Methylcobamide:Coenzyme M Methyltransferase Isozymes from Methanosarcina Barkeri

PHYSICOCHEMICAL CHARACTERIZATION, CLONING, SEQUENCE ANALYSIS, AND HETEROLOGOUS GENE EXPRESSION*

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A comparative study was made on the physicochemical characteristics of two isozymes of methylcobamide:coenzyme M methyltransferase (MT2). Both isozymes catalyzed S-methylation of 2-thioethanesulfonate (coenzyme M) and exhibited similar apparent Km values for coenzyme M of 35 μM (MT2-A) and 20 μM (MT2-M). Weak binding to methylcobalamin was indicated by the apparent Km of 14 μM for both isozymes. Cob(I)alamin was established as the major product of the reaction, demonstrating heterolytic cleavage of the methylcobamide carbon cobalt bond. The isozymes were shown to be zinc-containing metalloproteins. Metal ion chelators strongly inhibited both isozymes. A variety of coenzyme M analogs were tested for activity and/or inhibition. One alternative substrate 3-mercaptopropionate was discovered, with apparent Km 9 μM (MT2-A) and 10 μM (MT2-M). The results suggested an active site geometry in which coenzyme M is bound both by S-coordination to zinc, and electrostatic interaction of the sulfonate with a cationic group on the enzyme. Methanosarcina barkeri genes cmtA and cmtM encoding both isozymes were cloned and sequenced. Both genes encoded proteins with 339 amino acids and predicted molecular masses of 36–37 kDa. Active forms of both isozymes were expressed in Escherichia coli. A conserved segment with the potential for metal binding was found. The possibility of zinc involvement in catalysis of coenzyme M methylation is considered.

The methanogenic archaea are able to use for growth and methanogenesis only a relatively limited number of compounds with rather simple structures (1, 2). These compounds include substrates containing one-carbon groups such as carbon dioxide, methanol, acetate, and pyruvate. Depending upon the growth substrate, different pathways lead to the production of CH3-SCoM. The pathways of methanogenesis from carbon dioxide, methanol, acetate, and pyruvate have been described in detail (3, 5–7). Burke and Krzycki have purified and characterized a 29-kDa corrinoid protein and shown that it functions in CH3-SCoM formation from monomethylamine (8). However, the pathways of CH3-SCoM formation from this and other substrates are not fully defined. In the conversion of methanol, synthesis of CH3-SCoM proceeds by two sequential reactions (9), as shown in Reactions 1 and 2.

\[
\begin{align*}
\text{CH}_3\text{OH} + \text{cob(I)amide-MT1} + \text{H}^+ & \rightarrow \text{Co-cob(III)amide-MT1 + H}_2\text{O} \\
\text{HSCoM} + \text{Co-cob(III)amide-MT1} & \rightarrow \\
& \text{cob(I)amide-MT1 + CH}_3\text{-SCoM + H}^+ \\
\end{align*}
\]

The overall coupled reaction is given by Reaction 3.

\[
\text{CH}_3\text{OH} + \text{HSCoM} \rightarrow \text{CH}_3\text{-SCoM + H}_2\text{O}
\]

Reactions 1 is catalyzed by the oxygen-labile enzyme methanol:5-hydroxybenzimidazolylcobamide methyltransferase (MT1) (10). Activation of MT1 was found to require a reducing system (H2, hydrogenase, ferredoxin), ATP, and a separate methyltransferase activator protein (10, 11). In Reaction 2, the enzyme methylcobamide:CoM methyltransferase (MT2) catalyzes the transfer of the methyl group from the MT1-bound methylcobamide prosthetic group to coenzyme M. The ability to catalyze Reaction 4 is used as a means for routine assay of MT2 (12).

\[
\text{HSCoM} + \text{methylcobalamin} \rightarrow \text{CH}_3\text{-SCoM + cob(I)alamin + H}^+
\]

In contrast to MT1, MT2 is not inactivated by exposure to air. Hitherto, no organic or inorganic cofactors have been reported.

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The nucleotide sequences(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U38918 and U38919.

