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

(Received for publication, February 12, 1997, and in revised form, April 11, 1997)

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Most methanogens have a very limited substrate range, being capable of reducing only carbon dioxide to methane with electron donors such as hydrogen or formate. However, a variety of methylated compounds also serve as substrates for members of the family Methanosarcinaceae. This group is unique among methane-producing bacteria in the possession of pathways for the dismutation of methylotrophic substrates, such as methanol, methylamines, or methylated thiols, to methane and carbon dioxide (1, 2).

Reduction of methylotrophic substrates to methane occurs with the intermediate methylation of the thiol of 2-mercaptoethanesulfonic acid (coenzyme M, or CoM)1 (3). Methyl-CoM and 7-mercaptoheptanoylthreonine phosphate (HTP) are then converted to methane and the heterodisulfide of CoM and HTP by methylreductase (4, 5). The free thiols of CoM and HTP are regenerated with reducing equivalents gained from the concomitant oxidation of the methylotrophic substrate to CO2. The reduction of the heterodisulfide is a major site of energy conservation in methanogens (6).

Determination of the pathways of CoM methylation by methylotrophic substrates is therefore key to understanding methanogenesis from these substrates. The work of Vogels and co-workers (7, 8) with Methanosarcina barkeri demonstrated that two enzymes were required to methylate CoM with methanol, designated methyltransferase I (MT1) and methyltransferase II (MT2). MT1 is a two-subunit enzyme that binds 5-hydroxybenzimidazolylcobamide, the major corrinoid found in this organism (9). The carbon of methanol undergoes nucleophilic attack by the central cobalt atom of the enzyme-bound corrinoid when in the Co(II) state. The methylated Co(III) corrinoid then serves as a substrate for MT2, which acts as a methyl-cobamide:CoM methyltransferase with the subsequent formation of methyl-CoM and Co(I) corrinoid (10). Three isozymes or homologues of MT2 have been found (11, 12), but only the “M” isozyme, or MT2-M, of these interacts with MT1 in methanol metabolism (13). In addition to MT1 and MT2-M, several ancillary proteins can act in methanol:CoM methyl transfer. Oxidation of MT1 can leave the corrinoid in the inactive Co(I) form. A cellular activation system reactivates MT1 in a reductive process that also requires methanol and ATP (14–16). Most studies have required relatively crude protein fractions containing hydrogenase, ferredoxin, and a methyltransferase activation protein for study of in vitro methyltransferase II (MT2). Alcohols were methylated in the presence of MT2 and, although recently the pathway was reconstituted with only purified MT1 and MT2-M (17).

Methylamines utilized by M. barkeri include trimethylamine (TMA), dimethylamine (DMA), and monomethylamine (MMA). Cells grown on TMA produce intermediate pools of DMA and MMA, which are subsequently consumed (18). Studies with cell suspensions and fractions indicate that the conversion of each methylamine to methyl-CoM involves distinct proteins (19–21). However, a single MT2 isozyme, the “A” or amine isozyme (MT2-A), can be used for CoM methylation from all three methylamines (13). MT2-A was first identified by the general ability of MT2 isozymes to methylate CoM with free methylcobalamin (12). MT2-A shares 50% sequence similarity with MT2-M (10, 22), and both are 37-kDa zinc-binding polypeptides (10). MT2-A

MMA, monomethylamine; DMA, dimethylamine; TMA, trimethylamine; TCP, TMA corrinoid protein; TMA-52, 52-kDa polypeptide involved in TMA:CoM methyl transfer; MMCP, MMA corrinoid protein; MMAMT, MMA methyltransferase; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; MOPS, 3-(N-morpholino)propanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid.

* This work was supported by U.S. Department of Energy Grant DE-FG02-91ER200042. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: CoM, coenzyme M; HTP, 7-mercaptoheptanoylthreonine phosphate; MT1, methyltransferase I; MT2 methyltransferase II or methylcobamide:CoM methyltransferase; MT2-M, the M (methanol) isozyme of MT2; MT2-A, the A (amine) isozyme MT2.
Methyltransferases of Methanogenesis from Monomethylamine

EXPERIMENTAL PROCEDURES

Cell Cultures and Extract Preparation—*M. barkeri* MS (DSM 800) was cultured under anaerobic conditions in a phosphate-buffered medium supplemented to 80 mM with TMA, DMA, or methanol as described previously (20, 27). Cell extracts were prepared and stored at −70 °C in hydrogen-filled serum vials until use (25).

