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Transmethylation reactions during methanogenesis
from acetate in Methanosarcina barkeri

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The following pages and the accompanying two manuscripts encompass our report for this granting period.

Methanosarcina barkeri is a methylotrophic methanogen, capable of utilizing methylated compounds as methane precursors such as acetate, methanol, monomethylamine, dimethylamine, and trimethylamine. In many anaerobic environments, these compounds are the primary precursors of methane. Methyl groups entering metabolism in this organism can either be reduced to methane, oxidized to carbon dioxide, or enter anabolism. It may be expected that multiple methyl carriers and enzymes will be present which will dedicate methyl groups towards one set of reactions or the other.

Methanosarcina possesses an abundance of corrinoid, which acts as enzyme prosthetic group and functions in methyl transfer reactions. When this work was initiated only two corrinoid proteins had been described from Methanosarcina: methyltransferase I, which catalyses the first step in methanogenesis from methanol; and the corrinoid/iron sulfur protein, which is the primary methyl accepting group in the cleavage of acetyl-CoA during methanogenesis from acetate. Using our technique of in vitro methylation of corrinoid proteins by radioactive methanogenic substrates we have found several novel corrinoid proteins are present in this organism which can function as methyltransferases, which are methylated by growth substrate, and are apparently regulated by growth substrate.

Our original observations indicated that three chromatographically distinct proteins (with sizes of 480, 200, and 29 kDa) were methylated by $^{14}\text{CH}_3\text{COOH}$ in extracts of cells grown on acetate. Maximal methylation required inhibition by BES of the final step in methanogenesis, the conversion of methylCoM to methane. The 480 kDa protein was transiently methylated during methanogenesis even in the absence of BES, consistent with a potential role in methanogenesis. These three $^{14}\text{CH}_3$ -proteins were suspected to be corrinoid proteins because of the light lability of the methyl group. Two roles had been proposed by previous work done in the field for corrinoid proteins. The first role was the corrinoid protein which interacts

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with CO dehydrogenase in the cleavage of acetyl-CoA and subsequent methylation of THSP, the major pterin derivative in M. barkeri. This protein has been purified from Methanosarcina acetivorans, but had not previously been observed in Methanosarcina barkeri. The second was the CH₃-THSP:coenzyme M transmethylation reaction. This protein has not been isolated from Methanosarcina species, and its isolation from Methanobacterium has proven controversial. We have clearly ruled out the involvement of either protein in the first reaction, and at present feel the role of the 480 kDa protein is to function as a transmethylase specific for methanogenesis from acetate.

During this project period we have accomplished many of our immediate project goal objectives as outlined in our original proposal. We will continue with the remaining objectives essentially as outlined in the proposal, however, we are also pursuing several new lines of investigation which have been suggested by our results and relate to the topic of transmethylation reactions in M. barkeri.

We have isolated both the 480 kDa ¹⁴CH₃-protein and the 29 kDa ¹⁴CH₃-protein to homogeneity. Characterization of both proteins is described in the accompanying manuscript "Isolation of two novel corrinoid proteins from acetoclastic Methanosarcina barkeri." This was submitted to Journal of Bacteriology, and we have just received the notification that it will be accepted with minor revision. One 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". Briefly, we found the 480 kDa corrinoid protein possesses 6 bound corrins and is composed of 2 subunits of 41 and 30 kDa. The 29 kDa corrinoid protein is a monomer in solution with one bound corrin ring. Both carry entirely methylated corrin when isolated under dim red light. The 480 kDa corrinoid protein was found to give rise to two of the 4 electrophoretically distinct ¹⁴CH₃-protein bands in native gels we had identified as methylated by acetate. Neither the 480 kDa or the 29 kDa proteins possess significant quantities of iron, neither appears to bind corrinoid in the base-off form. This demonstrated neither protein is the corrinoid/iron sulfur protein that function with carbon monoxide dehydrogenase. Both isolated ¹⁴CH₃-proteins were demonstrated to form ¹⁴CH₄ when added to cell free extracts, and were methylated in cell extracts when methylreductase was inhibited, and demethylated by the subsequent addition of coenzyme M. We conclude that both proteins are novel proteins, not previously identified from a methanogen, which are intimately connected with the methanogenic pathway. The 200 kDa protein has proven to be too unstably methylated for further isolation.