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The abbreviations used are: coenzyme M or HSCoM, 2-methyl-2-thioethanesulfonate; MOPS, 3-(N-morpholino)propanesulfonic acid; MT2, methylcobamide:coenzyme M methyltransferase; MT1, methanol:5-hydroxybenzimidazolylcobamide methyltransferase; PCR, polymerase chain reaction; kb, kilobase pair(s); bp, base pair(s); 3-MPA, 3-mercapto propane acid.
Constraints on substrate binding at the active site, and the sensus zinc binding domain. The implications for geometric Analyses of the deduced amino acid sequences of both isozymes are differentially expressed depending upon the substrate available for growth (14, 15). The specific metabolic functions of both isoforms were recently delineated (8, 16). Conversions of monomethylamine and dimethylamine to CH$_3$-B$_12$ are strongly inhibited by metal ion chelators. The genes encoding the MT2 isozyme proteins catalyzing methyl transfer to HSCoM (16). Although cob(I)alamin was the predominant product found in nonenzymatic methyl transfer from methylcobalamin to thiol (13), herein we show that cob(I)alamin is by far the major product of the reaction catalyzed by MT2. A comparative study of the physicochemical properties of the two isoforms is presented that includes determination of kinetic parameters and characterization of methyl acceptor substrate specificities. The isoforms are shown to be zinc-containing metalloproteins that are strongly inhibited by metal ion chelators. The genes encoding MT2-A and MT2-M, which we designate as cmtA and cmtM, respectively (cmt = methylcobamide-CoM methyltransferase) were cloned in Escherichia coli. Furthermore, expression in E. coli is shown to produce both isoforms in an active state. Analyses of the deduced amino acid sequences of both isoforms are presented along with the tentative identification of a consensus zinc binding domain. The implications for geometric constraints on substrate binding at the active site, and the proposed function of zinc in methyltransferase catalysis are discussed. A preliminary account of this work has been presented (17).

**MATERIALS AND METHODS**

**General Procedures**—Methylcobalamin was obtained from either Aldrich or Sigma. Carbonic anhydrase was a product of Worthington Biochemicals. Unless otherwise specified, all commercially available reagents were of analytical grade. Synthesis of 2-thioethanephosphonic acid was carried out from 2-bromoethylphosphonic acid using thiourea reagents were of analytical grade. Synthesis of 2-thioethanephosphonic acid analysis yielded a value of 0.617 mg/ml 1 cm$^{-1}$ at 500 nm. The concentrations of 40 different elements were determined by inductively coupled plasma emission spectrometry using a Perkin Elmer 1100 Atomic Comp plasma emission spectrometer. Zinc concentration was also determined independently using the metallochromic indicator 4-(2-pyridylazo)resorcinol (PAR) (19). A value of $\lambda_{	ext{max}}$ at 560 nm was used as the molar absorptivity of the $2:\text{PAR}-\text{Zn}^{2+}$ complex. A sample of the probe to be analyzed (0.5–2 mg) was applied to a column of Sephadex G-25 (1.0 x 8.5 cm) prewashed with a solution containing 10 mM EDTA and 50 mM MOPS buffer, pH 7.2. Absorbance at 280 nm was measured on fractions (1 ml) eluted with 50 mM MOPS, pH 7.2, in the absence of EDTA. In the final reaction mixtures (1.0 ml) samples from each fraction (200–500 µl) were boiled for 10 min in the presence of 100 µM PAR, 150 µM methylmethanosulfonate, and 10% SDS, and the absorbance at 500 nm was recorded. When applied to carbonic anhydrase, a protein of known zinc content, the method yielded a value of 1.08 g-atoms of zinc/mol of protein.

**DNA Manipulations**—E. coli strains XL1-Blue MRF$^+$ and XLOLR (Stratagene, Inc.) were grown routinely on LB broth at 37°C with antibiotics, ampicillin, kanamycin, or tetracycline being added as required to final concentrations of 50, 75, and 15 µg/ml, respectively. Genomic DNA from M. barkeri was purified by phenol-chloroform extraction (20). Extraction and purification of plasmid DNA was performed using the Qiagen plasmid purifications kit (Qiagen). For the production of single-stranded DNA, the helper bacteriophage VCS-M13 was used, and single-stranded DNA was subsequently extracted by standard methods (20).

