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RamA, a Protein Required for Reductive Activation of Corrinoid-dependent Methylamine Methyltransferase Reactions in Methanogenic Archaea*[S]

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Archaeal methane formation from methylamines is initiated by distinct methyltransferases with specificity for monomethylamine, dimethylamine, or trimethylamine. Each methylamine methyltransferase methylates a cognate corrinoid protein, which is subsequently demethylated by a second methyltransferase to form methyl-coenzyme M, the direct methane precursor. Methylation of the corrinoid protein requires reduction of the central cobalt to the highly reducing and nucleophilic Co(I) state. RamA, a 60-kDa monomeric iron-sulfur protein, was isolated from *Methanosarcina barkeri* and is required for *in vitro* ATP-dependent reductive activation of methylamine:CoM methyl transfer from all three methylamines. In the absence of the methyltransferases, highly purified RamA was shown to mediate the ATP-dependent reductive activation of Co(II) corrinoid to the Co(I) state for the monomethylamine corrinoid protein, MtmC. The *ramA* gene is located near a cluster of genes required for monomethylamine methyltransferase activity, including MtbA, the methylamine-specific CoM methylase and the *pyl* operon required for co-translational insertion of pyrrolysine into the active site of methylamine methyltransferases. RamA possesses a C-terminal ferredoxin-like domain capable of binding two tetranuclear iron-sulfur proteins. Mutliple *ramA* homologs were identified in genomes of methanogenic Archaea, often encoded near methyltrophic methyltransferase genes. RamA homologs are also encoded in a diverse selection of bacterial genomes, often located near genes for corrinoid-dependent methyltransferases. These results suggest that RamA mediates reductive activation of corrinoid proteins and that it is the first functional archetype of COG3894, a family of redox proteins of unknown function.

Most methanogenic Archaea are capable of producing methane only from carbon dioxide. The Methanosarcinaceae are a notable exception as representatives are capable of methylotrophic methanogenesis from methylated amines, methylated thiols, or methanol. Methanogenesis from these substrates requires methylation of 2-mercaptoethanesulfonic acid (coenzyme M or CoM) that is subsequently used by methylreductase to generate methane and a mixed disulfide whose reduction leads to energy conservation (1–4).

Methylation of CoM with trimethylamine (TMA),[4] dimethylamine (DMA), or monomethylamine (MMA) is initiated by three distinct methyltransferases that methylate cognate corrinoid-binding proteins (3). MtmB, the MMA methyltransferase, specifically methylates cognate corrinoid protein, MtmC, with MMA (see Fig. 1) (5, 6). The DMA methyltransferase, MtbB, and its cognate corrinoid protein, MtbC, interact specifically to demethylate DMA (7, 8). TMA is demethylated by the TMA methyltransferase (MttB) in conjunction with the TMA corrinoid protein (MttC) (8, 9). Each of the methylated corrinoid proteins is a substrate for a methylcobamide:CoM methyltransferase, MtbA, which produces methyl-CoM (10–12).

CoM methylation with methanol requires the methyltransferase MtaB and the corrinoid protein MtaC, which is then demethylated by another methylcobamide:CoM methyltransferase, MtaA (13–15). The methylation of CoM with methylated thiols such as dimethyl sulfide in *Methanosarcina barkeri* is catalyzed by a corrinoid protein that is methylated by dimethyl sulfide and demethylated by CoM, but in this case an associated CoM methylase carries out both methylation reactions (16).

In bacteria, analogous methyltransferase systems relying on small corrinoid proteins are used to achieve methylation of tetrahydrofolate. In *Methylobacterium* spp., CmuA, a single methyltransferase with a corrinoid binding domain, along with a separate pterin methylase, effect the methylation of tetrahydrofolate with chloromethane (17, 18). In *Acetobacterium dehalogenans* and *Moorella thermoacetica* various three-component systems exist for specific demethylation of different phenylmethyl ethers, such as vanillate (19) and veratrol (20), again for the methylation of tetrahydrofolate. Sequencing of the genes

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[4] The abbreviations used are: TMA, trimethylamine; MMA, monomethylamine; DMA, dimethylamine; CoM, coenzyme M; MOPS, 4-morpholinepropanesulfonic acid; MAP, methyltransferase activation protein.

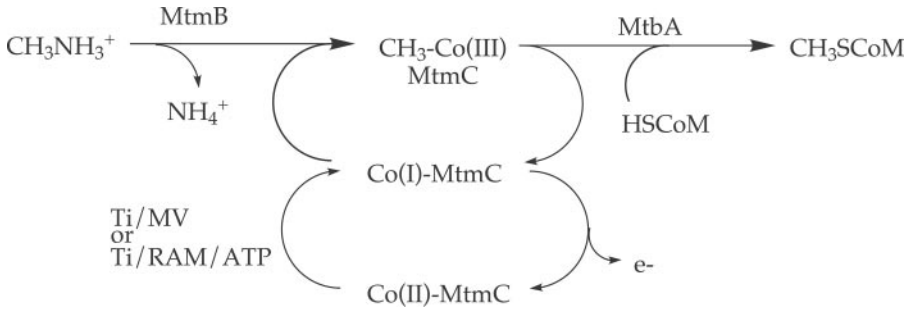

FIGURE 1. **MMA:CoM methyl transfer.** A schematic of the reactions catalyzed by MtmB, MtmC, and MtbA is shown that emphasizes the key role of MtmC in the catalytic cycle of both methyltransferases. Oxidation to Co(II)-MtmC of the supernucleophilic Co(I)-MtmC catalytic intermediate inactivates methyl transfer from MMA to the thiolate of coenzyme M (*HSCoM*). *In vitro* reduction of the Co(II)-MtmC with either methyl viologen reduced to the neutral species or with RamA in an ATP-dependent reaction can regenerate the Co(I) species. In either case *in vitro* Ti(III)-citrate is the ultimate source of reducing power.

encoding the corrinoid proteins central to the archaeal and bacterial methylotrophic pathways revealed they are close homologs. Furthermore, genes predicted to encode such corrinoid proteins and pterin methyltransferases are widespread in bacterial genomes, often without demonstrated metabolic function. All of these corrinoid proteins are similar to the well characterized cobalamin binding domain of methionine synthase (21, 22).

In contrast, the TMA, DMA, MMA, and methanol methyltransferases are not homologous proteins. The methylamine methyltransferases do share the common distinction of having in-frame amber codons (6, 8) within their encoding genes that corresponds to the genetically encoded amino acid pyrrolysine (23–25). Pyrrolysine has been proposed to act in presenting a methylammonium adduct to the central cobalt ion of the corrinoid protein for methyl transfer (3, 23, 26). However, nucleophilic attack on a methyl donor requires the central cobalt ion of a corrinoid cofactor is in the nucleophilic Co(I) state rather than the inactive Co(II) state (27). Subsequent demethylation of the methyl-Co(III) corrinoid cofactor regenerates the nucleophilic Co(I) cofactor. The Co(I)/Co(II) in the cobalamin binding domain of methionine synthase has an E_m value of −525 mV at pH 7.5 (28). It is likely to be similarly low in the homologous methyltrophic corrinoid proteins. These low redox potentials make the corrinoid cofactor subject to adventitious oxidation to the inactive Co(II) state (Fig. 1).

During isolation, these corrinoid proteins are usually recovered in a mixture of Co(II) or hydroxy-Co(III) states. For *in vitro* studies, chemical reduction can maintain the corrinoid protein in the active Co(I) form. The methanol:CoM or the phenylmethyl ether:tetrahydrofolate methyltransferase systems can be activated *in vitro* by the addition of Ti(III) alone as an artificial reductant (14, 19). In contrast, activation of the methylamine corrinoid proteins further requires the addition of methyl viologen as a redox mediator. Ti(III) reduces methyl viologen to the extremely low potential neutral species. *In vitro* activation with these agents does not require ATP (5, 7, 9).

Cellular mechanisms also exist to achieve the reductive activation of corrinoid cofactors in methyltransferase systems. Activation of human methionine synthase involves reduction of the co(II)balamin by methionine synthase reductase (29), whereas the *Escherichia coli* enzyme requires flavodoxin (30). The endergonic reduction is coupled with the exergonic methylation of the corrinoid with *S*-adenosylmethionine (27). An activation system exists in cellular extracts of *A. dehalogenans* that can activate the veratrol:tetrahydrofolate three-component system and catalyze the direct reduction of the veratrol-specific corrinoid protein to the Co(I) state; however, the activating protein has not been purified (31).

