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Involvement of Methyltransferase-Activating Protein and Methyltransferase 2 Isoenzyme II in Methylamine:Coenzyme M Methyltransferase Reactions in *Methanosarcina barkeri* Fusaro

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The enzyme systems involved in the methyl group transfer from methanol and from tri- and dimethylamine to 2-mercaptoethanesulfonic acid (coenzyme M) were resolved from cell extracts of *Methanosarcina barkeri* Fusaro grown on methanol and trimethylamine, respectively. Resolution was accomplished by ammonium sulfate fractionation, anion-exchange chromatography, and fast protein liquid chromatography. The methyl group transfer reactions from tri- and dimethylamine, as well as the monomethylamine:coenzyme M methyltransferase reaction, were strictly dependent on catalytic amounts of ATP and on a protein present in the 65% ammonium sulfate supernatant. The latter could be replaced by methyltransferase-activating protein isolated from methanol-grown cells of the organism. In addition, the tri- and dimethylamine:coenzyme M methyltransferase reactions required the presence of a methylcobalamin:coenzyme M methyltransferase (MT_2), which is different from the analogous enzyme from methanol-grown *M. barkeri*. In this work, it is shown that the various methylamine:coenzyme M methyltransfer steps proceed in a fashion which is mechanistically similar to the methanol:coenzyme M methyl transfer, yet with the participation of specific corrinoid enzymes and a specific MT_2 isoenzyme.

Methanosarcina barkeri is a methanogenic archaeon which is able to grow on acetate and various one-carbon compounds such as CO_2, methanol, and mono-, di-, and trimethylamine. The reduction of methanol and methylamines to methane occurs via *methyl*-coenzyme M (CH_3-S-CoM), the substrate of the final step in methanogenesis in all methanogens studied so far (13, 14). Synthesis of CH_3-S-CoM from methanol and coenzyme M (2-mercaptoethanesulfonic acid [HS-CoM]) is catalyzed by the concerted action of two methyltransferases. Firstly, methanol:5-hydroxybenzimidazolylcobamide (B_{12}-HBI) methyltransferase (MT_1) binds the methyl group of the substrate to its corrinoid prosthetic group (20). Secondly, the methyl group is transferred to HS-CoM by Co-*methyl*-5-hydroxybenzimidazolylcobamide:HS-CoM methyltransferase (MT_2) (18). MT_1 is catalytically active only when the central cobalt atom of its corrinoid prosthetic group is present in the highly reduced Co(I) state (B_{12}-HBI_s) (21). Enzyme molecules that are inactivated because of oxidation of their corrinoid group, however, can be reactivated by use of a reducing system (hydrogen, hydrogenase, and ferredoxin), ATP, and an enzyme named methyltransferase-activating protein (MAP) (3–5).

Naumann et al. (17) were the first to explore the methyl group transfer reactions from the three methylamines to HS-CoM in cell-free systems of *M. barkeri*. The ATP dependence of the reactions prompted the authors to propose that the conversions might take place in a way analogous to the synthesis of CH_3-S-CoM from methanol, though with the participation of differentially expressed methyltransferase systems. Subsequent investigations suggested that an isoenzyme of MT_2 that had previously been described, MT_2 isoenzyme II [MT_2(II)] (8), plays a role in the reactions (1, 7, 22).

In this paper, we present evidence that MAP functions in the methyl group transfer reactions from tri-, di-, and monomethylamine and that the trimethylamine:HS-CoM and dimethylamine:HS-CoM methyltransferase reactions are carried out by specific corrinoid proteins. In addition, it is shown not only that MT_2(II) is involved in methyl group transfer from monomethylamine as published before (1) but also that it quite specifically mediates the conversions of tri- and dimethylamine.

MATERIALS AND METHODS

Culture methods and preparation of cell extract. Cells of *M. barkeri* Fusaro (DSM 804) were cultured in a 20-liter fermentor in a mineral medium containing either 250 mM methanol or 100 mM trimethylamine as the carbon and energy source (10, 11). Cells were harvested anaerobically at the end of growth and stored under N_2 at −80°C until use.

Cell extracts were prepared by suspending the wet cells (1:1, wt/vol) in 50 mM *N*-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer, pH 7.0, containing 15 mM $MgCl_2$, 1 mM dithiothreitol, RNase (10 μg/ml), and DNase (10 μg/ml) and subsequently passing the suspension through a French pressure cell at 138 MPa under continuous flushing with N_2. Anaerobic centrifugation at 13,200 × *g* (20 min, 4°C) pelleted the cell debris and unbroken cells. The supernatant, referred to as cell extract, was collected in an anaerobic glove box (98% N_2/2% H_2), and stored under N_2 at −80°C. The protein concentration of the cell extract from methanol-grown cells was 18 mg/ml; the cell extract of trimethylamine-grown cells contained 46 mg of protein per ml.

Enzyme assays. Reaction mixtures were prepared in the glove box, and the reactions were performed in crimp-sealed 10-ml serum flasks (2). Unless stated otherwise, a typical reaction mixture (final volume, 200 μl) contained 50 mM TES-K^+ buffer (pH 7.0), 25 mM $MgCl_2$, 10 mM HS-CoM, 2 mM ATP, 1 mM 2-bromoethanesulfonic acid, 10 mM substrate (either methanol, monomethylamine, dimethylamine, or trimethylamine) and 40-μl amounts of each of the different enzyme fractions involved.

After being subjected to pressure (100% H_2 [10^5 Pa]), the vials were kept on ice. Fractions obtained from ammonium sulfate, column chromatographical, or fast protein liquid chromatography (FPLC) separations were tested for their stimulation of the HS-CoM conversion in 50-, 100-, and 100-μl amounts, respectively, together with 10 to 15 μl of crude extract. Here, cell extract was substituted for the other enzymic components (2, 18); the methyltransferase activity by the amount of extract alone was negligible. Reactions were started by putting the

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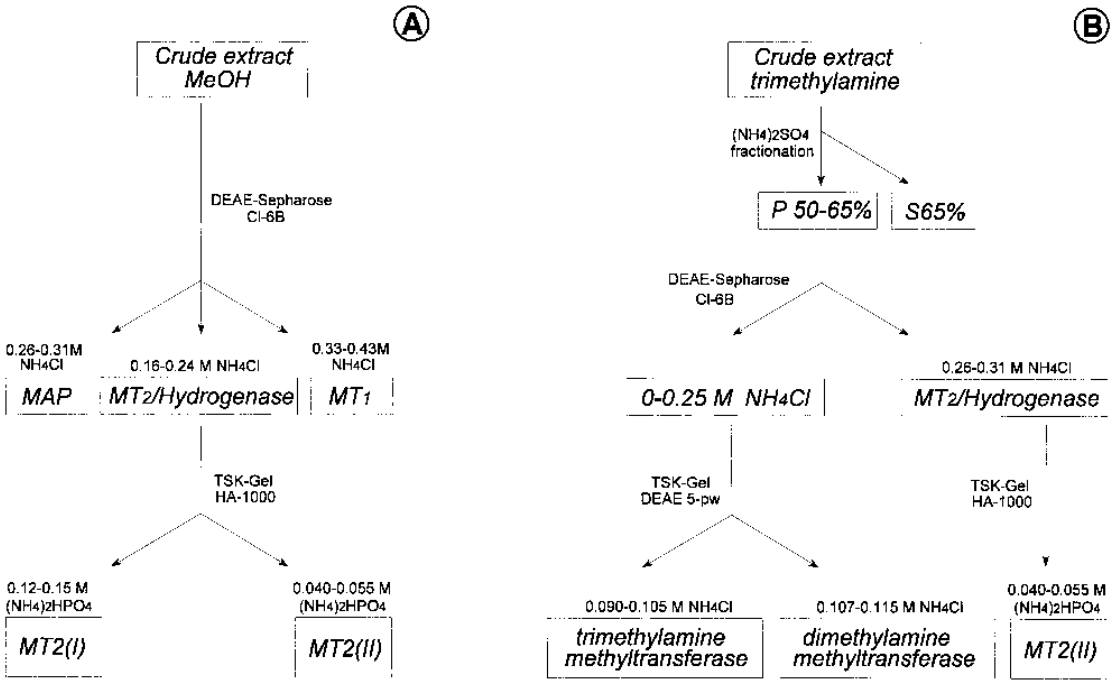