**PCR Amplification**—Approximately 20 ng of chromosomal DNA or plasmid DNA was added to a mixture (50 µl) containing 60 mM Tris-HCl, pH 9.0, 2 mM MgCl$_2$, the indicated oligonucleotide primers (0.5 mM each), 200 µM each dATP, dGTP, dCTP, dTTP, 10 mM each dATP, dGTP, dCTP, dTTP, 10 mM each of Ampli-Taq DNA polymerase (Perkin Elmer Corp.). A modified procedure was used to amplify DNA from plagues, as follows. Plaque cores were eluted with 200 µl of 10 mM Tris-HCl, 0.1 mM disodium EDTA, pH 8.0, and a 5 µl sample was added to the 50 µl PCR mixture supplemented with 0.1% Tween 20. Initial denaturation for 6 min at 94°C was followed by 34 amplification cycles (denaturation, 1 min at 94°C; annealing, 1 min at 46°C; extension, 45 s at 72°C) and a final incubation for 10 min at 72°C.

**Oligonucleotide Probes**—In order to generate probes for cloning the cmtA gene, we synthesized degenerate forward P2 and reverse P3 PCR primers based on the amino acid sequence of the NH$_2$ terminus and an internal peptide fragment generated by cyanogen bromide cleavage. In a similar fashion, forward P4 and reverse P5 primers were prepared for cmtM (Figs. 3 and 5). These primers have the following sequences: P2 (5'-AA(A/G)CA(A/G)CA(A/G)GT(A/T/G/C)GA(C/T/G/A)ATGCC-3'), P3 (5'-TC(A/G/T/G/C/G/T/G/C/G/ATCC-3'), P4 (5'-GA(A/G)GA(A/G/T/G/C/G/A/CG/C/T/G/G/ATCC-3'), P5 (5'-TG(C/T/G/T/G/A/TTT/T/G/G/CT/G/AGCC-3')) and P6 (5'-GCT/G/G/CT/G/G/ATCC-3'). When used in PCR with M. barkeri genomic DNA, the cmtA gene specific primers P2 and P3 amplified a fragment of 550 bp designated dsPCR-P23. In a similar manner, the primers P4 and P5, specific for the cmtM gene, amplified a fragment of 220 bp (dsPCR-P45). DNA nucleotide sequence analysis of the dsPCR-P23 and dsPCR-P45 amplification products showed corre-
RESULTS AND DISCUSSION

Identification of Cob(I)alamin as the Major Product of Methyl Group Transfer—The finding of trace levels of cob(I)alamin in non-enzymic reactions of methylcobalamin with thiols was taken to indicate that cob(I)alamin, and not cob(II)alamin, is the immediate product of methyl group transfer (13). In order to identify the products formed by the methyltransferase isozymes, reaction mixtures were monitored by UV-visible spectroscopy. As shown in Fig. 1, large amounts of cob(I)alamin were produced with relatively little generation of cob(II)-alamin. However, substantial amounts of cob(I)alamin were found in separate reactions (not shown) that contained either cobamide (as designated in Reactions 2 and 4). The findings strongly support the conclusion of heterolytic cleavage of the carbon-cobalt bond (13), thus indicating displacement of the methyl group by nucleophilic attack of thiolate.

Inhibition of MT2 by Metal Ion Chelators—Both MT2 isozymes were markedly inhibited in the presence of metal ion chelators. The dependence of methyltransferase activity on chelator concentration was assessed in standard reaction mixtures. Fifty percent inhibition was produced by EDTA at a level of 50 μM and by 1,10-phenanthroline at 70 μM. The strongest inhibitor was EDTA, which caused 50% reduction of activity at a concentration of about 5 μM. Inhibition by EDTA was relieved by excess addition of any of several different divalent metal ions, including Ca2+, Mn2+, and Ni2+. Chromatography of a 1-ml sample of enzyme containing 5 mM EDTA on a column (1.5 × 17.5 cm) of Sephadex G-25 resulted in 90% recovery of activity, indicating that upon removal of the chelator inhibition was reversible. The findings suggested the presence of an essential metal ion bound to the enzyme.

