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Reconstitution of Trimethylamine-Dependent Coenzyme M Methylation with the Trimethylamine Corrinoid Protein and the Isozymes of Methyltransferase II from *Methanosarcina barkeri*

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Reconstitution of trimethylamine-dependent coenzyme M (CoM) methylation was achieved with three purified polypeptides. Two of these polypeptides copurified as a trimethylamine methyl transfer (TMA-MT) activity detected by stimulation of the TMA:CoM methyl transfer reaction in cell extracts. The purified TMA-MT fraction stimulated the rate of methyl-CoM formation sevenfold, up to 1.7 μmol/min/mg of TMA-MT protein. The TMA-MT polypeptides had molecular masses of 52 and 26 kDa. Gel permeation of the TMA-MT fraction demonstrated that the 52-kDa polypeptide eluted with an apparent molecular mass of 280 kDa. The 26-kDa protein eluted primarily as a monomer, but some 26-kDa polypeptides also eluted with the 280-kDa peak, indicating that the two proteins weakly associate. The two polypeptides could be completely separated using gel permeation in the presence of sodium dodecyl sulfate. The corrinoid remained associated with the 26-kDa polypeptide at a molar ratio of 1.1 corrin/26-kDa polypeptide. This polypeptide was therefore designated the TMA corrinoid protein, or TCP. The TMA-MT polypeptides, when supplemented with purified methylcorrinoid:CoM methyltransferase (MT2), could effect the demethylation of TMA with the subsequent methylation of CoM and the production of dimethylamine at specific activities of up to 600 nmol/min/mg of TMA-MT protein. Neither dimethylamine nor monomethylamine served as the substrate, and the activity required Ti(III) citrate and methyl viologen. TMA-MT could interact with either isozyme of MT2 but had the greatest affinity for the A isozyme. These results suggest that TCP is uniquely involved in TMA-dependent methanogenesis, that this corrinoid protein is methylated by the substrate and demethylated by either isozyme of MT2, and that the predominant isozyme of MT2 found in TMA-grown cells is the favored participant in the TMA:CoM methyl transfer reaction.

Carbon dioxide is reduced to methane by the vast majority of methanogens in culture. *Methanosarcina barkeri* is one of the few species which is also capable of methylotrophic methanogenesis from substrates such as methanol, acetate, methylthiols, and methylamines (3). These compounds can be significant methane precursors in freshwater or marine ecosystems (32).

Methylotrophic substrates destined to become methane are first used to methylate the thiol of coenzyme M (2-mercaptoethanesulfonic acid; CoM). Methyl-CoM is then reduced to methane by methylreductase, a step which is linked to the major site of energy conservation in methanogens (9). Methanol, trimethylamine, and acetate are converted to methane with retention of most of the original hydrogen atoms of the methyl group (23, 30, 33).

CoM is methylated by acetate through the intermediacy of methyltetrahydrosarcinapterin (12, 15, 20, 27). In contrast, methanol is used to directly methylate CoM, as demonstrated by Vogels and collaborators (7, 8, 17, 28, 29). The reactions are summarized below:

$$H_+ + CH_3OH + MT1\text{-}[Co(I)] \rightarrow MT1\text{-}[Co\text{-}CH_3] + H_2O$$

$$HSCoM + MT1\text{-}[Co\text{-}CH_3] \overset{MT2}{\rightarrow} MT1\text{-}[Co(I)] + CH_3\text{-}S\text{-}CoM + H^+$$

where MT1 is methyltransferase I. MT1 is a 122-kDa corrinoid protein with subunits of 52 and 33 kDa. The corrinoid prosthetic group is methylated by methanol when in the Co(I) redox state. Methylated MT1 serves as substrate for CoM methylation by methyltransferase II (MT2) (22), a 37-kDa zinc binding polypeptide. Hydrogenase, ferredoxin, and a methyltransferase-activating protein are also required to supply an ATP-dependent activation system to reduce the central cobalt ion of the MT1-bound corrinoid to the Co(I) state (6–8).