As a second avenue to establishing the function of these proteins in M. barkeri we have compared patterns of protein methylation in cells grown on acetate or methanol. This study is described in the accompanying draft manuscript titled "Regulation of corrinoid proteins by Methanosarcina barkeri in response to growth substrate". This manuscript will be submitted to

the Journal of Bacteriology very shortly. We found the pattern to be very different between cells grown on either substrate. Most surprisingly, the 480 kDa corrinoid protein was methylated 100 times more strongly in cells grown on acetate than in cells grown on methanol, using either $^{14}\text{CH}_3\text{OH}$ or $^{14}\text{CH}_3\text{COOH}$ as the tracer. This indicated the 480 kDa protein was regulated in response to growth substrate. Western blots using antibody raised against the 480 kDa protein demonstrated the protein was much more abundant in acetate grown cells than in cells grown on methanol, trimethylamine, or hydrogen/carbon dioxide. This finding supports a unique role for the 480 kDa protein in metabolism of acetate grown cells, and thus further supports the hypothesis that this protein is involved in methane formation from acetate. The 29 kDa protein was found to be present in cells grown on both substrates, but two new electrophoretically distinct $^{14}\text{CH}_3$ -proteins were found in cells grown on methanol, which were termed MpM I and MpM II $^{14}\text{CH}_3$ -proteins. Both are corrinoid proteins which elute as a single 175 kDa $^{14}\text{CH}_3$ -protein from a gel permeation column. We are uncertain if MpM I and II are distinct proteins, or arise from the same protein. This protein may be the methyltransferase I described by previous workers. However, it has a different molecular weight and is not methylated in acetate grown cell extracts even by methanol. Therefore, we feel it may be a novel regulated protein involved in some other aspect of methanol metabolism. We intend on exploring this $^{14}\text{CH}_3$ -protein further by its isolation since it may represent a novel protein with a unique role in methanol metabolism.

The roles of the 480 and 29 kDa corrinoid proteins are currently being investigated by using interaction of either protein with methyl-accepting coenzymes from M. barkeri. We have demonstrated that the 480 kDa corrinoid protein can be methylated in a Sephadex G-25 protein fraction from acetate grown cells with $^{14}\text{CH}_3$ -THSP. Addition of HSCoM to a partially purified preparation of the 480 kDa $^{14}\text{CH}_3$ -protein resulted in demethylation of the protein. Interestingly, the 29 kDa $^{14}\text{CH}_3$ -protein could not be demethylated in this manner. We have raised mouse polyclonal antibodies to the 480 kDa and these were used in the manuscript describing regulation of this protein. We are now raising antibodies against both proteins in rabbits. These will be used to remove the 480 or 29 kDa proteins from cell extracts and directly test if the proteins are involved in methane formation from acetate or other substrates as described in our grant proposal.

We have also performed in vitro protein methylation studies with $^{14}\text{CO}_2$ in cells grown on hydrogen and carbon dioxide. Our initial studies indicate not much label is incorporated into either the 480 or 29 kDa protein in these extracts. Protein gels have indicated that neither the 480 or 29 kDa protein are abundant in cells cultured on hydrogen and carbon dioxide, but that the 29 kDa protein is found in cells grown on methyl containing substrates. This result indicates the 29 kDa protein plays a unique role in the metabolism of M. barkeri during growth on methyl containing substrates, such as methyl group oxidation. We will confirm this observation by assaying the 29 kDa protein in different extracts using Western blots and Elisa assays.

The identification of the corrinoid protein which participates in the cleavage of acetyl-CoA by carbon monoxide dehydrogenase during methanogenesis from acetate has been problematic in Methanosarcina barkeri strain MS. The isolated CO dehydrogenase has only two subunits of 92 and 18 kDa, and lacks a corrinoid protein. Using a radioactive alkyl halide we have been able to identify the very labile CO dehydrogenase:corrinoid protein complex in this strain. One of the first indications of corrinoid involvement in methanogenesis from acetate was light reversible inhibition of the process by alkyl halides. We have found that incubation of whole cells or extracts with ^{14}C -ethyl iodide results in the alkylation of a 1.6 MDa protein detectable by size exclusion HPLC. This protein coelutes with carbon monoxide dehydrogenase activity, and the 1.6 MDa complex possesses the two subunits of carbon monoxide dehydrogenase (92 and 18 kDa) and three other subunits of 70, 58, and 52 kDa. We have isolated an alkylated corrinoid from the complex. The strain MS CO dehydrogenase:corrinoid protein complex thus resembles that described by Ferry and coworkers, and by Grahame. Significantly, the complex does not have subunits corresponding to the 480 or 29 kDa proteins, confirming these abundant, regulated corrinoid proteins are not involved in the acetyl-CoA cleavage reaction. During isolation by traditional methods the alkylated 1.6 MDa protein disaggregates to the 230 kDa form of CO dehydrogenase and an alkylated 200 kDa protein which in turn rapidly loses the radiolabel.