Enzyme Assays—All assays and protein purifications were carried out under anaerobic conditions unless indicated otherwise. Gases were purchased from Linde Specialty Gases (Columbus, OH), and trace amounts of oxygen removed by passage through a 5–11 catalyst (Chemical Dynamics Corp., South Plainfield, NJ) before use. Routine assay of the MMA:CoM methyl transfer activity with purified components or during isolation of the MMAMT was carried out in N2-flushed 1.5-ml glass vials sealed with rubber stoppers. A typical reaction mixture contained the following components added to vials on ice in a total volume of 100 μl: 50 mM MOPS-HCl buffer, pH 7.0, 1.5 mM Tri[III]-citrate adjusted to pH 8.0 with a saturated Tris solution (28), 0.5 mM methyl viologen, 50 mM DMA, 2 mM CoM, 10 μg of purified MMCP, 10 μg of purified MT2-A, and a 50-μl column fraction or specified amount of purified MMAMT. The reaction was started by placing the vials in a 37 °C water bath. Samples (10 μl) were removed and analyzed for the free thiol of CoM using 5,5'-dithiobis(2-nitrobenzoic acid) (16, 29). Previously, this method has been shown to reflect the CoM-dependent disappearance of the thiol. CoM prepared extracts and is therefore an accurate measure of CoM methylation by MMA (20).

Methylation of MMCP with DMA was carried out using the same conditions described for the assay of MMAMT except that MT2-A and CoM were omitted from the reaction mixture, and 100 μg of MMCP and 120 μg of MMCP were used. The reaction was incubated at 37 °C for 30 min.

DMACoM or MMA-CoM methyl transfer activities of antibody-treated extracts were assayed by measurement of substrate-dependent disappearance of the free thiol of CoM as described previously (20).

Purification of Corrinoid Proteins and Methyltransferases—Purification of MMAMT was performed in an anaerobic chamber (Coy Laboratories, MI) with buffers and materials modified cycles of evacuation and flushing with Nz. Protein purification was initiated by applying 25 ml (13.6 mg/ml) of an extract of cells grown on MMA to a 10 × 2.5-cm chromatography column containing DE-52 (Whatman Inc., Fairfield, NJ) that had been equilibrated with 50 mM NaCl in 50 mM MOPS-HCl buffer, pH 7.8. Proteins bound to the matrix were eluted with a 250-ml linear gradient of 50–500 mM NaCl in the same buffer at a flow rate of 2 ml/min. Fractions that supported MMA-CoM methyl transfer activity when supplemented with MMCP and MT2-A eluted between 180 and 294 mM NaCl. The pool of fractions was diluted 10-fold in 50 mM MES-NaOH, pH 6.0, and applied to a Mono-Q HR 10/10 (Pharmacia Biotech Inc.) column equilibrated with 50 mM NaCl in 50 mM MES-NaOH, pH 6.0. Protein was eluted with an 80-ml gradient of 50–500 mM NaCl at a flow rate of 3 ml/min. Active fractions eluted between 180 and 200 mM NaCl. The fractions were pooled (4 ml) and then concentrated to 300 μl using Centricon-10 microconcentrators (Amicon Inc., Beverly, MA) and applied to a Superdex-200 gel filtration column (Pharmacia) equilibrated with 50 mM MOPS-HCl, pH 6.5, 100 mM NaCl. The peak of activity eluted at 58 ml. The active fractions were concentrated to 1 ml and applied to a Mono-Q HR 10/10 (Pharmacia Biotech Inc.) column equilibrated with 50 mM MOPS-HCl, pH 6.5, 100 mM NaCl. Fractions eluting between 205 and 221 mM NaCl of an 80-ml linear gradient of 100–500 mM NaCl were active in the MMA-CoM methyl transfer assay.

