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Coenzyme M Methylase Activity of the 480-Kilodalton Corrinoid Protein from *Methanosarcina barkeri*

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Activity staining of extracts of *Methanosarcina barkeri* electrophoresed in polyacrylamide gels revealed an additional methylcobalamin:coenzyme M (methylcobalamin:CoM) methyltransferase present in cells grown on acetate but not in those grown on trimethylamine. This methyltransferase is the 480-kDa corrinoid protein previously identified by its methylation following inhibition of methyl-CoM reductase in otherwise methanogenic cell extracts. The methylcobalamin:CoM methyltransferase activity of the purified 480-kDa protein increased from 0.4 to 3.8 μmol/min/mg after incubation with sodium dodecyl sulfate (SDS). Following SDS-polyacrylamide gel electrophoresis analysis of unheated protein samples, a polypeptide with an apparent molecular mass of 48 kDa which possessed methylcobalamin:CoM methyltransferase activity was detected. This polypeptide migrated with an apparent mass of 41 kDa when the 480-kDa protein was heated before electrophoresis, indicating that the α subunit is responsible for the activity. The N-terminal sequence of this subunit was 47% similar to the N termini of the A and M isozymes of methylcobalamin:CoM methyltransferase (methyltransferase II). The endogenous methylated corrinoid bound to the β subunit of the 480-kDa protein could be demethylated by CoM, but not by homocysteine or dithiothreitol, resulting in a Co(I) corrinoid. The Co(I) corrinoid could be remethylated by methyl iodide, and the protein catalyzed a methyl iodide:CoM transmethylation reaction at a rate of 2.3 μmol/min/mg. Methyl-CoM was stoichiometrically produced from CoM, as demonstrated by high-pressure liquid chromatography with indirect photometric detection. Two thiols, 2-mercaptoethanol and mercapto-2-propanol, were poorer substrates than CoM, while several others tested (including 3-mercaptopropanesulfonate) did not serve as methyl acceptors. These data indicate that the 480-kDa corrinoid protein is composed of a novel isozyme of methyltransferase II which remains firmly bound to a corrinoid cofactor binding subunit during isolation.

The methylation of coenzyme M (2-mercaptoethanesulfonic acid) (CoM) is the penultimate step of methane production from all substrates of methanogenic bacteria examined to date (14). The conversion of methyl-CoM to methane by methylreductase is then coupled to the major energy-conserving steps of methanogenesis (8, 9, 11). The elucidation of a methanogenic pathway from 1 of the 10 known substrates of methanogens therefore entails the identification of enzymes participating in the methylation of CoM from that substrate.

There are two types of CoM methylases present in methanogens. One type is represented by a membrane-bound protein found in both autotrophic and methylotrophic methanogens which mediates CoM methylation by a methylated pterin derivative, either methyltetrahydrosarcinapterin (methyl-THSP) in *Methanosarcina* spp. (3) or methyltetrahydromethanopterin in *Methanobacterium thermoautotrophicum* (15, 27). Transmethylation is apparently coupled to the translocation of sodium ions across the cytoplasmic membrane, thus forming a site of chemiosmotic energy conservation in methanogens (3). The protein has been purified from *Methanosarcina mazei* Gö1 (33) and *M. thermoautotrophicum* (15, 27), with variable numbers of subunits. Eight genes have been identified in the transcriptional unit encoding the methyltransferase in the latter organism (23, 39). Several subunits, including the 26-kDa subunit which binds the corrinoid, appear to be peripheral rather than intrinsic proteins (39). The membrane-bound methylpterin:CoM methyltransferase complex appears to function in direct methyl transfer during methanogenesis from acetate and CO_2 and may also function during oxidation of methanol or the methylamines to carbon dioxide (35).

A second type of CoM methylase is represented by soluble proteins lacking a prosthetic group with significant absorbance in the visible range. These proteins are termed isozymes of methyltransferase II (MT2). The methyl-donating compounds can be either free or protein-bound methylcobamides (26). Both known isozymes are monomeric polypeptides with molecular masses of approximately 35 to 40 kDa (17, 41) and are involved in CoM methylation from several different substrates. The isozyme most abundant in methanol-grown cells is termed the M isozyme (MT2-M). The second isozyme is most abundant in cells grown on methylamines and acetate and is called the A isozyme (MT2-A) (17, 44). The N-terminal sequences of the two isozymes differ by 50%, and the isozymes have different immunological and electrophoretic properties (17, 44).

All three known CoM methylases carry out the nonphysiological methylation of CoM by using free methylcobalamin. The methylcobalamin:CoM methyltransferase reaction mimics the physiological interaction of the CoM methylase with a corrinoid cofactor bound to another polypeptide. The methylcobalamin-dependent CoM methylation mediated by the methylpterin:CoM methyltransferase (43) is carried out by the subunit(s) which would normally interact with the corrinoid bound to the 26-kDa subunit (15) encoded by *mtrA* (23, 39). MT2-M methylates CoM by using the methylated corrinoid cofactor bound to methyltransferase I (42) during methanogenesis from methanol. MT2-A demethylates a 29-kDa corrinoid protein involved in methanogenesis from monomethylamine (6) and apparently interacts with other corrinoid-binding proteins during methanogenesis from dimethylamine (13) or trimethylamine (13, 44).

