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Methanococcus voltae is a marine methanogen which derives its energy from reactions derived from the reduction of carbon dioxide by hydrogen. We are interested in elucidating basic principles of energetics in this archaebacterium and have focused on active transort, ion translocation pathways and ATP generating systems. We have made significant progress in the elucidation and characterization of a novel P-type ATPase in this organism. The enzyme has been purified and partially characterized and the formation of an acyl phosphate intermediate demonstrated in its catalytic sequence. We have discovered an active transport system for HS-CoM, a novel methanogen coenzyme involved in a terminal step of methanogenesis. Mutants which are altered in this active transport system have been isolated and characterized.

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RESEARCH OBJECTIVES:

1) To characterize the cytoplasmic membrane of the archaebacterial methanogen Methanococcus voltae,

2) To determine the basis for methanogen resistance to bromoethane sulfonic acid,

3) To characterize methanogen transport of the coenzyme HS-CoM and methyl-S-CoM,

4) To characterize methanogen sodium pumps,

5. To characterize Methanococcus voltae ATPases,

PROGRESS REPORT DURING TERM OF THE AWARD:

Characterization of a M. voltae P-Type ATPase

Membrane-bound ATPase activity was detected in the methanogen Methanococus voltae. The ATPase was inhibited by vanadate, a characteristic inhibitor of E_1E_2 ATPases. The enzyme activity was also inhibited by diethylstilbestrol. However, it was insenstivite to N,N'-dicyclohexylcarbodiimide, ouabain, and oligomycin. The enzyme displayed a high preference for ATP as substrate, was dependent of Mg^{2+}, and had a pH optimum of approximately 7.5. The enyzme was completely solubilized with 2T Trition X01900. The enzyme was insensitive to oxygen and was stabilized by ATP. There was no homology with the Escherichia coli F_0F_1 ATPase at the level of DNA and protein. The membrane-bound M. voltae ATPase showed properties similar to those of E_1E_2 ATPases.

The vanadate-sensitive ATPase of Methanococcus voltae has been purified by a procedure which includes, purification of the cytoplasmic membrane by sucrose gradient centrifugation, solubilization with TritonX-100, and DEAE-Sephadex and Sephacryl S-300 chromatography. While the DEAE-Sephadex step provided a preparation consisting of two polypeptides (74 kDa and 52 kDa), the Sephacryl S-300 step yields a product with a subunit of 74 kDa. Incubation of either membranes or purified ATPase with [γ-^{32}P] ATP followed by acidic (pH-2.4) lithium dodecyl sulfate polyacrylamide gel electrophoresis demonstrated the vanadate-sensitive labeling of a 74 kDa acyl phosphate intermediate. These results indicate that the Mc. voltae ATPase is of the P type.

Discovery of HS-CoM and methyl-S-CoM transport systems

Mutants of Methanococcus voltae were isolated that were resistant to the coenzyme M (CoM; 2-mercaptoethanesulfonic acid) analog 2-bromeothanesulfonic acid (BES). The mutants displayed a reduced ability to accumulate [^{35}S]BES relative to the sensitive parental strain. BES inhibited methane production from CH_3-S-CoM in cell extracts prepared from wild-type sensitive or resistant strains. BES uptake required the presence of both CO_2 and H_2 and was inhibited by N-ethylmaleimide and several reagents that are known to disrupt energy metabolism. The mutants showed normal uptake of isoleucine and were not cross-resistant to either azaserine or 5-methyltryptophan and, thus, were neither defective in general energy-dependent substrate transport nor envelope permeability. Both HS-CoM and CH_3-S-CoM prevented the uptake of BES and proected cells from inhibition by it. We propose the M. voltae has an energy-dependent, carrier-mediated uptake system for HS-CoM and CH_3-S-coM which can also mediate uptake of BES.

The transport system for coenzyme M (2-mercaptoethanesulfonic acid, H-S-CoM), and methylcoenzyme M (2-(methylthio)ethanesulfonic acid, CH_3-S-CoM) in Methanococcus voltae required energy, showed saturation kinetics, and concentrated both forms of coenzyme M against a concentration gradient. Transport required hydrogen and carbon dioxide for maximal uptake. CH_3-S-CoM uptake was inhibited by N-ethylmaleimide and monensin. Both H-S-CoM and CH_3-S-CoM uptake showed sodium dependence. In wild type Mc. voltae H-S-CoM uptake was concentration dependent, with a V_{max} of 960 pmol/min/mg protein and an apparent K_m of 61 uM. Uptake of CH_2-S-CoM showed a V_{max} of 88 pmol/min/mg protein and a K_m of 53 uM. A mutant of *Mc. voltae* resistant to the coenzyme M analog 2-bromoethanesulfonic acid (BES) showed no uptake of CH_3-S-CoM but accumulated H-S-CoM at the wild type rate. Analysis of the intracellular coenzyme M pools in metabolizing cells showed an intracellular H-SOCoM concentration of 10.4 mM and CH_3-S-CoM concentration of 0.2 mM.

Since bromoethane sulfonate is an inhibitor of methane production (competitive with methyl-CoM), cells able to accumulate large internal pools of methyl-CoM via uptake of its precursor, HS-CoM, should be protected by BES by addition of HS-CoM to the growth medium. Marine methanogen enrichments were prepared from sediment samples from Sipperwissett marsh and Oyster pond inlet (Betsy's place). One of the SW enrichments was carried out using formate as substrate, while the others used H_2 and CO_2. One of the Betsy's place cultures was spread on bottle plates and a single colony type transferred to new medium. This culture contained two cell types, a non-methanogen long rod and a coccal shaped methanogen. All other cultures were a mixture of cell types with about 50% of the cells methanogens as judged by epifluoresence. Methane production for each enrichment was found to be BES sensitive with 1/2 maximal inhibition occurring at 5-20 uM BES depending on the enrichment. For each, half maximal protection from BES (40 uM) occurred at a HSCoM/BES molar ratio of 20 to 40. Since protected cells exhibited normal sensitivity to BES after removal of the HS-CoM, it was concluded that methane production in the presence of BES plus HS-CoM resulted from true protection and not growth of BES resistant mutants. For one of the enrichments it was shown that cells coming up after prolonged incubation in BES were BES-resistant. These results suggest that methanogens found in such enrichments are able to accumulate exogenous HS-CoM. Direct determinations of

HS-CoM uptake will be necessary to verify this suggestion. It is possible that uptake of this coenzyme is an important nutritional feature of some methanogens in their natural habitats.

PUBLICATIONS:

Santoro, N. and J. Konisky (1987). Characterization of bromoethanesulfonate-resistant mutants of Methanococcus voltae: Evidence of a coenzyme M transport system. J. Bacteriol. **169**:660-665.

Konisky, J. (1989). Methanogens for biotechnology: application of genetics and molecular biology. Applications for Biotechnology. Trends in Biotechnology, Elsevier **7**:88-92.

Dharmavaram, R. and J. Konisky (1987). Identification of a vanadate-sensitive membrane-bound ATPase in the Archaebacterium, Methanococcus voltae. J. Bacteriol., 169: 3921-3925.

Dharmavaram, R. and J. Konisky (1989). Characterization of a P-type ATPase of the archaebacterium, Methanococcus voltae. J. Biol. Chem, in press

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This award has supported three graduate students and one postdoctoral fellow.

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Characterization of Bromoethanesulfonate-Resistant Mutants of *Methanococcus voltae*: Evidence of a Coenzyme M Transport System

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Mutants of *Methanococcus voltae* were isolated that were resistant to the coenzyme M (CoM; 2-mercaptoethanesulfonic acid) analog 2-bromoethanesulfonic acid (BES). The mutants displayed a reduced ability to accumulate [^{35}S]BES relative to the sensitive parental strain. BES inhibited methane production from CH_3-S-CoM in cell extracts prepared from wild-type sensitive or resistant strains. BES uptake required the presence of both CO_2 and H_2 and was inhibited by *N*-ethylmaleimide and several reagents that are known to disrupt energy metabolism. The mutants showed normal uptake of isoleucine and were not cross-resistant to either azaserine or 5-methyltryptophan and, thus, were neither defective in general energy-dependent substrate transport nor envelope permeability. Both HS-CoM and CH_3-S-CoM prevented the uptake of BES and protected cells from inhibition by it. We propose that *M. voltae* has an energy-dependent, carrier-mediated uptake system for HS-CoM and CH_3-S-CoM which can also mediate uptake of BES.

Although remarkable progress has been made over the last few decades in elucidating certain aspects of methanogen metabolism (6, 28, 31), there is little doubt that the development of methods for the genetic manipulation of these microorganisms would expedite biochemical and physiological studies. An obvious handicap in "doing genetics" with methanogens is dealing with the technical requirements of working with anaerobes (18). With the development in recent years of reproducible methods for growing and plating methanogens (1, 2, 12), however, a major technical barrier has been removed, and genetic studies are under way in several laboratories.

Whitman and collaborators (16, 30) have defined the nutritional requirements and have established the optimal conditions for the growth of the marine methanococcus *Methanococcus voltae*. This hydrogen-oxidizing bacterium can be grown in both minimal and rich liquid and solid media. Its plating efficiency is high, and single-colony isolation is routine. Furthermore, generation times of 90 to 150 min are easily maintained in both small- and large-scale cultures, providing adequate cell mass for biochemical studies.

With regard to the exploitation of genetics, *M. voltae* is showing considerable promise. For example, Bertani has reported natural transformation in this organism (G. Bertani, Genetic Society of America Abstracts, 1986, abstr. 13.1), several genes have been cloned (20, 33), a plasmid has been identified in a closely related methanococcus (32), and auxotrophic mutants have been reported (G. Bertani and L. Baresi, 1986, Abstr. European Molecular Biology Organization Workshop on Molecular Genetics of Archaebacteria, 1985, abstr. A1).

Mutants that are resistant to 2-bromoethanesulfonic acid (BES), an analog of methyl-coenzyme M (CoM) and a cofactor in methanogenesis (3, 31), have been reported for several methanogens including *Methanosarcina barkeri* (26), *Methanobacterium thermoautotrophicum* (17), *Methanococcus vannielii* (10), and *Methanococcus voltae* (P. Gernhardt and A. Klein, Abstr. European Molecular Biology Organization Workshop on Molecular Genetics of Archaebacteria, 1985, abstr. B). In the case of the *Methanosarcina* mutants, it has been shown that methane formation from CH_3-S-CoM in extracts prepared from sensitive and resistant strains are equally sensitive to BES, suggesting that the mechanism of resistance involves a change in the cell envelope leading to decreased permeability to BES (25). A similar conclusion can be drawn from a report that a BES-resistant mutant of *M. voltae* is defective in uptake of CoM and that CoM itself can protect cells from the action of BES. These results suggest that in *M. voltae* BES is taken up by an uptake system which it shares with CoM (P. Gernhardt and A. Klein, Abstr. European Molecular Biology Organization Workshop on Molecular Genetics of Archaebacteria 1985, abstr. B3). In studies to generate suitable genetic markers, we also isolated *M. voltae* mutants that were resistant to BES. Their characterization is the subject of this report.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains used in this study are derivatives of *M. voltae* PS (DSM 1537). *M. voltae* was grown under a pressurized atmosphere (203 kPa) containing H_2 and CO_2 (80:20) (2). Medium used for liquid cultures was prepared as described by Whitman et al. (30), and agar medium was prepared anaerobically as described by Jones et al. (16). Complex medium (16) was used for all plating experiments and for maintenance of stock cultures. The culture conditions were identical to those described by Whitman et al. (30), except that cultures were grown at 35°C rather than 37°C. For preparation of solid media containing BES, filter-sterilized BES was added to the agar at a final concentration of 200 μM immediately before plates and overlays were poured.

Plating conditions and growth studies. Plating conditions were similar to those described by Jones et al. (16), except that the soft agar overlay method (16) was used rather than the spread plate method. Strains were purified with agar bottle plates (11). The percent plating efficiency is defined as

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(viable cell count/direct cell count) × 100. Direct microscopic counts of cells were made with a Petroff-Hauser counting chamber.

Growth in liquid culture was followed by measuring the A_{660} with a spectrophotometer (Spectronic 21; Bausch & Lomb, Inc., Rochester, N.Y.). All growth studies were performed with 5-ml cultures prepared in culture tubes (18 by 150 mm) sealed with butyl rubber stoppers (2).

Isolation of mutants. A wild-type culture was grown in defined medium until it reached the late logarithmic growth phase (optical density, 0.9 at 660 nm). Direct microscopic counts showed that this culture contained 3×10^9 cells per ml. The culture was diluted 10^7-fold, and samples from this dilution were used to inoculate 10 tubes of defined medium. The tubes were incubated at 35°C with shaking, and when the cultures reached an A_{660} of about 0.3, BES was added to a final concentration of 200 μM. This resulted in a slowing down of growth over a period of approximately 2 h and was followed by a 24-h incubation period in which no further growth occurred. Continued incubation, however, led to increased turbidity over a period of another 24 h. At this time (i.e., 48 h after the addition of the BES) putative mutants were purified by streaking samples from each of the 10 cultures for single-colony isolation on complex medium containing 200 μM BES. Two mutants were used in further studies.

Preparation of cell extracts and methylreductase assay. Cells were grown in 1-liter bottles (4) containing 200 ml of defined medium to an optical density of between 0.4 and 0.6. Extracts were prepared by a modification of the procedure described by Whitman and Wolfe (29). Cultures were brought into the anaerobic chamber (atmosphere, approximately 79% N_2, 19% CO_2, 2% H_2) and transferred to 50-ml polypropylene Oak Ridge-style centrifugation bottles (Du Pont Co., Biomedical Products Div., Newtown, Conn.) that had been equilibrated in the anaerobic chamber for at least 24 h. Next, the bottles were sealed and brought out of the chamber; and the cells were harvested by centrifugation for 20 min at 5,000 × *g*, washed once under anaerobic conditions with sterile defined medium, and then pelleted. After re-entry into the anaerobic chamber, the cell pellet was suspended in 3 to 5 ml of anaerobic breakage buffer saturated with H_2 gas. Breakage buffer consisted of 50 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate buffer (pH 6.8) containing 10 mM 2-mercaptoethanol and 20% (vol/vol) glycerol. The cell suspension was loaded outside the anaerobic chamber into a French pressure cell under a stream of H_2 and disrupted at 110 mPa, and the extract was collected under an H_2 atmosphere in a 10-ml serum vial containing 40 μg of DNase per ml. The broken cell mixture was then centrifuged for 20 min at 30,000 × *g* to remove unbroken cells and cell debris. The resulting supernatant solution was then decanted into a 10-ml serum vial inside the anaerobic chamber, stoppered, and stored at −20°C under an atmosphere of H_2 (200 kPa).

Assays for methylreductase were performed in calibrated, 5.7-ml stoppered vials as described by Nagle and Wolfe (22). The reaction mixture (200 μl) consisted of 96 mM potassium piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES; pH 6.3), 4 mM ATP (pH 7.0), 12 mM magnesium acetate, 1 mM CH_3-S-CoM, and cell extract (2 mg of protein). After the mixtures were prepared, they were placed on ice and flushed with H_2 for 10 min. Reactions were started by placing the vials in a 37°C water bath. At time intervals 20 μl of gas was removed from the vial, and the amount of methane formed was determined by gas chromatography. The reported rates were calculated from the linear portion of the time course obtained. Methane production in the absence of added CH_3-S-CoM as negligible.

Synthesis of [^{35}S]BES. [^{35}S]BES was synthesized by the procedure described by Marvel and Sparberg (21) with the following modifications. A total of 6.3 mg of sodium [^{35}S]sulfite (22 mCi/mmol; Amersham Corp., Arlington Heights, Ill.) was dissolved in 75 μl of water and injected into a 1.0-ml Recti-Vial (Pierce Chemical Co., Rockford, Ill.) which contained a mixture of 80 μl of dibromoethane–300 μl of absolute ethanol–75 μl of water at 80°C under a N_2 atmosphere. The resulting mixture was refluxed for 6 h at 80°C with frequent swirling. The mixture formed two phases and the upper phase (about 300 μl) was removed with a 500-μl Hamilton syringe and transferred to a clean 1-ml Reacti-Vial. The vial was placed at 35°C, and the contents were evaporated under nitrogen to dryness. The residue was taken up in 200 μl of absolute ethanol and refluxed for 4 h at 60°C with occasional mixing. The Reacti-Vial was then centrifuged for 1 min at 120 × *g*, and the supernatant was transferred to a 1-ml Reacti-Vial and stored overnight at −20°C under a N_2 atmosphere. The Reacti-Vial was then centrifuged for 1 min at 120 × *g*, the supernatant was removed, and the pelleted white precipitate was dried under nitrogen. Identity and purity of the product was confirmed by thin-layer chromatography on cellulose sheets (no. 13255; Eastman, Rochester, N.Y.) in two separate solvent systems of acetone-water-concentrated NH_4OH (16:3:1 and 16:3:0.3). A small sample containing a few crystals of the product was dissolved in a 0.1 M aqueous solution of nonradiolabeled BES and spotted on a cellulose thin-layer sheet (Eastman). After development, the plates were dried and sprayed with silver-fluoresceinate reagent to visualize the compounds (27). After the reagent had thoroughly dried, an autoradiogram was obtained by exposing Eastman Kodak XAR 5 diagnostic film to the sheet for 1 day. The results showed that the preparation was greater than 98% radiochemically pure.

Transport studies. The procedures for transport studies closely followed that described by Jarrell and co-workers (13, 15). Cells were cultured in 125-ml serum vials (Wheaton Scientific, Millville, N.J.) in 20 ml of defined medium at 200 kPa of pressure of an H_2-CO_2 (80:20) gas mixture. After growth of the cells to the mid-logarithmic growth phase (A_{660}, 0.4 to 0.6), they were concentrated by anaerobic centrifugation in sealed 50-ml Oak Ridge-style centrifugation bottles and washed once in the anaerobic buffer described by Jarrell and Sprott (15), which was modified to contain 100 μM NaCl. The cells were next suspended in 8 ml of anaerobic buffer. For assay of isoleucine uptake, 4 ml was transferred to a 60-ml serum vial under H_2-CO_2 (80:20). The vial was next incubated for 15 min at 35°C in a shaking water bath to allow for equilibration of the H_2-CO_2 gas mixture. Uptake was initiated by injecting 50 μl of the labeled substrate into the cell suspensions to final concentrations of 50 μM L-isoleucine (3.1 mCi/mmol) with a 50-μl, gas-tight syringe (The Hamilton Co., Reno, Nev.). At each time interval 0.4 ml was removed with a syringe and filtered (pore size, 0.5 μm, cellulose acetate; type EH; Millipore, Corp., Bedford, Mass.). The filters were next washed with 5 ml of anaerobic buffer and dried, and the amount of radioactivity on the filter was determined in a liquid scintillation spectrometer.