For the methanogen methylamine and methanol methyltransferase systems, an activation process is readily detectable in cell extracts that is ATP- and hydrogen-dependent (32, 33). Daas *et al.* (34, 35) examined the activation of the methanol methyltransferase system in *M. barkeri* and purified in low yield a methyltransferase activation protein (MAP) which in the presence of a preparation of hydrogenase and uncharacterized proteins was required for ATP-dependent reductive activation of methanol:CoM methyl transfer. MAP was found to be a heterodimeric protein without a UV-visible detectable prosthetic group. Unfortunately, no protein sequence has been reported for MAP, leaving the identity of the gene in question. The same MAP protein was also suggested to activate methylamine:CoM methyl transfer, but this suggestion was based on results with crude protein fractions containing many cellular proteins other than MAP (36).

Here we report of the identification and purification to near-homogeneity of RamA (reductive activation of methyltransfer, amines), a protein mediating activation of methylamine:CoM methyl transfer in a highly purified system (Fig. 1). Quite unlike MAP, which was reported to lack prosthetic groups, RamA is an iron-sulfur protein that can catalyze reduction of a corrinoid protein such as MtmC to the Co(I) state in an ATP-dependent reaction (Fig. 1). Peptide mapping of RamA allowed identification of the gene encoding RamA and its homologs in the genomes of *Methanosarcina* spp. RamA belongs to COG3894, a group of uncharacterized metal-binding proteins found in a number of genomes. RamA, thus, provides a functional example for a family of proteins widespread among bacteria and Archaea whose physiological role had been largely unknown.

EXPERIMENTAL PROCEDURES

Cell Cultures and Preparation of Extracts—M. barkeri MS (DSM 800) was cultured under anaerobic conditions in a phosphate-buffered medium supplemented with 80 mM MMA as described previously (37). Cell extracts were prepared as previously described (37).

Materials—Gases were purchased from Linde Specialty Gases (Columbus, Ohio) and passed through an R3-11 catalyst (Chemical Dynamics Corp., South Plainfield, NJ) to remove trace O_2 contamination. DE-52 cellulose was purchased from Whatman LabSales (Hillsboro, Oregon), whereas Mono Q 10/10 and Phenyl-Sepharose HP cartridges were obtained from GE Healthcare. UNO Q-2 and Q1 and Bio-Scale CHT2-I

hydroxyapatite and reagents for electrophoresis were purchased from Bio-Rad. Titanium (III) chloride (10% acidic solution), all other chromatographic materials, and all other reagents were purchased from Sigma-Aldrich.

Purification of Methyltransferases and Corrinoid Proteins Involved in MMA:CoM Methyl Transfer—MtmB, MtmC, and MtbA were either purified independently (5, 37), or MtmB and MtmC were co-purified aerobically from MMA-grown cells (23) in a purification scheme that also yielded MtbA. Cell extracts were applied to a DE-52 column (5 × 10 cm) equilibrated in 50 mM Tris HCl, pH 8, then eluted with a 2-liter gradient of 50–500 mM NaCl in 50 mM Tris, pH 8. MtmB, MtmC, and MtbA copurified from the column in a total of 240 ml between 260 and 310 mM NaCl. This sample was then loaded onto a Q-Sepharose column (15 × cm) equilibrated with 50 mM MOPS, pH 6.5, then eluted with a 1100-ml gradient with 100–500 mM NaCl in the same buffer. MtbA eluted first in a 170-ml volume between 295 and 360 mM NaCl followed by MtmC and MtmB, which eluted together in a 100-ml volume between 360 and 395 mM NaCl. This sample was then applied to a 400 ml (2.5 × 80 cm) Sephacryl S-100 column equilibrated in 50 mM MOPS, pH 7.0. MtmB and MtmC co-eluted after 200 ml in a 20-ml total volume. MtbA was also subjected to this same column and eluted in a 20-ml volume after 275 ml had eluted from the column. MtmB and MtmC were co-purified to near homogeneity with a Mono Q 10/10 chromatography column equilibrated in 50 mM MOPS, pH 6.5, then eluted using a 160-ml linear gradient from 100 mM NaCl to 500 mM NaCl in 50 mM MOPS, pH 7.0. The MtmB-MtmC fraction was then made anaerobic by flush evacuation followed by preincubation with 5 mM titanium (III) citrate prepared as described by Seefeldt and Ensign (38). Samples were then loaded onto a Sephacryl-100 gel filtration column (80 × 2.5 cm) which was pre-equilibrated in 50 mM MOPS, pH 7.0, and eluted with the same buffer. MtmB and MtmC, respectively, eluted after 200 and 260 ml of elution with the same buffer. The MtbA fraction was further purified on hydroxyapatite as described in Yeliseev *et al.* (39). The components for the TMA:CoM (MttB and MttC) and for the DMA:CoM (MtbB and MttC) methyl transfer reactions were isolated as previously described (7, 9).

Purification of RamA—MMA:CoM methylation reactions as catalyzed by MtmB, MtmC, and MtbA were used to locate peaks of RamA activity during column chromatography. Activity assays used to isolate RamA were conducted using stoppered 2.2-ml glass serum vials containing an atmosphere of H_2. A typical reaction contained 20 μg of purified MtmB, 10 μg of purified MtmC, 10 μg of purified MtbA, varying amounts of fractionated cell protein, 2 mM CoM, 100 mM MMA, 10 mM ATP, 20 mM $MgCl_2$, 1.5 mM Ti(III)-citrate, and 1 mM bromoethanesulfonate in a total volume of 125 μl. Reactions lacking MtmB, MtmC, or MtbA served as controls that the column fractions contained an activating protein for the reaction rather than one of these reaction components. After incubation at 37 °C, the remaining free thiol of unmethylated CoM was quantitated by removing 10 μl of reaction mixture and adding to 90 μl of 0.5 mM Ellman's reagent as described previously (37). This method has been previously been found to be an accurate measure of the MMA-dependent methylation of CoM in both whole and fractionated extracts of *M. barkeri* (5, 37).

RamA was purified from MMA-grown cell-free extract in a Coy anaerobic chamber containing 98% N_2 and 2% H_2 (Grass Lake, MI). The column matrices and buffers were made anaerobic by repeated cycles of evacuation followed by flushing with N_2. Purification was initiated by applying 1040 ml (21.84 g of total protein) of cell-free extract onto a 5 × 50-cm DE-52 cellulose column equilibrated in 50 mM Tris, pH 8.0. A 4500-ml linear gradient of 50–500 mM NaCl in 50 mM Tris, pH 8.0, was applied to the column at 4 ml/min. RamA activity eluted in 680 ml (3.74 g total protein) between 280 and 300 mM NaCl. The pooled active fractions were concentrated, diluted 4.5-fold with 50 mM MOPS, pH 6.5, then loaded onto an 80 × 5-cm Q-Sepharose column equilibrated in 50 mM MOPS, pH 6.5. A 2400-ml linear gradient from 50 to 500 mM NaCl in the same buffer was applied at 4 ml/min. RamA activity eluted in 400 ml (800 mg total protein) between 320 and 400 mM NaCl. The RamA active pool was concentrated to 30 ml, and two 15 ml samples were loaded onto an 80 × 2.5-cm Sepharose CL-6B column equilibrated in 50 mM MOPS, pH 7.0, containing 100 mM NaCl. RamA was eluted in 50 ml (670 mg total protein) after 360 ml of isocratic elution at 1 ml/min with the same buffer. The RamA activity was adjusted to pH 7.5 with 500 mM MOPS, pH 7.5, then loaded onto a 20 × 5-cm DEAE-Sepharose column equilibrated in 50 mM MOPS, pH 7.5, with 150 mM NaCl. A 2000-ml linear gradient from 150 to 450 mM NaCl in 50 mM MOPS, pH 7.5, was applied at 3 ml/min. RamA activity eluted between 245 and 308 mM NaCl. The fractions from 245 to 285 mM were chosen to minimize MtmC contamination that co-eluted with the later portion of the RamA activity. The collected fractions were concentrated from 260 ml (220 mg total protein) to 50 ml then diluted with 100 ml of 50 mM MOPS, pH 6.5. Three aliquots were then successively purified on a Mono Q HR 10/10 column. For each aliquot, a 120-ml gradient of 50 to 500 mM NaCl in 50 mM MOPS, pH 6.5, was applied at 2 ml/min. Three peaks of RamA activity eluted from each Mono Q run between 195 and 220 mM, 270 and 275 mM, and 290 and 298 mM NaCl. The first peak in each case contained the highest levels of activity, with the second and third peaks containing relatively little total activity (<10% in either peak). The major peaks of RamA activity from each of the three Mono Q runs were pooled (17 ml, 60 mg of total protein), then combined and adjusted to pH 8.0 with 500 mM Tris-HCl. This RamA active fraction was loaded onto a Bio-Rad Uno anion exchange column (two Bio-Rad Uno Q-2 and two Bio-Rad Uno Q-1 columns were connected in series, equally 6.6 ml of total matrix) equilibrated in 50 mM Tris, pH 8.0. After a 120-ml linear gradient from 50 to 300 mM NaCl in 50 mM Tris, pH 8.0, at 1.5 ml/min, RamA activity eluted as a single peak between 160 and 220 mM NaCl in 28 ml (33 mg of protein). The RamA active fraction was divided into three aliquots and directly applied to a 1-ml Bio-Rad CHT2-I hydroxyapatite column. The column was washed with 5 column volumes of 50 mM MOPS, pH 7.0, containing 5 mM potassium phosphate followed by a 30-ml gradient from 5 to 80 mM phosphate in 50 mM MOPS, pH 7.0, at 1 ml/min. RamA activity eluted in 12 ml (9 mg of total protein) between 40 and 53 mM phosphate. The RamA fractions from each of the three column