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In *Methanosarcina barkeri*, several corrinoid proteins are present which are methylated upon inhibition of methylreductase, presumably by interaction with accumulated methanogenic intermediates (7, 29). These proteins include the corrinoid protein involved in methanogenesis from monomethylamine (6). A second methylated protein is a 480-kDa corrinoid protein of unknown function. It is composed of equimolar amounts of 41- and 30-kDa polypeptides, termed the α and β subunits, respectively (7, 29). The corrinoid cofactor is bound to the β subunit (36). Addition of CoM to extracts in which methanogenesis has been blocked results in demethylation of the corrinoid bound to the 480-kDa protein, indicating an involvement in methane formation. Cells grown on acetate possessed approximately 40-fold more 480-kDa protein than cells grown on trimethylamine (28).

In this work, we examined methylcobalamin:CoM methyltransferase activities in extracts of *M. barkeri* grown on acetate or trimethylamine. We found an additional methyltransferase in cells grown on acetate. This is the 480-kDa corrinoid protein. The activity was catalyzed by a single subunit of the protein with sequence homology to the MT2 isozymes. The intact protein mediates CoM methylation from methyl iodide via the endogenous corrinoid cofactor.

MATERIALS AND METHODS

Chemicals. 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 prior to use. 3-(*N*-Morpholino)propanesulfonic acid (MOPS), CoM, 2-mercaptoethanol, mercapto-2-propanol ($CH_3CHOHCH_2SH$), dithiothreitol (DTT), L-homocysteine, 5,5′-dithio-bis(2-nitrobenzoic acid), methylcobalamin, and nitroblue tetrazolium were purchased from Sigma (St. Louis, Mo.). Methyl iodide was purchased from J. T. Baker Inc. (Phillipsburg, N.J.). Polyacrylamide gel supplies were purchased from Bio-Rad (Hercules, Calif.).

Gel electrophoresis. Nondenaturing gradient (5 to 22.5%) polyacrylamide gel electrophoresis (PAGE) was performed with the buffer system of Laemmli (30) in the absence of sodium dodecyl sulfate (SDS) with a Mini-slab electrophoresis system (Idea Scientific Co., Minneapolis, Minn.). This same buffer system was also used for denaturing 10% polyacrylamide gels containing either 0.1% SDS or 6 M urea. Samples for electrophoresis in urea gels were preincubated in 6 M urea for 15 min at 37°C. Samples for SDS electrophoresis were prepared in 10% 2-mercaptoethanol–0.1% SDS–10% glycerol–0.002% bromothymol blue and were heated at the temperatures indicated in Results for 15 min prior to electrophoresis. Molecular size markers (Bio-Rad) were phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and hen egg white lysozyme (14.4 kDa). Gels were either stained by using a colloidal Coomassie G-250 procedure (22) or activity stained as described below. The relative amount of Coomassie G-250 retained by 480-kDa subunits was determined by scanning gels with a Microscan 1000 gel imaging and analysis system (Technology Research Inc., Nashville, Tenn.).

Methylcobalamin:CoM methyltransferase gel activity stain. An activity stain was employed to detect the cob(I)alamin generated from the demethylation of methylcobalamin. Nondenaturing and SDS gels were washed three times in 50 ml of 50 mM MOPS buffer, pH 7.0, for 10 min and then equilibrated in 50 ml of 50 mM MOPS buffer with 0.25 mM methylcobalamin and 0.2 mM nitroblue tetrazolium at 22°C under dim red light with agitation. After equilibration, 1 mM CoM was added, and the gels were further incubated for 5 to 20 min. After color development, the gels were rinsed and equilibrated in MOPS buffer to remove cobalamin and oxidized nitroblue tetrazolium under dim red light with agitation. Development of activity bands required both CoM and methylcobalamin.

Purified enzymes and coenzyme. In vitro methylation with sodium [2-^{14}C]acetate, isolation, and N-terminal sequence analysis of the 480-kDa $^{14}CH_3$-corrinoid protein was as described previously (7, 29). The specific activity was 48,000 dpm/mg of protein. Unless stated otherwise, the protein was in the methylated form when added to reaction mixtures. The methyl group was removed by photolysis (29) when experiments with demethylated protein were performed. Spectra taken following photolysis indicated conversion of the methyl-corrinoid to the Co(II) form. MT2-A and MT2-M (6) were isolated from cells grown on acetate or methanol, respectively. Protein was determined by the Bradford method (4) with bovine serum albumin as a standard. THSP was isolated and methylated as described by Grahame (18).

Demethylation of the 480-kDa CH_3-corrinoid protein by CoM. Manipulations of the 480-kDa CH_3-corrinoid protein for spectral analysis were performed anaerobically under H_2 and dim red light. DTT, L-homocysteine, and CoM were added sequentially and anaerobically to the 480-kDa $^{14}CH_3$-corrinoid protein in 50 mM MOPS buffer, pH 7.0, at 22°C. The final concentration of each thiol after addition to the cuvette was 50 μM. Spectra were collected immediately after each addition by using a Beckman (Arlington Heights, Ill.) DU70 spectrophotometer with a scan rate of 300 nm/min.