The pathways of methanogenesis from trimethylamine (TMA), dimethylamine (DMA), and monomethylamine (MMA) are still being elucidated. A second isozyme of MT2 has been isolated (14) and has been found to participate in CoM methylation from the methylamines (4, 11). This is the A isozyme of MT2 (the amine isozyme; MT2-A). MT2-A show 50% sequence similarity to the M isozyme of MT2 (involved in methanol metabolism; MT2-M) (16, 22). Involvement of MT2-A in TMA metabolism was first postulated from the abundance of MT2-A in cells grown on TMA (31). Involvement of MT2-A in MMA metabolism was directly demonstrated by the stimulation of the rate of MMA:CoM methyl transfer by purified MT2-A, but not MT2-M, in cell extracts (4). Cell extracts depleted of MT2-A by affinity columns can methylate CoM with DMA or MMA only after addition of purified MT2-A, but not MT2-M, to the depleted extract (11). The involvement of MT2-A in DMA and MMA metabolism implies the involvement of corrinoid proteins in these pathways as well. The 29-kDa monomeric MMA corrinoid protein (MMCP) interacting with MT2-A during methanogenesis from MMA has been purified (4, 5, 18).

While corrinoid proteins mediating CoM methylation from DMA or MMA have a specific requirement for MT2-A, methane formation from TMA appears to be supported by either MT2 isozyme. Immunodepletion of MT2-A from extracts did not completely eliminate TMA:CoM methyltransferase activity, and supplementation of the immunodepleted extract with

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either highly purified MT2 isozyme reconstituted activity (11). This result also indicated that the methylation of CoM by TMA requires a corrinoid protein. Thus far, corrinoid proteins like MMCP or MT1 have shown a strict requirement for one or the other isozyme of MT2. However, the ability of the TMA:CoM methyl transfer pathway to utilize two different MT2 isozymes is consistent with either the involvement of multiple corrinoid proteins in TMA metabolism or the interaction of one corrinoid protein involved in the TMA pathway with either isozyme.

These ambiguities are resolved here by the further elucidation of the pathway of TMA:CoM transmethylation. A corrinoid protein was isolated which, when combined with either MT2 isozyme, mediated the methylation of CoM specifically with TMA.

MATERIALS AND METHODS

Cell cultures and preparation of extracts. *M. barkeri* MS (DSM 800) was cultured in a phosphate-buffered medium supplemented with 80 mM TMA or methanol in 15-liter carboys (19). Cells were lysed anaerobically at 20,000 lb/in^2 in a French pressure cell prior to ultracentrifugation at 150,000 × *g* (5). The supernatant was stored at −70°C in hydrogen-flushed stoppered serum vials until used.

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

Assay of TMA methyl transfer activity employed during purification. The TMA methyl transfer activity (TMA-MT) was detected by stimulation of the rate of TMA-dependent CoM methylation in cell extracts that were monitored for the loss of CoM thiol groups with DTNB (10), essentially as described previously (4) except that the BES concentration was 3.2 mM and 16 mM TMA was used. Other modifications are described in the text.

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

Purification of MT2-A and MT2-M was performed as described previously (4) using a methylcobalamin:CoM methyl transfer assay (14). The identity of the isozymes was confirmed with an affinity-purified MT2-A-specific antibody (10), which was the kind gift of David Grahame.

Measurement of TMA:CoM methyl transfer with purified proteins. Reactions were conducted in stoppered 2-ml serum vials under an atmosphere of H_2. The reaction mixtures contained final concentrations of 2 mM TMA, 2 mM CoM, 0.5 mM methyl viologen, 1.5 mM Ti(III) citrate in 50 mM MOPS buffer, pH 7.0, in a total volume of 125 μl. The amounts of MT2 isozyme or TMA-MT added are indicated in the text. The Ti(III) citrate solution was prepared as described by Seefeldt and Ensign (26). The 93 mM stock solution had a final pH of 8.8. Methyl viologen added to the reaction mixture was prereduced to the neutral species. Reduction to the blue monovalent cationic form of methyl viologen was achieved with addition of a slight molar excess of Ti(III) citrate to methyl viologen; methyl viologen was then further reduced by the addition of 2 μl of 93 mM Ti(III) citrate to 5 μl of 12.5 mM methyl viologen. The reactions were initiated by transferring the vials from ice to a shaking water bath at 37°C. Samples (10 μl) were removed at specific time points and mixed in wells of a microtiter plate containing 90 μl of 0.5 mM Ellman's reagent in 150 mM Tris-HCl, pH 8.0. The absorbances at 410 nm were measured by use of an MR700 enzyme-linked immunosorbent assay plate reader (Dynatech Laboratories, Inc.). Calculations of thiol concentrations were based on standard curves constructed with known amounts of CoM.