runs were pooled and adjusted to 600 mM $(NH_4)_2SO_4$. This fraction was then loaded onto a GE Healthcare Phenyl-Sepharose HP cartridge (1 ml) equilibrated in 50 mM MOPS, pH 7.0, with 600 mM $(NH_4)_2SO_4$. A 25-ml gradient from 600 to 0 mM ammonium sulfate and RamA eluted in a total of 6 ml between 240 and 320 mM ammonium sulfate.

ATP-dependent RamA Activation of Methylamine:CoM Methyl Transfer Activity—Unless otherwise stated, the activity of purified RamA for MMA:CoM methyl transfer was assayed under N_2 in a stoppered 2.2-ml vial. A typical reaction contained (unless otherwise indicated) 50 μg (1 nmol) of MtmB, 15 μg (0.5 nmol) of MtmC, 20 μg (0.5 nmol) of MtbA, 1.3 μg (21 pmol) of RamA, 4 mM HS-CoM, 100 mM MMA, 10 mM ATP, 20 mM $MgCl_2$, and 4 mM Ti(III)-citrate in a total volume of 125 μl. For other methylamines methyltransferase reactions MtmB and MtmC were omitted and replaced with 30 μg of MtbB and 11 μg of MtbC (for DMA:CoM methyl transfer) or 30 μg of MttB and 15 μg of MttC (for TMA:CoM methyl transfer), whereas the MMA was replaced with equivalent concentrations of DMA or TMA as appropriate. RamA-independent activation of MMA, DMA, or TMA methyltransferase reactions was performed by omitting RamA and MgATP and replacing them with 1 mM methyl viologen as previously described (5, 7, 9). In each assay, CoM methylation was monitored by measuring the loss of the free thiol of CoM with Ellman's reagent, as described above.

Other Analytical Techniques—Protein concentrations were determined by using the bicinchoninic acid protein assay (40) with reagent purchased from Pierce using bovine serum albumin (Sigma-Aldrich) as a standard. SDS gel electrophoresis was performed after Laemmli (41) using Mini-slab electrophoresis system (Idea Scientific Co., Minneapolis, MN). The molecular size markers (Bio-Rad) used were rabbit skeletal muscle myosin (200,000 Da), *E. coli* β-galactosidase (116,250 Da), phosphorylase B (97,400 Da), bovine serum albumin (66,200 Da), hen egg white ovalbumin (45,000 Da), bovine carbonic anhydrase (31,000 Da), soybean trypsin inhibitor (21,500), hen egg white lysozyme (14,400 Da), and bovine pancreas aprotinin (6,500 Da). A Sephacryl S-300 size exclusion matrix (2.5 × 88 cm, 430 ml) equilibrated in 50 mM MOPS, pH 7, flowed at 0.5 ml/min was used to determine the native molecular mass of RamA. The molecular mass standards used to generate the standard curve were blue dextran (2,000,000 Da), apoferritin (443,000 Da), β-amylase (200,000 Da), alcohol dehydrogenase (150,000 Da), bovine serum albumin (66,000 Da), carbonic anhydrase (29,000 Da), and ferricyanide (324 Da), purchased from Sigma-Aldrich.

The N terminus of RamA was determined by Edman degradation from 300 pmol of RamA in a liquid sample by the Protein Structure Laboratory at the University of California at Davis. RamA (10 μg) subjected to 12.5% denaturing polyacrylamide gel electrophoresis yielded a single 60-kDa polypeptide on detection with Coomassie staining. The sequence of the polypeptide was determined as described previously (42) by digestion with chymotrypsin and trypsin followed by liquid chromatography-tandem mass spectrometry.

UV-visible spectroscopy of RamA was performed in anaerobic sealed vials under an atmosphere of 98% N_2 and 2% H_2 using a Beckman Instruments DU-70 spectrophotometer using a 100-μl cuvette. Total iron bound by RamA was determined by the *o*-phenanthroline method (43). Acid-labile sulfide was measured as described by Beinert (44) using purified *Clostridium pasteurianum* ferredoxin as a standard (a kind gift of Richard Bare).

RamA-dependent Reduction of Co(II)-MtmC—Stoppered quartz cuvettes containing 100 mM Tris-HCl, pH 7.5, 22 mM $MgCl_2$, and 4.5 mM ATP were flush-evacuated under hydrogen or nitrogen, then supplemented with 1 mM Ti(III)-citrate. A Hewlett Packard 8453 photodiode array spectrophotometer was initially blanked with this solution before the addition of *M. barkeri* MtmC to a final concentration of 19 mM. Under these conditions Co(II)-MtmC was formed initially, and reduction to Co(I)-MtmC could be initiated by the addition of 0.12 mM RamA. The total reaction volume was 113 μl. The activity of RamA in the activation of MtmC and MtbC was followed by monitoring the increase in the Co(I) absorbance peak at 386 nm and a corresponding decrease in Co(II) absorbance at 475 nm. The $\Delta\epsilon$ values at 386 and 475 nm were determined by relating the differences in absorption at the two wavelengths of the respective absorption maxima to the concentration of MtmC.

RESULTS

Isolation of RamA—In previous work with the reconstituted MMA:CoM methyltransferase reaction, activity was completely dependent upon Ti(III)-citrate and methyl viologen (6). Methyl viologen was postulated to act as a redox mediator that facilitated reduction of MtmC to the Co(I) state required for subsequent nucleophilic attack on the methyl group of MMA. Reductive activation of the methyltransferase reaction with Ti(III)-citrate and methyl viologen is independent of ATP. However, soluble cell extracts of *M. barkeri* contained an activating protein or proteins that allowed methyl viologen-independent, but ATP-dependent, reductive activation of MMA:CoM methyltransferase activity by purified MtmB, MtmC, and MtbA with Ti(III)-citrate. The activating activity present in the extract was estimated by titration, and it was found that under conditions where the activating fraction was rate-limiting, the MtmB/MtmC/MtbA proteins in the assays were activated by 0.38 nmol of CoM consumed/min for each mg of extract protein added to the reaction. The MMA:CoM methyltransferase activity in the added crude fraction itself was negligible, and activity in this assay was further dependent upon the addition of the three purified methyltransferase components in addition to ATP.

To isolate the activating protein or proteins, the extract was fractionated by column chromatography, and fractions were tested for the ability to activate MMA:CoM methyl transfer dependent on MtmB, MtmC, and MtbA in the presence of Ti(III)-citrate and ATP. As described under "Experimental Procedures," eight successive columns were required to achieve a preparation of a single 60-kDa polypeptide that was designated as RamA and which was nearly homogeneous as judged by SDS-PAGE and staining with Coomassie Brilliant Blue (Fig. 2). In the final preparation, 3.8 mg of RamA were obtained from 21.8 g of soluble protein in the starting cell extract. A complete duplication of the purification procedure yielded similar amounts and quality of the purified RamA protein.