Identification of MT2 as a Zinc-containing Enzyme—In order to determine whether or not metals were present in the enzyme, samples of MT2-M were analyzed by plasma emission spectroscopy, as described under “Materials and Methods.” The only elements found at concentrations comparable to that of the protein were copper and zinc, accounting for 0.08 and 0.45 g-atom/mmol protein, respectively. No significant levels were found of any of the other elements tested, including Mg, Ca, Mn, Fe, Co, Ni, Se, and Mo. Exposure of the enzyme to 5 mM EDTA followed by gel filtration in the presence of EDTA (2 mM) had no effect on the level of either copper or zinc. Metal content of MT2-M measured by a separate method using a metallochromic indicator, as described under “Materials and Methods,” yielded a value of 0.5 g-atom zinc/mol MT2-M. By this method MT2-A was found to contain 0.9 g-atom zinc/mol. The levels of contaminating zinc in buffer solutions used with the enzymes were not detectable by either of the methods as employed. The results provide evidence that MT2 isozymes are zinc-containing metalloproteins, and suggest that inhibition by chelators may be due to interference with Zn2+ coordination by the...
Methyl Acceptor Substrate Kinetics and Specificity—In previous studies, methyl acceptor substrate activity was tested with several different thiol compounds (14, 15). However, until now no compounds have been reported to be active other than the natural substrate HSCoM. Thus, a high degree of substrate specificity is indicated. In order to characterize more fully the substrates specificity, and to determine the kinetic properties of alternative substrates, we have extended the range of compounds tested as methyl acceptors (Table I). With the exception of weak inhibition by 2,3-dimercaptopropanesulfonate, none of the compounds listed caused a decrease in activity when present at 20 mM in reaction mixtures containing 2 mM HSCoM. As shown in Table I, activity was found with two compounds other than HSCoM, i.e. with 3-mercaptopropanoic acid (3-MPA), and 2-thioethanephosphonic acid. Neither the higher homolog of coenzyme M, 2-thiopropanesulfonate, nor the lower homolog of 3-MPA, thioglycolate, was active, indicating that carbon chain length may be critical for interaction of the substrate at the active site. The data further suggest that the substrate must contain minimally a free sulfhydryl group positioned at a specific distance from an anionic moiety (e.g. carboxylate, phosphonate, or sulfonate).

Steady state kinetic experiments showed that both MT2 isozymes have similar values of K_m(app) for coenzyme M (35 μM for MT2-A, and 20 μM for MT2-M), as given in Table I. The apparent K_m values for 3-mercaptopropanoic acid were 300–500 times higher than the values for coenzyme M, possibly indicating a weaker affinity for the non-natural substrate. Nevertheless, both isozymes also displayed similarly high values of K_m(app) for 3-MPA (Table I). The methyl donor substrate kinetics were also similar in that both isozymes displayed rather high K_m(app) values for methylcobalamin of 14 mM (Table I). This finding indicates the possibility of weak binding to methylcobalamin. Low affinity for methylcobalamin has been previously considered to be a consequence of the fact that the physiological methyl donor substrates are different corrinoid proteins exhibiting specific recognition of MT2 isozymes via protein-protein interactions (16).

Cloning and Sequencing of MT2 Isozyme Genes cmtA and cmtM—A DNA library from M. barkeri consisting of genomic EcoRI fragments ranging from 4 to 6 kb was prepared in the λZAP Express vector, as described under “Materials and Methods.” Positive plaques containing the cmtA gene were screened initially by plaque hybridization using the 550-bp dsPCR-P23 amplification product as probe (Fig. 2). A second screening was performed by direct PCR amplification of the DNA eluted from 32 positive plaques using primers P2 and P3. From this, two positive clones Z-8 and Z-32 were purified for further characterization. The recombinant clone Z-8 was used for in vivo excision of the phagemid pMA81 (Fig. 2). Restriction mapping analysis indicated that the phagemid contained a 4.5-kb EcoRI insert (Fig. 2) that hybridized with the dsPCR-P23 probe. The presence of a region encoding MT2-A was further confirmed by PCR amplification with primers P2 and P3. The DNA sequence of cmtA was determined on both strands, as described under “Materials and Methods.” Analysis of the nucleotide sequence