Because we observed substantial background binding of [^{35}]BES to all membrane filters tested, we chose to determine its uptake using a centrifugation assay. After prepara-

tion of the anaerobic cell suspension as described above, 2-ml fractions were distributed into separate 60-ml serum bottles and equilibrated with the headspace H_2-CO_2 (80:20) gas mixture. [^{35}S]BES was then added at a final concentration of 10 μM (22 mCi/mmol). For sampling at each time, 1.8 ml was removed from one of the assay bottles and transferred to a 2.0-ml capped polypropylene Micro tube (Sarstedt, Inc., Princeton, N.J.). The tube was centrifuged for 5 min in a microcentrifuge (Eppendorf), and the pellet was washed twice in 2 ml of anaerobic buffer. The final pellet was suspended in 100 μl of anaerobic buffer and spotted onto a glass filter disk. The tube was then rinsed with 50 μl of the same buffer that was also spotted onto the glass filter disk. The filters were dried, and the amount radioactivity associated with the filters was determined by liquid scintillation counting.

Protein determination. Protein was assayed by the method described by Bradford (5), with bovine serum albumin used as the standard.

RESULTS

Isolation of spontaneous BES-resistant mutants. Two independent mutants of *M. voltae* were obtained that had spontaneously acquired resistance to 200 μM BES (see above). Both mutants grew without a lag in medium containing 200 μM BES. The growth rate of the parental strain in liquid culture containing 0.75 μM BES was reduced to 50% of that observed in the absence of the inhibitor. With the mutants 1.5 mM BES was required to reduce the growth rates to the 50% level. The mutants had a doubling time of approximately 2.6 h, whether grown in the presence or absence of 200 μm BES, which compares with a doubling time of approximately 2.1 for the parental BES-sensitive strain. Mutant and wild-type cultures reached similar final absorbance values. Similar plating efficiencies (80 to 95%) were obtained for the mutant and wild-type strains, and the plating efficiencies of the mutants were not influenced by the presence of 200 μM BES in the agar medium. As determined by phase-contrast and epifluorescence microscopy, the mutants appeared to have normal morphology.

Each mutant was subcultured 10 times in the absence of BES and then plated onto agar plates either containing or lacking 200 μM BES. We found no significant difference in the number of colonies obtained on plates in the presence or absence of BES for either mutant strain, indicating that the mutants were quite stable. The fraction of BES-resistant mutants in a wild-type culture was determined by plating cultures onto agar medium in the presence and absence of BES. The fraction of the parental strain represented by BES-resistant mutants was found to be 2×10^{-6}.

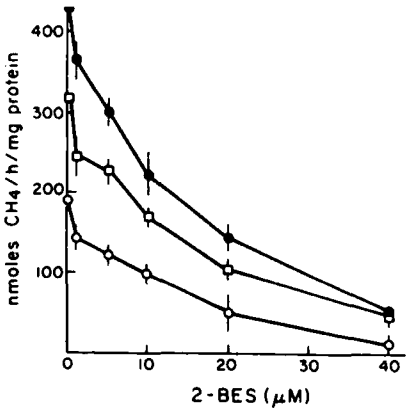

FIG. 1. Sensitivity of CH_3-S-CoM methylreductase to BES. Assays were performed as described in the text. Values represent the mean ± the standard error of the mean. Each assay was repeated by using at least three different extracts from each strain. Symbols: □, wild-type *M. voltae*; ○, BES^r-1 strain; ●, BES^r-2 strain.

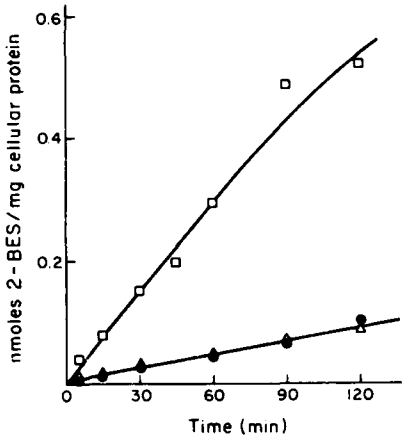

FIG. 2. Time course of BES uptake in strains of *M. voltae*. Cells were prepared and uptake was determined as described in the text. The uptake of BES (10 μM) was measured at 35°C by using the *M. voltae* wild type (□), the BES^r-1 strain (△), and the BES^r-2 strain (●).

CH_3-S-CoM methylreductase sensitivity to BES. Because the cellular target of BES is thought to be the CH_3-S-CoM reductase system (8, 25), it seemed possible that resistance might have been brought about by an alteration in some sensitive component of the reductase system itself. We determined the in vitro sensitivity of this enzyme system to BES (Fig. 1). Although the extracts displayed somewhat different specific activities in the absence of BES, mutant and wild-type extracts exhibited similar patterns of inhibition by BES over the entire range of concentrations examined. At 5 μM BES the rate of methane formation in each extract was about 50% of that observed in the absence of the inhibitor, while 40 μM BES led to almost complete inhibition. While methyl-reductase activity in the extract prepared from BES resistant mutant 1 (BES^r-1) was reproducibly slightly less than that obtained in extracts prepared from the parental strain, activity in the extract prepared from BES-resistant mutant 2 (BES^r-2) was reproducibly slightly higher. We obtained these results repeatedly using six independent extract preparations. The underlying basis for these differences is unknown.

Transport of BES and isoleucine. To investigate whether resistance correlated with a decrease in BES uptake into cells, accumulation of [^{35}S]BES by cells was measured (Fig. 2). Uptake by parental and mutant strains was linear for at least 30 min. The rate of accumulation of BES by the wild-type strain was about 5 pmol/min per mg of protein, whereas both mutants exhibited a drastically reduced capacity to accumulate the inhibitor. Although the amount of [^{35}S]BES taken up varied from culture to culture over a range of 5 to 30 pmol/min per mg of protein, in every set of experiments uptake by the mutants was drastically reduced relative to that by the parental strain. As compared with uptake in the presence of H_2 and CO_2, accumulation was

TABLE 1. Effect of ionophores and inhibitors on BES uptake[a]

Inhibitor	Concn	% uptake[b]
Control		100
CCCP	20 μM	30.2
Gramicidin	10 μg/ml	89.6
Monensin	20 μM	74.2
Nigericin	20 μM	50.8
Iodoacetate	100 μM	4.7
Sodium azide	10 mM	10.0
2,4-Dinitrophenol	1 mM	6.8
N-Ethylmaleimide	1 mM	10.2

[a] Cells were transferred to assay vials and incubated for 5 min with the indicated inhibitor prior to the addition of 10 μM BES.

[b] Control uptake was 0.327 nmol of BES per mg of cellular protein; this represents the total amount of BES bound in a 30-min assay.

much reduced in a H_2 (7%), N_2 (5%), or CO_2-N_2 (20:80) (14%) gas phase. It was not possible to determine uptake in the presence of a 100% CO_2 because the cells lysed under these conditions. Uptake was reduced to 15% when the headspace was air. The addition of sodium azide, 2,4-dinitrophenol, or iodoacetate greatly decreased the amount of BES taken up by *M. voltae* cells (Table 1). These agents have been shown to inhibit methanogenesis in whole cells, as well as methane production from CH_3-S-CoM by methyl-CoM-methylreductase in cell extracts of *M. thermoautotrophicum* (9); it is not known if their effects on BES uptake reflect a primary action of these inhibitors on methanogenesis. Incubation of cells with the protonophore carbonyl cyanide-*m*-chlorophenol hydrazone (CCCP) or with nigericin, which facilitates transmembrane H^+-K^+ exchange, resulted in an intermediate level of inhibition. Monensin and gramicidin were less inhibitory. It has previously been shown that gramicidin does not inhibit methane formation in *M. voltae* (14). *N*-Ethylmaleimide was strongly inhibitory, suggesting that uptake is carrier mediated.

Although amino acid transport in this organism is Na^+ dependent (7, 13), we found that uptake of BES was not significantly affected by the omission of Na^+ from the uptake assay reaction. For example, the uptake rate in cell suspensions lacking added Na^+ was reduced only 11%, while the addition of 400 μM Na^+ led to a 13% enhancement in the rate of uptake observed in the standard uptake medium (Na^+, 100 μM).

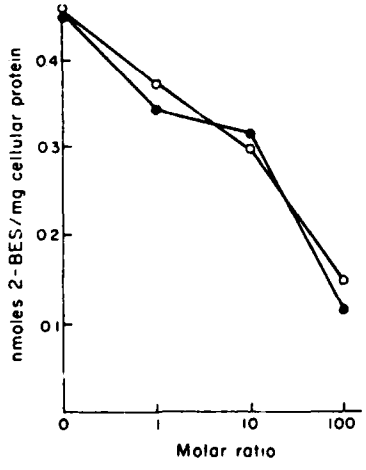

FIG. 3. Effects of HS-CoM and CH_3-S-CoM on BES uptake by wild-type *M. voltae*. HS-CoM or CH_3-S-CoM was added together with 10 μM BES to assay vials at the indicated molar ratios. Uptake was determined as described in the text. Symbols: ○, assay mixtures plus HS-CoM; ●, assay mixtures plus CH_3-S-CoM.

Effect of HS-CoM and CH_3-S-CoM on BES uptake. To ascertain the specificity of BES uptake by *M. voltae* competition studies were performed with nonradioactive HS-CoM or CH_3-S-CoM (Fig. 3). These substrates were added simultaneously with [^{35}S]BES at molar ratios of 1, 10, and 100 (nonradioactive compound, BES). Both HS-CoM and CH_3-S-CoM caused an inhibition in the amount of BES taken up by wild-type cells, and the amount of BES associated with the cells decreased with increasing concentrations of either CH_3-S-CoM or HS-CoM.

If HS-CoM and CH_3-S-CoM compete with BES for uptake, they might protect cells from its action. Indeed, protection from BES in vivo was apparent even when HS-CoM or CH_3-S-CoM were present at a molar ratio of 1 (Table 2). Neither HS-CoM nor CH_3-S-CoM enhanced the growth rate or the mass yield (Table 2) of wild-type *M. voltae*; the average doubling time for *M. voltae* was 5.5 h in defined medium and 5.1 and 4.7 h in defined medium containing HS-CoM or CH_3-S-CoM, respectively.

Uptake of isoleucine by wild-type and mutant strains. To determine if resistance to BES resulted from a general defect in nutrient uptake, we measured the rates of uptake of isoleucine by the wild-type and BES-resistant strains. The uptake of isoleucine by *M. voltae* assayed over a period of 2 min has been shown to reflect transport rather than metabolism of the label (13). Figure 4 shows the kinetics of isoleucine transport for the wild-type and mutant strains over a period of min. Both mutant and wild-type strains exhibited similar rates of isoleucine transport, corresponding to an averge rate of about 4 nmol/min per mg of protein.

The MICs of azaserine and 5-methyltryptophane were identical for the mutant and wild-type strains (20 μg/ml), indicating that resistance to BES is not due to a general alteration in cell permeability.

DISCUSSION

We have reported the isolation and characterization of mutants that exhibit a 2,000-fold increase in resistance to BES. Although the mutants arose spontaneously at a frequency of approximately 2×10^{-6}, and so probably arose via

TABLE 2. Effect of HS-CoM and CH_3-S-CoM on growth inhibition by BES

Concn (μM) of:			Absorbance[a]
BES	HS-CoM	CH_3-S-CoM	
			0.584
5			0.085
	500		0.574
		500	0.563
5	5		0.247
5	50		0.509
5	500		0.554
5		5	0.414
5		50	0.493
5		500	0.463

[a] Culture tubes containing 5 ml of defined medium were inoculated with the indicated compound(s) and 0.1 ml of a wild-type culture. All tubes were then incubated at 35°C for 24 h with shaking. The A_{600} was taken after 24 h. The values represent the average of two independent experiments. Anaerobic 10× stock solutions of BES, HS-CoM, and CH_3-S-CoM were prepared in defined medium.

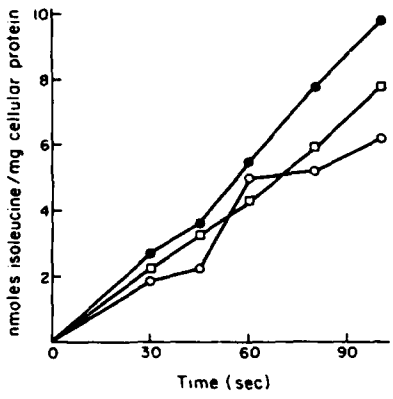

FIG. 4. Time course of isoleucine uptake in strains of *M. voltae*. Transport was determined as described in the text, except that the anaerobic assay mixture contained 50 mM NaCl. The uptake of isoleucine (50 μM) was measured by using the *M. voltae* wild type (□), the BES^r-1 strain (○), and the BES^r-2 strain (●).

a single mutation, further studies are necessary to define the mutants genetically. The mutants displayed normal cell morphology and plating efficiencies and grew at rates identical to that of the parental BES-sensitive strain, whether grown in the presence or the absence of BES. These properties are in contrast to those of BES-resistant mutants of *Methanobacterium thermoautotrophicum* which exhibited very low resistance, poor plating efficiencies, and altered cell morphology (19, 24). Spontaneous BES-resistant mutants have been isolated from another methanococcus, *Methanococcus vannielii* (10). Although these mutants were found to occur at almost identical frequencies as the *M. voltae* mutants reported here, the resistance levels of the *M. vannielii* mutants is about two orders of magnitude lower than what we have found for *M. voltae* (10).

Although mutant and wild-type cells differ greatly in sensitivity to BES in vivo, the in vitro sensitivity of their respective methyl-CoM-methylreductase systems to BES was similar. We show here that for each mutant resistance correlated with decreased uptake of the inhibitor and conclude that resistance is the result of a decreased capacity of mutants to accumulate the inhibitor. Because spent medium obtained after growth of the mutants is 200 μM BES retained the full capacity to inhibit growth of the wild-type strain (data not shown), we have ruled out the possibility that resistance involves BES inactivation. Similar results have been reported for BES-resistant mutants of *Methanosarcina* (25).

Wild-type *M. voltae* accumulated BES for up to at least 2 h. Based on an internal volume estimate of 1.37 μl/mg (dry weight) (13) and the assumption that the majority of the BES was intracellular, we estimated that the intracellular concentration of BES in the wild-type strain was approximately 200 μM after 1 h of accumulation. This represents a concentration that was approximately 20-fold greater than that present in the medium. Using the data of Balch and Wolfe (3), we estimate that the intracellular concentration of CoM in *M. voltae* is about 1,400 μM. The high intracellular CoM to BES ratio during the time course of these experiments may allow sufficient methanogenesis to support BES uptake over the 2-h assay period. The six- to sevenfold reduction of BES uptake in the mutants would lead to an even higher ratio of CoM to accumulated BES, thus rendering the cells resistant to BES action.

The finding that accumulation of BES by wild-type cells required both CO_2 and H_2 and was inhibited by several ionophores as well as agents known to effect methanogenesis strongly suggests that BES uptake is energy dependent. Given the uncertainty of the effects of these agents in this methanogen, however, this conclusion is made cautiously, and further documentation will be necessary. It has previously been shown that CoM uptake by *Methanobacterium ruminantium* is energy dependent (4). Although BES uptake by *M. voltae* exhibited a slight stimulation by Na^+, we did not observe a dramatic dependence on added Na^+. Consistent with this finding was our observation that monensin was only mildly inhibitory. These results are in contrast to the strong dependence on sodium for active transport of isoleucine in this organism (13, 15) and may reflect a basic difference in the energetics of the systems.

Our finding that the addition of either HS-CoM or CH_3-S-CoM protects cells from the inhibitory action of BES at a molar ratio of 1 and that their presence in the uptake assays leads to decreased uptake of BES argues that this organism possesses a HS-CoM/CH_3-S-CoM uptake system by which BES is able to enter the cells. Although growth of *M. voltae* is not stimulated by HS-CoM, it is possible that uptake of this compound is physiologically important in the natural habitat of this organism. At this time, however, we cannot rule out the possibility that uptake of these compounds is nonspecific and that they are taken up through a system with a primary role that is the uptake of some other substrate.

Although it is not established whether the in vivo action of BES is a result of a specific effect on methanogenesis (9), its capacity to alkylate cysteine residues (23), or both, we have shown that resistance can arise via a genetic change that affects its accumulation. A generalized defect in membrane energy coupling is ruled out by our finding that the mutants transport isoleucine normally. It is possible that that BES resistance arose via a specific alteration in the CoM–CH_3-S-CoM uptake system.

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Identification of a Vanadate-Sensitive, Membrane-Bound ATPase in the Archaebacterium *Methanococcus voltae*

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Membrane-bound ATPase activity was detected in the methanogen *Methanococcus voltae*. The ATPase was inhibited by vanadate, a characteristic inhibitor of E_1E_2 ATPases. The enzyme activity was also inhibited by diethylstilbestrol. However, it was insensitive to *N,N'*-dicyclohexylcarbodiimide, ouabain, and oligomycin. The enzyme displayed a high preference for ATP as substrate, was dependent on Mg^{2+}, and had a pH optimum of approximately 7.5. The enzyme was completely solubilized with 2% Triton X-100. The enzyme was insensitive to oxygen and was stabilized by ATP. There was no homology with the *Escherichia coli* F_0F_1 ATPase at the level of DNA and protein. The membrane-bound *M. voltae* ATPase showed properties similar to those of E_1E_2 ATPases.