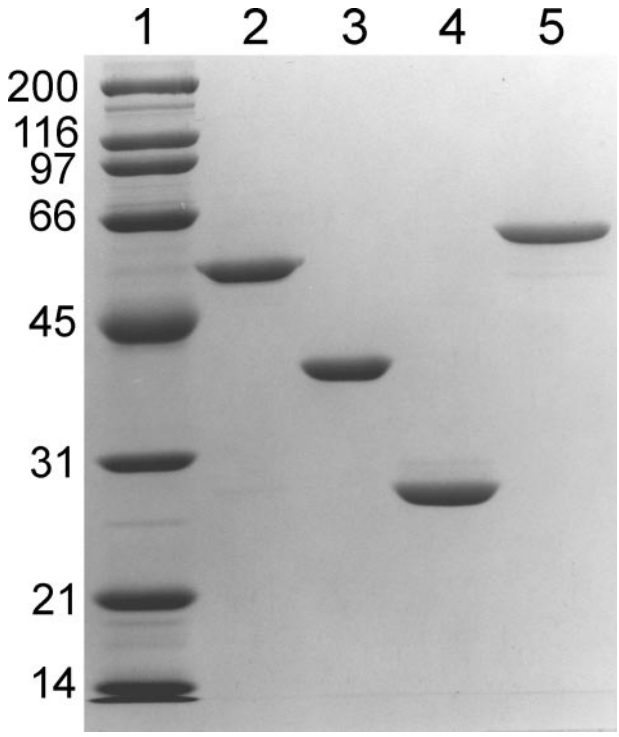

FIGURE 2. **Protein used to reconstitute the ATP-dependent MMA:CoM methyl transfer reaction.** The purity of the protein preparations used in these experiments was assessed by electrophoresis in a 11% polyacrylamine-SDS gel followed by staining with Coomassie Blue. *Lane 1* contains molecular mass markers with the sizes indicated on the *left* in kDa. *Lanes 2–5* were loaded with 5 μg of MtmB (*lane 2*), MtbA (*lane 3*), MtmC (*lane 4*), and RamA (*lane 5*).

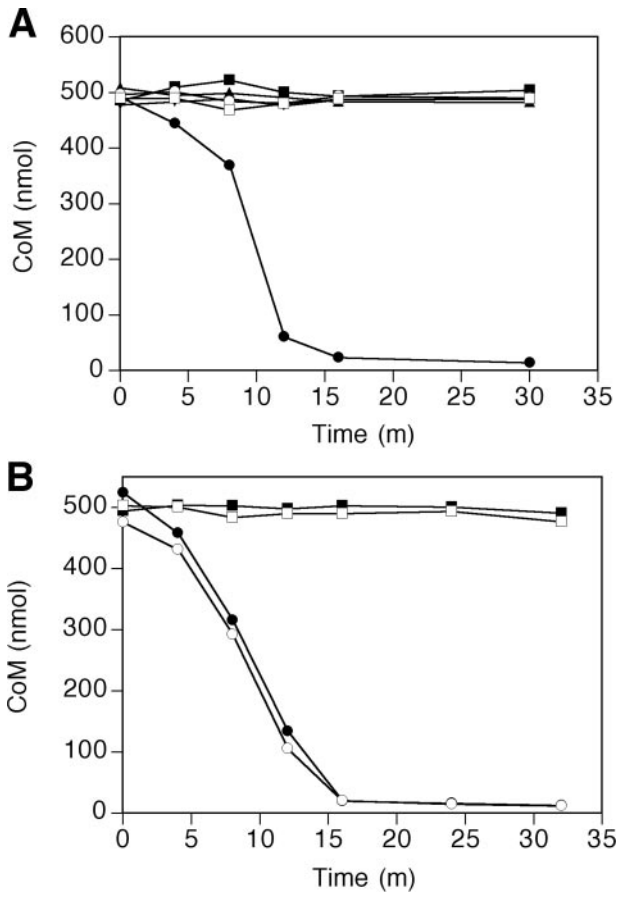

FIGURE 3. **RamA is sufficient to support ATP-dependent activation of MMA:CoM methyl transfer pathway.** The protein and chemical requirements for ATP-dependent MMA:CoM methyl transfer were tested. In *panel A*, a complete reaction is shown (*closed circles*) containing MtmB, MtmC, MtbA, RamA, Ti(III)-citrate and ATP under a nitrogen atmosphere. The loss of the free thiol of CoM via methylation was monitored using Ellman's reagent. No detectable activity was found when MtmB (*closed squares*), MtmC (*closed triangles*), MtbA (*closed inverted triangles*), RamA (*open circles*), or ATP (*open squares*) was deleted from reactions. In *panel B* the dependence on electron donor is shown. The reaction vials were filled with either nitrogen (*closed symbols*) or hydrogen (*open symbols*) and then supplemented with Ti(III)-citrate (*circles*) or no Ti(III)-citrate (*squares*). The concentrations of the reaction components are given under "Experimental Procedures."

Minimal Requirements for RamA-activated MMA:CoM Methyl Group Transfer—We tested the requirements for the purified protein and components of the MMA:CoM methyltransferase reaction as reconstituted with RamA (Fig. 3). During purification of RamA, the column fractions were analyzed under a hydrogen atmosphere to allow for the possibility that hydrogenase might be required for activation. However, the reaction could take place under nitrogen in the absence of hydrogen (Fig. 3, *panel A*) but only if Ti(III)-citrate was maintained. The activity was completely dependent on RamA addition, and under conditions where RamA was limiting, a rate of 56 nmol of CoM consumed/min·mg of RamA was observed, corresponding to 2.5% recovery of the initial activation activity in the starting material.

We found that, unlike activation with Ti(III)-citrate and methyl viologen, activation with Ti(III) and RamA was absolutely dependent upon the addition of ATP. In addition, the reaction required the further addition of purified MtmB, MtmC, and MtbA, thereby indicating that purified RamA fraction did not contain detectable amounts of these active proteins. Previous studies conducted with the MAP protein required the addition of a primary DEAE fractionation of cell extracts containing many uncharacterized proteins for activation activity (34–36). In contrast, no such fraction was required for the RamA-, MtmB-, MtmC-, and MtbA-catalyzed MMA:CoM methyl transfer activity using the proteins at the level of purity shown in Fig. 2.

RamA Affects Both Lag Times and Rates during Assay of MMA:CoM Methyl Transfer—If RamA functioned as an activation protein, we posited that lag times to the steady-state rate would depend upon the amount of RamA added to reactions. Furthermore, if the activated protein was unstable, the steady-state pool of active protein (and, thus, the steady-state methyltransferase rate) would depend also on RamA concentration. Therefore, increasing amounts of RamA were added to reaction vials containing 1000 pmol of MtmB and 500 pmol each of MtmC and MtbA in addition to Ti(III)-citrate and ATP (Fig. 4, *panel A*). RamA concentration affected the reaction rate under steady-state conditions. Observed maximum steady-state reaction rates were reached when 25 pmol was added to the reaction or 20 times less than the total amount of MtmC in the reaction. Even as little as 1 pmol of RamA was capable of supporting a low rate of MMA:CoM methyltransferase activity (Fig. 4, *panel B*). With increasing RamA, lag times were observed to decrease until reaching a point they were not detectable, which