In parallel experiments, 50-μl samples were removed after each thiol addition and frozen in liquid N_2 to determine protein retention of the methyl group by size exclusion high-pressure liquid chromatography (HPLC). HPLC separation was performed with a Beckman Instruments System Gold HPLC system equipped with a model 166 UV-visible detector and a model 171 flow cell radioisotope detector. Size exclusion chromatography was performed with a Supelco Progel-TSK G3000SWXL column (Supelco Inc., Bellefonte, Pa.) eluted at 0.5 ml/min with 0.1 M MOPS (pH 7.0)–0.2 M NaCl essentially as described previously (7, 29).

Assay of enzymatic activities. Methylcobalamin:CoM methyltransferase activity was determined under dim red light by using a dicyano derivatization assay (17) with 50 mM Tris HCl (pH 8.0)–10 mM CoM–1 mM methylcobalamin.

Methyl iodide:CoM methyltransferase reactions were performed under a hydrogen headspace in 2-ml stoppered vials. Reaction mixtures (200 μl) were prepared on ice and included 1 mM Ti(III) citrate, 10 mM $MgCl_2$, 10 mM ATP, 34 or 68 μg of 480-kDa CH_3-corrinoid protein, 25 mM methyl iodide, and various concentrations of CoM in MOPS buffer, pH 7.0. $MgCl_2$ and ATP were found not to be required in later tests but were included throughout the study for consistency. For some reactions methyl iodide was replaced with methanol, monomethylamine, dimethylamine, trimethylamine, acetate, acetyl phosphate, or acetyl-CoA (all at 10 mM) or with 2 mM methyl-THSP. For some reactions CoM was replaced with a tested thiol at 1 or 5 mM. Reactions were initiated by adding the enzyme and moving the assay vials from ice to an agitating 37°C water bath. Samples were removed anaerobically at various time points and diluted in 150 mM Tris, pH 8.0, with 0.5 mM 5,5′-dithio-bis(2-nitrobenzoic acid) for determination of the free thiol concentration (12). All reactions ascribed to the 480-kDa protein were shown to be strictly dependent on addition of the protein to the assay.

The effect of purified MT2-A or MT2-M on the methyl iodide:CoM reaction was tested by supplementing a reaction mixture containing the 480-kDa protein (methylcobalamin:CoM methyltransferase activity of 0.02 μmol/min) with either MT2-A or MT2-M (activity of 0.2 μmol/min).

HPLC analysis of CoM and CH_3-S-CoM following the methyl iodide:CoM methyltransferase reaction. A method based on a procedure for the detection of alkylsulfonates (31) was developed to detect CoM derivatives. Anaerobic reaction mixtures (100 μl) were prepared on ice as described above but contained 2 mM CoM, 25 mM methyl iodide, 0.1 mM DTT, and 34 μg of 480-kDa CH_3-corrinoid protein in 5 mM phosphate buffer, pH 7.0. Reactions were initiated by transfer to a 37°C water bath with agitation. After a 5-min incubation, reactions were terminated by the addition of an equal volume of anhydrous ethanol. Samples were centrifuged to remove precipitated protein, dried under vacuum, and suspended in 100 μl of 0.5 mM 2-sulfobenzoate, pH 3.0, for separation by anion-exchange HPLC.

Separation was performed on a 25-cm Supelcosil LC-SAX quaternary aminopropyl anion-exchange column (Supelco, Inc.) at 20°C; the column was equilibrated with 0.5 mM sulfobenzoate in deionized water, pH 3.0, and eluted with the same buffer at 1.0 ml/min. The detector was set at 275 nm. Negative peaks corresponding to displacement of the sulfobenzoate counterion by sulfonate derivatives or other anions were identified by using standard solutions of CoM, CH_3-S-CoM, CoM-homodisulfide, potassium phosphate, and potassium iodide in the elution buffer. Peaks arising from the enzyme preparation were identified by chromatography of samples containing only enzyme. CH_3-S-CoM was the generous gift of John Kemner. The homodisulfide of CoM was generated by oxidizing CoM under 100% O_2 (11).

RESULTS

Soluble proteins with methylcobalamin:CoM methyltransferase activity in cell extracts. In order to detect CoM methylases in cell extracts of *M. barkeri*, we utilized an activity stain employing the nonphysiological substrate methylcobalamin. Extracts of cells grown on acetate or trimethylamine were electrophoresed in a gradient polyacrylamide gel. When the gels were stained for methylcobalamin:CoM methyltransferase activity, multiple bands comigrating with purified MT2-A or MT2-M were observed in both extracts (Fig. 1A and B). However, when gel electrophoresis was performed in the presence of SDS (Fig. 1C), a major single polypeptide of approximately 37 kDa was observed for both isozymes, indicating that MT2-M and MT2-A migrate as multiple forms in the nondenaturing gel system.