FIG. 1. Flowchart illustrating the resolution of the methanol (A)- and tri- and dimethylamine (B)-converting systems from *M. barkeri* Fusaro. Enzyme fractions used in the experiments are boxed. MeOH, methanol; P 50–65%, 50 to 65% ammonium sulfate pellet; S65%, 65% ammonium sulfate supernatant.

vials in a water bath at 37°C. After the appropriate incubation periods, 15-μl aliquots of the reaction mixture were withdrawn with a gas-tight syringe and added to 1 ml of Ellman's reagent. The level of activity of methyl group transfer from the substrate to HS-CoM was routinely assayed by measuring the rate of decrease in HS-CoM.

Hydrogenase and MT_2 activities were assayed qualitatively with microtiter plates placed inside the anaerobic glove box. Hydrogenase activity in the enzyme fractions was screened by adding 10 μl of enzyme to 90 μl of 1 mM benzyl viologen in 100 mM TES-K^+ buffer (pH 7.0). MT_2 activity was detected by mixing 25 μl of enzyme fraction with 75 μl of a solution containing 2.5 mM methylcobalamin–5 mM HS-CoM in 100 mM TES-K^+ buffer (pH 7.0). Active fractions showed a color change of the reaction mixture from red (methylcobalamin) to brown [cob(II)alamin] within a 30-min incubation at ambient temperature (15). The quantitative determination of MT_2 proceeded as described by Harms and Thauer (9).

Fractionation of cell extracts. Since several enzymes involved in the transfer of the methyl group from the substrate (either methanol or methylamines) to HS-CoM are oxygen labile, all handlings were performed in the anaerobic glove box.

A schematic representation of the resolution of the trimethylamine-, dimethylamine-, and methanol-converting systems is given in the flowchart shown in Fig. 1.

Methanol-grown cells. The cell extract (30 ml) of methanol-grown cells was applied to a column (12 by 3.0 cm) packed with DEAE Sepharose Cl-6B and equilibrated in buffer A (50 mM TES-K^+ [pH 7.0], 15 mM $MgCl_2$, 1 mM dithiothreitol, 10% [vol/vol] ethylene glycol). After application of the sample, separation was performed at a flow rate of 2 ml/min by washing with 150 ml of buffer A, and this was followed by a 400-ml linear gradient of 0 to 0.6 M NH_4Cl in buffer A and a 100-ml wash with 0.6 M NH_4Cl in buffer A. The eluate was monitored at 280 nm, and 5-ml fractions were collected. MT_2-hydrogenase was present in the fractions that eluted between 0.16 and 0.24 M NH_4Cl (i.e., that were eluted at a NH_4Cl concentration from 0.16 to 0.24 M). MAP and MT_1 were recovered in the fractions that were eluted between 0.26 to 0.31 and 0.33 to 0.43 M NH_4Cl, respectively. The three collected pools were separately desalted and thoroughly washed in buffer A on an Amicon YM3 ultrafiltration membrane (Amicon, Lexington, Mass.), adjusted to a volume of 10 ml, and stored at −20°C under N_2 until use. The MT_1 fraction contained 8 mg of protein per ml; the MT_2-hydrogenase fraction contained 10 mg of protein per ml, and the MAP fraction contained 9 mg of protein per ml. The MT_2-hydrogenase fraction was purified further on a hydroxylapatite fast protein liquid chromatography (FPLC) column (7.5 by 0.75 cm) packed with TSK-Gel HA-1000. The column was equilibrated in buffer A lacking $MgCl_2$. Separation was carried out at a flow rate of 0.45 ml/min by washing with 12 ml of buffer, and this was followed by a 60-ml linear gradient of 0 to 200 mM $(NH_4)_2HPO_4$ in buffer A without $MgCl_2$. The eluate was collected in 2-ml fractions. Two distinct MT_2 peaks that eluted between 0.040 and 0.055 M $(NH_4)_2HPO_4$ and 0.120 and 0.150 M $(NH_4)_2HPO_4$ were obtained. The latter contained about 85% of the total MT_2 activity. Comparison of the separation characteristics of this fraction with those described by Grahame (8) shows it to contain the MT_2(I) isoenzyme involved in methanol conversion. The combined MT_2(I) fractions were desalted by ultrafiltration as described above, adjusted to a volume of 10 ml (0.7 mg of protein per ml), and stored under N_2 at −20°C.

Trimethylamine-grown cells. The cell extract of trimethylamine-grown *M. barkeri* (15 ml; 46 mg of protein per ml) was subjected to 50% ammonium sulfate fractionation by slowly adding an equal volume of neutralized saturated ammonium sulfate. After gentle stirring for an additional hour, the suspension was anaerobically centrifuged (18,000 × *g*; 20 min; 4°C). The supernatant was carefully decanted, adjusted to 65% ammonium sulfate saturation, and centrifuged as described above. The 50 to 65% ammonium sulfate pellet was resuspended in buffer A, thoroughly washed in the buffer on an Amicon YM3 ultrafiltration unit, and concentrated to a volume of 15 ml. Similarly, the 65% ammonium sulfate supernatant was washed, desalted, and concentrated to 15 ml (6 mg of protein per ml).

The 50 to 65% ammonium sulfate fraction was further resolved on a column (12 by 2.8 cm) packed with DEAE Sepharose Cl-6B equilibrated in buffer A. After application of the sample (15 ml; 30 mg of protein per ml), separation was carried out as described above. In this step, fractions that were eluted between 0 to 0.32 M NH_4Cl in the gradient step strongly stimulated the conversion of trimethylamine as well as of dimethylamine when assayed with crude cell extract. The fractions that eluted between 0.26 and 0.32 M NH_4Cl containing MT_2 and hydrogenase activity were combined and washed separately.