MMCP in the methylated or unmethylated state was isolated from extracts of *M. barkeri* as described previously (20, 26). MT2-A and MT2-M were aerobically purified from TMA- or methanol-grown cells, respectively, essentially as outlined by Yeliseev et al. (23). The two isozymes could be distinguished by their elution profiles on hydroxylapatite (20, 23). The specific activities at pH 7.0 of MT2-A and MT2-M were 34 and 6 μmol of methylcobalamin consumed/minute/mg, respectively, as measured by the cyanide derivatization assay described by Graham (12). A homogeneous preparation of TCP was the kind gift of D. J. Ferguson and was isolated by a modification of his published procedure (24). MT2-M, MT2-A, and MMCP were homogeneous preparations, and SDS-PAGE gels documenting their level of purity can be found in this paper and a previous paper (13).

Immunodepletion of MMCP from Extracts—Antiserum against MMCP was raised in rabbits inoculated with purified MMCP using standard techniques (30). IgG against MMCP was then further purified by affinity chromatography. The IgG fraction of 75 ml of antiserum was precipitated by the addition of ammonium sulfate to 50% of saturation. The precipitate was collected by centrifugation, and the pellet was resuspended in 150 mM NaCl in 10 mM potassium phosphate buffer, pH 7.0. The sample was dialyzed against phosphate-buffered saline overnight and passed through a 0.2-μm sterile filter. Purified MMCP (1.7 mg) was coupled to a 1-ml column of N-hydroxysuccinimide-activated Sepharose 4B (Pharmacia) according to the procedure recommended by the manufacturer. The IgG fraction (500 mg) was then applied in five aliquots to the affinity column following the procedure outlined by the manufacturer. Adsorption and subsequent elution of IgG was monitored by the absorbance at 280 nm of the column effluent. Unbound IgG was reapplied to the column in three or four subsequent runs until the effluent showed no α-MMCP activity as judged by an enzyme-linked immunosorbent assay (30).

Unbound IgG that eluted from the column was pooled and used as nonspecific IgG in control experiments as described below. Approximately 11 mg of α-MMCP IgG were recovered from 500 mg of the crude IgG fraction. MMCP was removed from extract using these antibodies in the following manner. An extract (3 mg of protein) of cells grown on TMA were added to 1.2 mg of affinity-purified α-MMCP IgG, and the mixture was incubated on ice for 3 h. A suspension (0.2 ml) of protein A-Sepharose (Sigma) containing approximately 1.2 mg of cross-linked protein A was added, and the mixture was incubated for 2 h at room temperature with gentle mixing. The protein A-Sepharose beads were removed by centrifugation at 10,000 × g for 3 min, and the supernatant was concentrated by ultrafiltration using a Centricron 10 microconcentrator (Amicon Inc.) at 12.5% of nonspecific IgG or an equivalent volume of 50 mM MOPS-HCl buffer in place of α-MMCP IgG. Both control extracts were subjected to the same incubations, treatment with protein A-Sepharose, and concentration steps so that direct comparisons could be made.

Analytical Procedures—SDS-PAGE was carried out according to the method of Laemmli (31) using 12% acrylamide and a Mini-Blab electro-
phoresis system (Idea Scientific Co., Minneapolis, MN). Molecular size
markers (Bio-Rad) used were myosin (200 kDa), β-galactosidase (116
dkDa), phosphorylase b (94 kDa), bovine serum albumin (66.2
kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin
inhibitor (21.5 kDa), and α-lactalbumin (14.4 kDa). Gels were stained for protein
visualization with Coomassie Brilliant Blue R-250. For Western blot
analysis (32), polypeptides were transferred from SDS-PAGE gels to
0.2-μm BA-S 83-supported nitrocellulose membrane (Schleicher and
Schuell) in a Bio-Rad Trans-Blot cell. Transfer was performed at 260
mA for 2.5 h in 25 mM Tris-HCl, pH 8.0, 90 mM glycine, and 40%
methanol. The membrane was blocked using 5% (w/v) nonfat dry milk
in 150 mM NaCl in 10 mM Tris-HCl (pH 8.0). Applications of primary
and horseradish peroxidase-linked secondary antibodies were also car-
ried out in the blocking buffer. Signal development employed 0.06%
4-chloro-1-naphthol, 0.01% H2O2 in Tris-buffered saline.

α-MMCP activity in antibody fractions was determined by enzime-
linked immunosorbent assay as described by Harlow and Lane (30).
Horse radish peroxidase-conjugated sheep α-rabbit IgG (Sigma) was
used as secondary antibody and was detected by reaction with 2,2'-
azino-bis-(3-ethylbenzthiazoline 6-sulfonic acid). Absorbance at 410 nm
was measured using an MR700 enzyme-linked immunosorbent assay
plate reader (Dynametech Laboratories, Inc.).