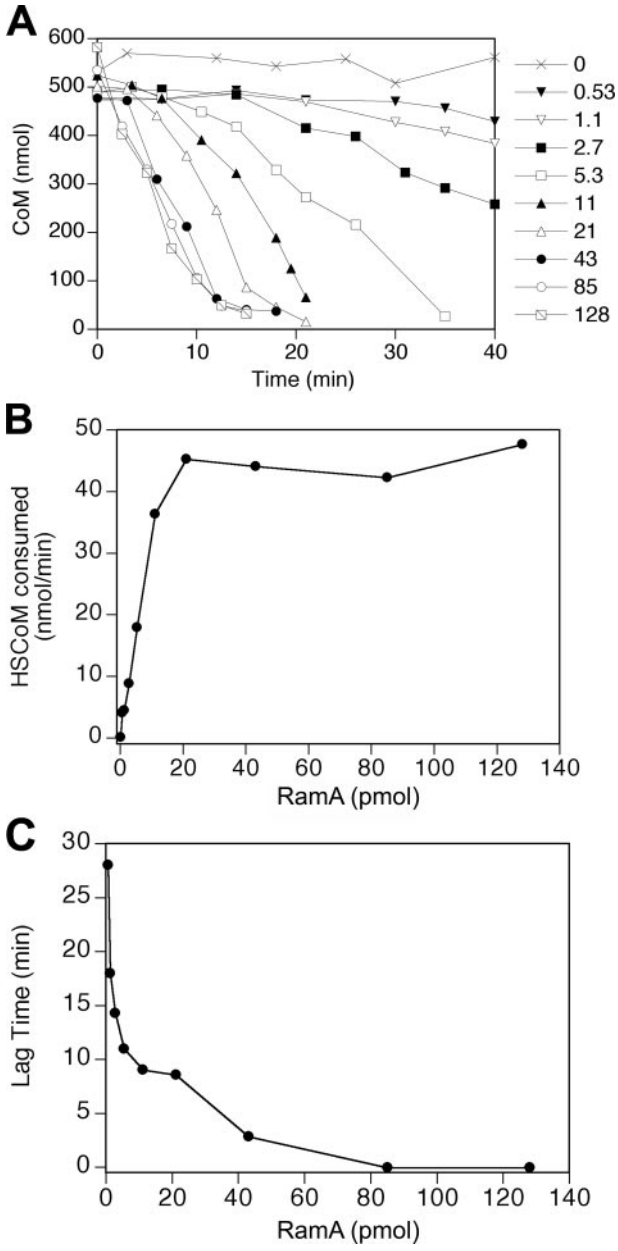

FIGURE 4. **RamA concentration affects both lag time and reaction rate for MMA:CoM methyl transfer.** *Panel A* shows the effects of increasing RamA concentrations on the reaction course of the methylation of CoM with MMA mediated by MtmB, MtmC, and MtbA. The legend on the *right* gives pmol of RamA added to a 0.125-ml reaction volume whose subsequent time course is represented by the indicated symbol. The reactions were incubated under a nitrogen atmosphere with all other protein and reactant conditions as under "Experimental Procedures." Each plot represents the average of duplicate reactions. *Panel B* shows the rate of CoM methylation, determined from the linear portions of the reactions, *versus* the amount of RamA present in a particular reaction. It was found that the rate of CoM methylation from MMA was linear with respect to RamA concentration at non-saturating concentrations of RamA. Saturating concentrations of RamA were reached when RamA and MtmC were in a 1:25 ratio. As little as 1 pmol of RamA was able to activate MMA:CoM methyl transfer in reactions containing 500 pmol of MtmC. *HSCoM*, thiol of coenzyme M. *Panel C* shows dependence of the lag time observed from the initiation of the reaction with MMA to the portion of the curve at which the reaction achieves maximum steady-state rate.

occurred upon the addition of 85 pmol of RamA to the reaction containing 1000 pmol of MtmB and 500 pmol each of MtmC and MtbA (Fig. 4, *panel C*).

TABLE 1

RamA activates TMA, DMA, or MMA methyltransferase systems

Each reaction was initiated by the addition of the indicated methylamine to the designated methyltransferase system. The rates of the reactions were monitored after activation by preincubation with either Ti(III)-citrate, ATP, and RAM, or methyl viologen was reduced with Ti(III) to the neutral colorless species in the absence of ATP. Each reaction contained 100 mM concentrations of the indicated substrate and was otherwise conducted with the protein and chemical concentrations indicated under "Experimental Procedures." ND, not detectable.

Substrate	Proteins	CoM methylation rates activated by Ti(III)citrates		
		+Methyl viologen	+ATP	
		−RAM	+RAM	−RAM
		nmol of CoM consumed/min·mg of total protein		
MMA	MtmB, MtmC, MtbA	425	698	ND
DMA	MtbB, MtbC, MtbA	224	411	ND
TMA	MttB, MttC, MtbA	563	664	ND

RamA Activates the TMA- and DMA-dependent CoM Methyl Transfer Reactions—The corrinoid proteins required for the TMA-, DMA-, and MMA-dependent CoM methyltransferase reactions are homologous, and additionally, are all demethylated by the same protein, MtbA, during CoM methylation (5–9). Like the MMA:CoM methyltransferase reaction, the DMA- and TMA-dependent CoM methyltransferases required activation by Ti(III)-citrate and methyl viologen. As observed with the MMA:CoM methyltransferase proteins, RamA and ATP could activate either DMA- or TMA-dependent CoM methylation with the purified methyltransferases specific for each reaction (Table 1). The rates achieved using RamA and ATP were similar to those achieved with the ATP-independent chemical activation using methyl viologen as a redox mediator.

RamA Mediates ATP-dependent Reduction of MtmC—The effect of RamA concentration on MMA:CoM reaction rates and lag times is consistent with a catalytic role in activating the reaction components. That RamA and ATP replaced the need for a redox-mediating dye in activation of MMA:CoM methyl transfer suggested a role in reducing MtmC to the active Co(I) state. Therefore, purified RamA was tested for the ability to mediate ATP-dependent reduction of MtmC with electrons provided by Ti(III)-citrate (Fig. 5). The redox state of MtmC was monitored by the characteristic UV-visible spectra of the bound corrinoid cofactor. Ti(III)-citrate alone was capable of reducing MtmC from the Co(III) to the Co(II) state; however, Co(I)-MtmC was not observed even after prolonged incubation with Ti(III)-citrate (data not shown). The addition of ATP without RamA did not cause further reduction of the corrinoid protein, but upon the addition of purified RamA, reduction of Co(II)-MtmC occurred, as signified by decrease of the absorbance peak at 475 nm (Fig. 5). Coincident with loss of the Co(II) signature peak was formation of an intense absorbance band at 386 nm, typical of the Co(I) form of the bound cofactor. We determined the extinction coefficients at 386 and 475 nm for both the initial Co(II) state and the final Co(I) state after the reaction reached saturation. These agreed well with previously determined coefficients for other small methyltrophic corrinoid proteins (31), and the $\Delta\epsilon$ were then used to estimate the rate of loss of the Co(II) substrate and formation of Co(I) product. Either extinction coefficient allowed estimation of the