Methanococcus voltae is a heterotrophic methanogen which uses hydrogen and carbon dioxide or formate as an energy source (37). Although many methanococci grow autotrophically in mineral medium, *M. voltae* requires acetate, leucine, and isoleucine for growth in mineral medium (37). This organism also carries out energy-dependent uptake of amino acids, and there is evidence that such transport is mediated by a transmembrane sodium gradient (21). Recently, the presence of an energy-dependent, carrier-mediated uptake system for HS-coenzyme M and CH_3-S-coenzyme M has been suggested (32).

The mechanism of coupling between methane formation and ATP synthesis in methanogens has been a topic of controversy. The concomitant decrease in ATP formation and membrane potential caused by the addition of protonophores to *Methanosarcina barkeri* has led to the conclusion that ATP synthesis is driven by a chemiosmotic gradient of protons generated by the reactions of methanogenesis (3). An earlier report that methanogenesis from H_2 and CO_2 as well as ATP synthesis can proceed in *Methanobacterium thermoautotrophicum* in the absence of a measurable membrane potential (33) has recently been clarified in studies with protoplasts of this methanogen. It has been shown that ATP synthesis, methanogenesis, and the membrane potential decrease in parallel in protoplasts treated with the protonophor- 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile (SF-6847) (27). The inability of the ionophore to reach the internal membranes in whole cells has been proposed to explain this difference in sensitivity. On the basis of evidence that electron transfer-driven ATP synthesis in *Methanococcus voltae* is not dependent on a proton electrochemical gradient, a molecular scheme in which ATP synthesis is coupled directly to electron transfer has been proposed (10, 25). Furthermore, the *M. voltae* ATPase is not considered to function physiologically as an ATP synthase; rather, it is believed to be involved in electrogenic sodium translocation (1, 8).

ATP-driven ion pumps fall into two classes (26). The F_0F_1 ATPases are structurally complex. They translocate protons and are inhibited by *N,N'*-dicyclohexylcarbodiimide (DCCD). They can carry out both ATP synthesis and ATP hydrolysis. The F_0F_1 ATPases characterized for bacteria, mitochondria, and chloroplasts display similar structural properties and a significant conservation of amino acid sequence (11). The characterization of an ATPase as an E_1E_2 type depends on either of two criteria: (i) inhibition of activity by vanadate or (ii) formation of an acylphosphate intermediate during ATP hydrolysis, with the phosphate binding to an aspartate residue (M. O. Walderhaug, G. Saccomani, T. H. Wilson, D. Briskin, R. T. Leonard, G. Sachs, and R. L. Post, Fed. Proc. 42:1275, 1983). The E_1E_2 ATPases function physiologically in the direction of ATP hydrolysis, vary with respect to cation specificity (depending on the system), and usually have a simple structure, often being composed of a single polypeptide chain.

In this paper we demonstrate the presence in *M. voltae* of a vanadate-sensitive, DCCD-resistant, membrane-associated ATPase. This is the first report of an ATPase with these properties in archaebacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. voltae* PS (DMS1537) was grown in a defined medium (37) supplemented with 0.05% yeast extract in a pressurized atmosphere of H_2-CO_2 (80 and 20% [vol/vol], respectively) at 32°C in 1-liter Wheaton bottles (1) containing 200 ml of growth medium. *Escherichia coli* W3110 was grown as described by Vogel and Steinhart (36).

Preparation of membranes. *M. voltae* cells were grown to late log phase (optical density at 660 nm, 0.6 to 0.8 with approximately 0.15 mg of cell protein per ml) and harvested aerobically by centrifugation at 4°C for 15 min at 5,000 × *g*. The harvested cells were suspended aerobically in twice the cell pellet volume of a mixture of 50 mM glycine–NaOH (pH 7.5)–50 mM KCl–5 mM $MgCl_2$ (G buffer), 1 mM ATP, and 10% glycerol. DNase (10 μg/ml) was added, and the cell suspension was passed through a French pressure cell at 110 MPa. The cell debris was removed by centrifugation at 4°C for 10 min at 10,000 × *g*. The supernatant was centrifuged at 4°C for 2 h at 100,000 × *g*, and the resulting pellet was washed once with and suspended in G buffer. The resulting membrane-containing fraction (15 to 20 mg of protein per ml) was loaded on a 30 to 60% (wt/wt in H_2O) sucrose gradient and centrifuged at 20°C for 16 h at 55,000 × *g* in a Beckman

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SW28 rotor. For inhibitor studies, the membrane fractions from the sucrose gradient were pooled and the sucrose was removed by washing with G buffer. For some experiments, membranes were prepared in an identical manner but with all steps carried out under anaerobic conditions. *E. coli* membranes were prepared as described by Vogel and Steinhart (36).

Enzyme assays. ATPase activity was measured in G buffer by the release of inorganic phosphate (9) or by the disappearance of ATP by the luciferin-luciferase method (23) with the Turner Designs model 20E Luminometer. A typical reaction mixture had a volume of 0.5 ml and contained 0.1 to 0.3 mg of protein. The reaction was started by the addition of 5 mM ATP and followed for 20 min at 37°C. All determinations were performed under protein-limiting conditions.

Protein determination. Protein was assayed by the Bradford method (5) with bovine serum albumin as a standard. The amount of protein present in the fractions from the sucrose gradients was estimated by absorbance at 280 nm.

Western blots. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was done with the Laemmli (24) buffer system. *E. coli* membranes were used as positive controls. Membranes were treated with sodium dodecyl sulfate (final concentration, 3.2%) and heated at 60°C for 30 min before being applied to the gel. Western blots (immunoblots) were performed as described elsewhere (6). After the proteins were transferred, the nitrocellulose was washed at room temperature as follows. The first wash was performed for 1.5 h in 10% fetal calf serum and phosphate-buffered saline (PBS) containing 10 mM sodium phosphate with 0.15 M sodium chloride. Next, immunoglobulin G (10 mg) raised against F_1 ATPase (provided by R. Simoni, Stanford University, Palo Alto, Calif.) was added to the 10% fetal calf serum–PBS, and the mixture was incubated for 3 h. The incubation was followed by two 20-min washes with PBS-Triton X-100 (0.05%) and a 10-min wash with PBS alone. The filter was then incubated for 20 min with 10% fetal calf serum–PBS which contained ^{125}I-protein A (5 μl contained 4 × 10^6 cpm). The filter was washed once again with PBS, PBS–Triton X-100, and PBS, as described above. It was then air dried and autoradiographed for 1 to 7 days at −70°C.

Southern blots. DNA from *M. voltae* and *E. coli* was extracted as described by Saito and Miura (31) except that lysozyme was not used and proteinase K was used instead of pronase during the extraction of *M. voltae* DNA. pRPG54 (15), a plasmid which has the entire *E. coli* ATPase operon, was labeled by nick translation with [α-^{32}P]dATP (29). *M. voltae* DNA was digested to completion with restriction enzyme *Hin*dIII, *Cla*I, *Eco*RI, or *Pst*I, electrophoresed on 1% agarose gels, and then transferred to nitrocellulose, as described by Southern (34). The filter was first incubated in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% Denhardt solution–100 μg of salmon sperm DNA per ml for 2 to 3 h at room temperature. The incubation was followed by hybridization with denatured ^{32}P-labeled pRPG54 DNA in 6× SSC–0.01 M EDTA–5× Denhardt solution–0.05% sodium dodecyl sulfate–100 μg of denatured salmon sperm DNA per ml at room temperature for 14 h. The stringencies of subsequent washes were varied by changing the temperature and salt conditions. These were carried out at 25, 37, and 50°C. The salt conditions used were 2×, 1×, and 0.1× SSC.

Staining for enzyme activity. ATPase activity was located on nondenaturing 10% polyacrylamide gels (28). The gel was incubated with 50 mM glycine–NaOH buffer (pH 7.5) containing 5 mM ATP, 5 mM $MgCl_2$, and 0.05% lead acetate for 40 min. It was subsequently developed in 0.1% sodium sulfide.

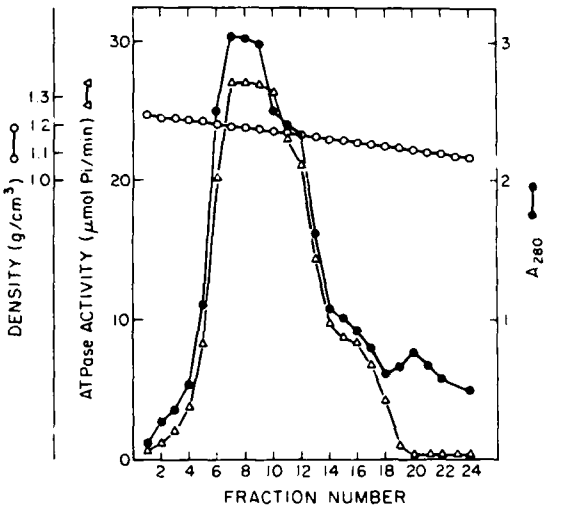

FIG. 1. Sucrose gradient centrifugation profile of the washed 100,000 × *g* membrane pellet.

Materials. Sodium orthovanadate, oligomycin, ouabain, diethylstilbestrol, and DCCD were purchased from Sigma Chemical Co. The luciferin-luciferase enzyme system was purchased from Turner Designs.

RESULTS

Characterization of ATPase activity. When *M. voltae* cells were broken in a French press and then subjected to centrifugation at 100,000 × *g*, 95% of the ATPase activity originally present in the crude cell extract was recovered in the pellet fraction. Sucrose gradient analysis of this pellet fraction yielded a major fraction of 280-nm-absorbing material whose density ranged from 1.16 to 1.20 g/cm³ (Fig. 1). Since the profile of incorporated mevalonic acid, a marker for membrane lipid (35), coincides with this material, it was apparent that the material represented membranes (N. Santoro and J. Konisky, unpublished experiments). As can be seen in Fig. 1, ATPase activity was localized to this fraction, which indicated that it was a membrane-associated enzyme.

The addition of 2% Triton X-100 to the membrane fraction resulted in complete solubilization of the ATPase (Fig. 2) and in an increase in the specific activity of the enzyme from 14.3 to 21.1 μmol/min per mg of protein. In the absence of detergent, G buffer containing 500 mM KCl solubilized 45% of the enzyme activity. The significance of this result has not been investigated further.

It was possible to specifically stain for ATPase activity on nondenaturing polyacrylamide gels. In such an analysis (data not shown), a single band of activity was observed, although we did on occasion observe additional weaker bands. The weaker bands may have represented enzymatically active degradation products of the native enzyme, or perhaps minor ATPase species. When assayed by staining on nondenatured polyacrylamide gels, both the cell extract and the 100,000 × *g* pellet fraction contained ATPase, while the 100,000 × *g* supernatant did not. In contrast, the pellet fraction obtained after treatment of the membrane with 2%

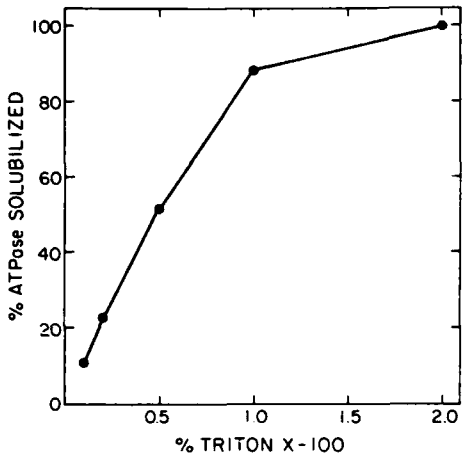

FIG. 2. Solubilization of *M. voltae* ATPase by Triton X-100. The 100,000 × *g* membrane pellet (protein concentration, 5 mg/ml) was treated with suspension buffer containing various amounts of Triton X-100 at 4°C for 30 min and centrifuged at 4°C for 2 h at 100,000 × *g* to obtain the soluble and membrane fraction.

Triton X-100 contained no assayable activity, while this procedure led to the solubilization of 100% of the ATPase activity. Furthermore, the washed membranes from fraction 8 of the sucrose gradient (Fig. 1) also contained ATPase. These results were consistent with the results of the sucrose gradient analysis and provided further evidence for the membrane association of this ATPase.

Properties of the membrane-associated enzyme. Washed membranes obtained from the sucrose gradients were used in these studies. ATP hydrolysis activity was optimum at pH 7.5 (Fig. 3) and required the presence of Mg^{2+}. Its absence from the assay mixture resulted in only 5% of the activity observed when 5 mM $MgCl_2$ was present. HSO_3^-, HCO_3^-, or SO_4^{2-} (25 mM) had no effect on ATPase activity. This finding was in contrast to results with *Methanosarcina barkeri* ATPase, which is stimulated by HSO_3^- (20).

The enzyme displayed a substrate preference for ATP (Table 1). Over the concentration range examined (2 to 10 mM), 5 mM ATP was optimal. When GTP and ITP were

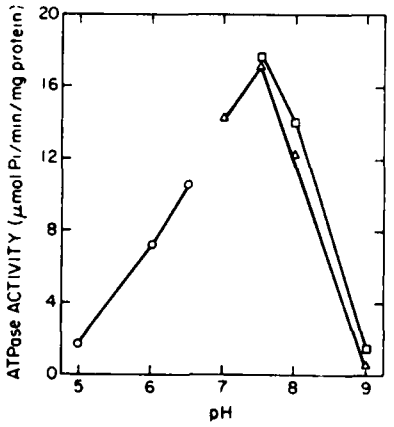

FIG. 3. Effect of pH on membrane-bound *M. voltae* ATPase activity. All buffers (0.1 M acetate [○], Tris [△], and glycine-NaOH [□]) contained 1 mM ATP, 5 mM $MgCl_2$, and 50 mM KCl.

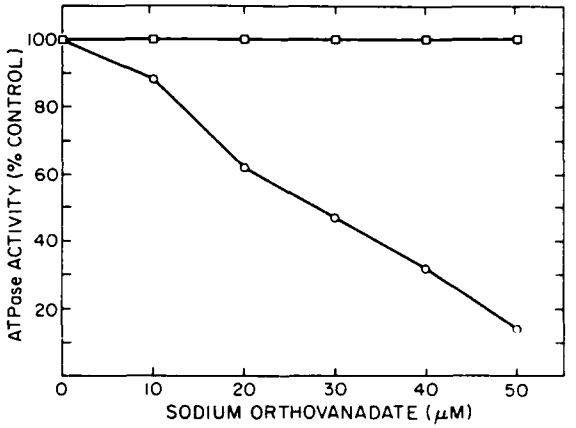

FIG. 4. Effect of sodium orthovanadate on ATPase activity. For *E. coli* ATPase (□), 100% activity represents 38 μmol of P_i/min per mg of protein. For *M. voltae* ATPase (○), 100% activity represents 16.5 μmol of P_i/min per mg of protein.

used as substrates, the enzyme activity was only 45 and 32%, respectively, as compared with that of ATP. UTP (15%) and TTP (7%) were poor substrates, and PP_i was not hydrolyzed, which ruled out the presence of a phosphatase activity. The ATPase activity was the same whether it was measured by P_i release or by decrease in ATP level.

We found that the inclusion of 1 mM ATP in buffer solutions greatly stabilized the ATPase. The membrane-bound enzyme was stable at 4°C in G buffer containing 1 mM ATP for at least 2 weeks. ATPase activity was not sensitive to the presence of oxygen, and we observed no difference in either enzyme level or inhibitor specificity in enzyme preparations made aerobically or anaerobically. Incubation of the membrane-bound enzyme for 10 min with trypsin (150 μg/ml) at 37°C resulted in complete loss of activity.

Sensitivity to vanadate and diethylstilbestrol. Several inhibitors of eubacterial and eucaryotic ATPases were tested for their effects on membrane-bound *M. voltae* ATPase. There was an 86% inhibition of *M. voltae* ATPase activity with 50 μM vanadate, an inhibitor of E_1E_2-type ATPases (Fig. 4). In contrast, 50 μM vanadate had no effect on the *E. coli* ATPase activity. Diethylstilbestrol, an inhibitor of vanadate-sensitive membrane ATPases of plants (2) and fungi (4), caused 92% inhibition of *M. voltae* ATPase activity at 150 μM (Fig. 5), while no effect was observed on *E. coli* ATPase activity. In contrast, DCCD, an inhibitor of F_0F_1 ATPases (13), had no effect on *M. voltae* ATPase activity up to a concentration of 100 μM (data not shown), but it completely abolished *E. coli* ATPase activity. Similarly, oligomycin

TABLE 1. Substrate specificity of *M. voltae* ATPase

Substrate (5 mM)	Sp act (μmol of P_i/min per mg of protein)	Relative activity (%)
ATP	16.0	100
GTP	7.17	45
ITP	5.15	32
UTP	2.34	15
TTP	1.17	7
CTP	0.93	6
Sodium PP_i	ND	ND

[a] ND, Not detected.

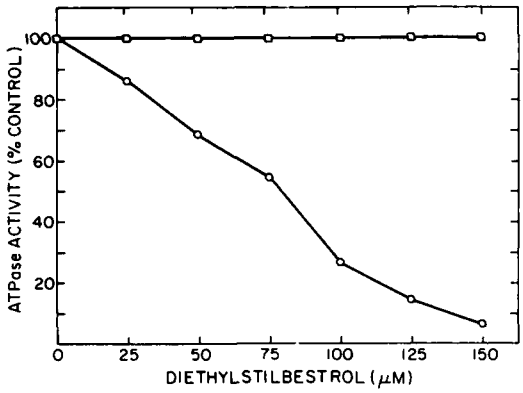

FIG. 5. Effect of diethylstilbestrol on ATPase activity. For *E. coli* ATPase (□), 100% activity represents 34 μmol of P_i/min per mg of protein. For *M. voltae* ATPase (○), 100% activity represents 18 μmol of P_i/min per mg of protein.

(10 μg/mg of protein), an inhibitor of the F_0 part of F_0F_1 ATPases of mitochondria and chloroplasts (13), had no effect on *M. voltae* and *E. coli* enzyme activity. The ATPase activities were also insensitive to the presence of ouabain (200 μM), an inhibitor of Na^+,K^+-ATPase (7). In all cases, identical results were obtained with the solubilized enzyme.

