol of polypeptide. The protein eluted as a 230-kDa protein during size exclusion chromatography, indicating a homotetrameric or possibly homopentameric configuration.
Three polypeptides are required to reconstitute DMA:CoM methyltransfer activity in a purified system: MtbB1, MtbA, and MtbC. The minimum requirements for DMA:CoM methyltransfer were examined using the purified proteins analyzed by SDS-PAGE as depicted in Fig. 2. 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. Time courses of CoM utilization were presented for the complete reaction (●), as well as the complete reaction minus MtbB1 (■), minus MtbA (○), or minus MtbC (▲).

The resolved DMA:CoM methyltransfer reaction was used to verify that MtbC was the protein responsible for activity of peak I that eluted from the DEAE-Sepharose column depicted in Fig. 4. 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 (data not shown). 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 methyltransfer 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.

**Specific Activities of Individual Protein Components in DMA:CoM Methyl Transfer**—Purified MtbB1 was titrated against a constant amount of purified MtbC (0.65 nmol) and purified MtbA (0.41 nmol), and the rate of DMA:CoM methyltransfer was measured (Fig. 6). Activity was seen to increase with increasing MtbB1 with saturation observed at approximately 0.6 nmol of MtbB1. This corresponds to an approximate molar ratio of 1:1 between MtbB1 and MtbC. No increase in activity was observed in reactions containing 1 nmol of MtbB1, 0.6 nmol of MtbC, and either 0.4, 0.8, or 1.2 nmol of MtbA, indicating that MtbA was also saturating under these conditions. These data allow estimation of the specific activities of both MtbB1 and MtbC in the DMA:CoM methyltransfer reaction when the other components are in excess. When MtbC and MtbA were not limiting, methylation of CoM occurred at an average specific activity of 2.2 μmol/min/mg MtbB1. In the presence of excess MtbB1 and MtbA, the methylation of CoM occurred at an average specific activity of 2.5 μmol/min/mg purified MtbC.

The specific activity of MtbC found in extracts of TMA-grown cells was determined in the presence of saturating amounts of MtbB1 and MtbA (1 and 0.4 nmol, respectively) as 42 nmol of CoM methylated per min/mg of protein. This indicates that an approximately 60-fold purification of MtbC was achieved during the course of MtbC isolation.

The specific activity of methyltransfer by MtbA was measured during the DMA:CoM methyltransfer reaction in the presence of excess MtbC and MtbB1 (0.6 nmol of both MtbB1 and MtbC with a range of 0.05 to 0.25 nmol MtbA) and found to be an average of 2.1 μmol/min/mg purified MtbA.

**MtbB1 Is a DMA:Cob(I)alamin Methyltransferase**—The re-

![Fig. 4. Stimulation of DMA:CoM methyl transfer activity in extracts by two separate peaks from anion exchange chromatography. An extract of TMA-grown cells was subjected to chromatography on a DEAE-Sepharose column, and the fractions were assayed for their ability to stimulate DMA:CoM methyltransfer in extracts. The reactions contained 50 μl of MMA-grown cell extract, 1.5 μl of TMA-grown cell extract, 61.5 μl of column fraction, 10 mM ATP, 20 mM MgCl2, 100 mM DMA, and 1 mM bromoethanesulfonate 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. Activity peaks I and II, described in the text, are indicated.](image-url)
requirement for a methylcorrinoid:CoM methyltransferase, a corrinoid protein, and MtbB1 for DMA:CoM methylation suggested that MtbB1 could function in the demethylation of DMA and methylation of the corrinoid prosthetic group of MtbC. To test this hypothesis, the ability of MtbB1 to methylate free cob(I)alamin 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 spectrophotometrically. Upon initiation of the reaction, a shift in the cob(I)alamin 553 nm absorbance peak occurred toward the methylcobalamin peak of 532 nm. An isosbestic point at 578 nm for time points following \( T_0 \) indicated that the transformation of cob(I)alamin to methylcobalamin proceeded without a detectable intermediate (Fig. 7A). The spectrum taken at \( T_0 \) may have contributions from cob(II)alamin since the reaction was initiated by introduction of hydroxocobalamin into the cuvette. The conversion of cob(I)alamin to methylcobalamin can be followed at 540 nm, the isosbestic point of Co(I)/Co(II) cobalamin (26). MtbB1 methylated 2.5 mM cob(I)alamin at a rate of 14.4 nmol/min/mg MtbB1 (Fig. 7B). No change in the cob(I)alamin spectrum was noted in the absence of MtbB1. The formation of methylcobalamin from DMA and cob(I)alamin as catalyzed by MtbB1 was confirmed by HPLC analysis (data not shown).