Size estimation of MMAMT and the complex formed with MMCP in
solution was performed using size exclusion HPLC. G4000SW XL and
G3000SW XL (Supelco, Bellefonte, PA) columns in series were equili-
brated with anaerobic 50 mM potassium phosphate buffer, pH 7, 100 mM
NaCl. The individual proteins to be tested were incubated at 22 °C
individually and in mixtures in 50 mM MOPS-HCl, pH 7.0, 100 mM
NaCl under a nitrogen atmosphere for 1 h. Samples were injected onto the
column being eluted at 1.0 ml/min with the elution buffer. Fractions (0.5 ml)
were collected for further analysis by SDS-PAGE.

The corrinoid cofactor bound to the MMCP used in these experiments
was in a mixture of the Co(II) and hydroxylated forms. Molecular mass
standards (Sigma) were thyroglobulin (689 kDa), apoferritin (450 kDa),
β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum
albumin (66 kDa), and carbonic anhydrase (29 kDa).

Corrinoid cofactor from MMCP was extracted with ethanol with
50–60% yield (25). Reversed phase HPLC of corrinoid cofactor was
performed essentially as described previously (25) with a Microsorb-MV
C-18 column (4.6 mm × 25 cm) (Rainin, Woburn, MA) equilibrated with
20% methanol in 25 mM sodium acetate, pH 6.0. Samples were
eluted with a linear gradient of 20–100% methanol over 60 min. The
retention time of methylocobalamin in this system was 22.5 min, while
that of hydroxyrobalamin was 12.6 min. As noted previously, methyl-
ated and hydroxylated corrinoid isolated from MMCP elute with very
similar retention times to the analogous cobalamin derivatives (25).

UV-visible spectroscopy was performed using a Hewlett-Packard
8453 photo diode array spectrophotometer. Protein was assayed using
bicinchoninic acid (33) with bovine serum albumin as standard.

RESULTS

Isolation of MMAMT, a Protein Essential for MMA:CoM
Methyl Transfer—Previous experiments indicated MMCP and
MT2-A required an unidentified protein in cell extracts to effect
CoM methylation with MMA (20). An assay for this putative protein
was devised that consisted of purified MT2-A, MMCP, Ti(III)-citrate,
and methyl viologen. Fractions from anaerobic column chromatographic separations of extract prepared with
MMA-grown cells were added to this assay mixture, and MMA-
dependent CoM methylation was monitored.

A single peak of MMA:CoM methyl transfer activity was detected upon initial
fractionation of an extract by anion exchange chromatography.
This peak was designated as the MMA methyltransferase activity or MMAMT. Methylation of CoM with MMA was abso-
lutely dependent on the pooled fraction, which was further
purified (Table I). The active peak from the final Mono-Q col-
umn was analyzed by SDS-PAGE and was comprised of a
single 52-kDa polypeptide (Fig. 1, lane 4). This band corre-
sponded to a major protein band that was observed in the extract of MMA-grown cells (Fig. 1, lane 3) but was much less
intense in the extract of cells grown on methanol (Fig. 1, lane
2). A polypeptide that comigrated with MMCP in SDS gels was
one of the last contaminants to be removed during the purifi-
cation of the MMAMT polypeptide (not shown).

Purified MMAMT from the Mono-Q fraction was used in the
remaining experiments described here. The isolated protein
was colorless; its UV-visible spectrum taken under aerobic
conditions possessed only a single peak at 280 nm, indicating
that this protein bound no detectable corrinoid.

Purified MMAMT, MMCP, and MT2-A Are Sufficient for
MMA:CoM Methyl Transfer—MMA-dependent methylation of
CoM by MT2-A and MMCP (see Fig. 1, lanes 5 and 6 for the purity of these preparations) required the presence of the
purified MMAMT. Increasing amounts of the MMAMT polypep-
tide were added to vials containing 0.27 nmol of MT2-A and
0.35 nmol of MMCP (Fig. 2). This resulted in a linear increase in the
MMA:CoM methyl transfer reaction rate until the approxi-
mate molar ratio of the MMAMT polypeptide to MMCP
was 2, after which saturation of the rate of methyl transfer was
observed. The specific activity at this ratio of MMCP, MT2-A,
and MMAMT was 0.6 μmol/min/mg of total protein.