Several bands of methylcobalamin:CoM methyltransferase activity that migrated at positions well above those of the MT2

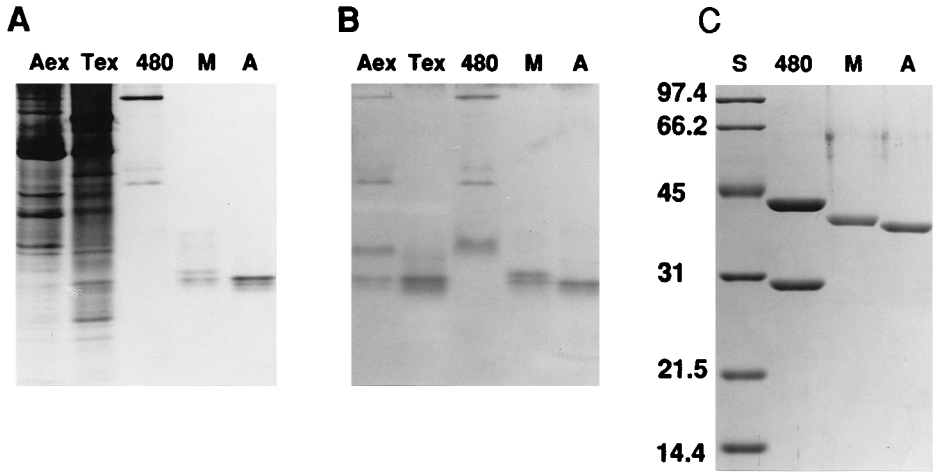

FIG. 1. Nondenaturing and denaturing PAGE of cell extracts, purified 480-kDa protein, MT2-A, and MT2-M. (A) A nondenaturing gradient polyacrylamide gel was stained for total protein with Coomassie G-250. The samples loaded were as follows: Aex, extract of acetate-grown cells (29 μg of protein); Tex, extract of trimethylamine-grown cells (31 μg of protein); 480, purified 480-kDa corrinoid protein (3 μg); M, MT2-M (1 μg); and A, MT2-A (1 μg). (B) A nondenaturing gradient polyacrylamide gel was stained for methylcobalamin:CoM methyltransferase activity. Lanes contain the same protein samples as in panel A, but at the following concentrations: Aex, 2.9 μg; Tex, 3.1 μg, 480, 0.1 μg; M, 0.05 μg; and A, 0.05 μg. (C) SDS–10% polyacrylamide electrophoretic gel of purified 480-kDa corrinoid protein and MT2-M and -A. The samples (purified proteins) loaded were as follows: 480, 480-kDa corrinoid protein (5 μg); M, MT2-M (1.4 μg); A, MT2-A (1.6 μg); and S, molecular mass standards. Numbers to the left indicate masses in kilodaltons. The gel was stained with Coomassie G-250.

isozymes were detected in the extract of acetate-grown cells. These bands were not present in the extract of trimethylamine-grown cells. The apparent regulation of these activity bands by the catabolic substrate, along with the pattern of the bands in the nondenaturing gel, indicated that the activity could be associated with the 480-kDa corrinoid protein. Previously, purified preparations of the corrinoid protein had been demonstrated to migrate as at least two bands composed of both subunits in this gel system (29). When the purified 480-kDa protein was electrophoresed and activity stained in the same nondenaturing gel system, it was found that the activity bands arising from the purified protein comigrated with those activity bands unique to the acetate-grown cell extract. No activity band corresponding to either MT2 isozyme was detected in the purified 480-kDa protein preparation.

Although the purified 480-kDa protein migrated as several bands in the nondenaturing gel, when SDS was included in the same buffer system only two polypeptides of 41 and 30 kDa were seen (Fig. 1C), as previously observed (29). Our combined results indicate that the major bands of methylcobalamin:CoM activity found in the nondenaturing gels of acetate-grown cell extracts arise from one or both of the subunits known to constitute the 480-kDa protein, rather than from the known isozymes of MT2.

The methylcobalamin:CoM methyltransferase activity of the 480-kDa corrinoid protein. The purified 480-kDa protein (in the presence of 10 mM CoM and 6 mM methylcobalamin) possessed 0.4 U of methylcobalamin:CoM methyltransferase activity per mg of protein (1 U = 1 μmol of methylcobalamin demethylated per min). The addition of denaturants to the protein increased the methylcobalamin:CoM methyltransferase activity. Preincubation of the 480-kDa corrinoid protein in 3 M urea for 15 min at 37°C followed by assaying in 3 M urea increased the activity by 3.1-fold. Preincubation of the 480-kDa corrinoid protein in 0.1% SDS for 10 min at 37°C followed by assaying in the same concentration of SDS increased the methyltransferase activity 9.5-fold. Stimulation of activity by either SDS or urea required the presence of the denaturant in the assay mixture.

The reaction was linear for up to 20 min in the presence or absence of either denaturant under anaerobic conditions. The same initial rates were measured under aerobic conditions, but the reaction ceased within 5 min.

Localization of methylcobalamin:CoM methyltransferase activity to the 41-kDa subunit of the 480-kDa protein. The increases in activity when the 480-kDa protein was treated with either urea or SDS indicated that limited denaturation or dissociation of the 480-kDa protein increased activity. We therefore examined the protein by Coomassie G-250 and activity staining after electrophoresis in gels containing either denaturant.