After washing with buffer A, 3-ml aliquots of the 0 to 0.25 M NH_4Cl fraction (9 mg of protein per ml) obtained from DEAE Sepharose Cl-6B chromatography were further resolved on an FPLC ion-exchange column (7.5 by 0.75 cm) packed with TSK-Gel DEAE-5pw and equilibrated in buffer A. Proteins were separated at a flow rate of 0.75 ml/min by a 15-ml wash with buffer A followed by a linear 105-ml gradient of 60 to 150 mM NH_4Cl in buffer A to collect 1.5-ml fractions. When assayed with crude extract, the fractions that were eluted between 0.090 and 0.105 M NH_4Cl strongly stimulated the trimethylamine:HS-CoM methyl group transfer reaction but not the dimethylamine or monomethylamine methyl group transfers. Likewise, the fractions eluting between 0.107 and 0.115 M NH_4Cl specifically enhanced the dimethylamine conversion. The trimethylamine (0.090 to 0.105 M NH_4Cl)- and dimethylamine (0.107 to 0.115 M NH_4Cl)-converting fractions were collected separately, washed, and concentrated to 3-ml volumes, containing 0.8 and 2.4 mg of protein per ml, respectively, as described above.

MT_2 obtained from DEAE Sepharose Cl-6B chromatography was further purified on the TSK-Gel HA-1000 FPLC hydroxylapatite column by the procedure described above for the separation of MT_2 isoenzymes from methanol-grown cells. In this case, only one peak of MT_2 activity, which was eluted between

0.040 and 0.055 M $(NH_4)_2HPO_4$ and accounted for MT_2(II), was detected. The peak was pooled, concentrated, washed with buffer A, and adjusted to a final volume of 8 ml (1 mg of protein per ml).

Preincubation of fractionated enzymes with ATP. Inside the glove box, 200 nmol of ATP was added to the enzyme fraction to be tested, and this was followed by a 10-min incubation at ambient temperature. Subsequently, excess ATP was removed by adding 400 nmol of glucose, hexokinase (22 U), and myokinase (15 U) to the preincubation mixture. After another 10-min incubation, the preincubated fraction was assayed with all other components except for ATP in the standard methyltransferase reaction described above.

Analytical methods. The concentration of HS-CoM was determined by mixing 15-μl samples with 1 ml of 0.48 mM 2,2′-dinitro-5,5′-dithiobenzoic acid (Ellman's reagent [6]) in 150 mM Tris-Cl^- buffer (pH 8.0) and immediately measuring the A_{412}. HS-CoM concentrations were quantified by comparison with a calibration curve made from a freshly prepared stock of the compound. Trimethylamine, dimethylamine, and monomethylamine concentrations were measured by gas chromatography. Samples (25 μl) of the reaction mixture were diluted 1:1 in 6 M KOH, and isopropylamine was added to a concentration of 6 mM as an internal standard. Aliquots (2 μl) of the prepared sample were analyzed on a Pye-Unicam GCD gas chromatograph equipped with a Hewlett-Packard 3390 A integrator and a column packed with 60/80 Carbopack B–4% Carbowax (20 M)–0.8% KOH (Supelco, Bellefonte, Pa.). The sample was injected at a temperature of 150°C and separated at 90°C and a flow rate of 50 ml of N_2 per min; detection was performed at 150°C with a flame ionization detector. The concentrations of the methylated amines were determined by comparing the detected peak areas with the calibration curves of the compounds.

The fractions obtained by TSK-Gel DEAE-5pw FPLC that stimulated the trimethylamine or dimethylamine:HS-CoM methyltransferase reactions were analyzed for the presence of corrinoids. Protein samples and the hydroxy-B_{12}-HBI standard were diluted 1:1 in 0.04% (wt/vol) KCN, boiled for 5 min, and centrifuged for 10 min in an Eppendorf centrifuge to remove precipitated protein. Samples and the standard were subjected to high-performance liquid chromatography (HPLC) at 40°C on a Hewlett-Packard HP 1090 Series II HPLC equipped with an HP 1046A programmable UV-visible light detector and a 5-μm LiChrosorb RP-18 column (150 by 46 mm; Alltech Europe, Eke, Belgium). After injection of the sample (250 μl), separation was achieved at a flow rate of 0.8 ml/min by elution with a 5-min linear gradient of 0 to 25% (vol/vol) methanol followed by a 10-min linear gradient from 25 to 45% (vol/vol) methanol and a 5-min wash with 45% (vol/vol) methanol. The solvents were buffered in 25 mM sodium acetate (pH 6.0). The eluate was scanned at three pilot wavelengths, 260, 361 and 518 nm, and complete UV-visible light spectra (210 to 600 nm) of the recognized peaks were taken. Corrinoids were identified by their retention times and UV-visible light spectra. The retention time of cyano-B_{12}-HBI was 11.72 min.

Protein concentrations were calculated with Bio-Rad dye reagent concentrate (Bio-Rad Laboratories GmbH, Munich, Germany), with bovine serum albumin used as a standard.

Analytical gel electrophoresis was performed with a Bio-Rad Mini Protean II assembly (Bio-Rad Laboratories) used according to the manual. Native polyacrylamide gel electrophoresis (PAGE) was performed on a 7% homogeneous slab gel, using urease (M_r, 545,000 and 272,000), bovine serum albumin (M_r, 132,000 and 66,000), chicken egg albumin (M_r, 45,000), bovine carbonic anhydrase (M_r, 29,000), and bovine α-lactalbumin (M_r, 14,000) as molecular weight standards. Sodium dodecyl sulfate (SDS)-PAGE was carried out on a 10% polyacrylamide gel using a mixture of rabbit muscle phosphorylase *b* (M_r, 97,000), bovine serum albumin (M_r, 66,000), chicken egg albumin (M_r, 45,000), bovine carbonic anhydrase (M_r, 29,000), soybean trypsin inhibitor (M_r, 21,000), and egg white lysozyme (M_r, 14,000) for calibration. After electrophoresis, the polyacrylamide gels were stained with Coomassie brilliant blue R-250.

Materials. HS-CoM, TES, 2-bromoethanesulfonic acid, and methylcobalamin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Hydroxy-B_{12}-HBI was purified as described by Daas et al. (2). Dithiothreitol was from Serva Feinbiochemika (Heidelberg, Germany). ATP, hexokinase, and myokinase were purchased from Boehringer (Mannheim, Germany). FPLC and liquid chromatography were performed with a Perkin-Elmer series 410 LC BIO Pump equipped with a Perkin-Elmer LC 90 BIO spectrophotometric UV detector. DEAE Sepharose Cl-6B was from Pharmacia LKB Biotechnology A.B. (Uppsala, Sweden). TSK-Gel DEAE-5pw and TSK-Gel HA-1000 FPLC columns were purchased from TOSO-HAAS (Stuttgart, Germany). Gases were supplied by Hoek-Loos (Schiedam, The Netherlands). To remove traces of oxygen, H_2-containing gases were passed over a BASF RO-20 catalyst at room temperature, and N_2 was passed over a prereduced BASF R3-11 catalyst at 150°C. The catalysts were a gift of BASF Aktiengesellschaft (Ludwigshafen, Germany).