Lack of homology with *E. coli* or *Neurospora* ATPase. Homology between the F_0F_1 eubacterial and eucaryotic ATPases has been observed at the level of DNA and protein primary structure (11, 14). To investigate the homology at the level of DNA, ^{32}P-labeled plasmid pRPG54, which contains the entire *E. coli* ATPase operon, was used to probe total *M. voltae* DNA. The homology at the protein level was tested by probing *M. voltae* membrane proteins with immunoglobulin G raised against *E. coli* F_1 ATPase with ^{125}I-protein A. No cross-reaction was observed in either case. Furthermore, the addition of the *E. coli* antibody to reaction assay mixtures did not inhibit *M. voltae* ATPase activity. These experiments ruled out the possibility that the *M. voltae* ATPase shared significant homology with the *E. coli* F_0F_1 ATPase.

Since the *M. voltae* ATPase displayed a pattern of inhibition similar to that observed with the *Neurospora crassa* plasma membrane ATPase (4), we examined the possibility that the two enzymes shared sufficient structural homology to be detectable by Western blot analysis, with the antibody raised against the fungal enzyme as the probe. No cross-reactivity was observed (R. M. Dharmavaram and K. Allen, unpublished observation).

DISCUSSION

On the basis of the sensitivity of *M. voltae* to diethylstilbestrol and vanadate and its resistance to DCCD, we suggest that the *M. voltae* ATPase is not of the F_0F_1 type and may be more closely related to E_1E_2 ATPases. In contrast, the ATPase of *Methanosarcina barkeri* is sensitive to DCCD and has other features which suggest that it is related to F_0F_1 proton-translocating ATPases (20). Membrane-associated, DCCD-sensitive ATPase activity has been reported in the thermophilic *Methanobacterium thermoautotrophicum* (12). This enzyme, which is insensitive to vanadate (30), is likely of the F_0F_1 type. These results raise the possibility that the mechanism of ATP formation in *M. voltae* may differ significantly from that utilized by these other methanogens.

There is evidence that the physiological function of the *M. voltae* ATPase is Na^+ translocation (8). This would provide a mechanism for the direct coupling of ATP hydrolysis to the generation of the sodium motive force which supports active transport of several amino acids in this organism (21). Several E_1E_2 ATPases have been shown to mediate ATP-dependent translocation of cations other than protons (26).

Since ATP synthesis in *M. voltae* is not diminished under conditions in which the transmembrane electrical potential is collapsed by the addition of the protononophore SF6847, substrate-level phosphorylation has been proposed as the physiological mechanism of ATP synthesis (10, 25). Such a mechanism would obviate the need for a reversible proton-translocating ATPase in this organism. The 80 to 90% inhibition of the *M. voltae* ATPase by vanadate or diethylstilbestrol and the total resistance of the enzyme to DCCD which we observed are consistent with the absence of an F_0F_1 ATPase in this methanogen. Nevertheless, we cannot at this time completely exclude the possibility that this methanogen contains minor levels of an ATPase of this type.

The most thoroughly studied E_1E_2 ATPases have been those of the plasma membranes of fungal, plant, and animal cells. Although reports of E_1E_2 ATPases in procaryotes have been limited to *Streptococcus faecalis* (17, 19), *E. coli* (18), and *Acholeplasma laidlawii* (22), it is likely that their distribution is more widespread. The presence of an E_1E_2 ATPase in *M. voltae* extends its distribution to the third biological kingdom (38). Comparison of the amino acid sequence of the *E. coli* K^+-ATPase with that of the Ca^{2+}-ATPase of the sarcoplasmic reticulum indicates significant sequence homology (18). The K^+-ATPase of *E. coli* displays a smaller, though still significant, degree of homology to the plasma membrane E_1E_2 ATPase of *N. crassa*, which itself manifests significant homology with Na^+-, K^+-, and Ca^{2+}-transporting ATPases of animal cells (16). Whether such homology reflects convergent or divergent evolution is impossible to assess.

Future characterization of the *M. voltae* enzyme and its structural gene will provide a third perspective on the utility of these enzymes and may lead to insights into the evolution of mechanisms of ion translocation across membranes.

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Characterization of a P-Type ATPase of the Archaebacterium Methanococcus voltae*

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SUMMARY

The vanadate-sensitive ATPase of Methanococcus voltae has been purified by a procedure which includes, purification of the cytoplasmic membrane by sucrose gradient centrifugation, solubilization with Triton X-100, and DEAE-Sephadex and Sephacryl S-300 chromatography. While the DEAE-Sephadex step provided a preparation consisting of two polypeptides (74 kDa and 52 kDa), the Sephacryl S-300 step yields a product with a subunit of 74 kDa. Incubation of either membranes or purified ATPase with [γ-^{32}P] ATP followed by acidic (pH-2.4) lithium dodecyl sulfate polyacrylamide gel electrophoresis demonstrated the vanadate-sensitive labeling of a 74 kDa acyl phosphate intermediate. These results indicate that the Mc. voltae ATPase is of the P type.

INTRODUCTION

The methanogen Mc. voltae is a strictly anaerobic archaebacterium that derives energy from reduction of CO_2 by H_2 or formate to form methane (1). Since uptake of amino acids in this methanogen has been shown to be coupled to a sodium gradient (2), it is important to establish the underlying mechanism whereby energy generated in reactions leading to methane formation is transduced to form ion gradients. The characterization of ion-motive ATPases in this organism is, therefore, relevant and important.

Ion-motive ATPases are divided into three major categories. The P type ATPases are sensitive to vanadate and form an acyl phosphate intermediate during ATP hydrolysis (3). We had previously suggested the presence of such an ATPase in Mc. voltae (4) based on sensitivity to vanadate. Such P type ATPases have been characterized in Schizosaccharomyces pombe (5), Neurospora crassa (6), Escherichia coli (7), Streptococcus faecalis (8) and animal cells (9, 10). These ATPases are usually composed of monomeric or multimeric complexes of a single catalytic polypeptide of about 100 kDa. They function physiologically in the direction of ATP hydrolysis and vary with respect to cation specificity.

The second category, F type ATPases, are inhibited by N-N'-

dicyclohexylcarbodiimide (DCCD) and azide (11). These ATPases function physiologically as ATP synthases in mitochondria (12), chloroplasts (13), and respiring bacteria (14). They have a complex structure consisting of a multisubunit catalytic F_1 component of $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$ composition (where the molecular weights of α and β are about 50-60 kDa, the γ about 30-36 kDa and the δ and ϵ subunits below 20 kDa) and a membrane component, F_o involved in H^+ translocation (14) whose subunit composition varies depending on the source.

The third category, V type ATPases, are also inhibited by DCCD and by nitrate and bafilomycin A_1 (15). These ATPases are found in the vacuoles of N. crassa and plants (16), lysosomes (17), endosomes (18), clathrin coated vesicles (19), secretory granules (20) and golgi vesicles (21). They are usually large (> 400 kDa) and are composed of two major polypeptide components of 70-89 kDa (A) and 60-65 kDa (B) along with several smaller peptides and couple the energy derived from ATP hydrolysis to translocation of protons (22).

ATPases have been partially characterized in several archaebacteria. In contrast to the reported Mc. voltae ATPase (4), the membrane-bound ATPase of Methanosarcina barkeri is insensitive to vanadate and sensitive to DCCD (23). The ATPase is solubilized from membranes by incubation in low ionic strength buffer with EDTA and can rebind to depleted membranes in a high

ionic strength buffer with Mg^{2+} reminiscent of the F type ATPases. The membrane associated ATPase of the thermoacidophilic archaebacterium Sulfolobus acidocaldarius has been purified and is inhibited by nitrate (24). Recently both the major polypeptides of 65 kDa and 51 kDa of this enzyme have been sequenced (25,26). Alignment of amino acid sequences has revealed that the Sulfolobus ATPase subunits (65 kDa and 51 kDa) are 50% identical with the A and B subunits of carrot vacuolar ATPase and only 25% identical to the β and α subunits of the F type ATPase from E. coli (27). The H^+-ATPase of Halobacterium halobium is insensitive to azide and sensitive to DCCD (28). This ATPase cross reacts with antibodies raised against Sulfolobus acidocaldarius and beet vacuolar H^+-ATPase (29).

This paper reports the purification of a vanadate-sensitive ATPase in Mc. voltae. We also demonstrate the formation of an acyl phosphate during ATP hydrolysis and insensitivity of the enzyme to Bafilomycin A_1 and NO^{3-}.

MATERIALS AND METHODS

Bacterial strain, growth conditions and membrane isolation: Mc. voltae PS (DSM 1537) was grown anaerobically in a defined medium in a 30L Braun fermentor and the membranes were prepared by sucrose gradient centrifugation as previously described (4).

Solubilization of Mc. voltae ATPase: The membrane fractions from the sucrose gradient were pooled and sucrose removed by 4 fold dilution with 50 mM Tris-Cl (pH-7.5), 5 mM $MgCl_2$ buffer and centrifugation at 100,000 X g for 2 h at 4°C. The resulting membranes were incubated with 1% Triton X-100 for 30 min. with stirring at 4°C at a final protein concentration of 5 mg/ml. The insoluble material was then removed by centrifugation at 100,000 X g for 2 h at 4°C.

DEAE-Sephadex chromatography: 174 mg (5 mg/ml) of solubilized membrane protein was applied to a 5 X 2.5 cm column equilibrated with 50 mM Tris-Cl (pH-7.5), 5 mM $MgCl_2$, 1% Triton X-100 and 10% glycerol (TMG buffer). The column was washed free of unbound protein with 15 ml of buffer. The bound protein was eluted by a 200 ml, 0-500 mM KCl gradient at a flow rate of 2.5 ml/hr. Fractions of 2.5 ml were collected and assayed for protein and for ATPase as described below.

Sephacryl S-300 chromatography: 1% octyl glucoside was added to

5.32 mg of the Triton X-100 solubilized membrane protein to disperse micelles. This was followed by dialysis against two changes of TMG buffer lacking 1% Triton X-100 to remove the detergents. The dialysate was concentrated using a Centricon microconcentrator (Amicon) to a volume of 0.8 ml. 0.2 ml (1.33 mg protein) of this concentrate was loaded on a 45 X 1 cm S-300 column equilibrated with TMG buffer lacking Triton X-100. Fractions of 0.2 ml were collected at a flow rate of 3 ml/hr. The same column was run three more times using 0.2 ml of the concentrate each time and the fractions from each run were collected on top of those from the previous run. The ATPase activity was assayed as described below. The S-300 column was calibrated by the following marker proteins: ferritin (450 kDa), catalase (240 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa) and cytochrome c (12.4 kDa).

Phosphorylation of Mc. voltae membranes: Phosphorylation was performed by the method of Post and Sen (30). The labeling was carried out at 0°C in a 1 ml reaction volume containing 40 μM of [γ-^{32}P] ATP (4500Ci/mmol), 40 μM $MgCl_2$, 30 mM Tris-Cl (pH-7.5), 2 mg carrier BSA and 100-120 μg native membranes. The reaction was initiated by the addition of radioactive ATP with constant stirring and terminated at the appropriate time by the addition of 25 ml of ice-cold 10% TCA containing 40 mM NaH_2PO_4, 5 mM $Na_4P_2O_7$ and 1 mM ATP. The quenched reaction mixture was centrifuged at 27,000 X g for 15 min at 0°C in a Sorvall SS-34

rotor. The supernatant was poured off and the inside of the tube was wiped with a tissue to remove the remaining supernatant. The pellet was suspended in 1 ml of 30 mM HCl with chilled glass rods and incubated at room temperature for 30 min. At that time 9 ml of 30 mM HCl was added and the pellet was recovered by the centrifugation step described above.

Lithium dodecyl sulfate-polyacrylamide gel electrophoresis: This was performed as described by Fairbanks and Avruch (31) except that the lithium salt of dodecyl sulfate (LDS) was used (32). Slab gels (5.6%) buffered to pH-2.4 with 50 mM Tris-citrate contained 0.2% LDS. The gels were polymerized on Gel Bond Pag film to facilitate handling. The membrane pellet obtained from the last centrifugation step described above was disaggregated in 1% LDS, 50 mM Tris-citrate (pH-2.4) 2 % β-mercaptoethanol, 4 M urea, 20 % glycerol and 10 μg/ml pyronin Y and incubated at room temperature for 15 min. The gels were electrophoresed at 150 V for 3-4 h at 4^{o}C and immediately dried on blotter paper and autoradiographed with Cronex Lightning Plus intensifying screen for 24-72 h at -70^{o}C.

Fluorescein 5'-isothiocyanate-labeling: Mc. voltae membranes were incubated with varying concentrations of FITC (dissolved in dimethylsulfoxide) at 37^{o}C in 30 mM Tris-borate (PH-9.2) and 1 mM EDTA for 40 min. The reaction was stopped by a ten fold dilution into the ATPase assay buffer (50 mM Tris-Cl pH-7.5, 5 mM $MgCl_2$

and 10 % glycerol). An identical set of reactions to which 5 mM ADP or 5 mM ATP was added before the addition of FITC was run simultaneously.

Assays: The ATPase was assayed by the release of inorganic phosphate as previously described (4). Solutions of Bafilomycin A_1 were prepared in dimethylsulfoxide as previously described (15). 20 μg of protein was used in the Bafilomycin A_1 experiment. Protein was measured by the modified Lowry assay (33).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis: This was performed as described by Laemmli (34) and the gels were stained with Coomassie brilliant blue-R-250.

Materials: Sodium orthovanadate was purchased from Sigma Chemical Co. [γ-^{32}P] ATP was from ICN Radiochemicals. DEAE-Sephadex and Sephacryl S-300 were from Pharmacia. Bafilomycin A_1 was obtained from Dr. K. Altendorf (Universität Osnabrück) and beet tonoplast ATPase from Dr. D.P. Briskin (University of Illinois).

RESULTS AND DISCUSSION

Purification of the ATPase: The specific activity of the sucrose gradient purified membranes was determined to be 0.25 μmol P_i/min/mg of protein. The addition of 1% Triton X-100 solubilized all of the enzyme activity present in the membranes and resulted in a specific activity of 0.44 μmol P_i/min/mg of protein. This corresponds to a 106% recovery of ATPase and a 1.8 fold purification.

When anion exchange chromatography with DEAE-Sephadex was performed, a single peak of vanadate-sensitive ATPase eluted at around 200 mM KCl (Fig. 1). The pooled ATPase containing fractions from this step had a specific activity of 3.00 μmol P_i/min/mg of protein. The result was an additional 6.8 fold purification of the enzyme and an overall recovery of 25%. The SDS-PAGE analysis of ATPase activity containing fractions revealed that DEAE-Sephadex chromatography was an efficient purification step as these fractions contained essentially only two polypeptides (74 and 52 kDa, Fig. 2).

Sephacryl S-300 gel filtration was also utilized to purify the active ATPase. Membranes subjected to sucrose gradient centrifugation had a specific activity of 0.5 μmol P_i/min/mg of protein. Extraction with 1% Triton X-100 resulted in a specific activity of 1.05 μmol P_i/min/mg of protein corresponding to a

124% recovery of the ATPase and a 2.1 fold purification. The extraction process was followed by: addition of 1% octyl glucoside to disperse micelles; dialysis to remove detergents; and Sephacryl S-300 gel chromatography. The pooled ATPase containing fractions from the final step had a specific activity of 17.5 μmol P_i/min/mg of protein. This resulted in an additional 16.6 fold purification of the ATPase and an overall recovery of 73.8%. It was observed that the ATPase eluted close to ferritin suggesting a molecular weight of about 450 kDa (data not shown). However, we cannot rule out the possibility of incomplete removal of detergent, and definitive assignment of the molecular mass of the holoenzyme must await further studies. Unlike the pooled fractions from DEAE-Sephadex chromatography, SDS-PAGE analysis of the purified ATPase from the Sephacryl S-300 column demonstrated a single protein of 74 kDa. Although we cannot exclude the possibility that the 52 kDa subunit observed after chromatography on DEAE-Sephadex is an unrelated contaminant, it is possible that it is a regulatory or structural (e.g. involved in anchoring the catalytic subunit to membrane) component of the ATPase.

Phosphoenzyme formation: When the Mc. voltae ATPase fractions at the various steps of purification were incubated with [γ-^{32}P] ATP, a 74 kDa protein was phosphorylated (Fig. 3). This protein was not labeled when the pooled ATPase fractions from DEAE-Sephadex were incubated with 50 μM vanadate before the addition of [γ-^{32}P] ATP (Fig. 3, lane 4). The radioactivity at the base of

the gel migrates ahead of the tracking dye and is observed even when the enzyme is boiled or treated with protease before labeling (data not shown). We have also noticed that the amount of radioactivity in the wells and at the base seems to vary with different batches of radioactive ATP.

When the Sephacryl S-300 purified Mc. voltae ATPase was phosphorylated and then treated with 0.25 M hydroxylamine (pH-5.2) after the TCA precipitation step, label was no longer associated with the 74 kDa protein (Fig. 3, lane 6). This result conclusively rules out the presence of phosphoserine or phosphothreonine intermediates which are both stable at acid pH, and resistant to hydrolysis by hydroxylamine (35).

The chemical nature of the phosphoryl ATPase linkage was further investigated by determining the stability of the intermediate at different pH values (Fig. 4). Mc. voltae membranes were phosphorylated, TCA precipitated and suspended in buffers of varying pH. It can be seen that the amount of radioactivity remaining associated with the 74 kDa protein decreases as the pH becomes more alkaline. The instability of the intermediate at alkaline pH makes it unlikely that it is a phosphohistidine or phospholysine (35). The pH stability profile is similar to the N. crassa plasma membrane ATPase which is also unstable at alkaline pH and exists as an acyl phosphate (6). Hence, the Mc. voltae acyl phosphate may well be an aspartyl

derivative.

