Transmethylation reactions during methanogenesis
from acetate in Methanosarcina barkeri

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Primary investigator: Joseph A. Krzycki
Associate Professor
Department of Microbiology
Ohio State University
Columbus OH 43210

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Background

Methanogenic archaeobacteria are key members of bacterial consortia breaking down organic matter in many strictly anaerobic environments and producing an alternative energy source, methane (2). Their catabolic production of methane is a unique biochemical process, employing several unusual cofactors and a variety of unusual enzymes (3). Most methanogens in culture have a limited substrate range, using only formate or H_2/CO_2 (4). In contrast, Methanosarcina barkeri MS (DSM 800) utilizes H_2/CO_2 , methanol, monomethylamine (MMA), dimethylamine (DMA), and trimethylamine (TMA), and acetate (5, 6, 7). M. barkeri possesses multiple methyltransferases which convert methylated substrates to methylated cofactors or proteins prior to their reduction to methane. Methylated substrates are also oxidized to CO_2 to provide reducing equivalents for reduction to methane or for biosynthesis (8).

Enzymes and cofactors involved in transmethylation reactions in M. barkeri.

This short review focuses on methyltransferase reactions leading to methane formation in Methanosarcina, and is summarized in fig. 1. Additional background information can be found in the appended manuscripts. Several recent reviews of the biochemistry of methanogenesis are available (3, 8, 9).

Methylreductase

The final methylated cofactor of methanogenesis from methanol (10, 11), TMA (12), acetate (13, 14), and CO_2 (15) has been demonstrated to be methyl-coenzyme M (CH_3CoM). Methylreductase, a nickel tetrapyrrole enzyme, converts 7-mercaptoheptanoylthreonine phosphate (HTP) and CH_3-CoM to methane and the heterodisulfide of CoM and HTP (16). Subsequent reduction of heterodisulfide by a membrane bound cytochrome b complex is key to chemiosmotic ADP phosphorylation (17, 18, 19). In extracts, methylreductase can be specifically inhibited by bromoethanesulfonic acid (BES), an analogue of CH_3CoM (20, 21).

Corrinoid

Corrinoid proteins participate in methyl-transfer reactions during methanogenesis from acetate, methanol, and CO_2 . Over 90% of the corrinoid in M. barkeri is associated with soluble proteins, the remainder is associated with membrane proteins (22). The majority of corrinoids from Methanosarcina are 5-hydroxybenzimidazolylcobamide derivatives (23), rather than 5,6 dimethylbenzimidazolylcobamide derivatives (such as vitamin B_{12}). The central cobalt ion is coordinated by the nitrogens of the tetrapyrrole ring. The purine can serve as the lower axial ligand, while the methyl group can occupy the upper axial position. The alkyl-Co bond can be broken by visible light, and consequently CH_3 -corrinoid proteins must be isolated in dim red light. The central metal ion can be in the Co (I), Co (II), or Co (III) redox states (24). Several corrinoid dependent methyltransferases react with their primary substrates when in the Co(I) state (25, 26, 27). The methylated corrinoid transfers the methyl group to a methyl-acceptor, and returns to the Co (I) state. Several corrinoid methyltransferases are Co (II) or Co (III) *in vitro* and require reductive activation, such as methyltransferase I (27) and CH_3 -THMP:CoM methyltransferase (28) which are discussed below.

Tetrahydrosarcinapterin (THSP)

Like tetrahydrofolate (THF), this pterin also binds formyl, methenyl, methylene, and methyl groups (29). It participates in methane formation from acetate and CO₂, and oxidation of methyl containing substrates to CO₂ (8). THSP is a diglutaryl derivative, and differs from tetrahydromethanopterin (THMP), the form found in Methanobacterium spp., which is a monoglutaryl derivative. The latter is not found in M. barkeri (29).

Membrane bound CH₃-THMP:CoM methyltransferase.

A corrinoid protein isolated from the membrane of Methanobacterium thermoautotrophicum methylates CoM with CH₃-THMP (30, 31, 32). In Methanosarcina, CH₃-THMP or CH₃-THF dependent methylation of CoM is found only in the membrane fraction (33, 34), the enzyme has not yet been assayed with CH₃-THSP. CH₃-THMP:CoM transmethylation has been linked to Na⁺ transport in membrane vesicles (34). The enzyme has not yet been isolated, but radiolabeling and propyl iodide inhibition indicate it is a corrinoid protein. It has been suggested to be directly involved in methanogenesis from acetate and CO₂, and in the oxidation of methanol (34).

Methyltransferase reactions during methanogenesis from acetate.