**Methylation of MtbC by MtbB1**—The methylation of free corrinoid cofactor by DMA with 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 \( \mu \)g of MtbC (1500 pmol of 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 II). 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 the resolved in vitro DMA:CoM methyl transfer system was analogous to the resolved three-component MMA:CoM methyl transfer system (11). In addition to the methylcorrinoid:CoM methyltransferase MtbA, each of these systems requires a methylamine-specific methyltransferase and a corrinoid protein. In the following experiments we examined the ability of these protein components to substitute for one another during CoM methylation from DMA or MMA.

MtbB and MtbB1 were incubated with either MtmC or MtbC in the presence of MtbA and CoM methylation from either DMA or MMA monitored (Fig. 8). Only the combination of MtbB1 and MtbC supported CoM methylation with DMA, whereas 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 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 the 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 corresponding methylamine substrates. Methylation of MtbC or MtmC was analyzed following extended incubation with either MtbB1 or MtmB in the presence of either DMA or MMA and Ti(III) citrate and methyl viologen (Table II). Fol-

![Fig. 6. 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 \( \mu \)g) and MtbC (0.65 nmol, 15 \( \mu \)g). Reactions were 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 \( \mu \)l.](image)

![Fig. 7. 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-h incubation in the presence of DMA and MtbB1. Reaction conditions are as described under “Experimental Procedures.” The spectra at \( T_0 \) and 120 min are indicated, and the remaining spectra were taken at 10-min intervals. B, the rate of cobalamin methylation determined using the extinction coefficient at 540 nm of 4.4 mmol cm\(^{-1}\).](image)

| Enzymes            | Substrate | Initial corrinoid | Methylated corrinoid pmol* |
|--------------------|-----------|-------------------|----------------------------|
| MtbB1 + MtbC       | DMA       | 1520              | 1250                       |
| MtbB1 + MtmC       | DMA       | 1210              | ND                         |
| MtbB1 + MtbC       | None      | 1520              | ND                         |
| MtmB + MtmC        | MMA       | 1210              | 1040                       |
| MtbB1 + MtmC       | MMA       | 1520              | 27                         |
| MtmB + MtmC        | None      | 1210              | ND                         |

*Amount of corrinoid attributed to MtbC or MtmC was based on a 1:1 ratio of corrinoid to protein.

**No other corrinoid peak was detectable during HPLC analysis of these fractions; recovery of total corrinoid averaged 80%.

**ND, below the limit of detection (20 pmol).**
The reactions also contained 4.5 mM Ti(III) citrate, 0.75 mM methyl MtbC or MtmC, viologen, 4 mM CoM, and 100 mM DMA or MMA, in a final volume of 125 μl. MtmB, MtbC, and MMA (♦); MtbB1, MtmC, and MMA (◊); MtbB, 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 DMA or MMA, 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.

Following incubation, the corrinoid prosthetic groups were aerobically extracted from the reaction mixture, and methylcorrinoid was quantitated by HPLC. In control reactions, MtmB methylated MtmC with MMA, as observed previously (11), and MtbB1 methylated MtbC with DMA as discussed above. No detectable unmethylated corrinoid remained in either of these reactions.