| Substrate analog | Activity | MT2-A | MT2-M |
|-----------------|----------|-------|-------|
| Methyl acceptor |          |       |       |
| 1. HS-CH2CH2SO3− | Yes      | (K_m(app) = 55 μM) | Yes | (K_m(app) = 20 μM) |
| 2. HS-CH2CO2−   | No       |       |       |
| 3. HS-CH2CH2HSO3− | No   |       |       |
| 4. HS-CH2CH2CO2− | Yes      | (K_m(app) = 9 mM) | Yes | (K_m(app) = 10 mM) |
| 5. HO-CH2CH2SO3− | No       |       |       |
| 6. H2N-CH2CH2SO3− | No     |       |       |
| 7. HS-CH2CH2OH   | No       |       |       |
| 8. HS-CH2CH2CH2SH | No     |       |       |
| 9. HS-CH2SO3−    | No       |       |       |
| 10. HS-CH2PO4H−  | Yesc    | Yes  |       |
| 11. HS-CH2CH2CO2− | No   |       |       |
| 12. H2SP        | No       |       |       |
| Methyl donor    |          |       |       |
| 13. O₂H₂B12     | Yesd    | Yes  |       |
| 14. CH3H2SP     | No       |       |       |

a Compounds were tested at 20 mM final concentration added to the standard assay in place of HSCoM, with the exception that H2SP and CH3H2SP were both 140 μM.
b K_m measurements for all methyl acceptor substrates were performed at 50 μM CH3-B12 in the presence of 1 mM dithiothreitol.
c Activity was lower than with either HSCoM or 3-mercaptopropionate.
d The K_m for methylcobalamin was determined under standard conditions in the presence of saturating HSCoM (2 mM).

FIG. 2. Strategy for cloning of cmtA. The steps employed to clone a 4.5-kb EcoRI fragment containing cmtA into pBK-CMV are shown schematically. Details of the procedures used are described under “Materials and Methods.” Results obtained at the various stages are presented under “Results and Discussion.”
Fig. 3. Nucleotide and deduced amino acid sequence of cmtA (MT2-A). The deduced amino acid sequence is shown directly below the second base of each codon. The stop codon is indicated by End. A potential zinc binding site TVLHICG is given in bold. The degenerate oligonucleotides P2 and P3 were designed from amino acid sequences within the shaded boxes. A sequence corresponding to the ribosome binding site is double underlined. The potential TATA box A and box B are marked above the sequences by asterisks and dots, respectively. The inverted repeats inv-1 and inv-2 are indicated by converging arrows below the sequence. A putative transcription terminator sequence is indicated by a dashed underline. Downstream direct tandem repeats are underlined.

In order to isolate the gene encoding MT2-M, an M. barkeri genomic library was constructed in the pBSK(-) vector containing HindIII fragments of size varying from 2 to 5 kb. The digoxigenin-labeled 220-bp dsPCR-P45 amplification product specific to MT2-M was used as a probe (Fig. 4). From a single positive colony, the plasmid pMET8 was isolated and subjected to restriction endonuclease digestions. The results showed that two fragments (3.4 and 3.6 kb) had been joined together into the same vector. Subcloning into pBSK(-) produced plasmid pMET82 (Fig. 4) that contained only the 3.6-kb HindIII fragment and was able to hybridize with the dsPCR-P45 probe and produce the 220-bp dsPCR-P45 product in PCR amplification.

The amino acid sequence deduced from DNA sequence analysis of the 5'-end matched perfectly the NH2-terminal region of MT2-M, confirming that the 3.6-kb HindIII fragment contained either a large portion or, as later demonstrated, the entire cmtM gene.