Acetate is activated to acetyl-CoA then cleaved by carbon monoxide dehydrogenase (CODH), associated with a corrinoid/Fe-S protein (35, 36) This reaction is analogous to that found in the synthesis of acetyl-CoA by Clostridium thermoaceticum (37). The CODH-corrinoid complex mediates exchange of CO and acetyl-CoA (38), and was recently demonstrated to directly methylate THSP with acetyl-CoA. CoM methylation by CH₃-THSP may be mediated the membrane bound corrinoid protein (39).

Methyltransferase reactions during methanogenesis from methanol.

Methanol is converted directly to CH₃-CoM by a two enzyme system, methyltransferase I (MTI) and methyltransferase II (MTII) (40). MTI is a 122 kDa corrinoid protein, while the 33 kDa MTII lacks a prosthetic group. A methylation activator protein and ATP reductively activate MTI to Co(I), which then reacts with methanol to form an enzyme bound methylated corrinoid (27). Transfer of the methyl group from the bound ¹⁴CH₃-corrinoid on MTI to CoM is mediated by MTII. MTII also methylates CoM with free CH₃-B₁₂ (40, 41).

Methyltransferases involved in methanogenesis from MMA, DMA, and TMA.

The enzymes methylating CoM with these methylamines have not been isolated. A TMA:CoM methyltransferase was studied in cell extracts of M. barkeri (12). Extracts of cells grown on TMA could make methane from all three substrates, whereas extracts of DMA grown cells had decreased activity of the TMA methyltransferase. The TMA methyltransferase system also produced DMA and CH₃CoM as products, indicating separate methyltransferases for TMA and DMA utilization. Our preliminary data indicates separate methyltransferases exist for each methylamine.

It has been suggested that use of methylamines is analogous to methanogenesis from methanol, and will involve 2 enzymes with similar mechanisms to MTI and MTII (42). M. barkeri has two isoforms of MTII (41). In cells grown on methanol, the predominant isoform is involved in methanogenesis from methanol, while the less abundant one does not have a demonstrated function.

This second isozyme is much more prevalent in cells grown on acetate (41), CO₂, or TMA . Compared to cells grown on these latter substrates, methanol grown cells make methane at a relatively slow rate from TMA, leading to the suggestion that this isozyme of MTII may be involved in methanogenesis from TMA (42).

Fig. 1. Schematic of methyltransferases involved in methanogenesis from methylamines, acetate, methanol and CO_2 . *Demonstrated or potential corrinoid protein.

Report of progress to July 1994.

We were awarded a DOE grant in August 1991 based on our discovery of a novel assay for corrinoid proteins involved in transmethylation reactions in *M. barkeri* (1). Several $^{14}\text{CH}_3$ -proteins were detected in extracts of acetate grown cells incubated with ^{14}C -2 acetate, H_2 , and ATP when methylreductase was inhibited using BES. Three $^{14}\text{CH}_3$ -proteins with molecular sizes of 480, 200, and 29 kDa were detected by size exclusion HPLC, while four $^{14}\text{CH}_3$ -protein bands were detected by native polyacrylamide gel electrophoresis (PAGE) followed by autoradiography. Two bands were correlated with the 480 and 29 kDa $^{14}\text{CH}_3$ -proteins, while two others were designated the unknown A and unknown B bands. All were coincident with reddish protein bands and the methyl group was susceptible to photolysis, indicating these were corrinoid proteins. That so many corrinoid proteins were methylated by acetate was an unexpected finding.

At the time of submission of our proposal we had partially purified the 480 and 29 kDa $^{14}\text{CH}_3$ -proteins and demonstrated the label was associated with $^{14}\text{CH}_3$ -corrinoid. The 480 kDa $^{14}\text{CH}_3$ -protein, but not the 29 kDa $^{14}\text{CH}_3$ -protein, was transiently methylated during methanogenesis from ^{14}C -2 acetate in a manner consistent with its involvement in methanogenesis (1).

One precaution we have maintained throughout this research is that non-physiological methylation of corrinoid proteins by accumulated intermediates of methanogenesis is possible. Methylation of any corrinoid protein by a particular substrate upon inhibition of methanogenesis can only indicate the presence of that protein as a potential methyltransferase. Other evidence must be gathered to indicate and prove involvement in methanogenesis from a particular substrate.

Concise listing of research progress by item for the project for period ending July 1994.

