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N^5-Methyl-Tetrahydromethanopterin:Coenzyme M Methyltransferase of *Methanosarcina* Strain Göl Is an Na^+-Translocating Membrane Protein

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To determine the cellular localization of components of the methyltransferase system, we separated cell extracts of *Methanosarcina* strain Göl into cytoplasmic and inverted-vesicle fractions. Measurements demonstrated that 83% of the methylene-tetrahydromethanopterin reductase activity resided in the cytoplasm whereas 88% of the methyl-tetrahydromethanopterin:coenzyme M methyltransferase (methyltransferase) was associated with the vesicles. The activity of the methyltransferase was stimulated 4.6-fold by ATP and 10-fold by ATP plus a reducing agent [e.g., Ti(III)]. In addition, methyltransferase activity depended on the presence of Na^+ (apparent K_m = 0.7 mM) and Na^+ was pumped into the lumen of the vesicles in the course of methyl transfer from methyl-tetrahydromethanopterin not only to coenzyme M but also to hydroxycobalamin. Both methyl transfer reactions were inhibited by 1-iodopropane and reconstituted by illumination. A model for the methyl transfer reactions is presented.

In methanogenic bacteria the reduction of $H_2 + CO_2$ to CH_4 is coupled with the generation of primary electrochemical proton and sodium gradients, which in turn are used for various energy-dependent reactions (3, 17). The location of the primary proton pump was identified (8), but the extrusion of Na^+ could not be assigned to one single enzymatic reaction. However, it was shown that it is coupled to the conversion of formaldehyde (via methylene-tetrahydromethanopterin [H_4MPT]) to the formal redox level of methanol, e.g., to methyl-coenzyme M (2-mercaptoethanesulfonate) (CoM-SH) (11, 18). This conversion is catalyzed by two enzymes, methylene-H_4MPT reductase and methyl-H_4MPT:CoM-SH methyltransferase (12, 26). Because of the observed Na^+ translocation, the responsible enzyme must be membrane associated. There is no evidence for such a membrane association of methylene-H_4MPT reductase. This enzyme was purified from the soluble fraction of cell extracts of several methanogenic bacteria (15, 16, 25). Its localization in the cytoplasm and the reversibility of the reaction catalyzed, as well as the low $\Delta G^{\circ\prime}$ value of the reaction, were taken as an indication against a possible function in energy conservation (26).

Comparatively little is known about the methyltransferase. Studies with *Methanobacterium thermoautotrophicum* by Sauer (21) are consistent with the location of the enzyme in the membrane. However, Kengen et al. (14) reported on a soluble enzyme in *Methanobacterium thermoautotrophicum* ΔH. Recently these investigators isolated a membrane-bound methyltransferase from the same organism (13), but a possible Na^+ dependence of the activity of this enzyme was not tested. Stupperich et al. (23) provided immunological evidence that the corrinoid protein discovered by Schulz and Fuchs (22) in membranes of methanogenic bacteria is identical with the methyltransferase of *Methanobacterium thermoautotrophicum*.

Using inverted-vesicle preparations of *Methanosarcina* strain Göl, we showed recently that the methyl transfer from methyl-H_4MPT to CoM-SH is associated with Na^+ translocation into the lumen of the vesicles and that methyl-H_4MPT could be replaced by methyl-tetrahydrofolate (H_4F) as the methyl group donor (2). We have now investigated the location of this enzyme in *Methanosarcina* strain Göl. We show that it is membrane bound and that its activity is Na^+ dependent and accompanied by sodium ion translocation in a corrinoid-dependent reaction.

MATERIALS AND METHODS

Organism and cultivation. *Methanosarcina* strain Göl (DSM 3647) was obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen and grown under strictly anaerobic conditions on the medium described by Hippe et al. (10) and supplemented with 1 g of sodium acetate per liter.