PAGE analysis of the 480-kDa protein in the presence of 6 M urea resulted in a smear of both protein and activity bands. In contrast, when the 480-kDa protein was incubated with SDS at 20°C and then subjected to SDS-PAGE, a band corresponding to a protein with an apparent molecular mass of 48 kDa was observed (Fig. 2A), in addition to the previously observed bands corresponding to the α and β subunits (with masses of 41 and 30 kDa, respectively). The polypeptide migrating at the position corresponding to 48 kDa possessed methylcobalamin:CoM methyltransferase activity (Fig. 2B), while neither of the bands migrating at positions corresponding to sizes of 41 and 30 kDa possessed activity. Densitometry of Coomassie G-250-stained SDS gels run with samples incubated in SDS at 20, 40, and 60°C prior to electrophoresis demonstrated that heat treatment in SDS converted the protein migrating with an apparent mass of 48 kDa into a form which migrated with an apparent mass of 41 kDa (Fig. 2B). Samples heated at 90°C displayed only the polypeptides migrating at positions corresponding to the 41-kDa α subunit and the 30-kDa β subunit. The 30-kDa band had the same intensity throughout the tested range of temperature. The polypeptide with an apparent molecular mass of 48 kDa possessing methylcobalamin:CoM methyltransferase activity thus appears to be a conformational isomer of the α subunit of the 480-kDa protein. No activity was detectable in the band corresponding to the 30-kDa β subunit at any tested temperature of incubation.

Similarity of the N-terminal sequences of MT2 isozymes and the α subunit of the 480-kDa corrinoid protein. The N-termi-

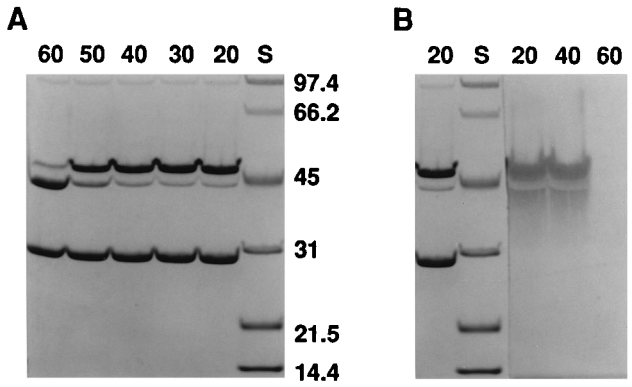

FIG. 2. Methylcobalamin:CoM methyltransferase activity staining of the 480-kDa protein following SDS gel electrophoresis. A single slab gel was cut into two portions for either Coomassie G-250 staining or methylcobalamin:CoM methyltransferase activity staining. Lanes were loaded either with molecular mass standards (lanes S) or with 5 μg of 480-kDa protein (lanes 60, 50, 40, 30, and 20). The numbers above the lanes in which the 480-kDa protein was loaded indicate the temperature at which the protein was incubated in the presence of 0.1% SDS prior to electrophoresis. (A) The gel was stained with Coomassie dye. The numbers to the right indicate masses in kilodaltons. (B) The two lanes to the left were stained with Coomassie G-250, while the three lanes to the right were stained for methylcobalamin:CoM methyltransferase activity.

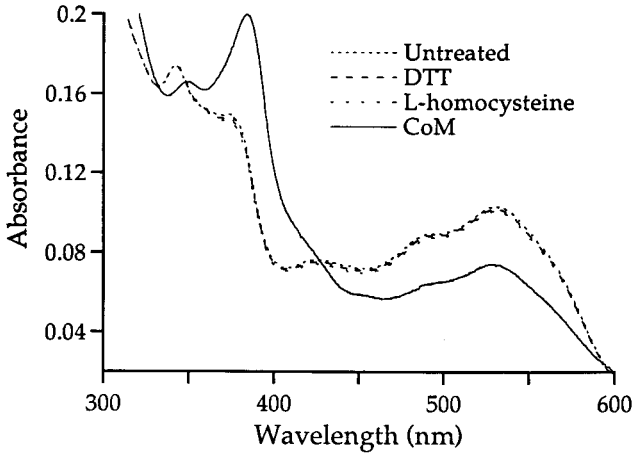

FIG. 4. UV-visible spectra of the 480-kDa CH_3-corrinoid protein before and after exposure to different thiols. The protein (1 mg/ml) was scanned as isolated (untreated) and then immediately after each sequential addition of 50 μM DTT, 50 μM L-homocysteine, and finally 50 μM CoM.

nal sequences have been obtained for both isozymes of MT2 (6, 44), as well as for the α subunit of the corrinoid protein. These sequences are aligned in Fig. 3. Overall, 9 of the first 19 amino acids in the sequences of the three polypeptides are either identical or otherwise positive in the Blosum 62 substitution matrix (24). Over this same region, 10 positions are identical for MT2-A and MT2-M. Six of these amino acids are identical in all three sequences. The N-terminal sequence of the 30-kDa subunit (MIRHIDLAVQNILEMKEKEPAKFKRL) possesses no significant similarity to that of either isozyme of MT2.

CoM-dependent demethylation of the 480-kDa CH_3-corrinoid protein. The 480-kDa protein possesses 6 mol of bound corrinoid cofactor per mol of enzyme (29). The methylcobalamin:CoM methyltransferase activity of the α subunit suggested that the intact protein should be able to methylate CoM by using the endogenous methylated corrinoid cofactor bound to the 30-kDa subunit. The 480-kDa CH_3-corrinoid protein was isolated following in vitro methylation in the presence of BES and [2-^{14}C]acetate (7). The 480-kDa protein (1 mg/ml or 12 μM methylated corrinoid) was monitored by UV-visible spectroscopy following addition of 50 μM DTT, L-homocysteine, or D-homocysteine (Fig. 4). No significant changes in the spectra of the methylated protein were observed. However, the addition of 50 μM CoM resulted in the demethylation of the protein, as indicated by the appearance of a strong absorbance peak at 384 nm corresponding to demethylated corrinoid in the Co(I) state.