In experiments in which methylamine concentrations were monitored during the course of the reactions, 12.5-μl samples of reaction mixture were removed and added to 2.5 μl of 2 M HCl. The samples were then centrifuged for 5 min at 10,000 × *g*. An aliquot (10 μl) of each supernatant was mixed with 16 μl of 4% KOH. Methylamines were then analyzed by gas chromatography as described previously (4). Retention times of methylamines on this system were 2.3 min (DMA) and 2.9 min (TMA).

Separation of the polypeptides in the TMA-MT protein. A Superdex-200 (16/60) size exclusion column (Pharmacia LKB Inc.) was equilibrated in 50 mM Tris-HCl, pH 8.0, containing 0.5% sodium dodecyl sulfate (SDS). The protein solution was diluted 1:1 in 100 mM Tris-HCl, pH 8.0, containing 2% SDS and 5% β-mercaptoethanol and loaded onto the column in a volume of 1 ml; the polypeptides were eluted with equilibration buffer at 0.5 ml/min. The A_{280} peaks which eluted were pooled and concentrated with Centricon YM-10 membranes for analysis by SDS-gel electrophoresis, by obtaining UV-visible spectra, or by determining cobamide content.

Other analytical techniques. Cobamide concentrations were determined by the dicyano derivatization method (15). Protein concentrations were determined by the method of Lowry et al. (24), with bovine serum albumin as the standard. UV-visible spectra were obtained with a Beckman DU-70 spectrophotometer equipped for use with 50-μl microcuvettes or a Hewlett-Packard model 8453 photodiode array spectrophotometer. SDS-gel electrophoresis was performed in accordance with the method of Laemmli (21) with a Mini-slab electrophoresis system (Idea Scientific Co., Minneapolis, Minn.). Molecular size markers (Pharmacia LKB Inc.) were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). Nondenaturing-gradient polyacrylamide gel electrophoresis (PAGE) was performed with the same buffer system but in the absence of SDS and β-mercaptoethanol. Two-dimensional gel electrophoresis was performed as described previously (18). To determine the native sizes of the proteins in the TMA-MT fraction, a Sepharose CL-6B column (1.6 by 84 cm) was adapted to a Bio-Rad medium-pressure liquid chromatography system (Bio-Logic) inside a Coy anaerobic chamber and equilibrated with 100 mM NaCl in 50 mM MOPS, pH 7.0. An aliquot (460 μg) of TMA-MT was loaded onto the column in a volume of 250 μl and eluted at a rate of 0.5 ml/min. Molecular weight standards used to generate the standard curve were blue dextran (2,000,000), thyroglobulin (669,000), apoferritin (443,000), B-amylase (200,000), alcohol dehydrogenase (150,000), bovine serum albumin (66,000), carbonic anhydrase (29,000), cytochrome *c* (12,400), and ferricyanide (324), all of which were purchased from Sigma Chemical Co.

RESULTS

Isolation of polypeptides involved in the TMA:CoM methyl transfer pathway. It was anticipated that several proteins might participate in the methylation of CoM with TMA. Therefore, individual components of the multicomponent reaction were detected by stimulation of TMA-dependent CoM methylation in a cell extract. This assay was a variation of that used previously to detect the corrinoid protein involved in MMA-dependent CoM methylation (4). An extract with a low level of TMA:CoM methyl transfer activity was obtained by diluting 1:32 (vol/vol) an extract of TMA-grown cells with an extract of cells grown on methanol. The trace amount of TMA-grown cell extract provided a low rate of TMA:CoM methyl transfer: from 2 to 10 nmol/min/mg of total protein. The methanol-grown cell extract lacked detectable TMA:CoM methyl transfer activity and maintained a low redox potential via hydrogenase and the hydrogen gas phase. With this assay, a single peak of activity was detected following initial chromatography of a TMA-grown cell extract on DEAE-Sephacel under anaerobic conditions. The pooled activity was further purified by low-pressure chromatography with a strong cation exchanger and gel permeation and, finally, by two passes on Mono-Q columns with medium-pressure chromatography (Table 1). The final fraction, termed the TMA-MT fraction, was used in the subsequent experiments described here. The dependence

TABLE 1. Purification of the TMA-MT activity from an extract of TMA-grown cells[a]

Fraction	Total protein (mg)	Total activity (μmol[b]/min)	Sp act (μmol/min/mg[c])	Recovery (%)
TMA extract	2,890	805	0.28	100
DEAE-Sephacel	890	69.2	0.41	46
Q-Sepharose	383	245	0.64	30
Sephacryl S-300	180	125	0.70	16
Mono-Q1	65	49.4	0.76	6.1
Mono-Q2	21	36	1.7	4.5

[a] For details, see Materials and Methods.
[b] Micromoles of CoM methylated.
[c] Per milligram of protein of the purification fraction that was dependent on the addition of TMA to the assay mixture.

of the rate of CoM methylation with TMA as the methyl donor on the TMA-MT fraction is illustrated in Fig. 1. In this particular experiment, a sevenfold stimulation over the background rate of TMA-dependent CoM methylation was observed with the greatest amount of TMA-MT fraction tested.