While ethyl iodide does not alkylate any other corrinoid protein when incubated under hydrogen, we have found that the other corrinoid proteins present in the cell are alkylated when ATP is added to extracts. The 480 kDa corrinoid protein is readily alkylated when incubated with ATP and hydrogen in a cell free extract. We feel the most likely explanation for this is the relatively high redox potential of the Co(II)/Co(I) transition in the carbon monoxide dehydrogenase corrinoid, which would allow Co(I) of this corrinoid protein to attack the alkyl halide in extracts incubated under hydrogen. The addition of ATP may allow the endergonic reduction of the other corrinoid proteins using hydrogen to the Co(I) state, allowing direct alkylation of the 480 kDa corrinoid protein. Alternatively, ATP may allow transfer of the alkyl group from the 1.6 MDa CO dehydrogenase:corrinoid protein complex to the 480 kDa protein. We are conducting experiments to differentiate these possibilities. The work involving ethyl iodide alkylation of the CO dehydrogenase:corrinoid protein complex, as well as the 480 kDa protein, is reaching publication stage, and a manuscript describing this work will be prepared next month.

We have obtained the N-terminal sequences of the polypeptides comprising the 480 and 29 kDa proteins and have made oligonucleotides to the 30 kDa subunit of the 480 kDa protein. We have succeeded in cloning M. barkeri genomic DNA into pUC 19 which hybridizes to two distinct oligos corresponding to the 30 kDa subunit. We are sub-cloning this gene, and have initiated sequencing studies to confirm this gene encodes the 30 kDa subunit of the 480 kDa protein. The gene sequence may provide important clues to the

precise function of the 480 kDa protein, and will be used in planned studies of transcriptional regulation of the protein. One long term goal of my laboratory is to clone and sequence several of the genes encoding corrinoid proteins from M. barkeri. Homologies among the genes may identify a common domain used to bind corrin rings to these proteins.

The determination of the stereochemistry of methyl transfer to both HSCoM and THSP from chiral labeled acetate is proceeding in collaboration with Dr. Heinz Floss. Thus far we have found that conversion of acetate to methyl-CoM proceeds with retention of configuration, indicating an even number of methyl group transfers occurs in the pathway up to the penultimate step in methane formation. The experiments with methyl-THSP are still in progress. Isolation techniques for the chirally labeled methyl-THSP have been perfected, and we should be able determine the chirality of this transfer within the next funding period. We intend on publishing a single manuscript describing the stereochemistry of transfer to both intermediates of methanogenesis.

Moneys from DOE have been used to initiate another project dealing with the transmethylation reactions of Methanosarcina. We have found that extracts of methanol grown cells lack the ability to convert trimethylamine (TMA) to methane. However, when methanol grown cell extracts are supplemented with small amounts of protein derived from a TMA extract, the methanol grown cell extracts will produce methane from TMA at a rapid rate. Using this as an assay system we have begun to isolate the transmethylases responsible for the conversion of TMA to methyl-coenzyme M, none of which have been previously identified. This method has been successful, and we have been able to purify the TMA:coenzyme M transmethylase activity approximately 10-fold. We intend on isolating this protein to homogeneity. Thus far the preparations have supported a suggestion made by earlier work done on this reaction, that the responsible enzyme binds a corrin prosthetic group.

Figure legends

Figure 1. Size exclusion HPLC of Sephadex G-25 protein fraction from methanol (MEX) or acetate (AEX) grown cell extracts incubated with either [^{14}C -2] acetate (*A) or $^{14}\text{CH}_3\text{OH}$ (*MeOH). Absorbance at 280 nm and radioactivity in the effluent were monitored. (A) AEX incubated with [^{14}C -2 acetate], 29.8 kdpm and 2.2 mg protein injected in 70 ul total volume. (B) AEX incubated with $^{14}\text{CH}_3\text{OH}$, 81 kdpm and 2.2 mg protein injected in 80 ul was injected. (C) MEX incubated with $^{14}\text{CH}_3\text{OH}$, 52 kdpm and 1.3 mg protein was injected onto the column.