With this ratio of proteins the requirements for MMA:CoM
methyl transfer were further investigated. MMCP and MT2-A
were absolutely required for the MMA-dependent methylation of
CoM, and no reaction was observed when these proteins were
omitted. MMA was the only methylamine to function as a
significant methyl donor in the reaction. DMA or TMA (50 mM)
were not required and did not stimulate the reaction. MT2-M
(1.35 nmol) did not substitute for MT2-A in the
MMA:CoM methyl transfer reaction. TCP (0.35 nmol), the
corrinoid protein involved in the TMA:CoM methyl transfer reaction
(24), did not substitute for MMCP in MMA:CoM methyl
transfer.

MMAMT Possesses MMA:MMCP Methyltransferase Activity—
MMA:MMCP reduced with Ti(III)-citrate and methyl viologen
was methylated with MMA when incubated in the presence of
MMAMT. Cofactor isolated from MMCP following incubation of the
corrinoid protein with Ti(III)-citrate, methyl viologen, and

| Fraction         | Amount of protein (mg) | MMA:CoM methyl transfer activityb (units/mg protein) | Total activity (units) | Recovery of the MMA:CoM methyl transfer activity (%) |
|------------------|------------------------|---------------------------------------------------|------------------------|-----------------------------------------|
| Crude extract    | 340                    | 0.15                                              | 51                     | 100                                     |
| DE-52            | 274                    | 0.054                                             | 14.8                   | 30                                      |
| Mono Q pH 6      | 15.8                   | 0.0                        | 7.5                    | 14.7                                    |
| Superdex-200     | 9.8                    | 0.45                                              | 4.4                    | 8.7                                     |
| Mono Q, pH 6.5   | 5.6                    | 0.49                                              | 2.7                    | 5.4                                     |

a MMA:CoM methyl transfer activity was assayed by monitoring MMA-dependent disappearance of free thiol of CoM as described under
“Experimental Procedures.”

b One unit is defined as 1 μmol of CoM consumed per min.

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**TABLE I**

**Purification of MMAMT**

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**RESULTS**

**Isolation of MMAMT, a Protein Essential for MMA:CoM Methyl Transfer**—Previous experiments indicated MMCP and
MT2-A required an unidentified protein in cell extracts to effect
CoM methylation with MMA (20). An assay for this putative protein
was devised that consisted of purified MT2-A, MMCP, Ti(III)-citrate,
and methyl viologen. Fractions from anaerobic column chromatographic separations of extract prepared with
MMA-grown cells were added to this assay mixture, and MMA-
dependent CoM methylation was monitored.

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MMA was not methylated as judged by reverse phase HPLC. However, the addition of MMAMT resulted in the methylation of the corrinoid, since the cofactor now eluted with the retention time of the methylated form. No hydroxylated corrinoid was detected following incubation with MMAMT and MMA.

Methylation of MMCP with MMA in the presence of the MMAMT polypeptide was confirmed by UV-visible spectroscopy (Fig. 3). The corrinoid from MMCP was extracted from samples of Ti(III)-citrate/methyl viologen-reduced MMCP incubated with MMAMT and MMA. The spectrum had the characteristic absorbance peaks of methylated corrinoid (34). The spectrum was not affected by the addition of cyanide (not shown), as expected for methylated corrinoid. In the absence of MMA, the spectrum of the extracted aerobic corrinoid was that of hydroxylated corrinoid. This form was sensitive to cyanalysis. The addition of cyanide led to the appearance of peaks at 361 and 549 nm, characteristic of the monocyano derivative of this corrinoid at pH 7.0. These experiments indicated that MMCP methylation required both MMA and MMAMT.