Another characteristic of the P-type ATPases is the rapid turnover of the phosphorylated intermediate. When Mc. voltae membranes were labeled for different times, it was determined by densitometry of the resulting autoradiograms that the labeling was essentially complete in 2.5 s (the shortest time tested, data not shown). Evidence for the rapid turnover of the intermediate was provided by a pulse-chase experiment (Fig. 5). Mc. voltae membranes were phosphorylated for 2 min with [γ-^{32}P] ATP, nonradioactive 5 mM ATP was added, and the chase was terminated after 20 sec by the addition of TCA. The chase with unlabeled ATP resulted in the complete loss of radioactivity in the 74 kDa protein. It would be expected that the turnover at 37^{o}C would be substantially higher. If we assume that the ATPase was fully labeled in 2.5 s, then the turnover time is no longer than 2.5 s at 0^{o}C, corresponding to a turnover rate of 24 min^{-1}. An analysis of the gel scans of Coomassie blue stained gels indicated that the 74 kDa ATPase polypeptide comprises about 5% of vesicle protein. From this information, we calculate an activity of 16.2 nmol of P_i/min/mg of membrane protein at 0^{o}C. This compares well to the value of 12 nmol P_i/min/mg determined by assaying membrane ATPase activity at 0^{o}C.

We had previously demonstrated that treatment of Mc. voltae membranes with 500 mM KCl resulted in the solubilization of 50%

of the ATPase activity (4). It was observed that both the pellet and supernatant obtained after 500 mM KCl treatment exhibit the same 74 kDa band upon phosphorylation, confirming our earlier finding (data not shown).

Our results are consistent with a mechanism in which ATP hydrolysis catalyzed by the purified Mc. voltae ATPase involves the formation of an acyl phosphate intermediate similar to that observed for the P-type ATPases of N. crassa (6), Kdp ATPase of E. coli (7), S. faecalis (8), H^+-K^+ATPase of gastric mucosa (9), Ca^+ATPase of sarcoplasmic reticulum (10) and Na^+-K^+ATPase of animal cells (36). Although these ATPases differ with respect to ion specificity, it is thought that they all share a common mechanism whereby phosphorylation and dephosphorylation of the enzyme is coupled to the conformational changes responsible for transport of ions (37).

Sensitivity to fluorescein 5' isothiocyanate : The fluorescent probe FITC binds to the lysine residue at the active site of the P type ATPases (38). The resulting inactivation is prevented by the addition of ATP or ADP and it has been suggested that the sensitive lysine residue is part of the substrate binding site. The FITC-reactive regions of the mammalian P type ATPases are highly conserved (39).

When solubilized Mc. voltae membranes were treated with

varying concentrations of FITC a gradual inactivation of the ATPase activity was observed. There was 92% inhibition of enzyme activity at 800 μM FITC (data not shown). This inactivation was prevented when 5 mM ATP or 5mM ADP was present in the reaction mix before the addition of FITC. This suggests that a lysine residue may be part of the substrate binding site. However, this conclusion is made with caution since the FITC concentration required to inhibit Mc. voltae ATPase activity is relatively high as compared to other P type ATPases and nonspecific inactivation is a possibility. For comparison, there is a 90% inhibition of Ca^{+}-ATPase from sarcoplasmic reticulum at 10 μM FITC (40).

Insensitivity to Bafilomycin A_1 and NO^{3-}: The macrolide antibiotic Bafilomycin A_1 inhibits a number of P type ATPases: for example, the Kdp ATPase of E. coli, Na^{+}-K^{+}ATPase of ox brain, Ca^{+}- ATPase of sarcoplasmic reticulum and H^{+}-ATPase of Neurospora in the μM range and the V type ATPases from Neurospora vacuoles, chromaffin granules, and Zea mays in the nM range (15).

However, Bafilomycin A_1 failed to inhibit the membrane-bound or Triton X-100 solubilized Mc. voltae ATPase up to a concentration of 1 mM (Fig. 6). This contrasted the results seen for beet tonoplast ATPase that was completely inhibited at a concentration of 1 μM. Our results are similar to those observed in the case of the P type ATPase of S. faecalis which is not inhibited by Bafilomycin A_1 up to a concentration of 1 mM (15).

Clearly sensitivity to Bafilomycin A_1 would seem not to be a firm diagnostic tool for the identification of P type ATPases.

We also observed a lack of inhibition of Mc. voltae ATPase activity by 100mM KNO_3 an inhibitor of V type ATPases. This result was in contrast to that of beet vacuolar ATPase which was 85% inhibited by 100 mM KNO_3.

This is the first report of the purification of a vanadate-sensitive ATPase that forms an acyl phosphate intermediate in a member of the archaebacterial kingdom. Future delineation of structure, biochemistry and function of Mc. voltae ATPase together with characterization of its structural gene will both clarify its role in methanogen energetics and further our understanding of the molecular mechanisms and evolution of these enzymes.

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FIGURE LEGENDS

Fig. 1. DEAE-Sephadex chromatography of Triton solubilized Mc. voltae membranes.

Fig. 2. SDS-PAGE. Lane 1. Sigma SDS-6H molecular weight markers- Myosin (205 kDa), β-galactosidase (116 kDa), Phosphorylase b (97.4 kDa), Bovine Albumin (66 kDa), Egg Albumin (45 kDa), Carbonic Anhydrase (29 kDa) Lane 2. 40 μg Triton X-100 solubilized membranes; Lane 3. 5 μg of fractions 32-38 from DEAE-Sephadex column; Lane 4. 2 μg of purified ATPase from Sephacryl S-300 column.

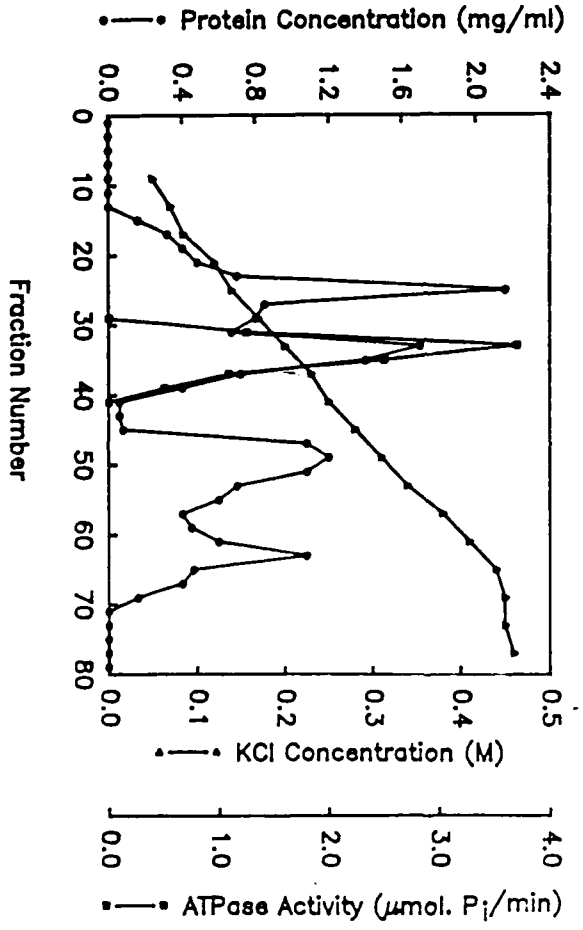
Fig. 3. Phosphorylation of enzymes fractions with [γ-^{32}P] ATP at different steps of purification. The numbers on the left side are the molecular masses of the marker proteins and the unidentified arrow represents the ATPase. Lane 1. 50 μg total membranes; Lane 2. 40 μg Triton solubilized membranes; Lane 3. 4.5 μg of fractions 32-38 pooled from DEAE-Sephadex column; Lane 4. Same as lane 3 except that the DEAE-Sephadex pool fractions were incubated with 50 uM vanadate for 10 min. at 37°C before addition of radioactive ATP; Lane 5. 2 μg of purified ATPase from S-300 column; Lane 6. Same as lane 5 except that the pooled ATPase fractions from Sephacryl S-300 were treated with 0.25 M

hydroxylamine (ph-5.2 with Tris base) after phosphorylation and TCA precipitation.

Fig. 4. Effect of pH on stability of phosphorylated ATPase:50 μg of membrane pellets obtained after TCA precipitation were treated with 1 ml of buffers of varying pH and incubated for 10 min. at 25°C. This was followed by addition of 9 ml of 30 mM HCl and centrifugation. The following buffers were utilized, Lane 1. 100 mM sodium citrate (pH-2) with HCl; Lane 2. 100 mM sodium citrate (pH-4 with HCl); Lanes 3. 100 mM Tris (pH-7.5 with HCl); Lane 4. 100 mM sodium borate (pH-9 with HCl); Lanes 5. 100 mM sodium carbonate (pH-11).

Fig. 5. Pulse chase experiment: Lane 1. 50 μg of Mc. voltae total membrane protein was phosphorylated for 10 sec. Lane 2. Membranes were phosphorylated for 2 min. with [γ-^{32}P]ATP, cold 5 mM ATP was added and the 20 sec chase was terminated by the addition of TCA.

Fig. 6. Effect of Bafilomycin A_1 on ATPase activity. 100% activity of membrane-bound Mc. voltae ATPase = 0.5 μmol P_i/min/mg of protein. 100% activity of beet tonoplast ATPase = 0.75 μmol P_i/min/mg of protein.


Protein Concentration (mg/ml)
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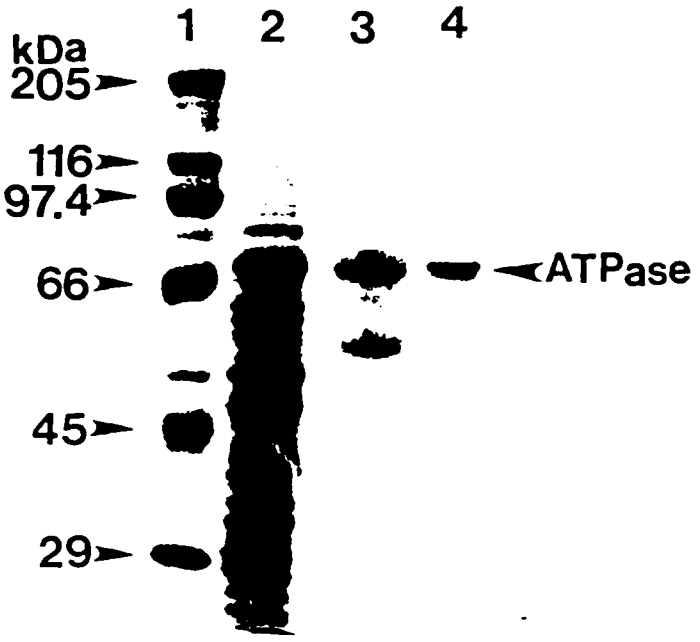
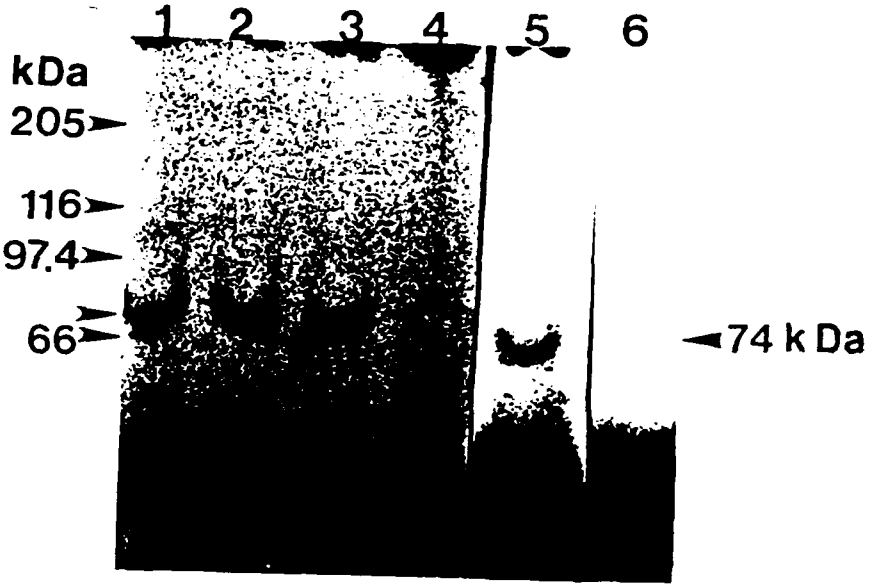
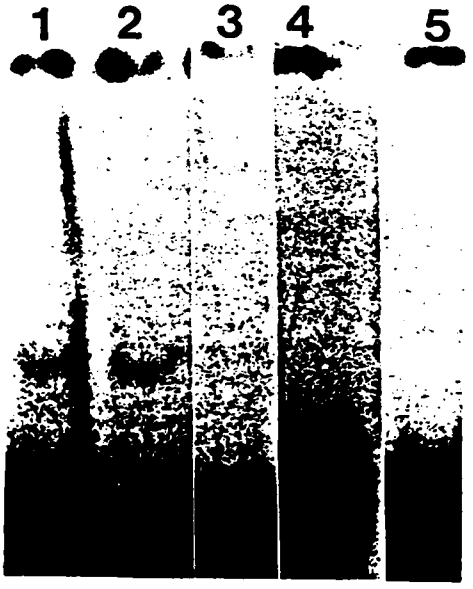
0.4
0.8
1.2
1.6
2.0
2.4
Fraction Number
0
10
20
30
40
50
60
70
80
0.0
0.1
0.2
0.3
0.4
0.5
KCl Concentration (M)
0.0
1.0
2.0
3.0
4.0
ATPase Activity (μmol. Pi/min)


1
2
3
4
kDa
205
116
97.4
66
45
29
ATPase


1
2
3
4
5
6
kDa
205
116
97.4
66
74 k Da


1
2
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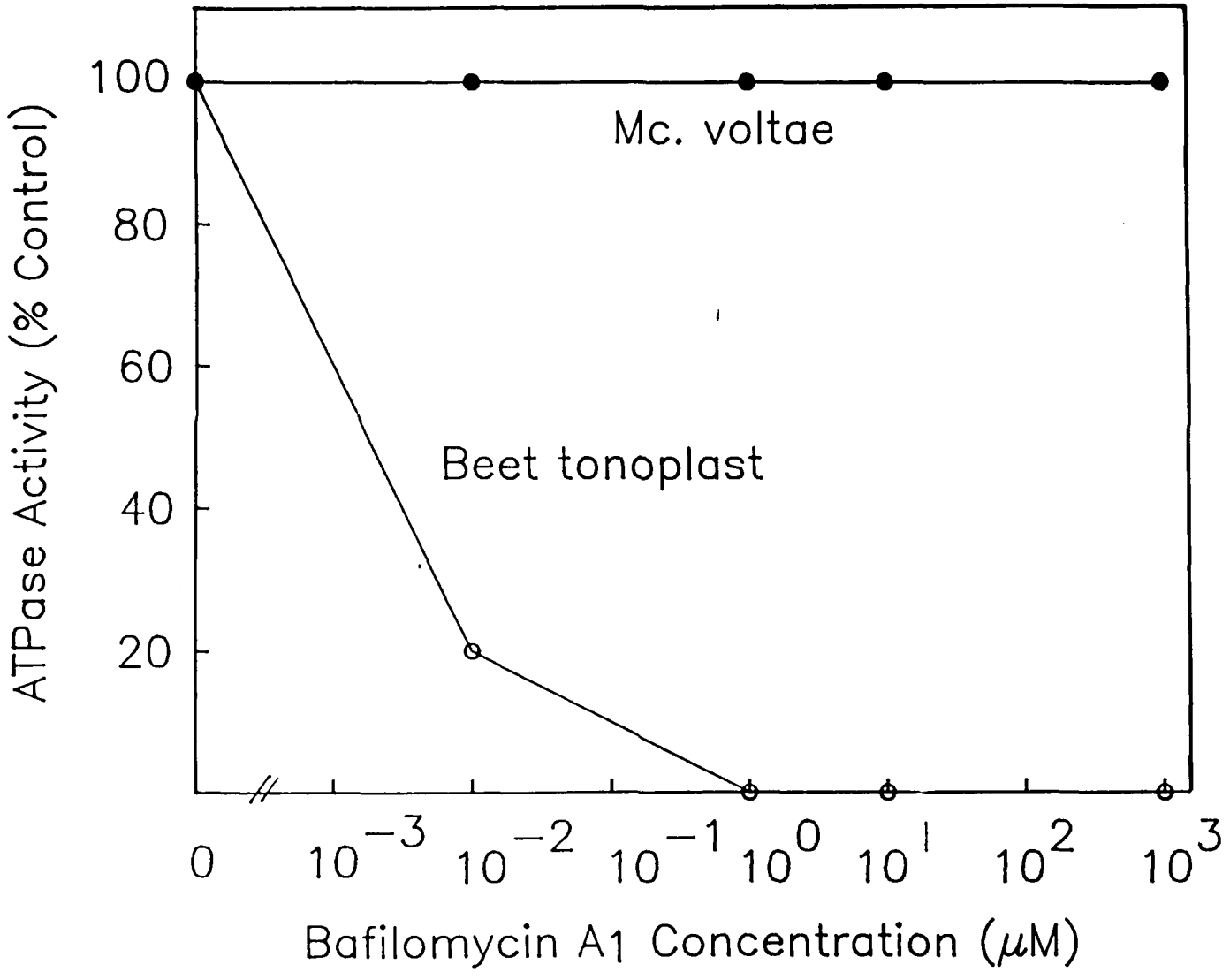
4
5


1
2


100
80
60
40
20
ATPase Activity (% Control)
Mc. voltae
Beet tonoplast
0
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
Bafilomycin A1 Concentration (μM)

Transport of Coenzyme M (2-mercaptoethanesulfonic acid) and Methyl coenzyme M ((2-methylthio)ethanesulfonic acid) in *Methanococcus voltae*

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A transport system for coenzyme M (2-mercaptoethanesulfonic acid, H-S-CoM), and methylcoenzyme M (2-(methylthio)ethanesulfonic acid, CH_3-S-CoM) in Methanococcus voltae required energy, showed saturation kinetics, and concentrated both forms of coenzyme M against a concentration gradient. Transport required hydrogen and carbon dioxide for maximal uptake. CH_3-S-CoM uptake was inhibited by N-ethylmaleimide and monensin. Both H-S-CoM and CH_3-S-CoM uptake showed sodium dependence. In wild type Mc. voltae H-S-CoM uptake was concentration dependent, with a V_{max} of 960 pmol/min/mg protein and an apparent K_m of 61 uM. Uptake of CH_3-S-CoM showed a V_{max} of 88 pmol/min/mg protein and a K_m of 53 uM. A mutant of Mc. voltae resistant to the coenzyme M analog 2-bromoethanesulfonic acid (BES) showed no uptake of CH_3-S-CoM but accumulated H-S-CoM at the wild type rate. Analysis of the intracellular coenzyme M pools in metabolizing cells showed an intracellular H-S-CoM concentration of 10.4 mM and CH_3-S-CoM concentration of 0.2 mM.