Figure 2. Autoradiography of labeled protein fractions from either methanol (MEX) or acetate(AEX) grown cell extracts incubated with either [^{14}C -2] acetate (*A) or $^{14}\text{CH}_3\text{OH}$ (*M) and electrophoresed in a non-denaturing 5-22.5% polyacrylamide gel. The lanes were loaded with the following protein fractions: (1) MEX/*M, 0.67 mg protein, 26 kdpm; (2) MEX/*M, 0.84 mg protein, 28. kdpm; (3) MEX/*A, 0.72 mg protein, 1.9 kdpm; (4) MEX/*A, 0.67 mg protein, 7.8 kdpm; (5) AEX/*M, 1.1 mg protein, 40.5 kdpm; (6) AEX/*M, 1.3 mg protein, 33.7 kdpm; (7) AEX/*A, 1.1 mg protein, 17 kdpm; (8) AEX/*A 1.26 mg protein, 10.7 kdpm.

Figure 3. Sepharose 6 B chromatography of the Sephadex G-25 protein fraction of a methanol grown extract incubated with $^{14}\text{CH}_3\text{OH}$. (A) Radioactivity elution profile from an 83.5 cm x 2.5 cm column eluted at 36 ml/hour. 7.5 ml of a labeled G-25 fraction (102 kdpm/ml) was applied to the column and eluted at 36 ml/hour. 5.7 ml fractions were collected. (B) Autoradiography of peaks from column. Fractions from the peak eluting in fractions 49-51 were collected and concentrated, as were fractions eluting in fractions eluting in fractions 56-61. These fractions were then loaded onto a 5-22.5% non-denaturing gel, electrophoresed and autoradiographed. The lanes were loaded with (1) G-25 protein fraction from an acetate grown extract incubated with ^{14}C -2 acetate, 10 kdpm (2) Fraction from 29 kDa peak, 5 kdpm (3) fraction from 175 kDa protein peak, 10 kdpm (4) G-25 protein fraction of methanol grown extract labeled with methanol, 12 kdpm (5) Fraction from 175 kDa protein peak in a separate Sepharose 6B fraction, 10 kdpm. Letters designate locations of radiolabelled bands (A) 480 kDa $^{14}\text{CH}_3$ -protein, (B) Unknown B protein, (C) MpM I $^{14}\text{CH}_3$ -protein (D) 29 kDa $^{14}\text{CH}_3$ -protein and (E) MpM II $^{14}\text{CH}_3$ -protein.

Figure 4. Reverse phase chromatography of the $^{14}\text{CH}_3$ -cofactors extracted from either the 480 kDa $^{14}\text{CH}_3$ -corrinoid protein or the 175 kDa $^{14}\text{CH}_3$ -protein Sepharose 6B fraction. The column was injected with (A) 1950 dpm of extracted $^{14}\text{CH}_3$ -corrinoid from the 480 kDa protein, or (B) 1950 dpm of the 175 kDa protein peak $^{14}\text{CH}_3$ -cofactor as isolated, or (C) 2600 dpm of the 175 kDa protein peak $^{14}\text{CH}_3$ -cofactor following exposure to light for 30 minutes on ice.

Figure 5. PAGE of extracts of grown on various substrates and purified 480 kDa $^{14}\text{CH}_3$ -protein. 250 ug of protein from 150,000x g supernatants of cell extracts was electrophoresed through a 5-22.5 % gradient polyacrylamide gel, and stained with Coomassie for total protein. Lanes were loaded with (480) 15 ug of purified 480 kDa protein; and extracts of cells cultured on acetate (A), trimethylamine (T), hydrogen and carbon dioxide (H), and methanol (M).

Figure 6. Western blot analysis of extracts of cells grown on various substrates. In part A, samples were electrophoresed through a non-denaturing 5% polyacrylamide gel prior to blotting. Lanes 1-3 contained 0.17, 0.35, and 0.7 ug of the purified 480 kDa preparation used to raise the antibody. The other lanes contained 130 ug of protein from extracts of cells grown on acetate (A), trimethylamine (T), methanol (M), and hydrogen/carbon dioxide (H). In part B, samples were electrophoresed through an SDS 12% polyacrylamide gel prior to blotting. Lanes 1-7 were loaded with 0.67, 0.56, 0.45, 0.33, 0.224, 0.112, and 0.045 ug purified 480 kDa protein. The gel was also loaded with 30 ug protein from extracts of cells grown on hydrogen/carbon dioxide (H), methanol (M), trimethylamine (T), and acetate (A).

Fig 3

Fig 3B

Fig 5

1 2 3 A T M H

Fig 6A

Fig 6B.