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MT2-M --AEFTLKTRLLAAL EGKPVD
        +• •   •++••+  •  ••
40kda MVSEMTPTRRVMAAV LGGRVDYVP
        +• ••  •+  •+    +•• +•
MT2-A --AEYTPKERLYRAL RKQQVDRMP
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FIG. 3. Aligned N-terminal sequences of MT2-A, MT2-M, and the 41-kDa subunit of the 480-kDa corrinoid protein. The alignment was obtained by using the program ClustalW (40) with the Blosum 62 substitution matrix on the World Wide Web server maintained at the Baylor College of Medicine. Identities (●) and substitutions with positive scores (+) in the Blosum matrix are indicated.

In order to confirm that demethylation of the protein had indeed occurred, samples of the 480-kDa $^{14}CH_3$-corrinoid protein were treated with either 50 μM D-homocysteine, L-homocysteine, DTT, or CoM and subjected to size exclusion HPLC analysis. The 480-kDa protein treated with the first three thiols did not lose the methyl group, and all radioactivity eluted with the protein. However, the addition of 50 μM CoM resulted in loss of the radioactive methyl group from the protein. All radioactivity eluted in the inclusion volume of the column, consistent with formation of $^{14}CH_3$-S-CoM.

Rapid CoM methylation via the endogenous corrinoid of the 480-kDa protein. The demethylation of the methylated 480-kDa protein left the bound corrinoid in the Co(I) state. Co b(I)amides are rapidly alkylated by methyl iodide (5), and methyl iodide has frequently been used as a methyl donor in the study of corrinoid-dependent methyltransferases (34, 38). Addition of methyl iodide to the 480-kDa corrinoid in the Co(I) state resulted in remethylation of the protein as detected by UV-visible spectroscopy (data not shown). Therefore, this compound was tested as a methyl donor for the formation of methyl-CoM (Fig. 5). The rate of CoM methylation by the 480-kDa $^{14}CH_3$-corrinoid protein was 2.3 ± 0.3 (n = 8) μmol/min/mg of protein with 25 mM methyl iodide and 1 mM CoM. The reaction was dependent on both the enzyme and methyl iodide. Methyl-THSP, methanol, monomethylamine, dimethylamine, trimethylamine, acetate, acetyl phosphate, and acetyl-CoA did not serve as methyl donors.

The reaction was catalyzed only by protein with the bound methylated corrinoid cofactor. When the endogenous methylated corrinoid was converted to Co(II) by photolysis under anaerobic conditions, it was inactive in the methyl iodide:CoM methyltransferase reaction. The reaction was initially linear but ceased after 1 to 2 min. The spectrum of the 480-kDa protein recovered at the end of the reaction indicated that the corrinoid cofactor was no longer methylated and was in a mixture of Co(II) and Co(III) states (data not shown). Ti(III) citrate in the presence or absence of methyl viologen was unable to reduce the Co(II) corrinoid protein to the Co(I) state.

Ti(III) citrate stimulated, but was not required for, the 480-kDa protein-dependent methylation of CoM from methyl iodide. ATP and $MgCl_2$ were not required for, and did not stimulate, the 480-kDa protein-dependent methylation of CoM

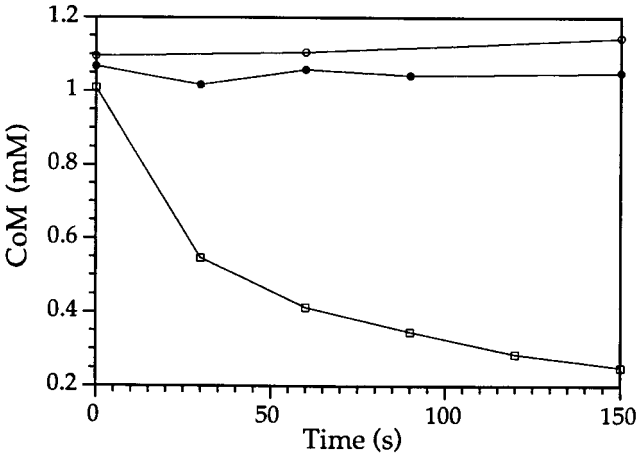

FIG. 5. The methyl iodide:CoM methyltransferase reaction mediated by the 480-kDa protein. Methyl iodide- and protein-dependent CoM disappearance was monitored by measuring loss of the free thiol from solution. The complete 200-μl assay mixture contained 68 μg of 480-kDa methyl-corrinoid protein, 1 mM CoM, and 25 mM methyl iodide (□) or 2 mM methyl-THSP (●). Controls with no enzyme but with 1 mM CoM and 25 mM methyl iodide had no activity (○).

by methyl iodide. Preincubation of the protein for 10 min in the presence of 25 mM methyl iodide did not change the initial rate of CoM methylation. The inclusion of 3.6 M urea or 0.1% SDS in the reaction mixture completely inactivated methyl iodide:CoM methyltransferase activity. Methyl group transfer required anaerobic conditions, and no reaction was detected in the presence of air. The addition of purified MT2-A or MT2-M to assay mixtures failed to stimulate the 480-kDa protein-catalyzed methyl iodide:CoM reaction. Neither MT2-A nor MT2-M could catalyze methyl transfer from methyl iodide to CoM under similar reaction conditions.