Composition of the TMA-MT fraction. SDS-PAGE of the TMA-MT fraction revealed two polypeptides with apparent molecular masses of 52 and 26 kDa (Fig. 2A). A densitometric analysis indicated that these polypeptides were present at a molar ratio of 3.6:1, respectively. Three independent purifications of TMA-MT were performed; both resulted in TMA-MT fractions containing only these two polypeptides. The TMA-MT polypeptides comigrated with prominent bands observed following electrophoresis of cell extracts of TMA-grown cells. Cell extracts of methanol-grown cells, which do not convert TMA to methane, did not appear to have prominent polypeptides of these molecular weights.

The temperature at which TMA-MT protein was heated (for 5 min) in sample buffer prior to SDS-PAGE was found to influence the electrophoretic migration of the smaller polypeptide. Samples heated at temperatures higher than 80°C yielded only the form of the polypeptide with an apparent molecular mass of 26 kDa. However, when samples were heated at temperatures below 60°C, no 26-kDa band was observed and only

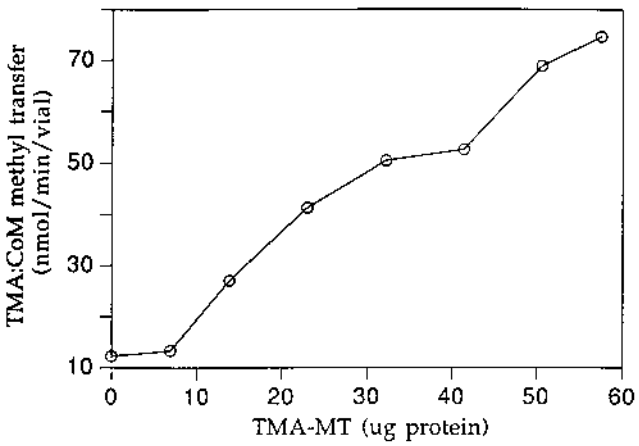

FIG. 1. Stimulation by the isolated TMA-MT fraction of TMA:CoM methyl transfer activity in cell extract. Increasing amounts of the TMA-MT fraction from the final Mono-Q purification step were assayed as described in Materials and Methods. The reaction mixture was supplemented with the indicated amounts of TMA-MT protein in 25 μl of MOPS buffer, pH 7.0. The activity corresponds to the TMA-dependent loss of free thiols of CoM and is not corrected for the background activity observed in the extract.

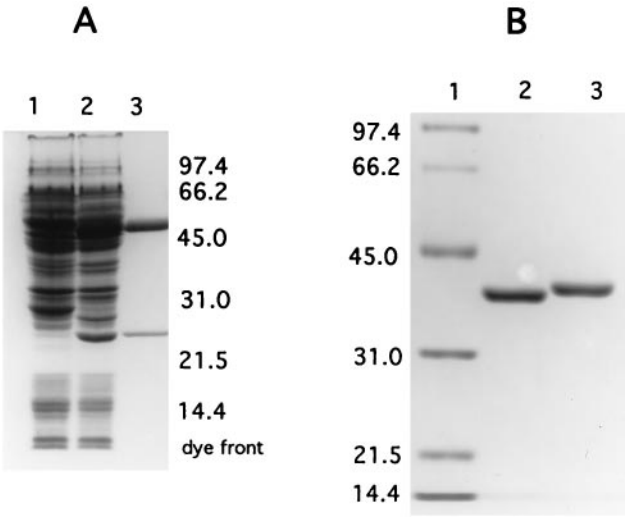

FIG. 2. SDS-gel electrophoresis of the protein components of the reconstituted TMA:CoM methyl transfer reaction. (A) Coomassie-stained 12% acrylamide gel of the TMA-MT fraction from the final Mono-Q purification step. Lanes: 1, 33 μg of protein from a methanol-grown cell extract; 2, 32 μg of protein from a TMA-grown cell extract; 3, 5 μg of protein from the TMA-MT fraction. The relative positions of standard proteins with the indicated masses in kilodaltons are indicated on the right. (B) Coomassie-stained 12% acrylamide gel of MT2-M and MT2-A preparations used in these experiments. Lanes: 1, molecular mass standards; 2, MT2-A (3 μg); 3, MT2-M (3 μg).

a form with an apparent molecular mass of 21 kDa was observed. When TMA-MT samples were heated at temperatures between 60 and 80°C, a mixture of both the 26- and 21-kDa bands was observed (data not shown).