RESULTS

Resolution of the enzyme system involved in the conversion of methanol. Cell extracts prepared from methanol-grown cells of *M. barkeri* Fusaro readily converted methanol and HS-CoM into CH_3-S-CoM. By the procedure developed by Daas et al. (2), the enzyme system involved in CH_3-S-CoM synthesis from *M. barkeri* MS could be resolved into three enzymic components, notably, MT_1, MT_2-hydrogenase, and MAP. Application of the procedure to the Fusaro strain of the organism yielded an identical separation into the three components, and as shown below, only the combination of these was able to reconstitute the methanol:HS-CoM methyltransferase reaction.

The MT_2-hydrogenase-containing fraction was further resolved by the method described by Grahame (8). By this method, two distinct MT_2 activities were obtained, with the major peak eluting between 0.12 and 0.15 M $(NH_4)_2HPO_4$. Since MT_2(I) is known to elute from hydroxylapatite at about this concentration (8), the MT_2 activity in this peak was attributed to MT_2(I).

Resolution of the enzyme system involved in the conversion of methylamines. Cell extracts of methanol-grown *M. barkeri* were incapable of the conversion of tri-, di-, or monomethylamine. The capability to use methylated amines as a substrate for growth was achieved only via an intermediary shift from methanol (250 mM) to acetate (120 mM) as the carbon and energy source. Acetate-adapted cells acquired the potential to utilize tri-, di-, and monomethylamine after subsequent transfers to media containing trimethylamine and progressively decreasing amounts of acetate. Once adapted to the methylamine, cell extracts of the organism grown on the substrate were capable of tri-, di-, and monomethylamine conversion with HS-CoM as the methyl group acceptor but lacked the activity of methanol:HS-CoM methyl transfer. In the presence of 2.25 mg of protein and 1 mM 2-bromoethanesulfonate, which is an inhibitor of the terminal methane-forming reaction, the rates of HS-CoM conversion from tri-, di-, and monomethylamine amounted to 89, 80, and 44 nmol/min · mg of protein, respectively. Reactions were strictly dependent on the presence of ATP (optimal concentration, 1 to 2 mM). However, even with an ATP concentration as low as 0.1 mM (20 nmol in the assay), HS-CoM (2,000 nmol) was completely converted to CH_3-S-CoM. This indicates that the compound is required only in catalytic amounts.

Our first approach to resolve the enzyme systems involved in the conversion of the three methylamines consisted of an ammonium sulfate fractionation of the cell extracts. It appeared that the enzyme fraction that was precipitated between 50 and 65% ammonium sulfate saturation was able to catalyze the conversion of tri-, di-, and monomethylamine (Fig. 2). However, the initial rates were low and the reactions took off after lagging. The lag period was relieved by the addition of carefully desalted 65% ammonium sulfate supernatant. In the presence of 25 μl of the supernatant, the reactions started immediately and the reaction rates compared well with those measured with crude extracts. The enzyme fraction that was pelleted at 50% ammonium sulfate saturation (200 μg of protein in the assay) did not show such an effect. The 65% ammonium sulfate supernatant alone was not capable of conversion of the methylamines.

The 50 to 65% ammonium sulfate fraction was subsequently subjected to DEAE Sepharose Cl-6B chromatography. When the column fractions were tested with a minimal amount of cell extract (10 to 15 μl), which by itself did not sustain the reaction, two fractions that strongly enhanced the conversion of tri- and dimethylamine were found. The fraction that eluted between 0.26 and 0.32 M NH_4Cl coincided with MT_2. This fraction also contained part of the viologen-reducing hydrogenase activity. The other was eluted as a broad band between 0 and 0.25 M NH_4Cl.

The fraction containing MT_2 was separated further on a hydroxylapatite FPLC column packed with TSK-Gel HA-1000

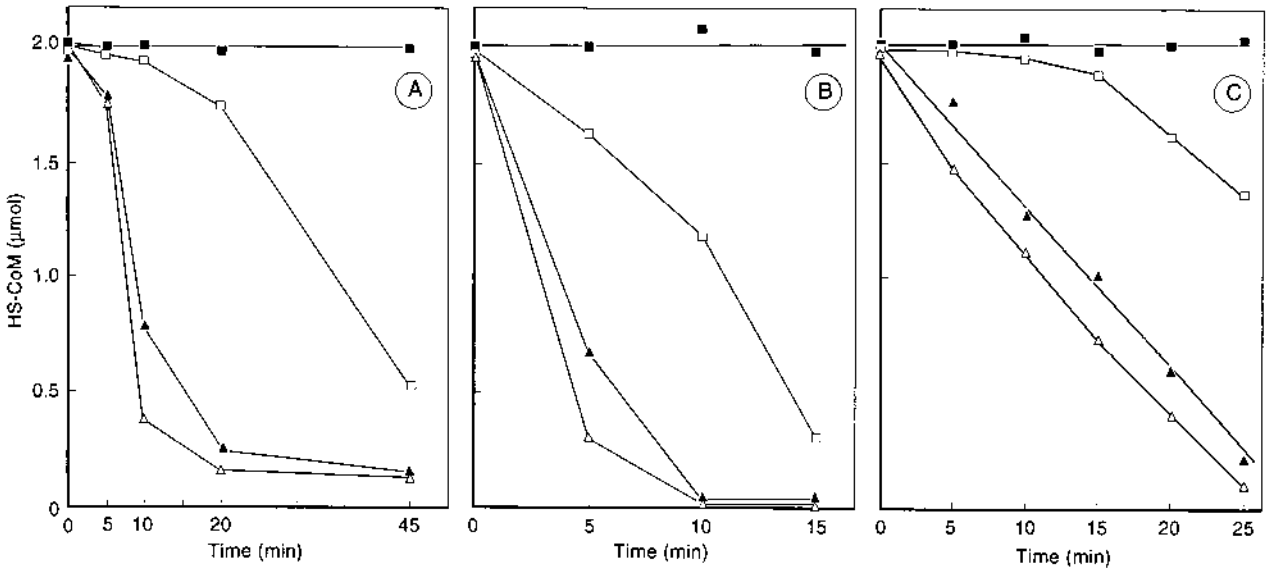

FIG. 2. The methyl group transfer reactions from trimethylamine (A), dimethylamine (B), and monomethylamine (C) to HS-CoM, catalyzed by crude cell extracts and by ammonium sulfate-fractionated extracts from *M. barkeri* Fusaro. The assays were performed as described in Materials and Methods in the presence of 10 mM trimethylamine (A), 10 mM dimethylamine (B), or 10 mM monomethylamine (C) together with 40 μl of cell extract (△), 40 μl of the 50 to 65% ammonium sulfate pellet (□), 40 μl of 65% ammonium sulfate supernatant (■), and 40 μl each of the ammonium sulfate pellet and supernatant fractions (▲).

as described above, yielding only one peak of MT_2 activity. By comparison with the chromatographical properties described by Grahame (8), we concluded that this should represent MT_2(II).