The methanogenic bacteria are strictly anaerobic archaebacteria which produce methane from H_2 and CO_2 and simple organic compounds such as formate, methanol,acetate and methylamines (23). CH_3-S-CoM (2-(methylthio)-ethanosulfonate) is the methyl donating substrate for the terminal reduction in methanogenesis (9,23). This reaction has been characterized using a highly purified methyl coenzyme M reductase from Methanobacterium thermoautotrophicum (strain Marburg) which has recently been found to catalyze the reduction of CH_3-S-CoM with H-S-HTP (mercaptoheptanoyl-O-phospho-L-threonine) to form CH_4 and CoM-S-S-HTP. The mixed disulphide is subsequently reduced to yield H-S-CoM (2-mercaptoethanesulfonic acid) and HS-HTP (7). While all examined methanogens have been found to contain coenzyme M (2), only the coenzyme M requiring rumen methanogen Methanobrevibacter ruminantium (formerly Methanobacterium ruminantium) has been shown to transport both HS-CoM and CH_3-S-CoM (3). HS-CoM was shown to be transported with an apparent K_m of 73 nM and a V_{max} of 312 pMol/min/mg dry weight, while uptake of CH_3-S-CoM had an apparent K_m of 50nM and a V_{max} of 320 pMol/min/mg dry weight (3). Uptake of coenzyme M at lower rates has been observed in two other methanogenic bacteria, Methanospirillum hungatii and Methanobacterium mobile, which show H-S-CoM uptake at 30% and 10% of the M. ruminantium rate, respectively (3).

The existence of a coenzyme M uptake system in Methanococcus voltae was suggested by analysis of 2-Bromoethanesulfonic acid

(BES) resistant mutants isolated in this laboratory (19). [^{35}S] BES was shown to be taken up by wild type, while uptake did not occur in BES resistant cells. Energy-dependant uptake of BES was found to be inhibited by addition of HS-CoM and CH_3-S-CoM, suggesting that these three coenzyme M derivatives were accumulated in Mc. voltae by a common carrier-mediated uptake system. To further characterize this uptake system, and to define further the phenotypic differences between BES^r and wild type Mc. voltae, the uptake of CH_3-S-CoM and H-S-CoM was studied. This analysis directly demonstrated an energy dependant-uptake system for coenzyme M derivatives in Mc. voltae, and provides further evidence that BES resistance in Mc. voltae is due to decreased uptake of this inhibitor into cells exhibiting the mutant phenotype.

Materials and Methods

Bacterial strains and growth conditions. Methanococcus voltae strain PS (DSM 1537) was grown in the defined media of Whitman (24) under a pressurized atmosphere of 80%H_2,20%CO_2 (v/v). Cultures were shaken at 80 rpm in 1 liter Wheaton bottles containing 200-300 ml of growth medium at 30°C. Methanobrevibacter ruminantium M1 (DSM 1033) was grown as described by Balch and Wolfe (3) in media supplemented with 2% rumen fluid and 630 nM HS-CoM under a pressurized atmosphere of 80%H_2,20%CO_2. The cultures were shaken at 100 rpm in 125 ml

serum vials which contained 20 ml of growth media at 42°C.

Anaerobic procedures. Anaerobic manipulations were performed in a Coy anaerobic glove box (Coy Laboratories, Ann Arbor, Michigan) with an atmosphere of 80%N_2:20%CO_2 supplemented with 3-5% H_2 (16). The oxygen level was monitored continuously with a Coy Gas Detector. Solutions were made anaerobic by flushing with N_2, 80%N_2:20%CO_2, or passage through the anaerobic interlock of the anaerobic glove box.

Synthesis of Ammonium 2-([^{3}H] methylthio)-ethanosulfonate. ^3H CH_3-S-CoM was synthesized according to the method of Gunsalus et al. (9) with the following modifications: 6.3 mMoles of 2-mercaptoethane sulfonic acid (H-S-CoM, sodium salt, Sigma) and 10 ml ammonium hydroxide (reagent grade) were sparged with N_2 for 30 minutes to render anaerobic. The ammonium hydroxide was anaerobically transferred to a flask containing the H-S-CoM. ^3H Iodomethane (4.9 Ci/mM, Amersham, Arlington Heights,Il), 5.1 umoles, was diluted into 6.295 mMoles iodomethane (Sigma) and transferred to the flask containing H-S-CoM in base. After incubation overnight under nitrogen in the dark the flask was vented to remove unreacted iodomethane and the product was evaporated to dryness. The ammonium salt was next dissolved in a minimal volume of water and recrystallized 5 times with acetone. The crystals were dissolved in water and passed through a Sephadex SP-C25-120 column (0.7x10 cm) and the second 1 ml

fraction to elute was lyophilized, dissolved in 200 ul water and column purified using a Whatman CF-11 cellulose acetate column (1x25 cm, acetone:water,16:3). The CH_3-S-CoM eluted as a sharp peak which co-migrated with non-labeled CH_3-S-CoM standards in two thin layer chromatography systems, acetone:water (16:3) and acetone:water:concentrated ammonium hydroxide (64:10:1). The thin layer sheets were dried and exposured to Kodak XR-5 film at -70°C. Label was determined to be 94% radiochemically pure. Specific activity of label as synthesized was 4 mCi/mmol.

Synthesis of [2-^{35}S]ammonium 2-mercaptoethanesulfonate. $H^{35}S$-CoM was synthesized by the procedure of Taylor and Wolfe (22) with the following modifications: 42 mg sodium 2-bromoethanesulfonate monohydrate (Sigma) was dissolved in 2 ml anaerobic ammonium hydroxide. This mixture was transferred anaerobically to a Douseal vial containing 9.25 mg [^{35}S] sodium sulphide nonahydrate (26 mCi/mmol; Amersham) and 38 mg unlabeled sodium sulphide (Sigma). The mixture was shaken for 3 h at 25°C in the dark. The vial was flushed with N_2 at 5 psi for 30 minutes to remove ammonium hydroxide and ammonium sulphide and the contents were transferred anaerobically to a teardrop flask filled with N_2 and then lyophilized. The product was dissolved in 1 ml water and crystallized with 15 ml acetone at 25°C. Crystals were harvested by centrifugation for 15 minutes at 5,000xg. The crystals were dissolved in 1 ml water and applied to a Whatman CF-11 cellulose acetate column (1.5x40 cm) equilibrated with acetone:water (16:3). H-S-CoM eluted as a

broad peak which was pooled and lyophilized. The product was 96% radiochemically pure as determined by thin layer chromatography in acetone:water (16:3) and acetone:water:concentrated ammonium hydroxide (64:10:1) followed by exposure to Kodak XR-5 film at-70°C. The specific activity of the label as synthesized was 5 mCi/mmol.

Measurement of H-S-CoM uptake. Cells were grown to mid-logarithmic growth phase (OD_{660}=0.3-0.5) and anaerobically harvested by centrifugation (15 minutes, 3,000xg, 15°C), washed and resuspended in anaerobic buffer containing 0.4 M sucrose, 100 mM HEPES, 10 mM KCl, 10 mM $MgCl_2$, pH=6.5 (11,19). Cells were resuspended to 0.6-0.8 mg protein/ml in 1.5 ml anaerobic transport buffer in serum vials and flushed with appropriate headspace gas, then pressurized to 30 psi. The redox potential of the suspensions was next lowered to that occuring under growth conditions by addition of Na_2S to a final concentration of 0.05% v/v. In low sodium assays, H_2S was used as the reducing agent (final concentration, 0.05%). Suspensions were equilibrated for 15 minutes at 25° C with shaking at 80 rpm. Assays were initiated by addition of labeled substrate. At each time point 1 ml of assay mixture was removed and rapidly diluted into 1 ml of reduced transport buffer containing 10 mM non-radioactive H-S-CoM, followed by vacuum filtration through Nucleopore PC 0.45 uM filter (Nucleopore, Pleasanton, CA). The filters were rinsed with 1 ml reduced transport buffer containing 10 mM non-radioactive H-S-CoM. Radioactivity was determined in a liquid

scintillation spectrometer.

Measurement of CH_3-S-CoM uptake in Methanococcus voltae.

To determine uptake, cells were grown, harvested and washed as described for H-S-CoM uptake experiments. Aliquots (5 ml) of cells at 0.4-0.6 mg protein/ml were transferred to 12.5 ml serum vials, flushed with desired headspace gas at 30 psi and reduced by addition of Na_2S solution to a final concentration of 0.05% (v/v). Cells were equilibrated at 30°C by shaking at 80 rpm for 15 minutes. Transport assays were initiated by addition of labeled substrate. At each time interval a 1.5 ml volume of headspace gas was removed, equilibrated to atmospheric pressure by expansion in a 5 ml syringe, and assayed for radioactive methane. Tritiated methane was detected by a liquid scintillation system (26) with the following modifications: a 7mM butyl rubber hypo vial septum (Pierce) was installed in a standard glass scintillation vial (25 ml) to allow gas injection. A 15ml aliquot of toluene-PPO scintillation cocktail was flushed for 1 minute at 5 psi with methane to achieve a constant partial pressure of methane above the liquid phase and insure uniform methane solubility in the scintillation fluid. Gas samples were added by injection, vials were shaken 5 minutes at 60 rpm at 25°C, and radioactivity was determined by scintillation counting. Uptake of ^3H CH_3-S-CoM was calculated from the rate of substrate conversion to radioactive methane. Counting efficiencies for ^3H CH_4 (27%) and ^{14}C CH_4 (56%) were determined. ^3H CH_3-S-CoM levels in cells were assayed by removal of a 1 ml aliquot of buffer at

each time point followed by rapid dilution of the sample into buffer containing 10mM CH_3-S-CoM followed by filtration over Millipore EH 0.4 uM or Nucleopore polycarbonate 0.4uM membrane filters. Filters were soaked overnight in Biofluor scintillation cocktail (NEN Research Products) before determining radioactivity.

Measurement of CH_3-S-CoM uptake by Methanobrevibacter ruminantium. Cells were grown to mid logarithmic phase (OD_{660nm}=0.4-0.8) and anaerobically harvested by centrifugation (15 minutes, 5,000xg,15° C). Cells were resuspended in coenzyme M free growth media (pH=7.1) (3,22) and 5 ml aliquots were dispensed into 12.5 ml serum vials. Transport was assayed and uptake calculated as described for Mc. voltae, except that cell suspensions were shaken at 120 rpm, 42°C (3).

Transport assays in low sodium. These assays were performed as described for standard assays except using a very low sodium buffer. This buffer is identical to standard transport buffer except for omission of NaCl and was prepared with deionized distilled water and stored in plastic vessels. Cell suspensions were reduced by addition of H_2S to a final concentration of 0.05% v/v. Sodium was added as anaerobic stock solutions of NaCl in assay buffer. The contaminating sodium level in this buffer was determined to be 0.14 uM by atomic absorption spectroscopy.

Determination of intracellular concentrations of H-S-CoM and CH_3-S-CoM in Mc. voltae. Mc. voltae was grown as described for transport assays and anaerobically harvested and washed once with transport buffer as described above. Cells were incubated under transport assay conditions for 15 minutes in 25 ml Balch tubes under an H_2:CO_2 atmosphere. Cells were pelleted at 3,000x g. 15 minutes, in a IEC HN-SII table top centrifuge while metabolizing H_2:CO_2 at 25° C. Cells were then resuspended in anaerobic distilled H_2O (40 ml/gram wet weight) and heated at 100°C for 15 minutes under a nitrogen atmosphere. The lysed cell suspension was then cooled and centrifuged twice anaerobically at 540,000x g at 4° C in a Beckman TL-100 ultracentrifuge. The supernatant was pooled and lyophilized anaerobically overnight. The dry supernatant, designated heat stable extract, was then analyzed for H-S-CoM and CH_3-S-CoM. H-S-CoM concentration was determined by dissolving the lyophilized supernatant in 400 ul anaerobic H_2O which was applied to a Whatman CF-11 cellulose acetate column (4x1.1 cm) equilibrated and developed with acetone:H_2O (16:3). H-S-CoM eluted as a broad peak, and was detected with Ellman reagent (8). Identity of Ellman positive material was confirmed by thin layer chromatography in acetone:H_2O:NH_4OH (64:10:1) and acetone:H_2O (16:3). The levels of CH_3-S-CoM in the heat stable extract was determined by following its conversion to CH_4 using cell free extracts prepared from M. thermoautotrophicum (10). The heat stable extract was dissolved in 50 mM phosphate buffer, pH 6.8, containing 10 mM $MgCl_2$ and 2mM ATP in calibrated vials.

Cell free extract (100 ul) was added to 500 ul of heat stable extract in the above buffer and the sealed vials were incubated for 90 minutes at 63°C under H_2 atmosphere to allow complete reduction of CH_3-S-CoM to methane. Methane was detected by gas chromatography as previously described (19), and the amount of CH_3-S-CoM present was calculated from the amount of methane formed. An average of 92% of added CH_3-S-CoM was converted to methane in this system as determined by conversion of ^3H CH_3-S-CoM to ^3H methane and by conversion of CH_3-S-CoM standards to methane.

Other analytical methods. Methanogenesis assays were performed on cells cultured, harvested and washed as described for CH_3-S-CoM uptake assays. Cell suspensions (2-3 mg protein) in anaerobic transport buffer were dispensed in calibrated vials and flushed with H_2:CO_2 for 1 minute. Methanogenesis rates were determined by gas chromatography as previously described (19). Protein was determined by modified Lowry assay (15) using bovine serum albumin as a standard. Concentrations of Na^+ and K^+ were determined by atomic absorption spectroscopy. [^3H] CH_4 standards were prepared by using a Methanobacterium thermoautotrophicum cell free extract (9,10) under H_2 atmosphere to reduce 500 nMol ^3H CH_3-S-CoM (specific activity 4 uCi/uM) to ^3H CH_4. 88% of substrate was converted to methane, yield= 443 nMol CH_4, specific activity equals 4 uCi/uM. [^{14}C] CH_4 standards (Amersham) were a generous gift of R.S. Wolfe.

Results

Kinetics of uptake of coenzyme M and methylcoenzyme M.

Uptake of [^{35}S] H-S-CoM was found to be linear for up to 10 minutes following addition of labeled substrate, while [^{3}H] CH_3-S-CoM uptake was linear for up to 45 minutes (Fig 1). Uptake of both H-S-CoM and CH_3-S-CoM was found to be concentration dependent and showed saturation kinetics in wild-type Mc. voltae. A linear least squares fit of a plot of substrate concentration/uptake velocity vs. substrate concentration (Hanes-Woolf plot) over a concentration range of 10 to 300 uM yielded an apparent K_m of 53 uM and V_{max} of 88 pmol/min/mg protein for CH_3-S-CoM (Fig. 2). A similar plot of H-S-CoM uptake data over a concentration range of 30 to 100 uM substrate showed a K_m of 61 uM and a V_{max} of 960 pmol/min/mg protein (Fig. 3).

The initial rate of H-S-CoM uptake was determined by a filtration assay as described in Materials and Methods. However, this method was not successful for examining the initial rate of CH_3-S-CoM uptake. While preliminary attempts to measure CH_3-S-CoM uptake by a filtration assay detected very little accumulation of CH_3-S-CoM, the ^{3}H CH_3-S-CoM in the assay buffer was found to be reduced rapidly to ^{3}H CH_4, which was detected by liquid scintillation counting (26). The rate of radioactive methane formation was found to be linear and dependent on concentration of cells and concentration of CH_3-S-CoM. We reasoned that the formation of ^{3}H methane was due to uptake of ^{3}H

CH_3-S-CoM followed by rapid reduction of the tritiated methyl group to tritiated methane. As we had been unable to detect an increase in the intracellular label concentration by a filtration assay similar to that used to assay H-S-CoM uptake, we concluded that the intracellular pool of tritiated CH_3-S-CoM was kept at a low and constant level. These observations led us to use the steady state assumption to determine the rate of CH_3-S-CoM uptake. The equation

$$^3H\ CH_3\text{-S-CoM}_{out} \xrightarrow{k_1} {}^3H\ CH_3\text{-S-CoM}_{in} \xrightarrow{k_2} {}^3H\ CH_{4out}$$

in which k_1 represents the rate of uptake and k_2 represents the rate of methanogenesis was used to derive the rate of uptake. The rate of methanogenesis in our assay buffer was found to be 13.5 nmol/min/mg protein with or without added CH_3-S-CoM (Table 3), which we assumed was at least two orders of magnitude greater than the rate of CH_3-S-CoM uptake. This assumption was considered reasonable as the rate of ^3H CH_3-S-CoM loss from the assay buffer was 23 pmol/min/mg protein when uptake was assayed at [CH_3-S-CoM] = 10 uM. We therefore concluded that the rate of ^3H methane formation, d[^3H] CH_4/dt is equal to the rate of ^3H CH_3-S-CoM uptake. To evaluate the CH_3-S-CoM conversion/methane formation assay, M. ruminantium M1, a rumen methanogen with an obligate growth requirement for coenzyme M (21) was assayed for uptake of CH_3-S-CoM. The rate of uptake (108 pMoles/min/mg dry wt, Fig. 4) was found to be sufficiently similar to the rate reported for CH_3-S-CoM uptake (80 pmol/min/mg dry wt) by Balch and Wolfe at this substrate concentration (10 uM) (3). This fact

verified the validity of this assay to determine CH_3-S-CoM uptake.