Nondenaturing gel electrophoresis of the TMA-MT activity under anaerobic conditions yielded a fast-migrating band and a slow-migrating band. Experiments with two-dimensional gels subjected to nondenaturing gel electrophoresis in the first dimension followed by an SDS gel in the second dimension revealed that the slower-migrating band was composed of both 52- and 26-kDa polypeptides. The faster-migrating band consisted only of the 26-kDa polypeptide. Approximately 30% of the 26-kDa polypeptide migrated with the 52-kDa polypeptide during nondenaturing PAGE.

The association of the 26-kDa polypeptide and the 52-kDa polypeptide was further demonstrated by chromatography of the TMA-MT fraction on a Sepharose 6B column under anaerobic conditions. Two well-separated and symmetrical protein peaks eluted from the column; the peaks represented proteins with apparent molecular masses of 280 and 33 kDa. The larger peak consisted primarily of the 52-kDa polypeptide, while the second peak consisted only of the 26-kDa polypeptide. However, some 26-kDa polypeptides also eluted with the 280-kDa peak. The molar ratio of 52- to 26-kDa polypeptide in the 280-kDa peak was 20:1. This indicates that 20% of the 26-kDa polypeptide remained associated with the 52-kDa polypeptide during gel permeation; the remainder eluted as a monomeric protein.

Reconstitution of the TMA:CoM methyl transfer reaction with purified proteins. The TMA-MT fraction was combined with another known participant in this reaction, MT2-A (Fig. 2 illustrates the purity of these preparations). In the presence of Ti(III) citrate and methyl viologen, these three proteins were found to mediate the TMA:CoM methyl transfer reaction. Three separate preparations of the TMA-MT fraction, whose specific activities varied from 600 to 150 nmol/min/mg of

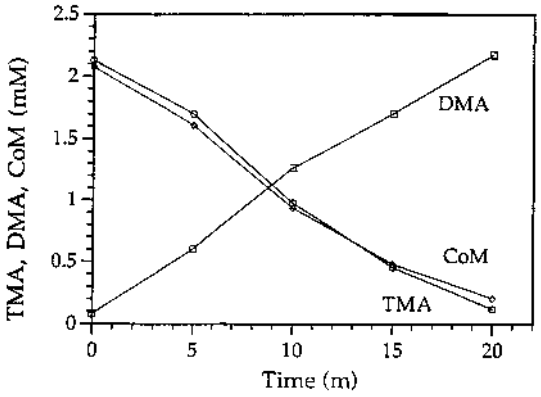

FIG. 3. Substrate and product stoichiometry of the TMA:CoM methyltransferase reaction mediated by the TMA-MT fraction and MT2-A. The reaction mixture initially contained 2 mM (each) CoM and TMA, 1.5 mM Ti(III) citrate, 0.5 mM methyl viologen, 80 μg of MT2-A, and 80 μg of the TMA-MT fraction. Samples (12.5 μl) were removed from the 250-μl reaction mixture at the indicated time points for measurement of the free thiol of CoM and methylamines.

TMA-MT protein when 40 μg of TMA-MT fraction was assayed with 40 μg of MT2-A, were made. Controls in which either MT2-A or the TMA-MT fraction was deleted from the reaction mixture did not methylate CoM with TMA. The reaction was strictly dependent on TMA. TMA concentrations higher than 2 mM did not increase the observed reaction rate. DMA and MMA did not serve as substrates for CoM methylation by the TMA-MT proteins and MT2-A.