Association of MMCP and MMAMT—The requirement of MMAMT for the MMA-dependent methylation of MMCP indicates interaction of these proteins. To determine if a stable association could occur between MMCP and MMAMT, comparison was made of the elution profiles of both proteins and their mixture from an anaerobic size exclusion HPLC column (Fig. 4A). Although MMCP migrates as a 29-kDa polypeptide in SDS-PAGE, MMCP eluted with a retention time of 20.5 min, corresponding to an apparent molecular mass of 42 kDa. MMAMT eluted with a retention time of 17.4 min, corresponding to a molecular mass of 170 kDa, indicating that MMAMT exists as a trimer or possibly a tetramer in solution. When equimolar mixtures of both proteins were chromatographed, two peaks eluted from the column. The last peak to elute had
pH 7.0, 38 m

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time points are the averages of duplicate reaction vials.
The rate of CoM methylation by methyl iodide was monitored by the disappearance of free thiol and is expressed as total thiol/vial. The citrate. The rate of CoM methylation by methyl iodide was monitored by the disappearance of free thiol and is expressed as total thiol/vial. The time points are the averages of duplicate reaction vials.

MMCP (Fig. 4B, lanes 5 and 6). However, the first peak eluted with a retention time of 16.5 min, faster than observed for MMAMT chromatographed by itself. This corresponded to an apparent molecular mass of 238 kDa. SDS-PAGE of fractions collected from this peak indicated the presence of MMAMT and MMCP, indicating that this peak was comprised of a complex of the two proteins (Fig. 4B, lanes 2–4). Densitometry of the Coomassie-stained gel indicated the ratio of the MMAMT polypeptide to MMCP in the peak was 3.5:1.

A stable association of MT2-A with either MMCP or MMAMT could not be demonstrated. MT2-A had no apparent effect on complex formation between MMAMT and MMCP or on their individual elution times. MT2-A continued to elute as a monomeric protein even in the presence of the other two proteins.

Methyl Transfer from MMCP to CoM Requires Only MT2-A—MT2-A can demethylate free methylcobalamin and methylate CoM. Presumably, the role of MT2-A in MMA-CoM methyl transfer is the demethylation of MMCP following MMCP methylation by MMAMT. This was indirectly tested by monitoring the suitability of methyl-MMCP as a substrate for CoM methylation by MT2-A in the absence of MMAMT. Methyl iodide serves as a nonphysiological methyl donor for many corrinoid proteins and will readily undergo nucleophilic attack by Co(I) corrinoid (35–37). Neither MT2-A nor MMCP could carry out methyl iodide:CoM methyl transfer when tested alone; however, in combination the two proteins mediated the reaction at a rate of 875 nmol/min/mg of total protein (Fig. 5). In order for MMCP to enter the Co(I)/methyl-cobalt catalytic cycle, it was added to the reaction mixture in the methylated form. MMCP in the Co(II) form was inactive in methyl transfer. This is consistent with the methylation of Co(I) corrinoid by methyl iodide, followed by catalysis of methyl transfer from methyl-MMCP to CoM by MT2-A.

MT2-M does not participate in MMA metabolism (20). Methyl iodide:CoM methyl transfer via MMCP was also specific for MT2-A; no detectable reaction occurred with MT2-M (Fig. 5). Interestingly, although MT2-M does not support CoM methylation with MMCP, MT2-M could inhibit the methyl iodide:CoM methyl transfer reaction mediated by MMCP and MT2-A. The addition of 1.5 nmol of MT2-M to a reaction mixture containing 0.27 nmol of MT2-A and 1.3 nmol of MMCP inhibited the reaction by 45%.

MMCP Is the Major Corrinoid Protein of MMA:CoM Methyl Transfer Detectable in Cell Extracts—The rate of MMA-dependent methylation of CoM in extracts is typically about 0.2 μmol/min/mg of protein. However, the final reconstituted system consisting of these three purified polypeptides mediated the reaction at a rate of 0.6 μmol/min/mg. It is possible that the MMCP and its associated methyltransferases were a minor pathway of CoM methylation from MMA. To determine if MMCP and its associated methyltransferases are the major route of MMA dependent CoM methylation, MMCP was specifically removed from extracts. Affinity-purified α-MMCP IgG was incubated with an extract of TMA-grown cells, then removed with Sepharose 6B coupled to protein A. Removal of MMCP protein was nearly complete, as indicated by Western analysis (Fig. 6B, lane 3). Extract treated with the nonimmune IgG fraction retained MMCP at a level similar to that of untreated extracts, as judged by densitometry of the corresponding signals on the Western blot (Fig. 6B, lanes I and 2). The MMCP-depleted extract had 9% of MMA:CoM methyl transfer activity remaining relative to extract treated with the nonimmune IgG fraction (Fig. 7). Activity could be completely restored to original levels by the addition of purified MMCP to the depleted extract.