Analysis of products of the methyl iodide:CoM methyltransferase reaction. Typically, reaction progress was monitored by the loss of the free thiol of CoM by using Ellman's reagent. In order to confirm that thiol loss was the result of methylation of CoM rather than formation of the CoM disulfide, analysis of the products was performed by HPLC with indirect photometric detection of anions (Fig. 6). When methyl iodide was omitted from reaction mixtures, only CoM (retention time of 13.5 min) and not CH_3-S-CoM (14.6 min) was detected (Fig. 6A). The doublet designated E (retention time of approximately 11 min) is an unidentified product of ethanol extraction of the 480-kDa corrinoid protein.

The inclusion of methyl iodide in reaction mixtures resulted in the formation of both CH_3-S-CoM and iodide (18 min) (Fig. 6B). In a typical reaction mixture initially containing 200 nmol of CoM, 94 nmol of CH_3-S-CoM and 113 nmol of CoM were measured at the end of the reaction. The appearance of CH_3-S-CoM always coincided with the appearance of iodide and was dependent on the inclusion of both methyl iodide and the 480-kDa corrinoid protein in the reaction. The disulfide of CoM eluted at approximately 24 min and was not detected following the methyl iodide:CoM methyltransferase reaction.

Thiols methylated by the 480-kDa corrinoid protein. In order to establish the specificity of CoM as a methyl acceptor for the 480-kDa protein, several thiols with structural similarity to CoM were tested with methyl iodide as the methyl donor. L-Homocysteine, DTT, 3-mercaptopropanesulfonate, 2-mercaptoethanolamine, 2-mercaptoacetate, and 3-mercaptopropionate were not methylated by the 480-kDa corrinoid protein at detectable rates. However, 1 mM 2-mercaptoethanol and mercapto-2-propanol did serve as substrates and were methylated at initial rates of 27 and 66%, respectively, of the rate observed with 1 mM CoM. Neither MT2-A nor MT2-M utilized these two compounds as methyl acceptors. Neither 2-mercaptoethanol nor mercapto-2-propanol followed typical Michaelis-Menten kinetics. Although active at lower concentrations, both compounds were inactive as methyl acceptors at a concentration of 4 mM.

The methyl iodide:CoM methyltransferase activity displayed saturation kinetics with increasing CoM concentration. In the presence of 25 mM methyl iodide, the enzyme had an apparent K_m for CoM of 1.3 mM and an apparent V_{max} of 5 μmol/min/mg of protein.

DISCUSSION

The 480-kDa corrinoid protein was first detected because it was one of several proteins that were methylated when methyl-CoM reductase was inhibited in otherwise methanogenic extracts of *M. barkeri* (7). The physiological function of this abundant corrinoid protein was uncertain. Here we have demonstrated that it catalyzes CoM methylation. Another protein methylated under these conditions is the 29-kDa corrinoid

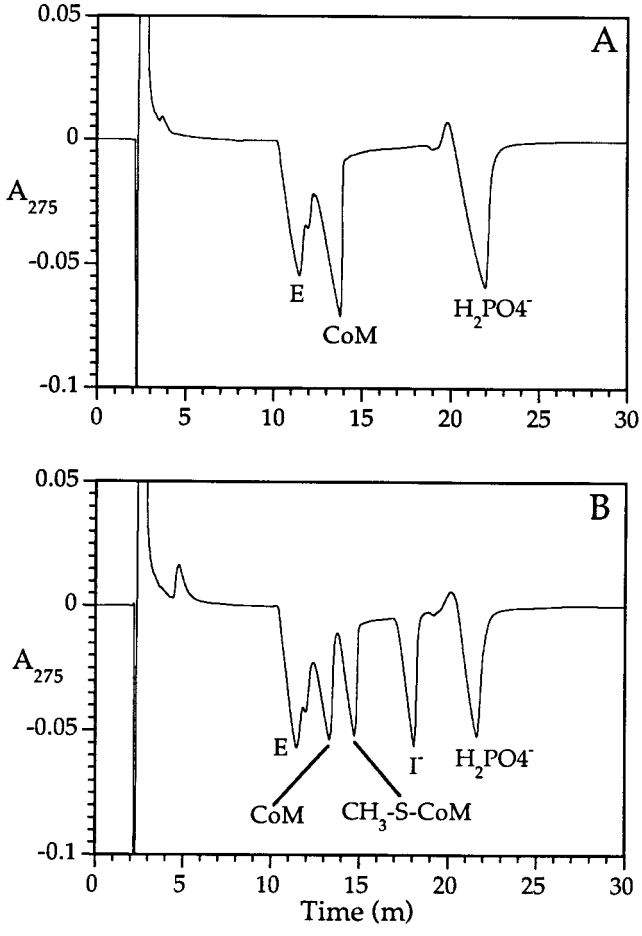

FIG. 6. Anion-exchange HPLC analysis employing indirect photometric detection of the reaction products of the methyl iodide:CoM methyltransferase reaction in phosphate buffer. The complete conditions are described in Materials and Methods. The negative doublet peak (E) was detected in controls with enzyme only. (A) Analysis of reaction mixture not supplemented with methyl iodide. (B) CoM, CH_3-S-CoM, and I^- were detected when the 480-kDa protein was incubated in the presence of methyl iodide.

protein involved in the CoM methylation during methanogenesis from monomethylamine (6). The function of both proteins in CoM methylation provides a possible explanation for why they are methylated when methyl-CoM reductase is inhibited. The accumulation of methyl-CoM may be sufficient to drive the methylation of a portion of the pool of either corrinoid protein in the Co(I) state.