TMA-dependent CoM methylation was strictly dependent on the addition of Ti(III) citrate and methyl viologen to the assay mixture. Either agent alone was insufficient. Best results were obtained when Ti(III) citrate was present at a threefold excess over methyl viologen and when these agents were incubated together prior to the addition of protein. Methyl viologen was immediately reduced to the blue monovalent cation, but was then further reduced by the Ti(III) citrate to the colorless neutral species, as evidenced by the loss of the methyl viologen absorbance peak at 605 nm. Ti(III) citrate preparations which were not freshly prepared were not capable of reducing methyl viologen to the neutral species and were inactive in promoting the TMA:CoM methyl transfer reaction. TMA-MT that was exposed to oxygen for 6 h at 4°C, and then made anaerobic by flush and evacuation, could still be activated by Ti(III) citrate and methyl viologen with little loss of original activity.

Substrates and products of the TMA:CoM methyl transfer reaction. The methylation of CoM by the TMA-MT polypeptides and MT2-A was typically monitored by observing the loss of the free thiol of CoM, using Ellman's reagent (10). Since this indirectly measures the demethylation of TMA and the subsequent methylation of CoM, the reaction rate was also monitored by observing the consumption of TMA and the production of DMA (Fig. 3). The rate of TMA utilization was very similar to that of the consumption of the free thiol of CoM (350 nmol/min/mg of TMA-MT protein). DMA was produced during the entire course of the reaction at a rate very near to that of TMA and CoM disappearance. After 20 min, 520 nmol of TMA and 470 nmol of CoM had been consumed and 520 nmol of DMA had been produced. No MMA was detected during the course of the reaction. These results indicate that the demethylation of 1 mol of TMA yields an equimolar amount of both DMA and methyl-CoM. The production of DMA was dependent on the addition of CoM to the reaction mixture.

The 26-kDa polypeptide is the TMA-specific corrinoid protein. The requirement for a methylcobamide:CoM methyltransferase such as MT2-A in the reconstituted TMA:CoM methyl transfer reaction indicated that one or both of the polypeptides in the TMA-MT fraction were corrinoid proteins. The anaerobically purified TMA-MT fraction had an orangish-red color, also indicating a corrinoid cofactor was present. This was confirmed by the UV-visible spectrum of the protein (Fig. 4B). The as-isolated scan of the Mono-Q2 fraction had absorbance peaks characteristic of protein-bound corrinoids. The maxima at 480 and 532 nm indicate that the corrinoids are present in a mixture of Co(II) and Co(III) redox states. The corrinoid cofactor was extracted and quantified by the dicyano method. The molar ratio was determined to be 0.92 nmol of corrinoid:1 nmol of 26-kDa polypeptide:4 nmol of 52-kDa polypeptide.

This ratio indicated that the 26-kDa polypeptide might bind the corrinoid cofactor present in the TMA-MT fraction. The two polypeptides form a loosely associated complex, however, and were not completely separated by gel permeation chromatography except in the presence of a denaturant. Chromatography of the TMA-MT fraction was performed with a medium-pressure gel permeation column eluted with buffer containing 0.5% SDS under aerobic conditions. The two polypeptides were cleanly separated and eluted as symmetrical peaks at 52.9 and 70.5 ml. The latter peak was found to contain entirely the 26-kDa polypeptide, while the former peak contained entirely the 52-kDa polypeptide (Fig. 4A). Each peak was pooled and concentrated, and the UV-visible spectra were collected. The 52-kDa polypeptide had a featureless spectrum except for the major absorbance peak centered at 276 nm (Fig. 4C). In contrast, the 26-kDa polypeptide clearly retained the corrinoid cofactor, with prominent peaks at 351 and 532 nm and a shoulder at 570 nm; these features are characteristic of protein-bound Co(III) corrinoid (Fig. 4D). The amount of corrinoid was quantitated by extraction and conversion to the dicyano form, and 1.1 nmol of corrinoid per nmol of 26-kDa polypeptide was found. Similar treatment of the 52-kDa polypeptide yielded no detectable corrinoid.

Relative efficacy of MT2 isozymes in TMA-dependent CoM methylation. The TMA-MT polypeptides were found to be capable of coupling TMA demethylation to CoM methylation with either purified isozyme of MT2 (Fig. 5). The concentration of each isozyme was varied while the amount of the TMA-MT fraction was held constant at 35 μg of protein (approximately 0.15 nmol of 26-kDa polypeptide). The concentrations of both isozymes were increased until they were no longer the rate-limiting components in the reaction. At saturation, MT2-A supported a maximal rate of 170 nmol/min/mg of TMA-MT protein. MT2-M supported a rate of 88 nmol/min/mg of protein. The reaction displayed a marked preference for the A isozyme. The amount of MT2-A required to reach half of the maximal rate was 0.35 nmol, or a molar ratio of MT2-A to the corrinoid protein of 2.3. The amount of MT2-M required to reach saturation was 1.5 nmol, corresponding to a molar ratio of MT2-M to corrinoid protein of 10.