1. We have purified and characterized the abundant 480 and 29 kDa corrinoid proteins, confirming these are not previously isolated proteins. They lack iron, and bind methyl-corrinoid in the base-on configuration. The 480 kDa protein consists of 30 and 41 kDa subunits and binds six corrins. The 29 kDa is a monomer with one bound corrin.
2. The methyl groups of the purified $^{14}\text{CH}_3$ -proteins are rapidly converted to $^{14}\text{CH}_4$ in cell extracts, and demethylated by addition of CoM to BES inhibited extracts, indicating a close relationship of these proteins with methanogenic pathways.
3. Extracts of methanol grown cells do not methylate the 480 kDa protein to a significant extent. The 480 kDa protein is 1-2% of the soluble cell protein of extracts of acetate grown cells, but is 5-10 fold less in extracts of methanol or H_2/CO_2 grown cells. It was nearly undetectable by the methods used in cells grown on TMA. The relative increase in synthesis of the 480 kDa protein when cells are grown on acetate is consistent with involvement in methanogenesis from this substrate.
4. We have identified a 175 kDa corrinoid protein from extracts of methanol grown cells which is methylated by $^{14}\text{CH}_3\text{OH}$ in extracts of cells grown on methanol. This protein is not methylated in extracts of cells grown on acetate.
5. We have cloned and sequenced the operon encoding the two subunits of the 480 kDa protein. We have identified a strong promotor sequence and Shrine-Delgarno

sequence upstream of the 40 kDa gene. This gene is followed by the gene encoding the 30 kDa gene, which in turn is followed a strong termination sequence.

6. The 30 kDa subunit of the 480 kDa protein possesses a region of high homology to the B₁₂ binding domain of E. coli B₁₂ dependent methionine synthase.

7. The methylated 480 kDa protein is rapidly demethylated by coenzyme M, resulting in Co(I) corrinoid bound to the protein. This can react rapidly with methyl iodide regenerating the methylated protein. The purified protein can catalyse the methylation of coenzyme M with methyl iodide at over 4 $\mu\text{mol}/\text{min}/\text{mg}$ protein.

8. We have identified the 1.6 MDa CODH:corrinoid protein complex from M. barkeri MS, and confirmed the corrinoid protein in this complex is not the 480 or 29 kDa corrinoid protein. The complex was identified by alkylation of the corrinoid by ethyl iodide in extracts incubated under H₂. Alkylation of all other known corrinoid proteins in extracts of acetate cells by ethyl iodide was ATP dependent, which will provide a ready assay for reductive activation of these proteins to the Co(I) state.

9. We developed a novel assay for methyltransferases involved in the conversion of TMA, DMA, or MMA to methane. These methyltransferases are distinct small proteins which may bind corrinoid.

10. We have partially purified the TMA methyltransferase using this assay.

11. We have preliminary evidence that the 29 kDa protein is the MMA methyltransferase. The MMA methyltransferase activity in TMA grown cells has a measured molecular mass of 29 kDa. A preparation of partially purified 29 kDa corrinoid protein stimulated methanogenesis from this substrate in extracts of methanol grown cells, and isolation of the MMA methyltransferase activity resulted in a preparation containing two major proteins, methyltransferase II, and the 29 kDa protein.

Papers resulting from project for period ending July 1994.

1. J. Kremer, X. Cao, and J. Krzycki. 1993. Isolation of two novel corrinoid proteins from aceticlastic Methanosarcina barkeri. J. Bacteriol. 175:4824-4823.

2. J. Kremer, S. Burchfield, C. Frazier, and J. Krzycki. 1994. Differential in vitro methylation and synthesis of soluble corrinoid protein from Methanosarcina barkeri grown on different substrates. J. Bacteriol. 176:253-256.

Presentations at scientific meetings for period ending July 1994.

1. L. Paul Pottenplackel, J. D. Kremer and J. A. Krzycki. Homology of the 480 kDa corrinoid protein of Methanosarcina barkeri to B₁₂ dependent methionine synthase. Annual Meeting of the American Society for Microbiology. 1994

2. J.A. Krzycki. In vitro methylation of corrinoid proteins following inhibition of methanogenesis in Methanosarcina. Invited seminar. Gordon Research Conference on Methanogenesis. 1993.

3. J.A. Krzycki. Proteins involved in methyltransfer reactions during growth on acetate. Invited seminar. Annual Meeting of the American Society for Microbiology. 1992

4. J. D. Kremer , X. Cao, and J. A. Krzycki. Purification of corrinoid proteins methylated by acetate from Methanosarcina barkeri. J. D. Kremer, X. Cao, and J. A. Krzycki. Annual Meeting of the American Society for Microbiology. 1992.

Detailed description of progress and preliminary data for the next project period.