The initial rate of HS-CoM uptake was determined to be 260 pmol/min/mg protein. Since the average number of cells per mg protein was 1.23×10^{10} cells/mg as determined by Petroff-Hausser chamber count, the observed uptake rate corresponds to an average rate of 210 molecules of H-S-CoM taken up per cell per second at a substrate concentration of 30 uM. Similarly the rate of CH_3-S-CoM uptake at 30 uM substrate (32 pmol/min/mg) was calculated to correspond to 25 molecules of CH_3-S-CoM per cell per second. For comparison, the rate of BES uptake in Mc. voltae at 10 uM substrate was reported to be 5.7 pmol/min/mg in wild type cells (19), corresponding to 5 molecules of BES taken up per cell per second. Uptake of CH_3-S-CoM by Mc. voltae required a gas phase of H_2 and CO_2 for maximal transport. Cells assayed under H_2 alone showed a 32% lower rate of uptake, and cells assayed under N_2:CO_2 of air showed no uptake activity. H-S-CoM uptake also required H_2:CO_2, and cells showed no significant uptake under N_2:CO_2 or air. Uptake of CH_3-S-CoM was completly inhibited by 1 mM N-ethylmaleimide, suggesting that uptake is carrier-mediated.

Inhibition of CH_3-S-CoM uptake by H-S-CoM and Bromoethane-sulfonate. The effect of HS-CoM and BES on CH_3-S-CoM uptake was examined. As can be seen, uptake of CH_3-S-CoM was reduced with increasing molar ratios of both HS-CoM and BES (Table 1). These

results together with the observation that BES uptake is inhibited by both CH_3-S-CoM and H-S-CoM (19) suggests that these three compounds are transported by a common uptake system.

Role of sodium in uptake and methanogenesis. Sodium has been demonstrated to play important roles in bioenergetics and transport in Mc. voltae (6,11,12). To examine a possible role of Na^+ in uptake of coenzyme M derivatives, uptake was monitored in low sodium anaerobic buffer. Both the uptake rate and the rate of methanogenesis decreased as the sodium concentration was decreased (Table 2 and 3).

Uptake of CH_3-S-CoM was blocked and uptake of H-S-CoM was reduced 42% by 20 uM monensin, a compound which mediates Na^+/H^+ exchange across membranes and collapses sodium gradients (17,18). Monensin (20 uM) also blocked methanogenesis from H_2:CO_2. From these data it is clear any role of sodium in uptake of CH_3-S-CoM cannot be separated from its requirement in methanogenesis. While the reduction in H-S-CoM uptake observed in low sodium buffer also might suggest a direct role of sodium ions in uptake, we cannot rule out that these results derive indirectly from the concomitant reduction in methanogenesis, causing a decrease in cellular energy levels coupled to methanogenesis.

Composition of intracellular coenzyme M pools. To determine if accumulation of H-S-CoM and CH_3-S-CoM occured against a concentration gradient, we analyzed the composition of intracellular coenzyme M pools. From the total amount of H-S-CoM

extracted from metabolizing cells the intracellular concentration of H-S-CoM was determined to be 10.4 mmolar +/- 0.93 mmolar (n=3). This was based on an average of 14.3 nmol H-S-CoM per mg dry weight, and a volume of 1.37 ul/mg dry weight (11).

To determine the intracellular concentration of CH_3-S-CoM in Mc. voltae, we determined the capacity of a heat stable extract (HSE) to serve as substrate for the CH_3-S-CoM dependent formation of CH_4 by cell free extracts (CFE) of M. thermoautotrophicum under H_2. CH_3-S-CoM is heat stable and is therefore not destroyed by heating to 100 °C under 100% N_2. When the Mc. voltae extract was assayed in this way, methane production required H_2, was a function of the amount of heat stable extract added and was not stimulated by H-S-CoM (Table 4). The amount of methane produced from the added heat stable extract was significantly greater than the background amount of methane produced from the small amount of CH_3-S-CoM present in M. thermoautotrophicum CFE. Using this assay we determined the intracellular concentration of CH_3-S-CoM to be 0.20 mmolar +/- 0.03 mmolar (n=3). This was calculated from an average of 0.26 nmoles methane formed/mg dry weight of Mc. voltae extracted (Table 4).

Uptake of coenzyme M derivatives by BES resistant M. voltae. Since we had previously determined that two BES-resistant mutants of Mc. voltae showed decreased ability to take up BES (19), it was of interest to determine the capacity of a

BES-resistant mutant to transport H-S-CoM and CH_3-S-CoM. As can be seen in Fig. 5, the mutant tested (BES^r-1) did not show transport of CH_3-S-CoM, but did transport H-S-CoM at approximately wild-type rates (Fig 6).

Discussion

Uptake of CH_3-S-CoM and H-S-CoM by Mc. voltae shows characteristics of a carrier-mediated, energy dependent process. H-S-CoM, CH_3-S-CoM and BES were accumulated at different rates, the relative rates of uptake were H-S-CoM > CH_3-S-CoM > BES. Based on our determination of intracellular levels of H-S-CoM and CH_3-S-CoM, in a typical transport assay with 30 uM substrate, transport is active, occuring against a H-S-CoM concentration gradient of 347:1 and a CH_3-S-CoM concentration gradient of 7:1 (intracellular:extracellular concentration). While the concentration of H-S-CoM in cells increases from 10.4 mM to 11.4 mM in 15 minutes under our assay conditions, uptake of CH_3-S-CoM probably did not result in an increase of the intracellular CH_3-S-CoM pool as methanogenesis rapidly converts the CH_3-S-CoM to CH_4 and H-S-CoM. Mc. voltae has also been shown to accumulate BES against a concentration gradient (19).

The observation that transported CH_3-S-CoM equilibrated with the intracellular CH_3-S-CoM pool as substrate for the methylreductase complex suggests that chemical modification coupled to an energy consuming group translocation is not the mechanism of transport. In M. ruminantium transported H-S-CoM

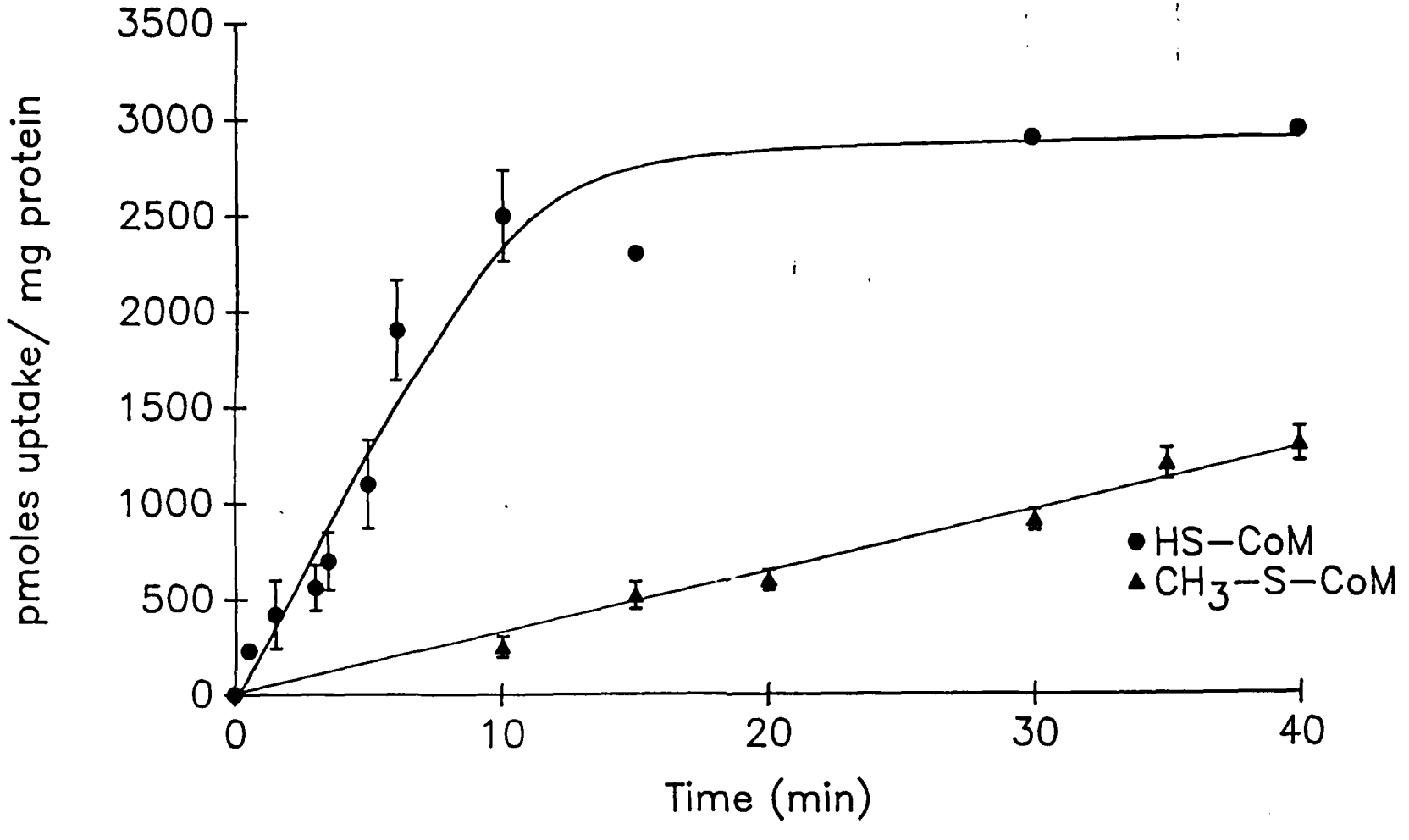
and CH_3-S-CoM could be detected intracellularly in unmodified form, with CH_3-S-CoM as the major stable intracellular form of transported labeled coenzyme M (3). In Mc. voltae the composition of coenzyme M pools were examined by detecting coenzyme M synthesized by the cells under metabolizing conditions instead of examining the composition of transported coenzyme M pools. We found H-S-CoM to be the major component of the intracellular pool (10 mM) with CH_3-S-CoM present at lower levels (0.2 mM).

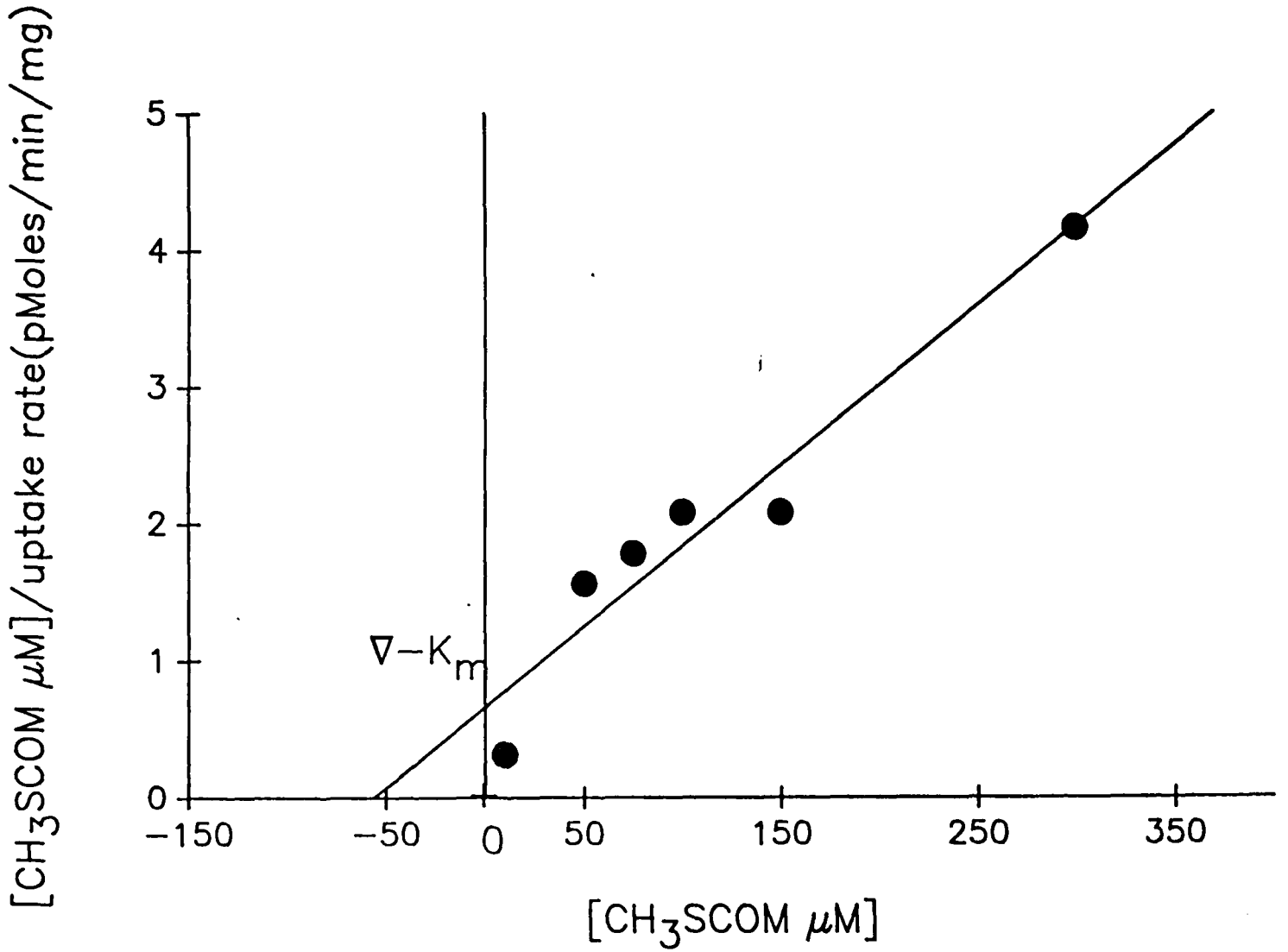
Sodium has been shown to have an important role in bioenergetics and transport of amino acids (6,11,12) in Mc. voltae. BES uptake has been shown to be slightly stimulated by increasing Na^+ concentration (19). We examined the role of Na^+ in uptake of H-S-CoM and CH_3-S-CoM by using a very low sodium transport assay. Using this procedure we were able to show a sodium dependence of coenzyme M uptake, in agreement with the inhibition of uptake observed in the presence of monensin. These results raise the possibility that the uptake of coenzyme M derivatives may be coupled mechanistically to a sodium gradient. However, we feel that a determination of the exact basis for the observed Na^+ dependence will require the development of a responsive membrane vesicle system. Such a system would allow us to distinguish between effects of sodium ions on uptake of coenzyme M per se and effects of Na^+ on methanogenesis and cellular metabolism.

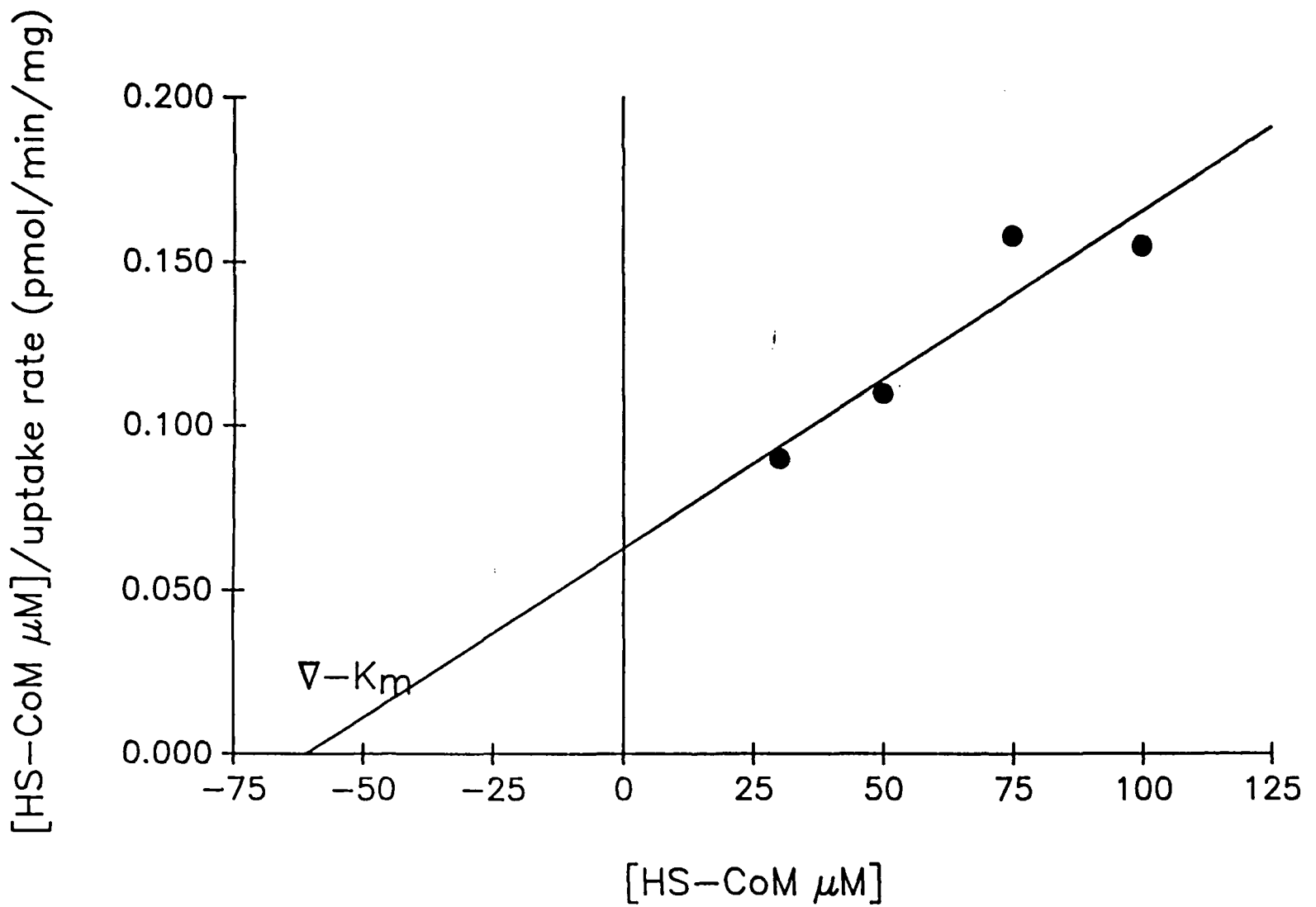
The physiological significance of coenzyme M uptake under