References

1. Abbanat, D. R. and J. G. Ferry. 1991. Resolution of component proteins in an enzyme complex from Methanosarcina thermophila catalyzing the synthesis or cleavage of acetyl-CoA. Proc. Natl. Acad. Sci. U. S. A. 88:3272-3276.
2. Bechler, B., V. Müller, and G. Gottschalk. 1992. N5-methyl-tetrahydromethanopterin:coenzyme M methyltransferase of Methanosarcina strain Gö1 is an Na⁺-translocating membrane protein. J. Bacteriol. 174:7656-7660.
3. Blaylock, B. A. 1968. Cobamide-dependent methanol-cyanocob(I)alamin methyltransferase of Methanosarcina barkeri. Arch. Biochem. Biophys. 124:314-324.
4. Blaylock, B. A. and T. C. Stadtman. 1963. Biosynthesis of methane from the methyl moiety of methyl-cobalamin. Biochem. Biophys. Res. Comm. 11:34-38.
5. Bradford, M. M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.
6. Cao, X. and J. A. Krzycki. 1991. Acetate-dependent methylation of two corrinoid proteins in extracts of Methanosarcina barkeri. J. Bacteriol. 173:5439-5448.

7. Dangel, W., H. Schulz, G. Diekert, H. König, and G. Fuchs. 1987. Occurrence of corrinoid-containing membrane proteins in anaerobic bacteria. Arch. Microbiol. 148:52-56.
8. DeMoll, E., D. A. Grahame, J. M. Harnly, L. Tsai, and T. C. Stadtman. 1987. Purification and properties of carbon monoxide dehydrogenase from Methanococcus vanniellii. J. Bacteriol. 169:3916-3920.
9. DiMarco, A. A., T. A. Bobik, and R. S. Wolfe. 1990. Unusual coenzymes of methanogenesis. Annu. Rev. Biochem. 59:355-394.
10. Eikmanns, B. and R. K. Thauer. 1985. Evidence for the involvement and role of a corrinoid enzyme in methane formation from acetate in Methanosarcina barkeri. Arch. Microbiol. 142:175-179.
11. Ellermann, J., R. Hedderich, R. Böcher, and R. K. Thauer. 1988. The final step in methane formation. Investigations with highly purified methyl-CoM reductase (component C) from Methanobacterium thermoautotrophicum (strain Marburg). Eur. J. Biochem. 172:669-677.
12. Ferry, J. G. 1992. Methane from acetate. J. Bacteriol. 174:5489-5495.
13. Fischer, R., P. Gärtner, A. Yeliseev, and R. K. Thauer. 1992. N⁵-methyltetrahydromethanopterin:coenzyme M methyltransferase in methanogenic archaeobacteria is a membrane protein. Arch. Microbiol. 158:208-217.

14. Fischer, R. and R. K. Thauer. 1988. Methane formation from acetyl phosphate in cell extracts of Methanosarcina barkeri. Dependence of the reaction on coenzyme A. *Febs Lett.* **228**:249-253.
15. Frasca, V., R. V. Banerjee, W. R. Dunham, R. H. Sands, and R. G. Matthews. 1988. Cobalamin-dependent methionine synthase from Escherichia coli B: electron paramagnetic resonance spectra of the inactive form and the active methylated form of the enzyme. *Biochemistry.* **27**:8458-8465.
16. Grahame, D. A. 1989. Different isozymes of methylcobalamin:2-mercaptoethanesulfonate methyltransferase predominate in methanol- versus acetate-grown Methanosarcina barkeri. *J. Biol. Chem.* **264**:12890-12894.
17. Grahame, D. A. 1991. Catalysis of acetyl-CoA cleavage and tetrahydrosarcinapterin methylation by a carbon monoxide dehydrogenase-corrinoid enzyme complex. *J. Biol. Chem.* **266**:22227-22233.
18. Grahame, D. A. and T. C. Stadtman. 1987. Carbon monoxide dehydrogenase from Methanosarcina barkeri. Disaggregation, purification, and physicochemical properties of the enzyme. *J. Biol. Chem.* **262**:3706-3712.
19. Grahame, D. A. and T. C. Stadtman. 1987. In vitro methane and methyl coenzyme M formation from acetate: evidence that acetyl-CoA is the required intermediate activated form of acetate. *Biochem. Biophys. Res. Commun.* **147**:254-258.