DISCUSSION

The involvement of MT2 isozymes (that is, methylcorrinoid:CoM methyltransferases) in TMA conversion to CoM (11) entailed the participation of a corrinoid binding protein in the pathway, since little free corrinoid is present in *M. barkeri*. That corrinoid binding polypeptide is identified here for the first

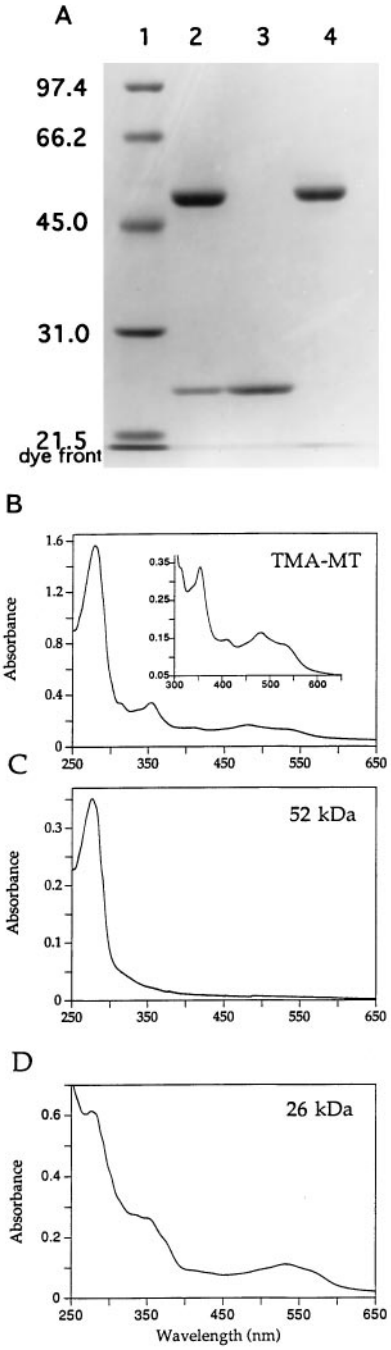

FIG. 4. The 26-kDa polypeptide binds the corrinoid cofactor present in the TMA-MT fraction. The two polypeptides of the TMA-MT fraction were separated on a gel permeation column eluted with a buffer containing SDS. (A) SDS–10% polyacrylamide gel of TMA-MT before and after gel permeation in the presence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

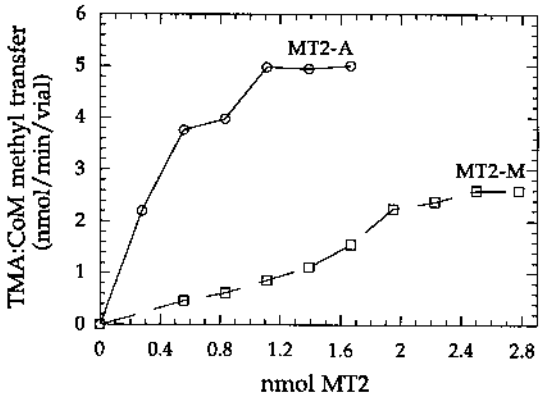

FIG. 5. Relative activities of different MT2 isozymes with TMA-MT for the transfer of methyl groups from TMA to CoM. The amount of TMA-MT was held constant at 35 μg of protein, while the amount of either MT2-A or MT2-M was varied over the indicated range. The molecular mass of both isozymes was taken as 37 kDa, and the total assay volume was 125 μl.

time. The isolated TMA-MT fraction consists of a 26-kDa corrinoid binding polypeptide weakly associated with a 52-kDa polypeptide. The 26-kDa polypeptide is therefore designated TCP (trimethylamine corrinoid protein). The TMA-MT fraction containing these two polypeptides, along with either MT2 isozyme, could mediate CoM methylation by TMA.

With the isolation of TCP and MMCP (4), two different corrinoid proteins involved in the methylamine pathways have been purified. The N termini of the polypeptides in the TMA-MT fraction have been obtained, and they are both different from the N terminus of MMCP (data not shown). TCP does not stimulate MMA-dependent CoM methylation in cell extracts, nor does the reconstituted TMA:CoM methyl transfer activity utilize MMA or DMA. MMCP specifically stimulates MMA- but not TMA-dependent CoM methylation in cell extracts. The participation of MT2-A in the methylation of CoM from TMA, DMA, and MMA had raised the possibility that a single corrinoid protein exists which participated in all three pathways. The activities of MMCP and TCP show that at least two methylamine pathways are mediated by separate corrinoid proteins. It remains to be seen whether an independent corrinoid protein exists for the DMA:CoM methyl transfer pathway.