The corrinoid-binding polypeptide involved in DMA:CoM methyl transfer has yet to be clearly identified. Since MT2-A was demonstrated by immunodepletion of extracts to be involved in both DMA- and MMA-dependent CoM methylation, it remained a possibility that the same corrinoid protein was also involved in both catabolic reactions. The extracts immunodepleted of MMCP above were also tested for DMA:CoM methyl transfer activity (Fig. 7). DMA-dependent CoM methylation was virtually unaffected by removal of MMCP, and the levels of activity were similar in extracts treated with either immune or non-immune IgG. This indicates that the role of MMCP in methylene metabolism is specifically MMA utilization and that the corrinoid-binding polypeptide involved in DMA metabolism is distinct from MMCP.

DISCUSSION

This is the first reconstitution of MMA:CoM methyl transfer using only purified proteins. The involvement of MMCP and MT2-A had been previously demonstrated, but these two proteins were insufficient to reconstitute MMA:CoM methyl transfer in vitro (20). In this work, we were able to identify and purify to homogeneity from extracts of cells grown on MMA an abundant 52-kDa polypeptide, MMAMT, which...
Fig. 7. MMCP is the major route of MMA- but not DMA-dependent CoM methylation detectable in cell extracts. Untreated extract or extracts treated with either nonspecific IgG or anti-MMCP IgG were assayed for MMA:CoM and DMA:CoM methyl transfer activities as described under “Experimental Procedures.” Reaction mixtures (125 μl) contained 50 mM MOPS-HCl, pH 7.0, 2 mM CoM, 20 mM MMA or DMA, 10 mM ATP, 20 mM MgCl2, 1 mM bromoethanesulfonic acid, 0.8 mg of extract sample, and, where designated, 20 or 40 μg of purified MMCP.

Acted along with MMCP and MT2-A in the methylation of CoM with MMA.

In many of the studies of methyltransferases involved in methylocrotrophic methanogenesis, heterogeneous protein fractions have been used to supply a methyltransferase activation protein and reducing power to effect the ATP-dependent reductive activation of corrinoid proteins (14, 16). We were able to reconstitute methyl transfer with only three proteins, and activity was not dependent on ATP. This was possible since Ti(III)-citrate and methyl viologen were used for reductive activation of MMCP. Ti(III)-citrate is capable of effecting the reduction of methyl viologen to the neutral species (24), which has a midpoint potential of −730 mV (38). This is sufficient to effect reduction to the Co(I) state in the corrinoid-dependent methyltransferases whose midpoint potentials have been measured, which range from −426 to −526 mV (39–43). Methyl viologen greatly stimulates the MMA:CoM methyl transfer reaction and may circumvent a kinetic barrier in the direct reduction of MMCP to Co(I) with Ti(III)-citrate.

The specific activity that we have observed for the MMA:CoM methyl transfer reaction is lower than expected from the rate found with unfractionated extract. This accounts for the low apparent recoveries of MMAMT during purification. Similarly low specific activities have been observed with the purified proteins and protein fractions catalyzing CoM methylation with either TMA (21, 24) or in most cases with methanol (7, 15, 21, 24). Several explanations for the low specific activity with the MMA methyltransferases (and the resultant low apparent recovery of total activity from the extract) are possible. Reductive activation of MMCP to the Co(I) state with Ti(III)-citrate and methyl viologen could be incomplete, leaving only a fraction of MMCP in the active state. Alternately, a protein stimulating the reaction could be lost during purification. Our results indicate that such a protein would not be involved in direct methyl transfer from MMA to CoM but, for example, could promote the reductive activation of MMCP. In addition, the components of the in vitro reaction have not yet been optimized. However, despite the low specific activity of the reconstituted system, MMCP is the major route of methanogenesis from MMA detectable in cell extracts. Removal of MMCP from extracts with affinity-purified IgG significantly inhibited MMA-dependent methylation of CoM. Supplementation of the MMCP-depleted fraction with highly purified MMCP resulted in reaction rates even higher than those observed before removal of MMCP.