20. Hogenkamp, H. P. C. "Reactions of alkyl ligands coordinated to cobalamins and cobaloximes." In B₁₂, ed. D. Dolphin. 295-323. New York: John Wiley & Sons, Inc., 1982.

21. Hogenkamp, H. P. C., G. T. Bratt, and A. T. Kotchevar. 1987. Reaction of alkylcobalamins with thiols. *Biochemistry*. **26**:4723-4727.

22. Jetten, M. S. M., W. R. Hagen, A. J. Pierik, A. J. M. Stams, and A. J. B. Zehnder. 1991. Paramagnetic centers and acetyl-coenzyme A/carbon monoxide exchange activity of carbon monoxide dehydrogenase from Methanothrix soehngenii. *Eur. J. Biochem.* **195**:385-391.

23. Jetten, M. S. M., A. J. M. Stams, and A. J. B. Zehnder. 1989. Purification and characterization of an oxygen-stable carbon monoxide dehydrogenase of Methanothrix soehngennii. *Eur. J. Biochem.* **181**:437-441.

24. Keltjens, J. T. and G. D. Vogels. 1988. Methanopterin and methanogenic bacteria. *BioFactors*. **1**:95-103.

25. Kengen, S. W. M., P. J. H. Daas, E. F. G. Duits, J. T. Keltjens, C. van der Drift, and G. D. Vogels. 1992. Isolation of a 5-hydroxybenzimidazolyl cobamide-containing enzyme involved in the methyltetrahydromethanopterin:coenzyme M methyltransferase reaction in Methanobacterium thermoautotrophicum. *Biochim. Biophys. Acta.* **1118**:249-260.

26. Kremer, J. unpublished results.

27. Krzycki, J. and J. G. Zeikus. 1980. Quantification of corrinoids in methanogenic bacteria. *Curr. Microbiol.* 3:243-245.
28. Krzycki, J. A., L. E. Mortenson, and R. C. Prince. 1989. Paramagnetic centers of carbon monoxide dehydrogenase from acetoclastic Methanosarcina barkeri. *J. Biol. Chem.* 264:7217-7221.
29. Krzycki, J. A. and J. G. Zeikus. 1984. Acetate catabolism by Methanosarcina barkeri: hydrogen-dependent methane production from acetate by a soluble cell protein fraction. *Fems Microbiol. Lett.* 25:27-32.
30. Krzycki, J. A. and J. G. Zeikus. 1984. Characterization and purification of carbon monoxide dehydrogenase from Methanosarcina barkeri. *J. Bacteriol.* 158:231-237.
31. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)*. 227:680-685.
32. Lovenberg, W., B. B. Buchanan, and J. C. Rabinowitz. 1963. Studies on the chemical nature of Clostridial Ferredoxin. *J. Biol. Chem.* 238:3899-3913.
33. Lovley, D. R., R. H. White, and J. G. Ferry. 1984. Identification of methyl coenzyme M as an intermediate in methanogenesis from acetate in Methanosarcina spp. *J. Bacteriol.* 160:521-525.

34. Lowry, O. H., R. N. J., A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin reagent. *J. Biol. Chem.* **193**:265-275.

35. Lundie, L. L. J. and J. G. Ferry. 1989. Activation of acetate by Methanosarcina thermophila. Purification and characterization of phosphotransacetylase. *J. Biol. Chem.* **264**:18392-18396.

36. Müller, V., M. Blaut, and G. Gottschalk. 1988. The transmembrane electrochemical gradient of sodium as driving force for methanol oxidation in Methanosarcina barkeri. *Eur. J. Biochem.* **172**:601-606.

37. Pol, A., R. A. Gage, J. M. Neis, J. W. M. Reijnen, C. van der Drift, and G. D. Vogels. 1984. Corrinoids from Methanosarcina barkeri. The .beta.-ligands. *Biochim. Biophys. Acta.* **797**:83-93.

38. Pol, A., C. van der Drift, and G. D. Vogels. 1982. Corrinoids from Methanosarcina barkeri: structure of the α -ligand. *Biochem. Biophys. Res. Commun.* **108**:731-737.

39. Ragsdale, S. W., P. A. Lindahl, and E. Münck. 1987. Mössbauer, EPR, and optical studies of the corrinoid/iron-sulfur protein involved in the synthesis of acetyl coenzyme A by Clostridium thermoaceticum. *J. Biol. Chem.* **262**:14289-4297.