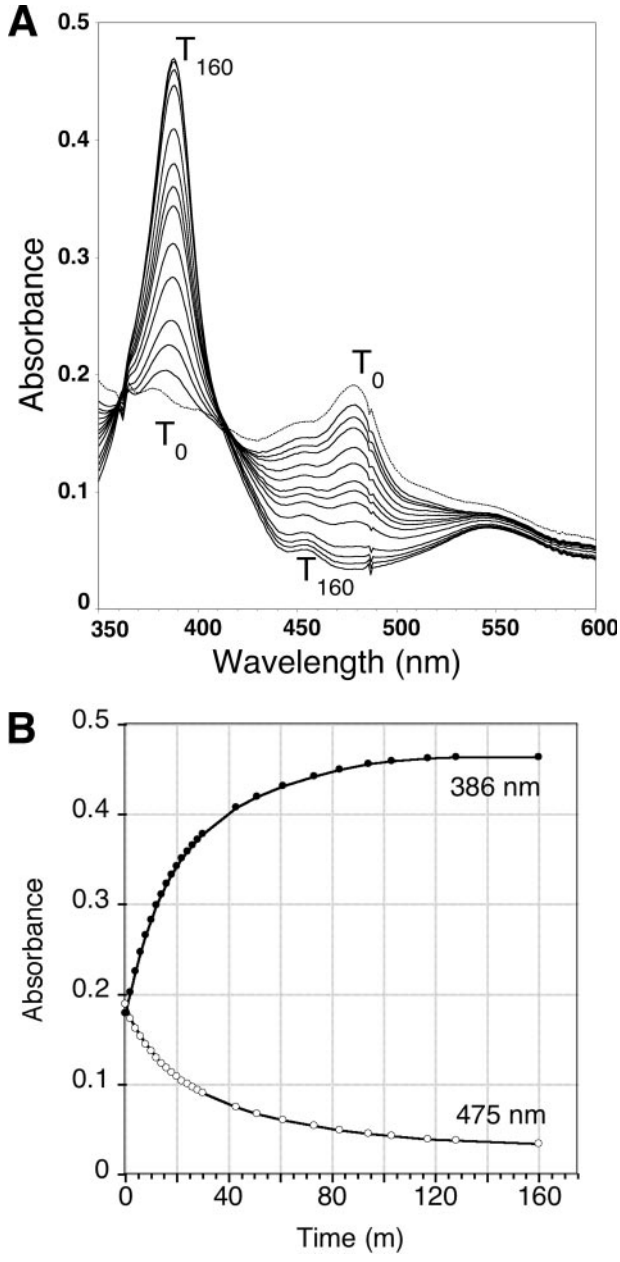

FIGURE 5. **Reduction of Co(II)-MtmC to Co(I)-MtmC in the presence of RamA, ATP, and Ti(III)-citrate.** *Panel A*, reduction of MtmC is monitored with UV-visible spectroscopy. The cuvette initially contained 100 mM Tris-HCl buffer, pH 7.5, 22 mM $MgCl_2$, 4.5 mM ATP, and 1 mM Ti(III)-citrate and served as a blank spectrum. Subsequently, MtmC (19 mM) was added, and the resultant Co(II)-MtmC spectrum (T_0) was recorded after 1 min of incubation. The Co(II)-MtmC spectrum was stable until the addition of 0.12 mM RamA, at which time conversion to Co(I)-MtmC was observed as marked by a decrease in absorbance centered at 475 nm due to loss of the Co(II) species and the development of the intense 386-nm absorbance band due to Co(I)-MtmC. Spectra were recorded at the time points indicated in *panel B* (not all spectra are shown for clarity) until the final spectrum was recorded at 160 min after the addition of RamA. In *panel B* a replot of the data from *panel A* is shown, with absorbance at 386 nm (*closed circles*) and 475 nm (*open circles*) illustrating cessation of the reaction as monitored by both wavelengths.

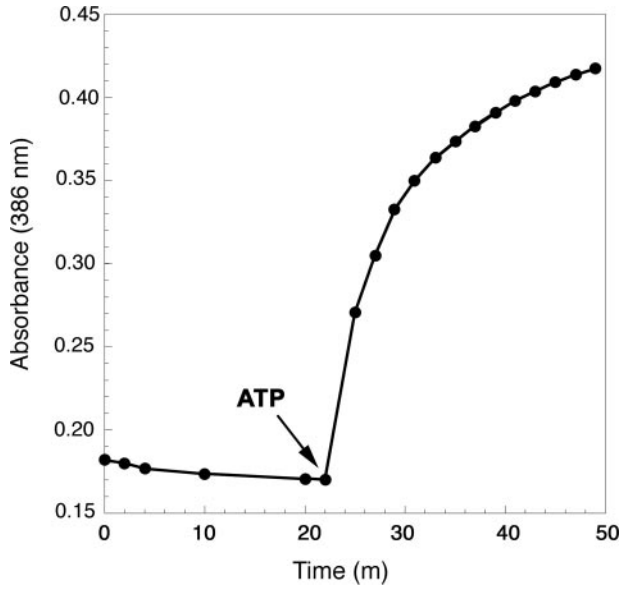

FIGURE 6. **ATP is required for RamA mediated reduction of MtmC.** The conversion of MtmC from the Co(II) to the Co(I) form was monitored by the increase in absorbance at 386 nm. At time 0, the reaction cuvette contained 20 mM MtmC, 1 mM Ti(III)-citrate, 0.12 mM RamA, and 20 mM $MgCl_2$ in 100 mM Tris-HCl, pH 7.5. At 22 min, 4.5 mM ATP was added to initiate the reaction.

turnover rate as 1 min^{-1} at 23 °C. This number is comparable with the apparent activation rate of 3 min^{-1} found at 37 °C with the same RamA:MtmC ratio during activation of the MMA:CoM methyl transfer reaction (Fig. 4).

Reduction of MtmC to the Co(I) state was not observed if ATP was not added to reactions that contained RamA. However, upon the addition of ATP, reduction of Co(II)-MtmC to the Co(I) form occurred, as evidenced by the increase in absorbance at 386 nm (Fig. 6).

RamA Is a Monomeric Iron-Sulfur Protein—The native molecular mass determinations of two different purified RamA preparations averaged 71 kDa when determined by gel filtration chromatography. As this is very near the mass of the denatured enzyme in SDS page, RamA appears to be predominantly a monomer in solution. A UV-visible spectrum of the protein revealed a broad absorbance from 350 to 450 nm, indicative of the presence of iron-sulfur clusters (Fig. 7). No increase was observed in the as-isolated sample upon the addition of potassium ferricyanide, suggesting the clusters were fully oxidized in the as-isolated form (not shown). The addition of dithionite to the protein resulted in bleaching in this region, indicating that these clusters were redox-active and could be reduced in the absence of ATP. Analysis of purified RamA revealed 8.0 mol of acid-labile sulfide and 6.5 mol of iron per mol of monomer, suggesting the presence of two Fe_4S_4 clusters or one Fe_4S_4 cluster with an additional Fe_3S_4 cluster.

RamA Is Encoded by a Gene with a C-terminal Ferredoxin-like Domain—The N-terminal amino acid sequence of RamA was determined by Edman degradation to be MYGIALDLGTSGFRTQLIDLETKETLKTVITMGHPLPGGN and was used to identify possible *ramA* genes in sequenced *Methanosarcina* spp. genomes (supplemental Fig. S1 and Table S1). Initially, the *Methanosarcina mazei* genomic sequence (45) was examined, which led to identification of MM1440 (GenBank™ NP_633464), whose predicted product differed from the N terminus of the isolated protein by only 2 of 40 residues. Examination of the *M. barkeri* Fusaro genome (46) similarly revealed another gene (Mbar_A0840, GenBank™ AAZ69816) encoding a protein whose N terminus was 95% identical to that determined for the isolated *M. barkeri* MS RamA protein. To

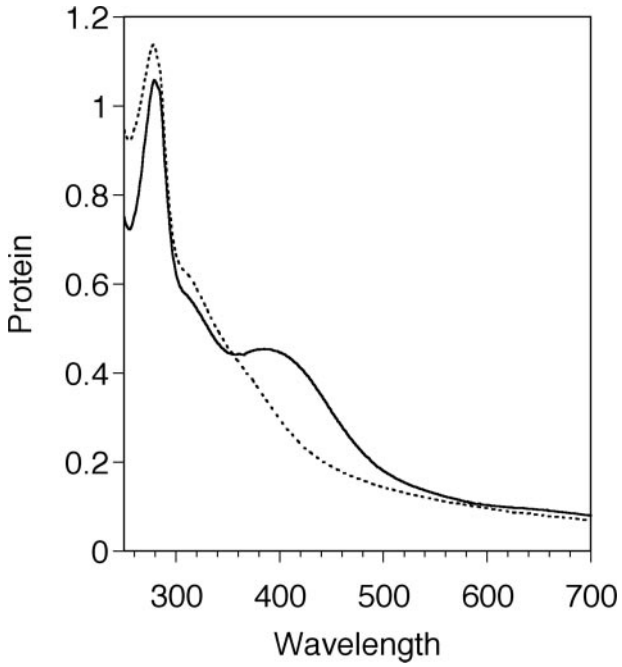

FIGURE 7. **RamA is a redox-active protein.** UV-visible spectra of RamA (0.8 mg/ml) were taken as isolated (*solid line*) in 50 mM Tris-HCl, pH 8.0, and after the addition of 0.5 mM dithionite (*dotted line*). The broad absorbance from 350 to 500 nm is indicative of iron-sulfur clusters. The bleaching observed upon the addition of dithionite suggests the clusters are redox-active.

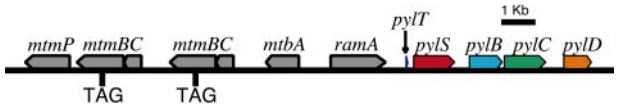

FIGURE 8. **Location of *ramA* in the sequenced genome of *M. barkeri* Fusaro.** On the *left* are flanking genes encoding the methyltransferases and corrinoid protein required for CoM methylation with MMA. On the *right* are the *pyl* genes responsible for the genetic encoding and biosynthesis of pyrrolysine. The UAG codons within the *mtmB* transcripts are translated as pyrrolysine.

further confirm the identity of the RamA gene, we PCR-amplified, cloned, and sequenced the gene directly from *M. barkeri* MS (also known as strain DSM800), the strain from which it was isolated (GenBank™ accession number FJ477063). Degenerate PCR primers were designed from the first eight residues of the N-terminal sequence of the isolated RamA protein and the final seven residues from the *M. barkeri* Fusaro *ramA* sequence and used to obtain the *M. barkeri* MS *ramA* gene from genomic DNA. The N terminus predicted for the sequenced PCR product was in complete agreement with all 40 residues determined for the N-terminal sequence for RamA as isolated from *M. barkeri* MS (Fig. S1). The sequence predicts a product whose molecular mass is 59,046 Da. The C terminus of the protein was found to be similar to Fe_4S_4 ferredoxins (Fig. S1). Two sequences that might bind tetranuclear iron-sulfur clusters were identified; one was identical to that found in many ferredoxins ($CX_2CX_2CX_3CP$), whereas the second had an insertion of two additional residues between the first and second cysteine residues ($CX_4CX_2CX_3CP$).