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

**DISCUSSION**

This work describes 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 (Fig. 9). In each of these pathways, a small corrinoid protein (26–29 kDa) specific for that pathway interacts with two methyltransferases to initiate CoM methylation from the methylocorrinoid substrate. The first methyltransferase methylates the corrinoid protein with the methylation from the methylotrophic substrate. The second methyltransferase catalyzes the demethylation of the corrinoid protein and methylation of CoM.

Several lines of evidence support this model of DMA:CoM methylation reaction in M. barkeri. MtbC is a 24-kDa corrinoid protein that specifically stimulates 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(III)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 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 methyltransferase, MtbA.

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Wassenaar et al. (21, 22) have postulated the existence of a different pathway of DMA:CoM methylation in M. barkeri (Fig. 1B). 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. Their proposed pathway does not include a small corrinoid protein such as MtbC, rather, DMAMT was reported to bind 0.45 mol of corrinoid/mol of polypeptide and was suggested to serve both as a corrinoid-binding protein and as 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 protein, MtbC. The complete sequence of DMAMT has not yet been published. We have obtained the full sequence of MtbB1, and it has no detectable homology with any corrinoid-binding protein (30) (see also Genbank™ accession number AF102623). The specific activity of DMA demethylation measured for purified MtbB1 is comparable to that reported for DMAMT. The native structure of the two proteins may also be different. DMAMT is reported to be a dimer, whereas MtbB1 is a tetramer or pentamer.

If both DMAMT and MtbB1 represent gene products that are homologous across their entire length, 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 MtbA homolog (31). This observation indicates that the DMA methyltransferase-corri-
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It is difficult to determine if a small corrinoid protein is functionally involved in the DMAMT-dependent methylation of CoM. Wassenaar et al. (22) acknowledged that a small corrinoid protein could have been added to their assays as part of non-homogeneous MAP and MtbA preparations. 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 Fig. 9.

Recently, the genes encoding two polypeptides required to initiate TMA:CoM methylation, MtbB and MtbC (see Fig. 9), were cloned and sequenced (30) (see also GenBank accession number AF102623). 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 MtbC obtained in this study indicate that each of these open reading frames encode proteins involved in the DMA-dependent methylation of CoM. Interestingly, the genes mttB, mttC, mttB1, and mttB2 are encoded on a single transcript found in TMA-grown cells (30). The cotranscription of the genes encoding MtbC 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 mttB1 and mtc genes are cotranscribed with genes for initiating TMA catabolism. Since M. barkeri generates DMA as an intermediate of methanogenesis from TMA, cotranscription of both TMA and DMA methyltransferase genes would be an efficient means of coordinating expression of these methyltransferases.

The protein sequence predicted for MtbC from its gene, mbtC, is approximately 50% similar to the predicted protein sequences from the genes encoding the methylocrophic corrinoid proteins of TMA (MttC) (30), MMA metabolism (MmcC) (31), and methanol metabolism (MtcA) (16). Each of these corrinoid proteins is in turn homologous to the cobalamin-binding domain of cobinamide-dependent methionine synthase and mutases (16, 32–34). The homology of the corrinoid protein isolated here, MtbC, to these other small discrete corrinoid proteins involved in methanogenesis from methylocrophic substrates is consistent with our results demonstrating a role for MtbC in a CoM methylation pathway, that is DMA:CoM methyl transfer.

MtbB, MtbB1, and MtbB2 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 methylene methyltransferases are not homologous (30, 32), see also GenBank accession number AF102623. This may explain the presence of the different, yet homologous, methylocrophic corrinoid proteins of methylene-dependent methanogenesis. Although each corrinoid protein serves as a methyl donor for the same CoM methylease, Mtha, the differences in primary sequence must be necessary in order to optimize interaction of a particular corrinoid protein with one of the non-homologous methylene/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 methyamine 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 methylate rapidly 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 the unique role of the corrinoid-binding protein MtbC in greatly, and specifically, enhancing the interaction of corrinoid with the DMA:corrinoid methyltransferase, MtbB1.

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