40. Raybuck, S. A., S. E. Ramer, D. R. Abbanat, J. W. Peters, J. W. H. Orme-Johnson, J. G. Ferry, and C. T. Walsh. 1991. Demonstration of carbon-carbon bond cleavage of acetyl coenzyme A by using isotopic exchange catalyzed by

the carbon monoxide dehydrogenase complex from acetate-grown Methanosarcina thermophila. J. Bacteriol. 173:929-932.

41. Scherer, P., V. Höllriegel, C. Krug, M. Bokel, and P. Renz. 1984. On the biosynthesis of 5-hydroxybenzimidazolylcobamide (vitamin B₁₂-factor III) in Methanosarcina barkeri. Arch. Microbiol. 138:354-359.

42. Stupperich, E., A. Juza, C. Eckerskorn, and L. Edelman. 1990. An immunological study of corrinoid proteins from bacteria revealed homologous antigenic determinants of a soluble corrinoid-dependent methyltransferase and corrinoid-containing membrane proteins from Methanobacterium species. Arch. Microbiol. 155:28-34.

43. Terlesky, K. C., M. J. K. Nelson, and J. G. Ferry. 1986. Isolation of an enzyme complex with carbon monoxide dehydrogenase activity containing corrinoid and nickel from acetate-grown Methanosarcina thermophila. J. Bacteriol. 168:1053-1058.

44. van de Wijngaard, W. M. H., R. L. Lugtigheid, and C. van der Drift 1991. Reductive activation of the corrinoid-containing enzyme involved in methyl group transfer between methyl-tetrahydromethanopterin and coenzyme M in Methanosarcina barkeri. Antonie von Leeuwenhoek. 60:1-6.

45. van der Meijden, P., H. J. Heythuysen, A. Pouwels, F. Houwne, C. van der Drift, and G. D. Vogels. 1983. Methyltransferases involved in methanol conversion by Methanosarcina barkeri. Arch. Microbiol. 134:238-242.

46. van der Meijden, P., B.W. te Brömmelstroet, C. M. Poirot, C. van der Drift, and G. D. Vogels. 1984. Purification and properties of methanol:5-hydroxybenzimidazolylcobamide methyltransferase from Methanosarcina barkeri. J. Bacteriol. 160:629-635.
47. Wood, H. G., S. W. Ragsdale, and E. Pezacka. 1986. The acetyl-CoA pathway of autotrophic growth. Fems Microbiol. Rev. 39:345-362.

Table I. Purification of the 480 kDa $^{14}\text{CH}_3$ -protein.

Step	Protein (mg)	Radioactivity (dpm)	Specific activity (dpm/mg)	Recovery ^a dpm (%)
<i>M. barkeri</i> extract	2653.6	1.20×10^9	4.52×10^5	
Sephadex G-25	1968.0	8.12×10^6	4.13×10^3	
DEAE Sephacel pH 6.5	66.8	3.82×10^5	5.71×10^3	100
DEAE Sephacel pH 8.5	32.9	3.74×10^5	1.14×10^4	98
Ultrafiltration				
Sepharose CL-6B	18.26	3.00×10^5	1.65×10^4	79

Table II. Purification of the 29 kDa $^{14}\text{CH}_3$ -protein.

Step	Protein (mg)	Radioactivity (dpm)	Specific activity (dpm/mg)	Recovery ^a dpm (%)
<i>Ms. barkeri</i> extract	2653.6	1.20×10^9	4.52×10^5	
Sephadex G-25	1968	8.12×10^6	44.1×10^3	
DEAE Sephacel pH 6.5	135.2	1.12×10^6	8.32×10^3	100
DEAE Sephacel pH 8.5	40.7	7.88×10^5	1.93×10^4	70
Ultrafiltration				
Sephacryl S-100	8.32	6.03×10^5	7.25×10^4	54

Purification procedures were carried out under dim red light in an anaerobic chamber. Details of *in vitro* methylation of protein and purification of the radiolabelled proteins are described in Materials and Methods. ^aRecovery of radioactivity is expressed relative to DEAE fraction.

Figure Legends

Figure 1. DEAE Sephacel separation of $^{14}\text{CH}_3$ -proteins and labeled metabolites from G-25 protein fraction. 203 ml (1812 mg) of protein fraction from a G-25 Sephadex column step was loaded onto a 5 x 58 cm DEAE Sephacel column. 8.7 ml fractions were collected.