The methyl iodide:CoM methyltransferase reaction has a relatively high apparent K_m for CoM. This may be due to the nature of the assay, since oxidation of the intermediate Co(I) state can inactivate the enzyme. At higher CoM concentrations, there may be less inactivation, since higher steady-state concentrations of Co(I) could lower the redox potential of the reaction mixture. The apparent K_m value may also reflect the use of the nonphysiological methyl donor methyl iodide. In any case, it is clear that the 480-kDa protein exhibits strong specificity for CoM as a methyl acceptor. Several other small thiolated compounds could not effect demethylation of the endogenous corrinoid. A homolog of CoM, 3-mercaptopropanesulfonate, which retains both functional groups of CoM but has one additional methylene group, was inactive. Interestingly, 2-mercaptoethanol and mercapto-2-propanol were active as methyl acceptors at lower concentrations but not at higher concentrations. Neither MT2-A nor MT2-M possesses the ability to use these compounds as methyl acceptors. However, unlike the 480-kDa protein, both of these isozymes can use 3-mercaptopropionate as a substrate (20).

The 480-kDa protein mediates methylation of CoM by using either methylcobalamin or methyl iodide. Methylation of CoM with methylcobalamin requires only the α subunit of the 480-kDa protein, since the methylcobalamin:CoM methyltransferase reaction was catalyzed by a form of this subunit in SDS gels. The β subunit binds the corrinoid in the 480-kDa protein, since this subunit has the corrinoid binding sequence of methionine synthase (10, 36, 37). These observations explain the increase in methylcobalamin:CoM methyltransferase activity in the presence of SDS or urea. Dissociation of the subunits exposes the site of the α subunit which interacts with the corrinoid tightly bound to the β subunit, allowing interaction of the α subunit with free methylcobalamin.

The methyl iodide:CoM methyltransferase reaction appears to require the corrinoid bound to the β subunit in association with the α subunit. Although separation of the subunits by SDS enhanced methylcobalamin:CoM methyltransferase activity, it completely inhibited methyl iodide:CoM methyl transfer. The latter reaction also had a requirement for bound methylated corrinoid and was extremely oxygen sensitive. This is consistent with the involvement of the corrinoid bound to the β subunit. Corrinoid-dependent methyltransferases are in the Co(I) state following methyl group transfer (1, 2, 16, 19, 21, 25, 33). In this strongly nucleophilic, but oxygen-sensitive, state, they can be remethylated by the substrate. Following transfer of the methyl group from the bound corrinoid of the 480-kDa protein to CoM, the cofactor is converted to the Co(I) form, which can then be remethylated by methyl iodide. The 480-kDa corrinoid protein in the Co(II) state is inactive in methyl iodide:CoM methyl transfer. When the reaction ceased, all protein-bound corrinoid had been converted to the Co(II) or Co(III) state.

The 480-kDa corrinoid protein is the fourth enzyme discovered in methanogens that is capable of performing the penultimate reaction in the production of methane. The sequences of the genes encoding the 480-kDa protein have recently been obtained (37). The sequence of the gene for either subunit is distinct from those of the subunits of the *M. thermoautotrophicum* pterin:CoM methyltransferase gene (23, 39), or the genes encoding MT2-M or MT2-A. However, the α subunit of the 480-kDa protein does bear similarity to the isozymes of MT2. All three are soluble proteins with similar sizes, are regulated by the growth substrate, and mediate similar reactions. Both MT2-A and MT2-M associate with a corrinoid protein during methanogenesis from different catabolic substrates. However, this association is not strong enough to survive the rigors of isolation, and both isozymes are purified without their respective associated corrinoid proteins. The available N-terminal sequences for the three polypeptides indicate a possible homology. We compared the complete amino acid sequence of the α subunit with sequences recently obtained for MT2-A and MT2-M (32). The α subunit exhibited an average of 30% identity and 47% similarity over the entire length of either MT2-A or MT2-M. On the basis of these considerations, we propose that the large subunit of the 480-kDa protein represents a novel isozyme of MT2. However, unlike other MT2 isozymes, it remains strongly bound to its metabolic partner, the corrinoid-binding β subunit, during isolation.

The demonstration that the 480-kDa protein is a CoM methylase ensures its function in a methanogenic pathway. However, the important unsolved question is the nature of the physiological methyl donor. Multiple pathways to methane exist in *M. barkeri*, and the protein may function in the catabolism of one of several substrates. Growth on acetate appears to derepress catabolic pathways for substrates other than acetate (6, 44). Two enzymes of monomethylamine metabolism, MT2-A and the 29-kDa corrinoid protein (6), were proteins of uncertain function originally discovered in cells grown on acetate (7, 17). Similarly, at present the physiological function of the CoM methylase activity of the α subunit of the 480-kDa corrinoid protein is inapparent, but it may prove to be in an unelucidated pathway of methanogenesis from a methylated substrate of *M. barkeri.*

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