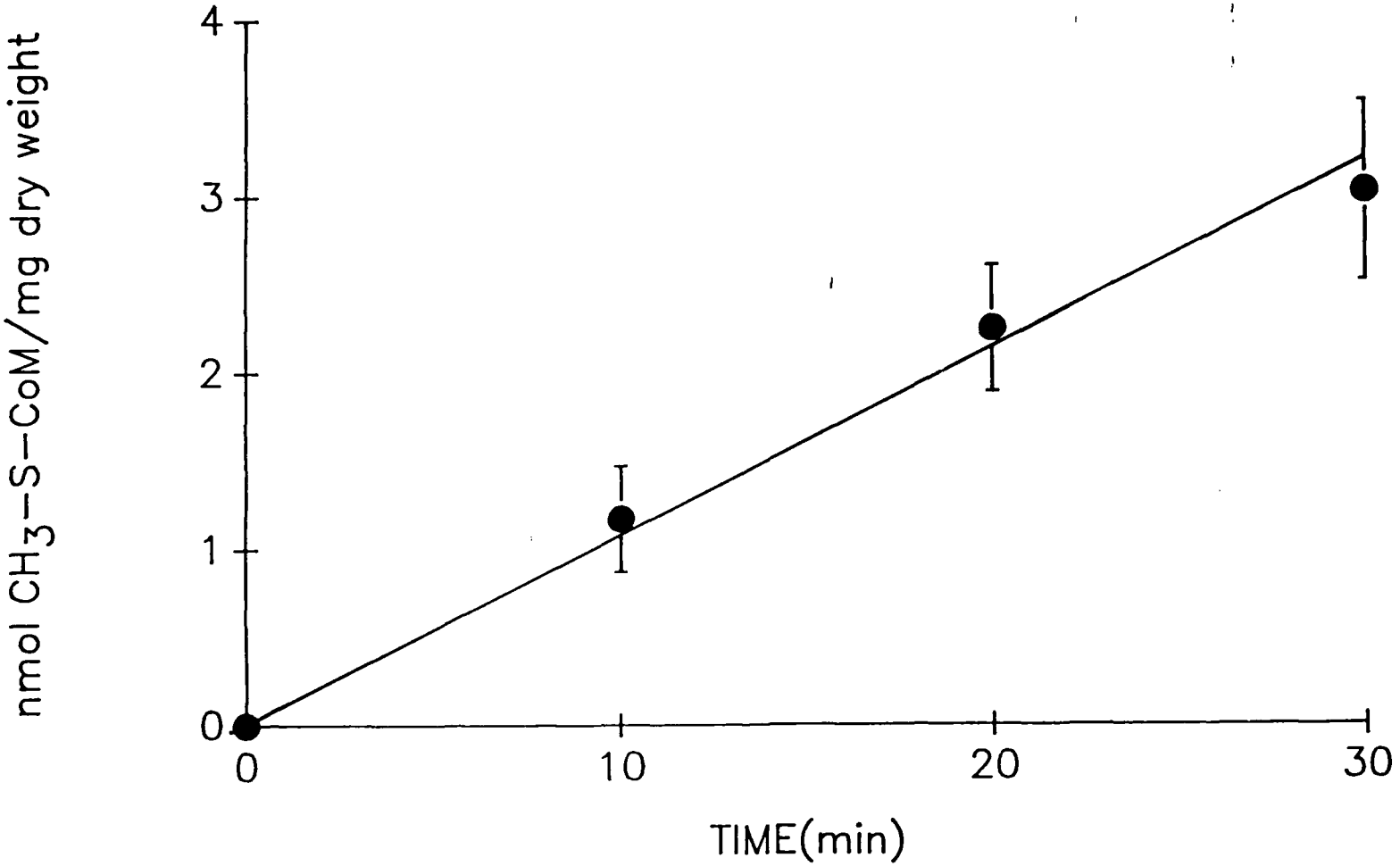
natural conditions is unknown. Autolysis of Mc. voltae occurs under laboratory conditions, and given osmotic and temperature fluctuations autolysis probably occurs in its natural estuarian environment. The coenzyme M transport system would allow this organism to take advantage of locally concentrated pools of coenzyme M released by cell lysis.

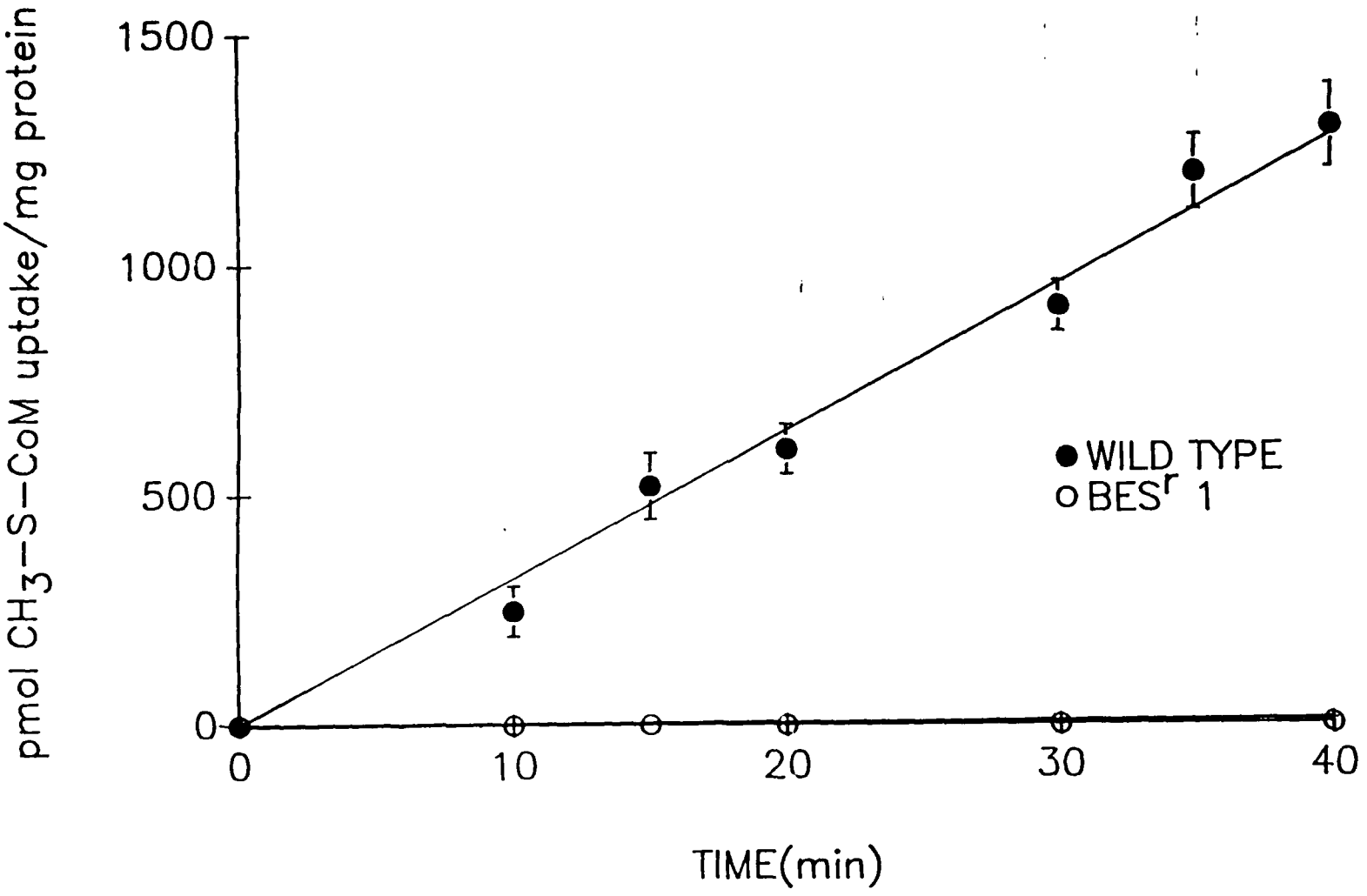
The uptake of coenzyme M may also provide a sulfur source for Mc. voltae in its natural environment. Coenzyme M has the highest percentage of sulfur (45%) of organic coenzymes (25) and has been detected in marine sediments (16). Mc. voltae has been found to excrete methionine (20) which has been suggested to be an important intermediate in the formation of organic thiols in marine sediments (16). It is possible that Mc. voltae may accumulate and use organic thiols as sulfur sources, and perhaps coenzyme M is transported by a system which in nature accumulates this or some other organic thiol. Further work is needed to determine if coenzyme M and other organic thiols found in marine sediments are involved in natural sulfur cycles.


3500
3000
2500
2000
1500
1000
500
0
pmoles uptake/ mg protein
0
10
20
30
40
Time (min)
HS–CoM
CH3–S–CoM


[CH3SCOM μM]/uptake rate(pMoles/min/mg)
5
4
3
2
1
0
−150
−50
0
50
150
250
350
∇−Km
[CH3SCOM μM]


[HS–CoM μM]/uptake rate (pmol/min/mg)
0.200
0.150
0.100
0.050
0.000
∇–Km
–75
–50
–25
0
25
50
75
100
125
[HS–CoM μM]


nmol CH_3–S–CoM/mg dry weight
4
3
2
1
0
0
10
20
30
TIME(min)


pmol CH_3—S—CoM uptake/mg protein
1500
1000
500
0
0
10
20
30
40
TIME(min)
WILD TYPE
BES^r 1

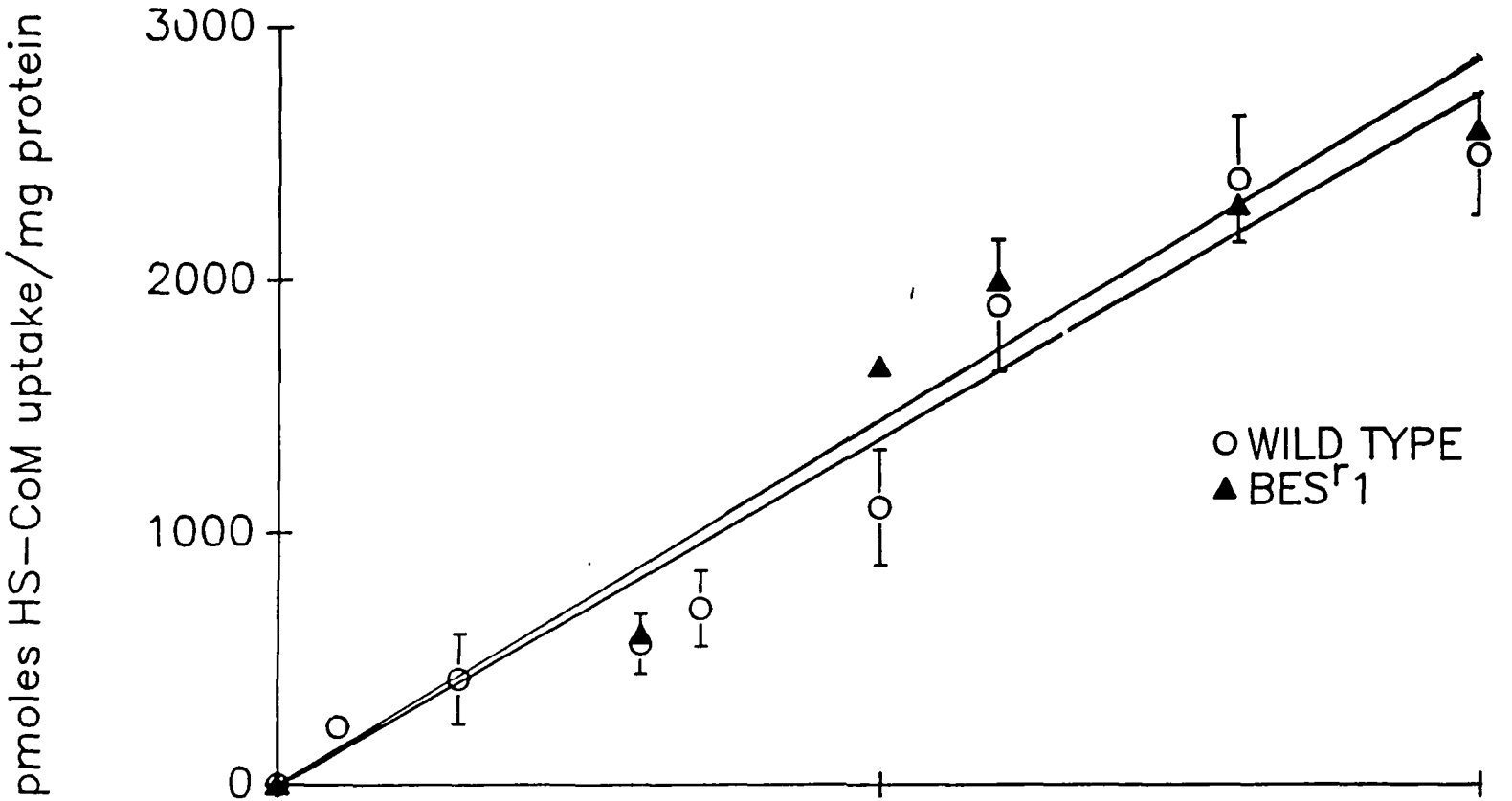

3000
2000
1000
0
0
5
10
pmoles HS–CoM uptake/mg protein
TIME (min)
WILD TYPE
BES^r1

Fig. 1. Time course of H-S-CoM and CH_3-S-CoM uptake. Uptake was assayed under H_2:CO_2 at 30°C and the concentration of substrates was 30 uM. Time points without error bars are averages of duplicate assays, all other points are averages of 6 assays showing standard deviation. Uptake determined as described in Materials and Methods.

Fig. 2. Determination of K_m and V_{max} for CH_3-S-CoM uptake in wild type Mc. voltae. Uptake rates werecalculated from the amount of label transported in 30 minutes under H_2:CO_2 atmosphere in standard transport buffer at 30 °C. Points represents average of 4 assays. K_ms determined in separate assays ranged from 48 to 53 uM. The slope of the line is $1/V_{max}$.

Fig. 3. Determination of K_m and V_{max} for H-S-CoM uptake in wild type Mc. voltae. Uptake rates were calculated from the amount of label accumulated in 10 minutes under H_2:CO_2 in standard transport buffer at 30°C. Points represent average of 4 assays. K_ms in separate assays ranged from 60 to 63 uM. The slope of the line is $1/V_{max}$.

Fig. 4. Uptake of CH_3-S-CoM by M. ruminantium M1 assayed under H_2:CO_2 at 42°C. The concentration of the substrate was 10 uM. Uptake was calculated from the rate of conversion of labeled CH_3-S-CoM to methane as described in Materials and Methods.

Fig. 5. Uptake of CH_3-S-CoM by BES^r 1 and wild type Mc. voltae assayed under H_2:CO_2 at 30°C. The concentration of the substrate was 30 uM. Time points without error bars represent averages of duplicate assays. Uptake was calculated as described in Materials and Methods.

Fig. 6. Uptake of H-S-CoM by BES^r 1 and wild type Mc. voltae assayed under H_2:CO_2 at 30°C. The concentration of substrate was 30 uM. Time points for BES^r 1 represent averages of duplicate assays. Uptake was determined by the rapid dilution/ filtration assay as described in Materials and Methods.

TABLE 1. Effect of coenzyme M derivatives on CH_3-S-CoM uptake[a]

Addition	concentration	% uptake[b]
H-S-CoM	10uM	56
H-S-CoM	50uM	36
H-S-CoM	100uM	29
BES	10uM	63
BES	50uM	41

[a] Cells were grown and harvested as described in Materials and Methods and incubated with the indicated inhibitor for 5 minutes under H_2:CO_2 prior to addition of 10 uM CH_3-S-CoM.

[b] Control uptake is 26 pmol/min/mg cellular protein. Values are averages of 4 assays and were calculated from uptake over 30 minutes.

TABLE 2. Effect of sodium concentration on CH_3-S-CoM and H-S-CoM uptake

	reductant	sodium	% uptake[a]
CH_3-S-CoM			
	Na_2S	4mM	100
	H_2S	4mM	100
	H_2S	70uM	44
	H_2S	40uM	9
	H_2S	0.1uM	0
H-S-CoM			
	Na_2S	4mM	100
	H_2S	32uM	26

Reductant was added to 0.05% v/v. Cells were incubated for 15 minutes, 30°C prior to addition of substrate to 30 uM.

[a] Control uptake= 32 pmol/min/mg cellular protein (CH_3-S-CoM), 260 pmol/min/mg H-S-CoM.

TABLE 3. Effect of sodium concentration and CH_3-S-CoM concentration on methanogenesis from H_2:CO_2

condition	rate of methanogenesis (nmol/min/mg)
400 mM NaCl	173
4 mM Na^+	13.5
4 mM Na^+, 300 uM CH_3-S-CoM	13.5
660 uM Na^+	4.5
132 uM Na^+	2.0
0.14 uM Na^+	0.0

Cell suspensions were reduced with H_2S (0.05% v/v) and incubated under H_2:CO_2 prior to assaying methane formation. Values are averages of three trials.

Table 4. Stimulation of methane formation by addition of Mc. voltae heat stable extract

Heat stable extract (mg cells extracted)	Additions: CH_3-S-CoM (nmol)	Additions: HS-CoM (nmol)	nmol CH_4[a]	[CH_3-S-CoM][b]
-	-	-	1.11	-
5	-	-	2.21	0.17
10	-	-	3.89	0.21
16	-	-	5.56	0.21
-	1.0	-	1.98	-
-	2.0	-	3.09	-
-	-	2.0	0.96	-
-	-	20.0	1.01	-

[a] nmoles methane formed in 90 minutes at 63°C under 100% H_2. Methane formation required H_2 and M. thermoautotrophicum cell free extract.

[b] concentration of CH_3-S-CoM calculated by the following formula:

$$\frac{(\text{nmol } CH_4 \text{ formed} - \text{background } CH_4)(1.37 \text{ ul/mg dry weight})^{-1}\ (0.32)^{-1}}{\text{mg dry weight extracted}} = [CH_3\text{-S-CoM}]\ (\text{mM})$$

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