Figure 2. Non denaturing polyacrylamide gel electrophoresis of purified $^{14}\text{CH}_3$ -proteins. Each of the following were 5-22.5% polyacrylamide gradient gels. A) Coomassie-stained gel of isolated 480 and 29 kDa proteins. The unknown A protein was seen as a faint band in this preparation of the 480 kDa protein. The lanes were loaded with: (1) 200 μg protein of a Sephadex G-25 extract; (2) 12 μg of 480 kDa protein; (3) 12 μg of 29 kDa protein. (B) Preparation of 480 kDa protein showing higher amount of unknown A form. Lane 1, 500 μg of protein fraction from Sephadex G-25 step; lane 2, 12 μg of 480 kDa protein. (C) Autoradiogram of non-denaturing gradient gel of protein fraction from Sephadex G-25 and 480 kDa $^{14}\text{CH}_3$ -protein demonstrating the four $^{14}\text{CH}_3$ -protein bands seen in extracts methylated with ^{14}C -2 acetate. Lane 1, 500 μg of a Sephadex G-25 extract, 5400 dpm; Lane 2, 12 μg of 480 kDa protein, 300 dpm.

Figure 3. SDS-polyacrylamide gel electrophoresis of the 480 kDa and 29 kDa $^{14}\text{CH}_3$ -proteins. Lanes were loaded with the following samples: A) Molecular size markers listed in Materials and Methods; B) 3.2 μg of 480 kDa protein; C) 2.2 μg of 29 kDa protein.

Figure 4. Two-dimensional electrophoresis of a 480 kDa preparation with a large amount of the Unknown A form present. The first dimension was carried out using non-denaturing electrophoresis of 41 μ g of 480 kDa protein in a 5-22.5% polyacrylamide gel. The lane containing the 480 kDa and Unknown-A proteins was excised and fixed onto a 12% acrylamide-SDS gel with the uppermost part of the gel to the right for electrophoresis in the second dimension. A preparation electrophoresed only in the first dimension was stained with Coomassie and is visible at the upper part of the figure with the locations of the Unknown A protein (B) and the major 480 kDa protein band (C) marked. The second dimension was electrophoresed with molecular weight markers on the left hand side (A).

Figure 5. UV-Visible spectra of $^{14}\text{CH}_3$ -corrinoids extracted from the purified $^{14}\text{CH}_3$ -proteins. Samples were shielded from light during the extraction of cofactor. Scans were acquired of the cofactor under the conditions indicated as described in Methods and Materials. 50 μ l of each corrinoid was scanned as isolated and after addition of KCN or HCL as indicated. (A) The cofactor of 0.65 mg of the 480 kDa $^{14}\text{CH}_3$ -protein was extracted as described in Materials and Methods. (B) Cofactor extracted from 0.27 mg of the 29 kDa $^{14}\text{CH}_3$ -protein. A dilution of 20% occurred during addition of KCN to the isolated corrinoid.

Figure 6. UV-visible spectra of the 480 kDa and 29 kDa $^{14}\text{CH}_3$ -proteins before and after 3.0 hour exposure to light. Proteins as isolated (—), proteins after exposure to light (---). (A) 75 μ l (40 μ g, 660 dpm) of 480 kDa protein. The spectra are shown in expanded form in the inset to emphasize peaks due to the corrinoid. (B) 75 μ l (40 μ g, 2900 dpm) of 29 kDa protein. Spectra were taken under a 3% H_2 :97% N_2 atmosphere.

Figure 7. Photolysis of $^{14}\text{CH}_3$ -proteins under anaerobic conditions. Aliquots (50 μl) of the 480 kDa protein (27 μg , 440 dpm) or the 29 kDa protein (27 μg , 1960 dpm) in 1.6 ml tubes with a H_2 gas phase were exposed to a 100-W tungsten bulb placed 24 cm away while on ice. At the indicated time points aliquots were removed to new tubes and wrapped in foil prior to being returned to the ice. At the end of the incubation period the radioactivity remaining in all samples was determined. Controls not exposed to light lost no radioactivity from solution.

Figure 8. Demethylation of $^{14}\text{CH}_3$ -proteins formed in 25 mg protein/ml extract incubated in the presence of 0.2 mM BES, 12.5 mM ATP, and 2 mM ^{14}C -2 acetate (25 $\mu\text{Ci}/\mu\text{mol}$) at 37°C . During incubation 50 μl aliquots were removed every three minutes, spun in a 1.0 ml Sephadex G-25 spin column, and then electrophoresed on a 5-22.5 % non-denaturing polyacrylamide gel. At 9.5 minutes 5 mM HSCoM was added to the extract. A control vial showed no change in the methylation of any protein during incubation over the same time period.

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