The 0 to 0.25 M NH_4Cl fraction from DEAE Sepharose Cl-6B that stimulated both the tri- and dimethylamine conversion was purified further on an FPLC ion-exchange column packed with TSK-Gel DEAE-5pw. The fraction that was eluted between 0.090 and 0.105 M NH_4Cl strongly stimulated the trimethylamine:HS-CoM methyltransferase reaction, but not the dimethylamine or monomethylamine:HS-CoM methyltransferase reaction, when assayed with limited amounts of crude extract. Native PAGE indicated that the fraction essentially contained only two proteins in about equal amounts, showing apparent molecular masses of 270 and 300 kDa (Fig. 3). HPLC analysis after cyanolysis of the protein fraction unequivocally demonstrated that it harbored cyano-B_{12}-HBI (Fig. 4B). A second fraction that was eluted from the TSK-Gel DEAE-5pw FPLC column between 0.107 and 0.115 M NH_4Cl specifically stimulated dimethylamine conversion. HPLC analysis of the cyanolyzed sample revealed the presence of two chromophoric compounds, notably, cyano-B_{12}-HBI and factor F_{430} (Fig. 4C). Upon native gel electrophoresis of the fraction, two predominant protein bands could be detected: a major band, showing an apparent M_r of 130,000, and a minor band, with a molecular mass of about 100,000 kDa (Fig. 3). Denaturing SDS-PAGE indicated the former to be composed of three subunits (66, 42, and 31 kDa). These data are fully consistent with the presence of factor F_{430}-containing CH_3-S-CoM reductase (12). A more careful analysis of the patterns of the enzymes that were eluted from the TSK-Gel DEAE-5pw FPLC column, however, showed that the dimethylamine-converting activity always coincided with the 100-kDa band, which consequently represents dimethylamine methyltransferase.

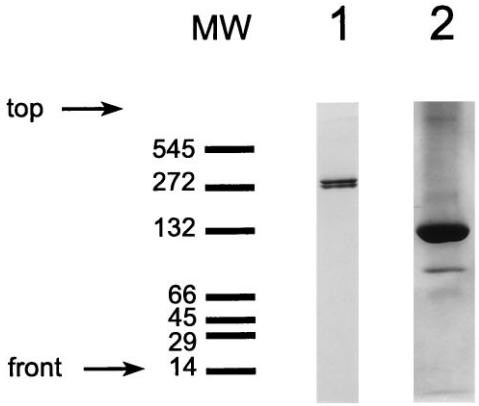

FIG. 3. Native PAGE of trimethylamine methyltransferase (lane 1; 0.090 to 0.105 M NH_4Cl fraction from TSK-Gel DEAE-5pw chromatography; 3 μg of protein) and dimethylamine methyltransferase (lane 2; 0.107 to 0.115 M NH_4Cl fraction; 11 μg of protein). The numbers at the left are the molecular weights (in thousands) of the protein standards.

Reconstitution of the trimethylamine, dimethylamine, and methanol:HS-CoM methyl transfer reactions and evidence for the role of specific MT_2 isoenzymes in the processes. Reconstitution experiments established that trimethylamine conversion could be brought about only by the combination of three fractions obtained during purification of extracts prepared from trimethylamine-grown cells: (i) the 0.090 to 0.105 M NH_4Cl pool from TSK-Gel DEAE-5pw chromatography, (ii) the MT_2(II) pool from TSK-Gel HA-1000 chromatography, and (iii) the 65% ammonium sulfate supernatant (Fig. 5A). A similar combination of fractions also actively catalyzed the methyl group transfer from dimethylamine to HS-CoM if fraction i above was replaced by the 0.107 to 0.115 M NH_4Cl fraction from TSK-Gel DEAE-5pw (Fig. 5B). Likewise, the methanol:HS-CoM methyltransferase reaction could be reconstituted by three protein fractions isolated from methanol-grown cells, notably, MT_1, MT_2(I), and a protein fraction harboring MAP (Fig. 5C).

The role of the MT_2 isoenzymes in the reactions proved to be quite specific. MT_2(II) isolated from trimethylamine-grown

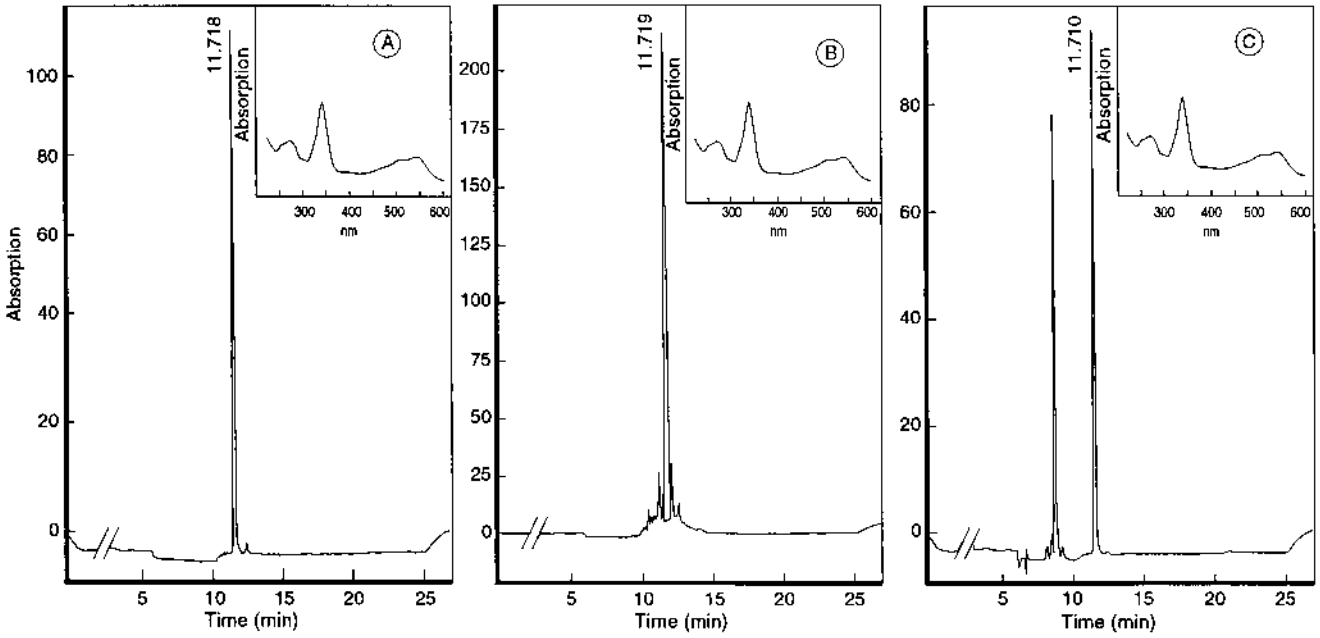

FIG. 4. HPLC analysis of corrinoids. Corrinoid extraction and analysis took place as described in Materials and Methods. The figure shows the HPLC profiles of the cyano-B_{12}-HBI standard (A), trimethylamine methyltransferase (0.090 to 0.105 M NH_4Cl fraction from TSK-Gel DEAE-5pw chromatography) (B), and dimethylamine methyltransferase (0.107 to 0.115 M NH_4Cl fraction from the TSK-Gel DEAE-5pw column) (C). In the insets, the UV-visible light spectra (200 to 600 nm) of the peaks eluting at 11.72 min are shown. Spectral analysis of the peak in panel C having a retention time of 8.91 min demonstrated that it represents factor F_{430}.

cells was active only in the conversion of trimethylamine (Fig. 5A) and dimethylamine (Fig. 5B), but it was inactive in the methanol:HS-CoM methyltransferase reaction (Fig. 5C). Conversely, MT_2(I) from the methanol-grown cells showed activity in the methanol, but not in the tri- and dimethylamine, methyl transfer steps. The combination of the two MT_2 isoenzymes did not further stimulate the reactions, but rather it could have had some inhibitory effect (Fig. 5A and B).