We have isolated both the 480 kDa $^{14}\text{CH}_3$ -protein and the 29 kDa $^{14}\text{CH}_3$ -protein to near homogeneity. Characterization of both proteins is described in the accompanying preprint. This was recently accepted by the Journal of Bacteriology. A reviewer called the paper "a beautiful piece of work" and commented that it was "carefully performed and all aspects are well interpreted" and that it "adds significantly to our understanding of corrinoid proteins in methanogens". The 480 kDa corrinoid protein possesses 6 bound corrins and is composed of 2 subunits of 41.4 and 30.4 kDa in equimolar stoichiometry, and a probable $\alpha_6\beta_6$ or $\alpha_7\beta_7$ configuration. The 29 kDa corrinoid protein is a monomer in solution with one bound corrin ring. Both proteins carried entirely $^{14}\text{CH}_3$ -corrinoid when isolated anaerobically under dim red light from extracts incubated with ^{14}C -2 acetate and BES. The ^{14}C -methyl group on both proteins was completely photolabile. Photolysis resulted in Co(II) corrinoid. We were unable to localize the corrin to either the 41 or 30 kDa subunit of the 480 kDa protein using SDS or urea gels and columns. However, in the next year of our current project period we will attempt to identify the subunit by reaction with formaldehyde. This may allow us to detect a monomeric subunit with cross-linked corrinoid in SDS gels. Preliminary evidence indicates the 30 kDa protein binds the corrin ring since this subunit possesses high homology to methionine synthase B₁₂ binding domain.

The purified 480 kDa $^{14}\text{CH}_3$ -corrinoid protein gives rise to the 480 kDa and the Unknown A $^{14}\text{CH}_3$ -protein bands in native PAGE/autoradiography. Neither the 480 kDa nor the 29 kDa protein possesses high amounts of iron, both appear to bind methyl-corrinoid in the base-on form. Therefore neither protein resembles the corrinoid/iron sulfur protein that associates with CODH (43, 44). When the isolated 480 and 29 kDa $^{14}\text{CH}_3$ -proteins were added to cell free extracts, the methyl groups on both were converted completely to methane. The 480 and 29 kDa proteins are methylated when methylreductase is inhibited by addition of BES to extracts. We also demonstrated they are demethylated by the subsequent addition of CoM, indicating these proteins donate and receive methyl groups from methanogenic intermediates. We concluded that the 480 and 29 kDa corrinoid proteins are novel proteins, not previously isolated from a methanogen, which are intimately connected with the methanogenic pathway. The 200 kDa $^{14}\text{CH}_3$ -protein has proven too unstable for isolation.

As described in the second appended manuscript, we compared protein methylation in extracts of cells grown on acetate or methanol. The key observation in this paper is that methylation of the 480 kDa corrinoid protein was 100 fold higher in extracts of cells grown on acetate than in extracts of cells grown on methanol, using either $^{14}\text{CH}_3\text{OH}$ or $^{14}\text{CH}_3\text{COOH}$ as the radiolabel. The increased amount of methylation was in part due to regulation of synthesis of this protein. Immunoblots using mouse polyclonal antibody raised against the 480 kDa protein

demonstrated that the protein is 5-10 times more abundant in acetate grown cells than in cells grown on methanol or H_2/CO_2 . Cells grown on TMA had little detectable 480 kDa protein. Regulation of the synthesis of the protein by catabolic substrate argues against an anabolic function, since (aside from the synthesis of acetyl-CoA) major anabolic reactions would be similar in cells grown on acetate, methanol, or TMA. This result further supports the hypothesis that the 480 kDa protein is involved in methane formation from acetate.

Two previously undetected $^{14}CH_3$ -protein electrophoretic bands, termed MpM I and MpM II, were found in extracts of cells grown on methanol following incubation with $^{14}CH_3OH$. Both are corrinoid proteins which elute in a single 175 kDa $^{14}CH_3$ -protein peak from a gel permeation column. We are uncertain if the MpM I and MpM II $^{14}CH_3$ -protein bands are distinct proteins, or arise from the same $^{14}CH_3$ -protein. These $^{14}CH_3$ -proteins may be derived from MTI (methanol utilization). However, it is not methylated in extracts of acetate grown cells by $^{14}CH_3OH$ which convert methanol to methane and presumably have MTI. Therefore, it may be a novel regulated corrinoid protein involved in some other aspect of methanol metabolism.

Fig. 2. Size exclusion HPLC of proteins labeled by ^{14}C -2 ethyl iodide in extracts of acetate grown cells under H_2 (A) or under H_2 and ATP (B). Sizes of ethylated peaks are indicated.