We confirmed that the sequenced *M. barkeri MS ramA* gene encoded the isolated RamA protein by MS-MS sequencing of peptides from chymotryptic and tryptic digests of the highly purified RamA protein (Tables S2 and S3). Peptides were found that covered 78% of the predicted product of the *ramA* gene (Fig. S2), and these peptides exactly matched the predicted sequence of the *ramA* gene product.

The *M. barkeri* MS *ramA* gene has multiple homologs in every Methanosarcinaceae genome whose sequence is available (supplemental Table 1). Genes were identified in *M. mazei* (MM1440), *M. barkeri* Fusaro (Mbar_A0840), and *Methanosarcina acetivorans* (MA0150) genomes whose predicted gene products are 93–97% similar to that produced by the entire length of *ramA* from *M. barkeri* MS. Fig. 8 shows the local gene organization surrounding the closest *ramA* homolog from *M. barkeri* Fusaro, whose product is overall 97% identical to the predicted *M. barkeri* MS RamA protein sequence. In *M. barkeri* Fusaro, the *ramA* gene is found sandwiched by a cluster of genes required for conversion of MMA to methyl-CoM. This cluster of genes includes those encoding the monomethylamine methyltransferase (*mtmB*), the MMA corrinoid protein (*mtmC*), and the methylamine-specific cobamide:CoM methyltransferase (*mtbA*). Furthermore, nearby are also the *pyl* genes required for the biosynthesis and genetic encoding of pyrrolysine. Very similar gene arrangements surround the closest *ramA* homologs found in each of the other *Methanosarcina* spp. genomes that have been sequenced. *Methanococcoides burtonii*, a close relative of *Methanosarcina* spp., also had its most similar *ramA* homolog found adjacent to an open reading frame predicted to encode a methylamine methyltransferase; in this case it is the *mttB* gene encoding trimethylamine methyltransferase (Table S1).

Aside from the RamA homologs encoded near the methylamine gene clusters, another set of RamA homologs (designated here "RamM") were found that were ~95% similar to one another yet ~65% similar to the actual RamA genes (supplemental Table 1). The RamM genes were found in each *Methanosarcina* spp. genome adjacent to genes specific for methanol:CoM methylation. RamM genes were found in *M. acetivorans* (MA4380), *M. mazei* (mm1071), and *M. barkeri* (Mbar_A1055). Additionally, other RamA homologs in *Methanosarcina* spp. were found, but these were not near corrinoid proteins or their associated methyltransferases. An exception was a gene encoding a Ram-like homolog found in *M. barkeri* Fusaro (Mbar_A3583) near the genes most similar to the methylthiol:CoM methyltransferase, a corrinoid-dependent methyltransferase homologous to methylamine corrinoid proteins and methylcobamide:CoM methyltransferases (47, 48). A RamA homolog (MA0849) was also found in *M. acetivorans* adjacent to a gene encoding a methylcobamide:CoM methyltransferase homolog.

RamA Homologs Are Widely Distributed in Genomes Near Corrinoid Proteins or Their Associated Methyltransferases—A wide number of genomes possess *ramA* homologs. Indeed, the methanosarcinal *ram* genes are part of a recognized group, COG3894, which is annotated as uncharacterized metal binding proteins (www.ncbi.nlm.nih.gov/COG (49)). We surveyed this group as well as ran BLAST searches against completed archaeal and bacterial genomes for RamA homologs using the data base maintained by the National Center for Biotechnology Information. We then examined the adjacent genes for homologies and predicted function. Many genomes were found that possessed *ramA* homologs near genes encoding small corrinoid

proteins or their associated methyltransferases (Table S4). The corrinoid proteins were most often homologous to MtmC, although in a few instances the adjoining genes encoded subunits of the corrinoid-iron sulfur proteins of acetyl-CoA synthase/decarbonylase (50, 51). The methyltransferases found encoded near RamA genes were often similar to the family of proteins comprised of methylcobamide:CoM methyltransferases, such as MtbA and CmuA. Many representatives in the Gram-positive group *Firmicutes* and Gram-negative Alphaproteobacteria of *ramA* homologs fit this trend. Only one or two examples were found in the bacterial Chloroflexi and the Fibrobacter/Acidobacteria groups. Besides the Methanosarcinales, *ramA* homologs with associated corrinoid related genes were found in the crenarchaeote *Staphylothermus* and the euryarchaeote *Archaeoglobus fulgidis*. Examples of *ramA* homologs were found in some genomes that were not apparently near corrinoid-related genes; however, these were far less prevalent than those near corrinoid proteins or their associated methyltransferases.

Alignments of most of the above *ramA* homologs found outside the Methanosarcinales revealed that only the first 400 residues of the methanosarcinal RamA protein are conserved (Fig. S3 and Table S4). The C-terminal region containing the tetranuclear iron-sulfur clusters (~130 residues) are not present in most examples found outside of *Methanosarcina* spp. and close relatives. However, the predicted bacterial and crenarchaeal proteins had instead an N-terminal domain with homology to cd00207, a family of proteins binding Fe_2S_2 clusters (Fig. S3). Alignments of several of these proteins revealed that only the conserved cysteine residues for a single iron-sulfur cluster could be detected in the N-terminal domain ($CX_5CX_2CX_{16-44}C$). All bacterial instances were found to possess only genes encoding homologs of RamA bearing the Fe_2S_2 ferredoxin signature in the N-terminal domain. Several crenarchaeaotal and euryarchaeotal genomes have *ram* gene homologs whose predicted products have N termini bearing an Fe_2S_2 binding signature. Only in *Methanosarcina* spp., *Methanosphaera stadtmanae*, and *Methanobrevibacter smithii* genomes were *ramA* homologs found whose products are predicted to possess a C-terminal tetranuclear iron-sulfur domain.

Distant Relationship of RamA to Sugar Kinases—A psi-BLAST search with residues 1–450 *M. barkeri ramA* gene product (that is, excluding the C-terminal ferredoxin-like domain) revealed a relationship to xylulose kinase from a number of different sources, a member of the FGGY family of carbohydrate kinases. These proteins share a conserved-fold that binds and hydrolyzes ATP (52). The best alignments following 14th iterations of psi-BLAST possessed an expect value of <10e-124 and aligned nearly the entire length of the *ramA* product. The most highly conserved region between xylulose kinase and RamA was the first 25 residues (Fig. S4), which included a highly conserved aspartate residue (D-7 in the *ramA* gene product) that is part of the MgATP binding pocket (52).

DISCUSSION

RamA is a protein that mediates activation of methylamine:CoM methyl transfer reactions when reconstituted with highly purified proteins and that has characteristics unlike any other such activation protein previously described. RamA replaces an otherwise obligate *in vitro* requirement in this system for a reduced low potential redox dye but only by the introduction of a new dependence of the reaction on ATP. Highly purified preparations of RamA increasingly shortened the lag time to steady-state CoM methylation from MMA with purified MtmB, MtmC, and MtbA. These results were indicative of a catalytic role for RamA in the activation of the methyltransferase reaction. In keeping with this expectation, RamA mediates the reduction of purified MtmC from the Co(II) to the Co(I) form in reactions containing those two proteins at high levels of purity (Fig. 2). This ability accounts for RamA activation of the MMA:CoM methyltransferase reaction. RamA is, thus, the first member of COG3894, a large group of uncharacterized electron transfer proteins, to have a demonstrated function.

Highly purified RamA was also capable of activating the TMA and DMA:CoM methyltransferase reaction catalyzed by the purified TMA and DMA methyltransferases (MttB and MtbB) and their respective cognate corrinoid proteins (MttC and MtbC). This result, therefore, suggests that RamA is capable of reducing the MttC and MtbC corrinoid proteins as it does MtmC. Our preliminary data does indeed indicate that RamA reduces purified MtbC to the Co(I) form; however, it cannot activate the methylthiol:CoM methytransferase.[5] The ability of RamA to recognize multiple corrinoid proteins involved in the methylamine:CoM methyl transfer pathways yet discriminate against others has precedent in the methylamine:CoM methyltransferase pathways, as MtbA, a methylcobamide:CoM methylase, will utilize the TMA, DMA, and MMA corrinoid proteins as substrates but not the methanol corrinoid protein MtaC (10).