The stoichiometry of the trimethylamine and dimethylamine conversions catalyzed by the respective resolved enzyme sys-

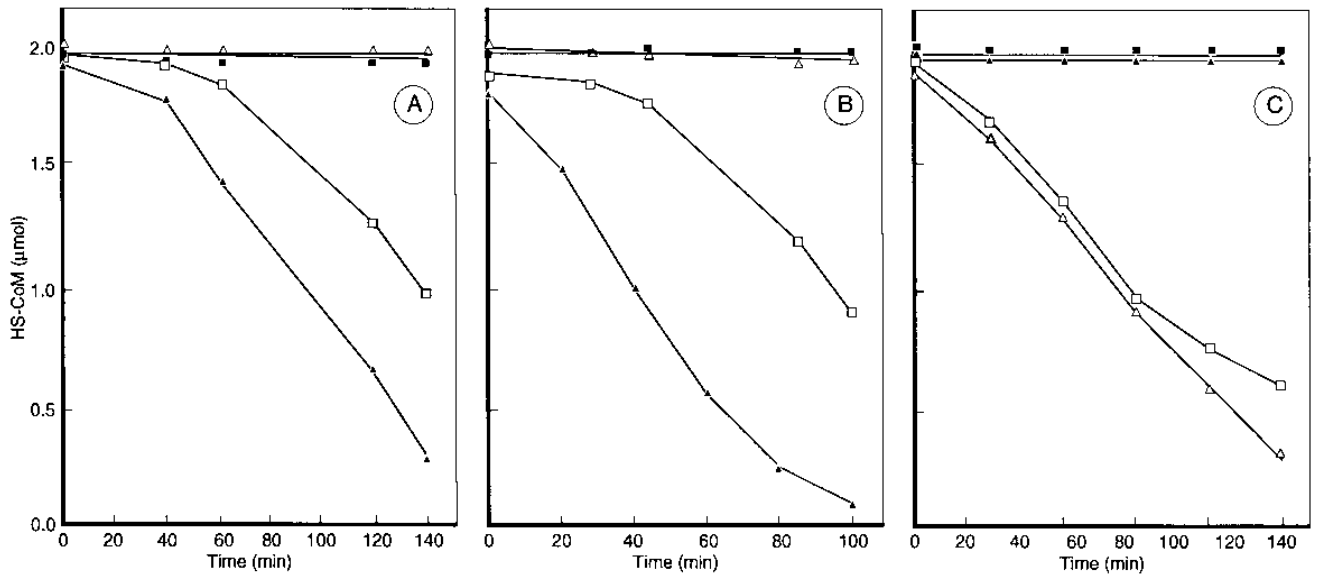

FIG. 5. Reconstitution of the enzyme systems and evidence for the role of specific MT_2 isoenzymes involved in the methyl group transfer reactions from trimethylamine (A), dimethylamine (B), and methanol (C) to HS-CoM. The assays were performed as described in Materials and Methods in the presence of 10 mM trimethylamine (A), 10 mM dimethylamine (B), or 10 mM methanol (C) and 30-μl amounts each of the following enzymic components: 65% ammonium sulfate supernatant (A and B) and either trimethylamine methyltransferase (A) or dimethylamine methyltransferase (B) or the MAP plus MT_1 fractions from methanol-grown cells (■). The assay mixtures were supplemented with 30 μl (each) of MT_2(II) (▲), MT_2(I) (△), or both MT_2(II) and MT_2(I) (□), isolated from trimethylamine- and methanol-grown cells, respectively. No reactions were observed when either the methyltransferase or the 65% ammonium sulfate supernatant was omitted (A and B) or in the absence of MT_1 or MAP (C). Protein concentrations of the enzyme fractions used were as specified in the Materials and Methods; enzyme denotations are explained in Fig. 1.

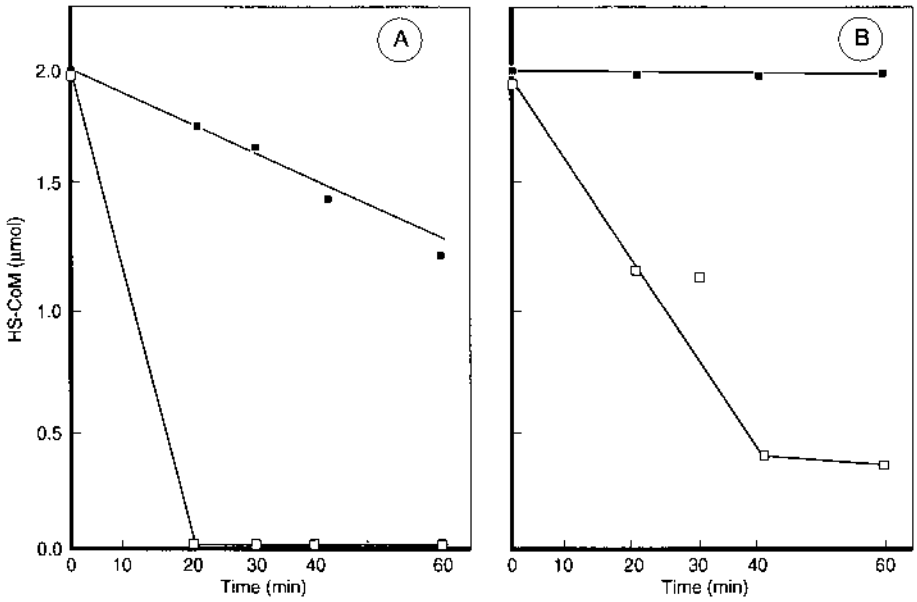

FIG. 6. Effect of preincubation with ATP of the enzymic components involved in the trimethylamine:HS-CoM (A) and dimethylamine:HS-CoM (B) methyltransferase reactions. Preincubations with ATP and the subsequent assays without ATP were performed as described in Materials and Methods in the presence of 10 mM trimethylamine (A) or 10 mM dimethylamine (B) and 40-μl amounts of the enzymic components. In the curves with solid squares, the 0 to 0.25 M NH_4Cl and MT_2-hydrogenase fractions from DEAE-Sepharose Cl-6B chromatography were preincubated with ATP. In the curves with open squares, ATP-preincubated 65% ammonium sulfate supernatant was used. No reactions were observed when ATP pretreated with glucose-hexokinase-myokinase was added to the preincubations or to the assays.

tems was determined by simultaneously measuring the concentrations of HS-CoM, trimethylamine, dimethylamine, and monomethylamine during the incubation periods. In the trimethylamine-converting reaction, the consumption of 1 mol of trimethylamine paralleled the production of 1 mol of dimethylamine and the consumption of 1 mol of HS-CoM. Similarly, the consumption of 1 mol of dimethylamine by the dimethylamine-converting system is accompanied by the simultaneous consumption of 1 mol of HS-CoM and the production of 1 mol of monomethylamine. These data are in agreement with the transfer of one methyl group from trimethylamine or dimethylamine by the respective systems to HS-CoM, resulting in the simultaneous production of dimethylamine or monomethylamine, respectively.