The current data allow the roles of MMAMT and MT2-A in the MMA:CoM methyl transfer reaction to be further defined. The role of MMAMT in MMA:CoM methyl transfer is the methylation with MMA of the corrinoid cofactor bound to MMCP, i.e., the protein acts as an MMA:MMCP methyltransferase. Methylation of MMCP did not require the presence of MT2-A. The MMAMT polypeptide could facilitate MMCP methylation with MMA in several ways. MMAMT binds MMCP, and it could further bind MMA and activate the methylamine so that nucleophilic attack by Co(I) of MMCP is facilitated. The interaction of MMAMT and MMCP could also result in kinetic and/or thermodynamic effects that favor the Co(I) state of the MMCP-bound corrinoid. In any case, the involvement of MMAMT in methanogenesis from monomethylamine is clear. We recently found that MMAMT and MMCP lie adjacent on the chromosome and are co-transcribed.2

The complex formed between MMCP and MMAMT is consistent with MMAMT function as an MMA:MMCP methyltransferase. However, it must be stressed that the stoichiometry observed in this complex (3–4 MMAMT monomers/MMCP monomer) may only represent the most stable complex detectable by the means employed. Complexes with higher amounts of MMCP may form that dissociate during size exclusion HPLC. Supporting this possibility is the observation that MMAMT-to-MMCP monomer ratios of 2 yielded maximal rates in the reconstituted MMA:CoM methyl transfer reaction.

Once methylated by MMA, MMCP serves as methyl donor to CoM. The role of MT2-A in the MMA:CoM methyl transfer reaction is that of a methyl-MMCP:CoM methyltransferase. This reaction can be carried out in the absence of MMAMT, as demonstrated by the methyl iodide:CoM methyl transfer reaction mediated by MMCP and MT2-A. That MT2-A serves to methylene CoM with methyl-MMCP was not unexpected, since MT2 isozymes such as MT2-A and MT2-M catalyze rapid methylation of CoM with non-protein-bound methylcobalamin (7, 12), but direct methyl transfer from MMCP to CoM had not been previously demonstrated. The specificity of MMCP for MT2-A in either the methyl iodide- or MMA-dependent reactions is striking. Another MT2 isozyme, MT2-M, did not act as a methyl-MMCP:CoM methyltransferase, indicating that specific interaction of determinants on MMCP and MT2-A are required for the enzymatic transfer of the methyl group from the corrinoid to CoM. It was interesting in this regard to observe that MT2-M could inhibit methyl transfer from MMCP to CoM by MT2-A. The simplest explanation for inhibition is that MT2-M can also interact with MMCP but does not allow the proper orientation of CoM and methyl-corrinoid needed for methyl transfer. The inhibition by MT2-M of MMA:CoM methyl transfer would not present a problem in vivo, since MT2-A is by far the predominant MT2 isozyme found in cells grown on methylamines (25).

A direct comparison of the methyltransferases involved in CoM methylation from both MMA and TMA is now possible (Fig. 8). Like MMA:CoM methyl transfer, the TMA:CoM methyl transfer reaction requires three polypeptides (24). Aside from MT2-A, the TMA-dependent reaction requires a corrinoid-binding protein (TCP), and a 52-kDa polypeptide (TMA-52). However, both sets of methylamine methyltransferases are very specific for their substrates. The MMA:CoM methyltransferases do not utilize either DMA or TMA, while the TMA:CoM methyltransferases do not utilize DMA or MMA. Since both methylamines can donate methyl groups to CoM via

2 S. Burke and J. Krzywosz, manuscript in preparation.
The smaller subunit has the corrinoid binding motif found in cobalamin-dependent methionine synthase (44) and the corrinoid-binding subunit of a recently discovered CoM methylase (11, 35). The sequences of MtaB and MtaC do not contain the N-terminal sequences we have obtained for TCP, TMA-52, MMAMT, and MMCMP. However, homology of the N termini of TCP and MMCMP to MtaC is detectable.3

The similarities among methylotrophic paths to the methylation of CoM are striking and indicate that a general model of methylotrophic methanogenesis can be proposed. Central to each pathway is a distinct corrinoid protein. The corrinoid protein appears to determine specificity for the MT2 isozyme, which methylates CoM with the methylated corrinoid protein. Aside from the corrinoid protein and the MT2 isozyme, a third polypeptide is found in each methyl transfer system. In the MMA system, this larger polypeptide is MMAMT, which acts to methylate the corrinoid protein with the methylotrophic substrate. This may also be the role of the largest polypeptides of the methanol:CoM and the TMA:CoM methyl transfer proteins.

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