Alkylation of corrinoid proteins by ethyl iodide.

The involvement of corrinoid proteins in methanogenesis from acetate was indicated by specific inhibition of this pathway by propyl iodide(45, 46). Reversal of inhibition by light was consistent with cleavage of a cobalt-carbon bond. Experiments with intact cells indicated the primary site of inhibition was a corrinoid accepting methyl groups following the splitting of acetyl-CoA by CODH(46), a second corrinoid protein was predicted in further studies (47). CODH isolated from *M. barkeri* MS, *Methanococcus*, or *Methanotherix* lacks corrinoid and is a 2 subunit enzyme with a mass of approximately 230 kDa(48, 49, 50, 51). However, a very high molecular weight, CODH:corrinoid complex consisting of 5 subunits can be isolated

from Methanosarcina thermophila (52) and Methanosarcina barkeri strain NIH (39). We conducted experiments with commercially available ^{14}C -2 ethyl iodide to ascertain if any of the corrinoid proteins we had identified corresponded to the corrinoid protein which associated with CODH. We established that ^{14}C -2 ethyl iodide also inhibited acetate dependent methanogenesis in cell suspensions in a light reversible manner. HPLC analysis of extracts of acetate grown cells incubated with inhibitory concentrations of ^{14}C -2 ethyl iodide indicated that the 480, 200 and 29 kDa proteins were alkylated (see fig. 2B for a representative chromatogram). In addition, a protein whose molecular size was estimated at 1.6 MDa was alkylated, as well as a radiolabeled peak that eluted in the void volume of the size exclusion HPLC column (> 7 MDa). Radioactivity on all CH_3CH_2 -proteins was lost upon photolysis. The >7 MDa peak has not been identified, however, the 1.6 MDa $^{14}\text{CH}_3\text{CH}_2$ -protein peak was further investigated. Only the 1.6 MDa peak was alkylated when extracts were incubated under H_2 with ^{14}C -2 ethyl iodide (fig. 2A). We had occasionally observed small amounts of radioactivity incorporated into this protein when extracts were incubated with ^{14}C -2 acetate in the presence of BES. CODH activity was analyzed in the fractions from the size exclusion HPLC column and was observed with retention times corresponding to 1.6 MDa and approximately 200 kDa (fig. 3). The 1.6 MDa $^{14}\text{CH}_3\text{CH}_2$ -protein peak with CODH activity was collected from the column and extracted with ethanol. The radioactivity was found to be present on a ethanol soluble cofactor which lost radioactivity upon exposure to light. The UV-visible spectra of the ethylated cofactor had an absorbance maxima at 508 nm which shifted to 450 nm at pH 1, indicating the cofactor was an ethylated corrinoid (fig 4a). The ethylated corrinoid was resistant to cyanolysis in the dark, but upon photolysis in the presence of KCN converted to a monocyanocorrinoid (fig 4b). SDS PAGE of the 1.6 MDa $^{14}\text{CH}_3\text{CH}_2$ -protein showed subunits with masses of 92, 66, 58, 52, and 19 kDa (fig 5). The smallest and largest subunits comigrated with the subunits of isolated CODH from M. barkeri MS. This pattern of subunits closely resembles the CODH:corrinoid protein complex from M. thermophila and M. barkeri NIH. The molecular mass of the CODH:corrinoid protein from strain NIH is also estimated at 1.6 MDa. This indicates that the CODH:corrinoid complex exists in extracts of M. barkeri, and that the site of alkylation on the complex is the corrinoid rather than on CODH itself. Neither the 480 or the 29 kDa corrinoid protein is the CODH associated corrinoid, since the subunits of neither protein were associated with the CODH:corrinoid complex (fig 5). This confirms our conclusions based on biochemical analysis of the 480 and 29 kDa proteins.

Attempts to purify the complex on Sepharose 6B demonstrated that the complex rapidly dissociated. The predominant CODH activity was found to elute with a molecular weight of 230 kDa, and radioactivity was spread from the high to low molecular weight fractions.