The complete sequence of DNA containing cmtM is shown in Fig. 5. A single open reading frame of 1,017 bp was found encoding a protein of 339 amino acids with a calculated molecular mass of 35.9 kDa. The deduced methanogenic amino acid composition was very close to the composition of the protein obtained by hydrolysis and amino acid analysis (data not shown).
Degenerate oligonucleotides P4 and P5 were devised from the (MT2-M) sequence identical to the consensus 5'-ATGC'-3' (26). As in the case of cmTA sequences capable of secondary structure formation were found in the promoter region (Fig. 5). One of these (inv-1) was localized at positions 250–295, and another (inv-2) was found at positions 191–219. A third region located at positions 1434–1448 was found immediately downstream from the stop codon at the positions of the proposed transcription start sites (box B) and termination sequences shown in Figs. 3 and 5. The sequence is annotated as described in the legend to Fig. 6. Based on the content of charged amino acids, the calculated net charges at pH 9.0 were -18 and -17 for MT2-A and MT2-M, respectively. This finding is consistent with previous results, which showed that MT2-A has greater electrophoretic mobility at pH 9 than MT2-M (14). A large number of the identical amino acids were found grouped in clusters. These conserved regions might be involved with catalytic functions common to both isozymes. Conversely, variation in other parts of the protein might be related to their immunological and phosphoacceptor substrate specificities (14, 16). The sequence TVLHICG located in the carboxy-terminal region (residues 236–242) is the longest stretch of identical amino acids. The primary structures of zinc-containing proteins frequently contain closely spaced sulfhydryl and imidazole groups at the site of metal ion binding (28). Thus, the conserved TVLHICG sequence is a potential candidate for binding of zinc.

The GenBank/EMBL data base was searched for translated sequences homologous to MT2 isozymes. A FASTA homology search showed 21.7% identity with 8 gaps within a 335-amino acid region overlap between MT2-M and the uroporphyrinogen decarboxylase from Anaecystis nidulans R2 (29). Significant homology was also revealed between MT2-A and the Rhodobacter capsulatus uroporphyrinogen decarboxylase (30). However, 12 gaps were required yielding 24% identity overall. Moreover, both MT2 isozymes showed high levels of homology (~30% identity and 53% similarity) to the 40-kDa subunit of the M. barkeri 480-kDa corrinoid enzyme.

Functional Expression of cmTA and cmTM Genes in E. coli—In order to determine whether or not the cloned M. barkeri genes were expressed in E. coli, cell extracts were prepared from E. coli containing plasmids pMA81 (cmTA), pMET82 (cmTM), and pMET81 (control). The plasmid pMET81 was used as a negative control because it contained an anonymous 3.4-kb HindIII fragment. Western blot analyses revealed that both cmTA genes were expressed in E. coli, and that both protein products exhibited electrophoretic mobilities identical to those found with M. barkeri extracts. A band at the position corresponding to MT2 was absent in the extract from cells containing pMET81. The results are presented in Table II. Extracts from cells containing pMA81 and pMET82 were both capable of rapidly catalyzing methyl group transfer from methylocobalamin to HSCoM (Table II). However, activity was not detectable in extracts of E. coli cells harboring pMET81. Although neither cmTA nor cmTM was under the control of the lacZ promoter (see Figs. 2 and 4), the results show that both archaeal genes are recognized by the E. coli transcriptional apparatus, and that substantial levels of both isozymes are produced in an active form. These findings suggest that in the A+T-rich 5'-untranslated region there may be a sequence that mimics the E. coli promoter consensus sequence, thereby providing a site for initiation of transcription. Such a sequence has been reported in Methanococcus vannielii at which E. coli RNA polymerase binds and initiates transcription of the hisA gene (31).
TABLE II

Methylcobamide-Coenzyme M methyltransferase activity in E. coli expressing MT2-A and MT2-M

| Bacterial strain | DNA (cell-free extract) | MT2 specific activitya |
|------------------|-------------------------|------------------------|
| M. barkeri (trimethylamine-grown) | | 2.2 |
| M. barkeri (methanol-grown) | | 7.5 |
| E. coli XLOLR/pMA81 (contA) | | 1.21 |
| E. coli XL1-Blue/pMET82 (cmtM) | | 0.154 |
| E. coli XL1-Blue/pMET81 (control) | | 0.000 |

a Activity measured under standard assay conditions.

ligands has been described in transition metal complex systems (32, 33). Furthermore, the involvement of zinc in the mechanism of methylthioether formation at a specific cysteine residue in the repair of DNA methylphosphotriesters by the E. coli Ada protein has received considerable attention (34–38). In the Ada system, spectroscopic evidence shows that zinc binds to, and activates the sulfhydryl of cysteine 69 for nucleophilic attack on the methyl group of a DNA methylphosphotriester, forming the methylthioether (35). Further studies may now be considered in order to determine whether or not the MT2 isozymes operate by an analogous mechanism.

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