Evidence for the role of MAP. The methyl group transfers from tri-, di-, and monomethylamine catalyzed by the crude and resolved systems always required the presence of catalytic amounts of ATP. In addition, in the resolved systems, a component(s) present in thoroughly washed 65% ammonium sulfate supernatant was required for activity (Fig. 1 and 4). Above, it was mentioned that the 50 to 65% ammonium sulfate pellet of crude cell extracts could be separated into two parts, the 0 to 0.25 M eluate and the MT_2-hydrogenase fraction, by DEAE Sepharose Cl-6B chromatography. The combination of the two fractions was preincubated with ATP, and excess ATP was subsequently removed by a glucose-hexokinase-myokinase trap. The same was done with the 65% ammonium sulfate supernatant. After this step, reaction mixtures were prepared both from the ATP-preincubated protein fraction and from the nonpreincubated counterpart, and the trimethylamine and dimethylamine conversions were measured without the addition of ATP to the mixture. It appeared that ATP was no longer necessary when ATP-preincubated 65% ammonium sulfate supernatant was added to the assays (Fig. 6). The residual activity in the trimethylamine:HS-CoM methyltransferase reaction with the combined ATP-preincubated Sepharose fractions is likely to be the result of some contamination of the active component in the rather crude enzyme pool. Treatment of the supernatant with air (overnight at 4°C) completely destroyed its activity. In contrast, a similar treatment of the 50 to 65% ammonium sulfate pellet or its subsequently purified enzymic constituents caused at most a short lag in the tri- or dimethylamine methyltransferase reactions, when assayed with anaerobically stored 65% ammonium sulfate supernatant. The role of the oxygen-labile component present in the latter is highly reminiscent of that of MAP in the methanol:HS-CoM methyltransferase reaction (2, 3, 5). Indeed, the MAP fraction isolated from methanol-grown cells was able to substitute for the ammonium sulfate supernatant in the conversion of trimethylamine (Fig. 7A) and in dimethylamine methyl transfer (Fig. 7B). Conversely, the 65% ammonium sulfate supernatant was able to substitute for the MAP fraction in the reconstituted methanol:HS-CoM methyltransferase system (Fig. 7C).

DISCUSSION

CH_3-S-CoM synthesis from methanol in *M. barkeri* is catalyzed by a complex system in which no fewer than five proteins participate (2, 4, 8, 14, 17–22) (Fig. 8A). The system comprises two distinct methyltransferases: MT_1, which accepts the methyl group from the substrate and binds it to the corrinoid prosthetic group, and MT_2, which mediates in the methyl group transfer between MT_1 and HS-CoM. The other three enzymic components, hydrogenase, ferredoxin, and MAP, function in the reactivation of oxidatively inactivated MT_1. Hydrogen, hydrogenase, and ferredoxin, which enhances the rate of reactivation but which is not absolutely required, deliver the reducing equivalents to convert the corrinoid cobalt to the active Co(I) state (2). MAP and ATP play a role in facilitating this thermodynamically unfavorable reduction. In the process, MAP is phosphorylated by ATP, and phosphorylated MAP

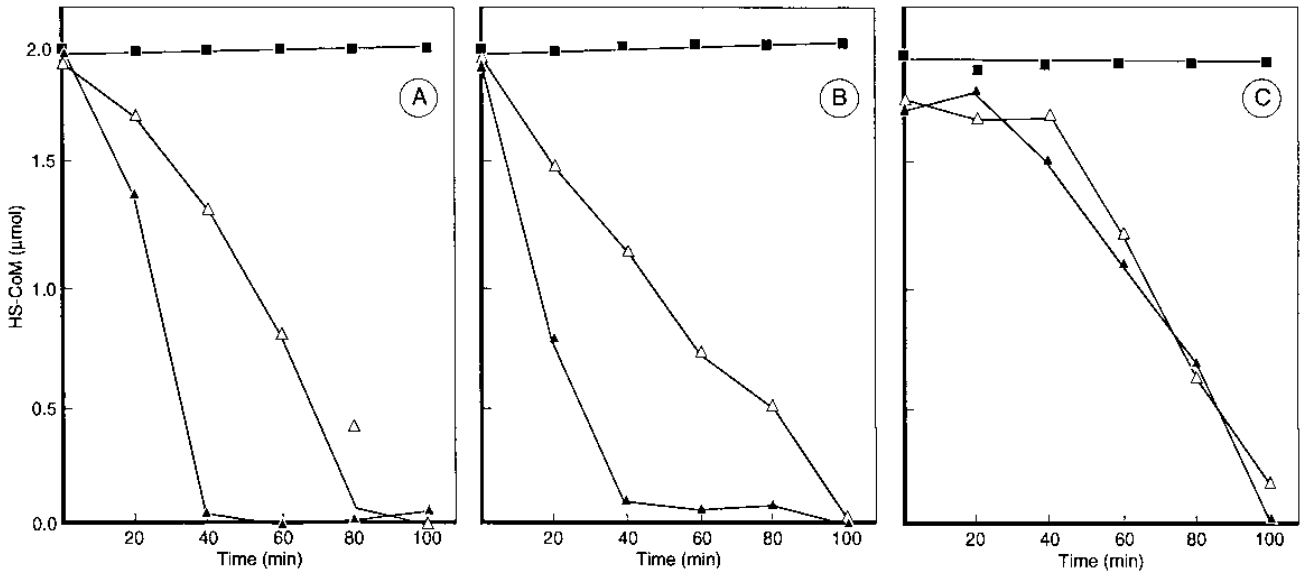

FIG. 7. Capability of the 65% ammonium sulfate supernatant and MAP fractions to substitute in the methyl group transfers from trimethylamine (A), dimethylamine (B), and methanol (C) to HS-CoM. Reactions were performed as described in Materials and Methods in the presence of 10 mM trimethylamine (A), 10 mM dimethylamine (B), or 10 mM methanol (C). The reactions indicated by solid squares contained 40-μl amounts each of MT_2-hydrogenase and 0 to 0.25 M NH_4Cl pool (A and B) or MT_2-hydrogenase and MT_1 (C) obtained by DEAE-Sepharose Cl-6B chromatography of extracts from trimethylamine- or methanol-grown cells, respectively. The reaction mixtures were supplemented with either 40 μl of 65% ammonium sulfate supernatant (▲) or 40 μl of MAP fraction (△) derived from trimethylamine- or methanol-grown cells.

induces a conformational change of MT_1 that makes the reduction feasible (2, 3, 5).