The involvement of a corrinoid protein in TMA:CoM methyl transfer indicates a mechanism similar to that of methanol:CoM methyl transfer, that is, TCP carries the corrinoid cofactor which is methylated by TMA and demethylated by MT2 for the subsequent methylation of CoM. However, the role of the 52-kDa polypeptide in the TMA:CoM methyl transfer reaction is not as clear. We did not observe activity without the 52-kDa polypeptide in the TMA-MT fraction. The 52-kDa polypeptide eluted as a 280-kDa protein, and TCP eluted as a monomer, but some TCP was always present in small amounts with the 52-kDa polypeptide. The copurification of the two polypeptides and their weak association indicate that the 52-kDa polypeptide is probably also involved in the TMA pathway. The direct involvement of both polypeptides in TMA-dependent CoM methylation would be consistent with the dimeric nature of MT1, which interacts with MT2-M during the methanol-dependent methylation of CoM (28). A simple hypothesis is that the 52-kDa polypeptide acts to methylate TCP with TMA prior to CoM methylation by MT2.

In cell extract, the TMA:CoM methyl transfer reaction requires the presence of hydrogen and ATP for reductive acti-

vation of the transmethylases mediating the reaction (25). Reconstituted TMA:CoM methylation required Ti(III) citrate and methyl viologen. This low-potential reducing system replaced cellular-ATP-dependent reductive activation and allowed the reaction to be mediated by TMA-MT and MT2. Corrinoid-dependent methyltransferases are active in the extremely nucleophilic Co(I) state, which is converted to the methylated Co(III) state upon interaction with a methyl donor (1, 2). The presence of Ti(III) citrate can result in the direct reduction of even free cobalamin to Co(I) (13). However, Ti(III) citrate was unable to activate the TMA-MT fraction without methyl viologen as a mediator. Methyl viologen was further inactive unless it was reduced completely to the colorless neutral species. The requirement for the neutral species of methyl viologen indicates that a polar agent may be excluded from the site of reduction on TCP.

This is the first demonstration of CoM methylation by a methylamine in a completely purified system. However, the rate at which TMA-MT and MT2-A catalyze CoM methylation was lower than expected, since cell extracts mediate the reaction at 400 nmol/min/mg of protein (4). Assuming that the TMA-MT polypeptides constitute 5% of the soluble protein in the cell (which is a typical value for the major catabolic enzymes in methanogenesis), only 2 to 5% of the expected activity was achieved with the resolved system. Isolated enzymes of methanogenesis often have lower-than-expected levels of specific activity. For example, a specific activity of 800 nmol/min/mg of MT1 protein was reported for methanol:CoM methyl transfer (28). The TMA-MT fraction also has a higher level of specific activity when assayed in the presence of cell extract (see Table 1). This may reflect the presence of components in the cell extract which stimulate activity of the TMA-MT proteins, such as the cellular reductive activation system. In addition, MT2-A must associate with TCP during TMA-dependent CoM methylation; higher concentrations of protein might increase the association of the proteins significantly.

It was initially surprising to find that either MT2-A or MT2-M supported TMA:CoM methyl transfer activity in the MT2-A-depleted cell extract, since MT1 and MMCP show a marked ability to use only one of the MT2 isozymes. Our current results show that these results can be explained by the ability of one corrinoid protein, TCP, to interact with either MT2-A or MT2-M. TCP must possess determinants which allow it to interact with either isozyme. This flexibility could fulfill a useful physiological function. Cells growing primarily on methanol contain little MT2-A; primarily they contain MT2-M. If TMA-MT can function with either MT2 isozyme, induction of only these components of the pathway could assist the transition from growth primarily on methanol to growth on TMA.

Although either isozyme of MT2 can interact with TCP, it is clear that, on a molar basis, the affinity of the corrinoid protein is greater for MT2-A than for MT2-M. The strict requirement of the MMA and DMA pathways of CoM methylation for MT2-A, as well the greater affinity of the TMA pathway for MT2-A, further emphasizes that this enzyme can be considered the amine isozyme of MT2.

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