The gene encoding RamA reveals traits consistent with the protein function in the reductive activation of methylamine corrinoid proteins. Methanogens have been noted to cluster genes required for methylamine metabolism on their genomes (6, 53). The deduced primary sequence of the *M. barkeri* MS RamA protein is nearly identical to a gene within the methylamine cluster in the *M. barkeri* Fusaro, *M. mazei*, and *M. acetivorans* genome. It is notable that flanking each side of the *ramA* gene in these genomes are genes encoding proteins acting in all methylamine:CoM methyltransferase pathways. These genes include *mtbA*, encoding the CoM methylase that can be used in common with either the TMA, DMA, or MMA corrinoid proteins, and the *pyl* gene cluster, which is essential for biosynthesis and co-translational insertion of pyrrolysine into the TMA, DMA, and MMA methyltransferases (54). The deduced primary sequence of RamA also supports its involvement in the ATP-dependent reduction of corrinoid proteins. RamA possesses an N-terminal motif associated with the binding of MgATP found in xylulose kinase and associated proteins. Furthermore, RamA carries a redox-active ferredoxin-like domain at its C terminus. The presence of two cysteine clusters within this domain that can both bind a tetranuclear iron-sulfur cluster supports the interpretation that two Fe_4S_4 clusters are present in the protein. However, the somewhat lower than

[5] J. Soares, T. Ferguson, T. Tallant, and J. Krzycki, unpublished data.

expected iron bound to the isolated protein (6.5 rather than 8 irons/monomer) may indicate one cluster could be present as a Fe_3S_4 center. A further study will be required to resolve this issue.

All published genomes of the *Methanosarcinaceae* have multiple copies of genes homologous to *ramA*. As in *M. barkeri* Fusaro, the *ramA* homolog most identical to *M. barkeri* MS *ramA* was found in the monomethylamine methyltransferase gene cluster in *M. acetivorans* and *M. mazei*. In *Mc. burtonii* the closest *ramA* homolog is near *mttB*, the TMA methyltransferase gene. In contrast, *ramA* homologs with less similarity to *M. barkeri ramA* are found near other methylotrophic corrinoid proteins (Table S1). These observations suggest the possibility that these proteins might be isozymes that also act as corrinoid protein reductases but with primary specificity for other methyltransferase systems. During isolation of RamA, some minor peaks of activation activity were observed to separate from the major peak of RamA activity. This may be due RamA complexes with methylamine corrinoid proteins; however, it is possible these minor activity peaks could represent the gene products of *ramA* homologs whose primary partner is another methyltransferase system but which have cross-reactivity with the methylamine corrinoid proteins.

The characteristics of the purified RamA protein suggest it is distinct from the MAP protein that was isolated from *M. barkeri* growing on methanol and which was suggested to be involved in the activation of the methanol:CoM methyltransferase (34, 35). Unfortunately, a gene encoding MAP was never identified due to the limiting amounts of the isolated protein, so a direct comparison of the sequences of the two proteins is not possible. MAP and RamA were reported to have similar native masses, but MAP was reported to be a heterodimer of 60- and 30-kDa subunits. We could not find a ~30-kDa subunit in any of our purified RamA preparations (Fig. 2), although it may be present at concentrations that were far substoichiometric to the RamA polypeptide and, so, undetectable in our Coomassie-stained SDS gels. Occasionally, a 31-kDa contaminant was present in our MtmC preparations (see for example Fig. 2), but this was lacking in many preparations of MtmC employed in this study (not shown), for example, those in which it was reduced upon the addition of purified RamA and ATP.

Most significantly, MAP lacked any chromogenic prosthetic group when examined by UV-visible spectroscopy (35), suggesting the absence of the iron-sulfur clusters that are readily apparent in the UV-visible spectrum of RamA. The amount of MAP isolated (40 μg) may have been simply too low to allow assessment of the iron-sulfur cluster feature in the spectrum, but it also cannot be excluded that MAP is a distinct protein from RamA protein. In this regard it is notable that one of the *ramA* homologs in the sequenced genomes of the Methanosarcinaceae is found encoded adjacent to the methanol methyltransferase genes. This RamM homolog also has signatures for a C-terminal ferredoxin sequence. Although not diagnostic of function, this genomic context is suggestive that this homolog could act with the methanol corrinoid proteins and that previous work with MAP might have simply missed the iron-sulfur clusters due to the low amount finally isolated as a pure protein. Unfortunately, the activities observed with MAP are not useful in resolving the question of the identity of RamA (or RamM) and MAP. MAP activation with any methyltransferase could not be observed except with the addition of portions of a primary DEAE fractionation of cell extract; indeed most experiments with MAP were conducted with only primary DEAE fractions from cell extracts that contained MAP (34). Even under this condition, MAP could not achieve the direct reduction of Co(II) to Co(I) corrinoid protein but, rather, appeared to cause conversion of base-on Co(II) corrinoid protein to the base-off form.

Under the conditions of our *in vitro* assay, we employed Ti(III)-citrate as an electron donor, which has a redox potential of −750 mV. In principle this low potential should not require additional energy input (such as ATP hydrolysis) for reduction of the corrinoid protein, even with a redox potential for the Co(II)/Co(I) corrinoid couple lower than −500 mV. However, as Ti(III)-citrate cannot reduce the corrinoid protein without a redox dye as mediator, this suggests the role of RamA is essentially to replace that mediator but also that RamA function obligately requires ATP. There are three possible general scenarios by which RamA might operate that could require ATP and that can be tested in future experiments. First, it is possible that RamA binds the corrinoid protein and in an ATP-dependent manner which, although it does not cause a change in RamA redox potential, causes a conformation change of MtmC that allows its reduction by Ti(III)-citrate (or a low potential cellular reductant). That a low potential donor would be an obligate requirement for activation seems unlikely, as hydrogen is sufficient for reduction of the corrinoid protein to the Co(I) form in cell extracts supplemented with ATP. An alternative hypothesis is RamA may bind and/or hydrolyze ATP in such a way as to lower the redox potential of the reduced iron-sulfur clusters in the C-terminal ferredoxin domain, allowing these clusters to directly reduce MtmC. Such a scenario could involve RamA autophosphorylation and/or a conformational change that exposes the clusters for electron transfer. This would allow higher potential electron donors in the cell to fuel the lower potential reduction of the corrinoid protein. This would be generally analogous to the strategy employed by benzoyl-CoA reductase or 2-hydroxyglutaryl-CoA dehydrase-activating enzyme in carrying out low potential reductions (55–57). Finally, RamA may bind the corrinoid protein and in an ATP-dependent fashion raise the Co(II)/Co(I) potential to allow reduction of the corrinoid protein with a higher potential redox center, such as the C-terminal ferredoxin-like domain of RamA. Of these three hypotheses, the latter two seem most likely, as they would permit an ATP-dependent activation of the methyltransferase system under *in vivo* conditions where the redox potential could not achieve direct reduction of the corrinoid protein.

The widespread nature of genes encoding RamA homologs suggests that many members of COG3894 protein family have found utility among both bacteria and Archaea. Our results with the methylamine methyltransferase system lead us to suggest that many of these proteins function in corrinoid-dependent methyltransferase activation, presumably by mediating the ATP-dependent reduction of corrinoid proteins. A large number of *ramA* homologs are found adjacent to genes homologous to those for methylotrophic corrinoid proteins and/or corrinoid/cobalamin-dependent methyltransferases. As many prokaryotes cluster genes used in similar physio-

logical tasks, this common genomic context for *ramA*-like genes near corrinoid-dependent methyltransferase genes is consistent with a role in the activation of such methyltransferases. It is striking that many of the bacterial RamA homologs are predicted to possess an N-terminal domain binding a single dinuclear iron-sulfur cluster rather than the C-terminal dual iron-sulfur clusters found in methanosarcinal RamA. This suggests that the function of the protein family inevitably requires electron transfer but that a single electron center is sufficient. This may be in keeping with a function of many members of this protein family in one-electron reduction, *i.e.* the formation of Co(I) corrinoid cofactors from the inactive Co(II) forms and the subsequent activation of the methyltransferase reactions in which they participate.

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