The explorative study with crude cell extracts of *M. barkeri* by Naumann et al. (17) indicated that the conversion of trimethylamine occurs by three consecutive demethylation reactions, each proceeding in a way resembling the methanol:HS-CoM methyl transfer. The work by Burke and Krzycki (1) proved this to be true for the monomethylamine:HS-CoM methyl transfer step (Fig. 8B). In that study (1), a 29-kDa corrinoid protein was methylated by the substrate; the MT_2(II) isoenzyme subsequently transferred this methyl group to HS-CoM. Apart from the two purified components, crude extract had to be added to allow the reactions to proceed, indicating that the process involves more components. The present study establishes and refines the analogy with the methanol:HS-CoM methyltransferase reaction as to the conversions of methylamines. The enzyme systems involved in the demethylation of trimethylamine and dimethylamine could each be separated into three fractions: (i) a specific corrinoid enzyme, (ii) the MT_2(II) fraction from hydroxylapatite FPLC, and (iii) the 65% ammonium sulfate supernatant. In the trimethylamine-converting system, the corrinoid protein was present in the 0.090 to 0.104 M NH_4Cl fraction eluted from the TSK-Gel DEAE-5pw FPLC column. Native PAGE demonstrated the presence of only two protein bands, showing apparent molecular masses of 270 and 300 kDa (Fig. 3). Subsequent resolution of the two proteins indicated that both catalyzed the trimethylamine:HS-CoM methyl transfer (data not shown). Therefore, the proteins may represent either two different isoenzymes or posttranscriptionally modified forms of a common enzyme. The corrinoid protein required for the dimethylamine conversion was recovered in the 0.107 to 0.115 M NH_4Cl eluate from the column described above. Native PAGE revealed the presence of two predominant proteins in this fraction. The major protein was a 130-kDa CH_3-S-CoM reductase which was inactive in dimethylamine conversion. Dimethylamine-converting activity consistently coincided with the presence of the minor protein band present in the preparation, showing an apparent M_r of 100,000. This protein should harbor the B_{12}-HBI found after HPLC analysis of the protein fraction.

From the reconstitution experiments, it can be seen that the trimethylamine:HS-CoM methyltransferase reaction is strictly dependent on the presence of MT_2(II) in the reaction mixture. No evidence for a stimulatory role of MT_2(I) on the reaction was found (Fig. 5). This partly contradicts the results reported by Ferguson et al. (7), who observed that the trimethylamine:HS-CoM methyl transfer in *M. barkeri* MS was stimulated by both MT_2(I) and MT_2(II). The specific activity of the latter in our hands may be related to a difference in the bacterial strains used or to the fact that our experiments were performed with a more completely resolved enzyme system.

Furthermore, the trimethylamine and dimethylamine conversion in reconstituted systems needed a component which is present in the 65% ammonium sulfate supernatant. The component reduced the lag phase of the trimethylamine-to-HS-CoM and dimethylamine-to-HS-CoM methyltransferase reactions (Fig. 2). Preincubations showed this component to be the primary site of the action of ATP (Fig. 6). Moreover, the 65% ammonium sulfate supernatant could replace the MAP fraction in the methanol conversion. This demonstrates that the component present in the supernatant is MAP or a MAP-like protein. The requirement for the supernatant in the monomethylamine conversion (Fig. 2C) shows that MAP plays an essential role in that reaction as well.

In conclusion, in order to convert its methylated substrates (methanol, methylamines) into CH_3-S-CoM, *M. barkeri* has developed a common strategy taking advantage of the supreme catalytic properties of the corrinoid in methyl group transfer (16) (Fig. 8). In order to keep the group activated, the organism employs a sophisticated ATP-dependent activation system featuring MAP. Yet, a number of substrate-specific variations with a common theme are found. First of all, each specific methyl group transfer reaction requires its own corrinoid protein. Furthermore, two MT_2 isoenzymes for *M. barkeri* have

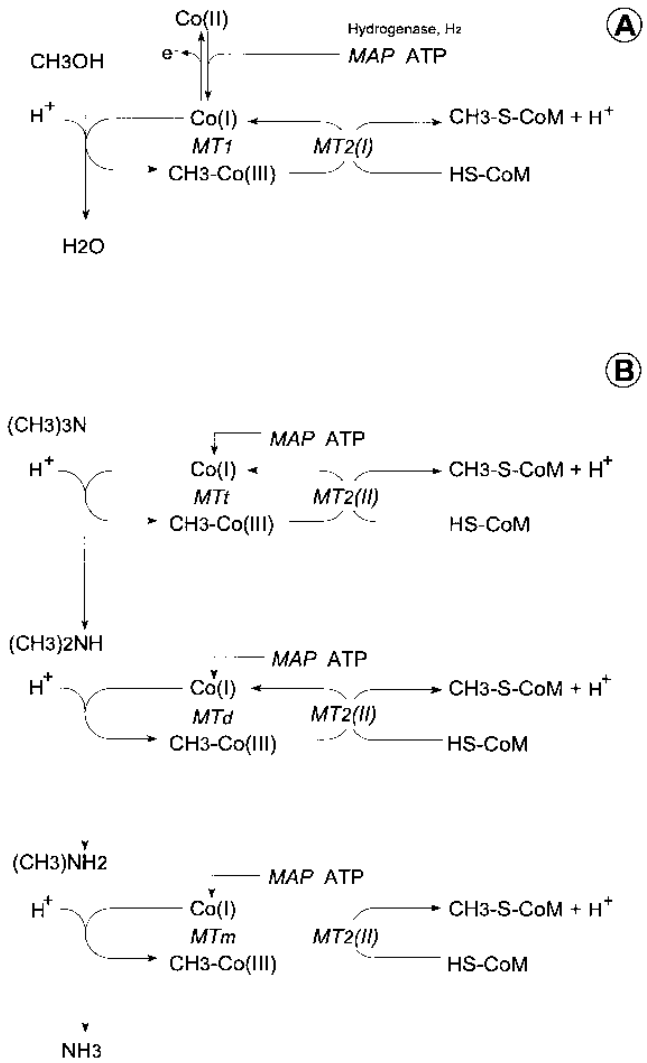

FIG. 8. Analogy between the corrinoid enzyme-dependent methyl group transfers from methanol (A) and tri-, di-, and monomethylamine (B) to HS-CoM. Abbreviations: *MTt*, trimethylamine:B_{12}-HBI methyltransferase; *MTd*, dimethylamine:B_{12}-HBI methyltransferase; and *MTm*, monomethylamine:B_{12}-HBI methyltransferase. The role of MAP in the reductive activation of the corrinoid proteins involved in the conversion of the methylamines (Fig. 7B) is proposed to be the same as that in the methanol methyl group transfer (Fig. 7A) but is represented in a more simplified way for the sake of clarity of the scheme.

been characterized (8, 22). One [MT_2(I)] functions in the methanol conversion, while the other [MT_2(II)] was demonstrated to act in monomethylamine methyl transfer (1, 22). The findings presented in this paper show that the tri- and dimethylamine:HS-CoM methyl transfer reactions are strictly dependent on MT_2(II) (Fig. 5). This demonstrates that MT_2(II) is specifically involved in methanogenesis from di- and trimethylamine, making it the specific MT_2 isoenzyme for all methylated amines in *M. barkeri* Fusaro.

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