When extracts of acetate grown M. barkeri were incubated under hydrogen with ^{14}C -2 ethyl iodide only the 1.6 MDa CODH:corrinoid protein complex was alkylated. Ethyl iodide is most rapidly attacked by corrinoid in the extremely nucleophilic Co (I) redox state (53). This indicates that in extract incubated with H_2 only the CODH:corrinoid complex is in the Co(I) state. This is consistent with the

relatively high measured E_0' (pH 7.8) of -486 mV (54) and E_0' of -438 mV (55) for Co(II)/Co(I) couple in the corrinoid/Fe-S proteins associated with CODH. The E_0' of the Co(II)/Co(I) couple in corrinoid can be as low as -600 mV (24). This indicates all other corrinoid proteins in the extract must have a redox potential significantly lower than that of the corrinoid in the CODH complex. Interestingly, the 480 and 29 kDa corrinoid proteins, as well as the >7 MDa and 200 kDa $^{14}\text{CH}_3$ -proteins, were alkylated by ^{14}C -2 ethyl iodide when ATP was also added to extracts incubated with H_2 (fig. 2b). Labeling of the 480 and 29 kDa proteins was confirmed by native PAGE/autoradiography. This indicates these corrinoid proteins undergo an ATP dependent reductive activation process to the Co(I) state which then allows reaction with ethyl iodide. MTI has been demonstrated to undergo ATP dependent reductive activation to the Co (I) state (27). ATP dependent ethyl iodide alkylation of these proteins will be a useful assay to study how methanogens are capable of generating extremely low potential reducing equivalents for reductive activation reactions.

Fig. 3. Size exclusion HPLC of an extract of acetate grown cells incubated with H₂ and ¹⁴C-2 ethyl iodide, analysed for CODH activity. Sizes of CODH peaks are indicated, radioactivity eluted only with the 1.6 MDa peak.

Fig. 4. UV-Vis spectra of ¹⁴CH₃CH₂-corrinoid extracted from 1.6 MDa ¹⁴CH₃CH₂-CODH:corrinoid protein complex. Spectra were taken aerobically under conditions indicated.

Fig. 5. SDS PAGE of purified CODH (1), 480 kDa protein (2), and partially purified 1.6 MDa CODH:corrinoïd protein complex (3). Numbers on right side indicate approximate subunit molecular mass in kDa. Dye front is indicated (d). Top of picture coincides with top of separating gel.

Cloning and sequence analysis of the genes encoding the 480 kDa corrinoid protein.

The N-terminal sequences of the polypeptides comprising the 480 and 29 kDa proteins have been determined to 40 amino acids. Oligonucleotides were made using the sequence of the 30 kDa subunit of the 480 kDa protein and used to clone a 4.5 kb EcoR I/Hind III fragment into pUC 19 which contains the gene encoding the 30 kDa subunit of the 480 kDa protein. Insert sequence of this plasmid (termed pCo4500) contained the 40 amino acids of the N-terminus of the 30 kDa subunit of the 480 kDa gene. The length of the entire gene was 274 amino acids, followed by a typical archaeobacterial terminator poly T region. A second genomic fragment was found using oligonucleotides complementary to the N-terminus of the 40 kDa subunit. The 3.7 kB Pst I fragment was cloned into pUC 19 and called ppt3700. This fragment contained the the 40 amino acids of the N-terminus of the 40 kDa subunit. Overlap between ppt 3700 and pCo4500 gave the complete sequence of the 40 and 30 kDa genes in a continuous operon with the 40 kDa gene at the start of the operon. In front of this gene was an archaeobacterial box A promoter element (TATATATA) spaced 22 bases from a TGC sequence which often coincides with the transcriptional start site. A consensus box B element was present as well. Approximately 30 bases following the sequence encoding the 356 amino acids of the 40 kDa subunit is the start of the 30 kDa gene.

The 30 kDa subunit has a region of strong homology to the 28 kDa tryptic fragment of methionine synthase which binds B₁₂ (figure 6). This region also corresponds to a region of homology observed for the coenzyme B₁₂ dependent enzymes glutamate mutase and methylmalonyl-CoA mutase. Thus the 30 kDa subunit may bind the corrinoid of the 480 kDa protein.

(Figure 6) The 30 kDa subunit of the 480 kDa corrinoid protein has sequence similarity to a tryptic fragment of E. coli methionine synthase which binds B₁₂

30 kDa met H	80	KLISALLEKG	KDPTDLVINA	LMPGIQTVCE	LYDIGESYVP	EILLANEALM
	681	EAR-----QQA	TRPIEVIEGP	LMGGMNVVGD	LFGECKMFLP	QVKSARVMK
30 kDa met H	130	KGVE-----LC	QKKKGEVPSQ	GKVVSIVVIG	DLTDIGKNIV	AAILRANGFE
	727	QAVAYLEPFI	EASKEQGKTN	GKMVIATVKG	DVHDIGKNIV	GVVLQCNNEY
30 kDa met H	175	VIDLGRDVTV	EAAVEAVKST	KANLVTGTTL	MSTTKGGLKA	SQMLWNPRG-
	777	IVDLGVMVPA	EKILRTAKEV	NADLIGLSGL	ITPSLDEMNV	VAKEMERQGF
30 kDa met H	224	--PLACGGAA	VDR-----RF	VDTF-GNSVY	GRTPLDAVKI	AKEICEGKSW
	827	TIPLLLGGAT	TSKAHTAVKI	EQNYSGPTVY	VQNASRTVGV	VAALLSDTQR
30 kDa met H	266	EEARNELY				
	877	DDFVARTR				

+ indicates conserved substitutions.

* indicates identities.

Demethylation of the 480 kDa protein by coenzyme M.

We have found that the methylated 480 kDa protein can be rapidly demethylated by coenzyme M. When a 1 mg/ml solution of the purified protein is mixed with 50 micromolar coenzyme M, the spectrum of the enzyme undergoes rapid changes indicative of the formation of Co (I) corrinoid, notably a peak at 385 nm which rapidly decayed upon exposure to oxygen. Demethylation by homocysteine or dithiothreitol at equivalent concentrations was not observed, indicating the function of this protein is in the methylation of coenzyme M, i.e. methanogenesis (see figure 6A below)

figure 6a

Upon formation of Co(I) the corrinoid is susceptible to methylation by methyl iodide. In the presence of 30 mM methyl iodide methylation of coenzyme M is rapidly catalysed by the protein, at observed rates up to 4.4 $\mu\text{mol}/\text{min}/\text{mg}$ purified 480 kDa protein. The methylation of coenzyme M is detected using Ellman's reagent to measure the resultant loss of the free thiol. The K_m for coenzyme M was estimated as 1.3 mM. This is comparable to enzymes such as methylreductase, which has a K_m of 1.6 mM for methylcoenzyme M.

Development of a rapid assay for methylamine methyltransferases.

We have developed a new assay for methyltransferases involved in methane formation from TMA, DMA, and MMA based on the following considerations. Extracts of cells grown on methanol evolve 30 to 80 nmol CH_4 /min/mg protein from methanol, while TMA and DMA are not converted to methane. MMA is converted to methane at rates from 5 to 30% of the rates of methanol dependent methanogenesis measured in the same extract. In contrast, extracts of cells grown on TMA are able to convert MMA, DMA, and TMA to methane at equivalent rates of 20 to 40 nmol/min/mg protein. TMA and methanol (and presumably MMA and DMA) are converted to CH_3CoM prior to CH_4 formation (10, 58). The lack of methanogenesis from TMA or DMA, and the relatively low rate of methane formation from MMA, in extracts of methanol grown cells indicate that the methyltransferases for methylation of CoM by TMA, DMA and MMA are at low activity or are absent. We have found that when methanol grown cell extracts are supplemented with small amounts of protein derived from TMA grown cells, the extract of methanol grown cells will produce methane from TMA, DMA, or MMA at markedly increased rates. This is illustrated for TMA in fig. 7. The response to increasing amounts of protein from the TMA grown cells was linear from 5 to 20 μl of TMA extract added to 200 μl of a 15 mg protein/ml extract of methanol grown cells. Higher amounts of extract of TMA grown cells resulted in saturation of the assay. The specific activity of methane formation from TMA in 200 μl of extract of methanol grown cells supplemented with 20 μl of TMA grown cells was actually twice that of TMA dependent methanogenesis from 200 μl of extract of TMA grown cells. Addition of 20 μl of TMA extract to 200 μl of MOPS buffer resulted in negligible methanogenesis.

This is a general assay for methyltransferases required for the methylamine dependent methylation of CoM that are absent or present at low activity in extracts of methanol grown cells. The assay shows no dependence on low molecular factors present in extracts of TMA grown cells. The methyltransferase activities are O_2 sensitive. Methanogenesis above background rates in the assay is strictly dependent upon the addition of the methylamine under study.

Anaerobic separation of an extract of TMA grown cells on a Sephacryl S-300 HR gel permeation column is illustrated in fig. 8. Fractions were analyzed by mixing 50 μl of each column fraction with 200 μl of a 20 mg protein/ml methanol extract in the presence of H_2 , ATP, and the indicated methylamine. Methane evolved after 30 min was measured. All three methyltransferase activities eluted closely together as proteins with estimated molecular masses of 29 kDa (MMA dependent), 18 kDa (TMA dependent), and 12 kDa (DMA dependent). Their elution was coincident with a bright red color, indicating abundance of corrinoid in these fractions. The specific activity of each methyltransferase was determined as nmol CH_4 evolved from amine/min/mg protein of the fraction added to the assay. The specific activities in the extract prior to fractionation were 80 (MMA methyltransferase), 150 (DMA methyltransferase), and 141 (TMA methyltransferase). An average 4 fold purification was obtained for each activity after fractionation. Recoveries of the individual methyltransferase activities averaged 60%. Methanogenesis from the active fractions did not occur without addition of the indicated methylamine. This

experiment illustrates that specific proteins are responsible for stimulating methanogenesis from these methylamines in extracts of methanol grown cells.

We have partially purified the TMA methyltransferase activity using anion exchange and hydroxylapatite (fig. 9). Following hydroxylapatite the preparation had 6 bands in native Coomassie stained gels. The purified TMA methyltransferase did not stimulate methane formation from MMA or DMA, indicating the TMA methyltransferase is a separate protein or group of proteins from the other methyltransferase activities. UV-Vis spectra of the partially purified TMA methyltransferase indicated the presence of a corrinoid.

Evidence that the 29 kDa corrinoid protein from acetate grown cells is a constitutive MMA methyltransferase.

We have observed that an intense band co-migrates with the purified 29 kDa corrinoid protein in native PAGE of extracts of TMA grown cells. In addition, the molecular mass of the MMA methyltransferase activity is measured as 29 kDa. This indicates that the 29 kDa corrinoid protein originally identified in extracts of acetate grown cells might be constitutive amounts of a MMA methyltransferase. We recently tested this directly by measuring the ability of a preparation of 80% homogenous 29 kDa protein to stimulate methanogenesis from MMA in an extract of methanol grown cells. Addition of the 29 kDa protein preparation to the methanol extract stimulated the formation of methane from MMA (fig 10) three fold. This preparation of the 29 kDa protein had a MMA methyltransferase specific activity of 850 (corrected for MMA dependent methanogenesis without the 29 kDa protein). Neither DMA nor TMA dependent methanogenesis was stimulated by this same preparation of the 29 kDa protein. This indicates that the 29 kDa may be a MMA methyltransferase, and that this enzyme is a separate activity than either the DMA or TMA methyltransferases. This is suggestive evidence that the 29 kDa corrinoid protein is involved in methane formation from MMA.

Using the described assay for monomethylamine methyltransferase, we have isolated two proteins from cells grown on trimethylamine which stimulate methane formation from monomethylamine in an extract of methanol grown cells. The N-termini of these proteins correspond to the 29 kDa corrinoid protein, and the isozyme of methyltransferase II which is most abundant in cells grown on trimethylamine. We are currently attempting to obtain conditions under which these two protein will catalyse the formation of methylcoenzyme M from monomethylamine.

Fig. 7. Assay for TMA methyltransferase using an extract of methanol grown cells. A. Extract (200 μl , 3.4 mg protein) from a methanol grown cell (MEOHX) was supplemented with 0, 5, 10, or 20 μl (45 $\mu\text{g}/\mu\text{l}$) of an extract of TMA grown cells (TMAX). TMA dependent methanogenesis from 200 μl of TMAX is also shown. B. The specific activity of methanogenesis (normalized to total TMAX and MEOHX protein in assay) is shown vs. mg of TMAX protein added to the vial. The specific activity increased to from 0.3 to 27 nmol/min/mg with addition of 20 μl of TMAX to MEOHX. Assay of 200 μl of TMAX from part A gave a specific activity of 14 nmol/min/mg. All are averages of duplicate vials with H_2 , ATP, Mg^{++} , and 10 mM TMA. Methanogenesis was dependent on TMA.

Fig. 8. Sephacryl-300 separation of an extract of TMA grown cells. The fractions were tested for MMA, DMA, and TMA methyltransferases by addition to an extract of methanol grown cells supplemented with the appropriate methylamine. The assay vials were incubated for 30 m, then analysed for methane. The active fractions did not evolve significant methane without addition of methylamines.

Fig. 9. Purification of TMA methyltransferase on hydroxylapatite following initial purification on DE-52. The column was eluted with pH 7 KPO_4^- at the indicated concentrations. TMA methyltransferase was assayed by the ability of fractions to stimulate methane formation from TMA in an extract of methanol grown cells. No DMA or MMA methyltransferase was detected in the fraction following this purification.

Fig. 10. Stimulation of MMA dependent methanogenesis in extracts of methanol grown cells by a preparation of partially purified 29 kDa protein (amounts added to extract indicated in ug). No DMA or TMA methyltransferase activity was present.

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