Preparation of washed inverted vesicles. Cells of *Methanosarcina* strain Göl were harvested in the late logarithmic growth phase by centrifugation in anaerobic 400-ml airtight bottles (20 min at 12,000 × *g*) and washed once with 40 mM potassium phosphate (pH 6.9) containing 20 mM $MgSO_4$, 0.1 M sucrose, 5 mM dithioerythritol, and 1 mg of resazurin per liter. The pellet was then resuspended in the same buffer but with 0.4 M sucrose instead of 0.1 M sucrose. This cell suspension was treated with pronase (2.5 mg/liter of cell culture) at 37°C for 7 to 10 min. The reaction was stopped with phenylmethylsulfonyl fluoride (final concentration, 100 μM), and the suspension was cooled on ice. The protoplasts were centrifuged at 27,000 × *g* for 15 min at 4°C, suspended in the same buffer supplemented with phenylmethylsulfonyl fluoride (final concentration, 100 μM) and a few crystals of DNase, and then passed through a French pressure cell at 65 MPa. The unbroken protoplasts and cell debris were removed by centrifugation at 6,000 × *g* for 30 min. The vesicle preparation was washed by ultracentrifugation at 120,000 × *g* for 1 h at 4°C. The sedimented material was diluted with the same potassium phosphate buffer and centrifuged at 38,000 × *g* for 30 min. This washing procedure was repeated once or twice, depending on the conditions.

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Assay conditions. The assays were performed at 37°C in 2.3-ml glass vials filled with 40 mM potassium phosphate buffer (pH 6.9) containing 20 mM $MgSO_4$, 0.4 M sucrose, 5 mM dithioerythritol, 5 mM potassium 2-bromoethanesulfonate, and 1 mg of resazurin per liter under an atmosphere of molecular hydrogen. Further additions as described for each experiment were made anaerobically by syringe.

Enzyme assays. All enzyme assays were performed by using 40 mM potassium phosphate (pH 6.9) containing 20 mM $MgSO_4$, 0.4 M sucrose, 5 mM potassium 2-bromoethane sulfonate, and 1 mM dithioerythritol. To monitor the activity of methyl-CoM formation from formaldehyde + H_2 + CoM-SH, of methyl-H_4MPT:CoM-SH methyltransferase, and of 5-hydroxybenzimidazolylcobamide (methyl-B_{12}-HBI):CoM-SH methyltransferase, vesicle suspensions were incubated at 37°C and 5-μl portions were withdrawn and analyzed for thiol groups with Ellman's reagent as described previously (9). CH_3-H_4F and methyl-5,6-dimethylbenzimidazolylcobamide (CH_3-B_{12}) were used as the methyl group donors for the methyl-H_4MPT:CoM-SH methyltransferase and the CH_3-B_{12}HBI:CoM-SH methyltransferase, respectively, at the concentrations described in the figure legends.

Methylene-H_4MPT reductase and the coenzyme F_{420}-dependent hydrogenase were assayed at room temperature in 1.7-ml glass cuvettes gassed with N_2 or H_2 and filled with 1 ml of anaerobic 40 mM potassium phosphate buffer (pH 6.9) containing 20 mM $MgSO_4$, 0.4 M sucrose, 5 mM dithioerythritol, and 20 μM $F_{420}H_2$ or 20 μM F_{420}. The methylene-H_4MPT reductase activity was determined by monitoring the methylene-H_4MPT-dependent oxidation of $F_{420}H_2$ spectrophotometrically at 420 nm (ε_{420} = 32 $mM^{-1} \cdot cm^{-1}$ at pH 7.0); the reaction was started by addition of formaldehyde. The F_{420}-dependent hydrogenase was measured after the reduction of F_{420} at 420 nm with molecular hydrogen as the reductant.

Preparation of H_4MPT, F_{420} and $F_{420}H_2$. F_{420} [the (*N*-L-lactyl-γ-L-glutamyl)-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin 5′-phosphate] was isolated from *Methanosarcina barkeri* and reduced to $F_{420}H_2$ with $NaBH_4$ as described previously (7). H_4MPT was purified anaerobically in a glove box as described previously (4).

Measurement of sodium transport. The experiments to measure sodium transport were done as described previously (2) with 2 mM $^{22}Na^+$.

Extraction and quantification of corrinoids. Corrinoids were extracted from vesicles of *Methanosarcina* strain Gö1 after three washes and thermal denaturation of the membranes, and the total corrinoid content in this fraction was quantified after conversion to the dicyano form. Therefore, 0.02% (wt/vol) KCN was added to the vesicle fraction in 40 mM potassium phosphate buffer (pH 6.9), and the mixture was incubated in the dark for 30 min at room temperature. The pH was adjusted to 4 with acetic acid, and the suspension was then incubated at 100°C for 20 min. After the mixture had been cooled to room temperature, 4 to 5 mg KCN was added, the pH was adjusted to 10 to 11 with KOH, and this solution was incubated for 30 min in the dark. The denatured proteins were pelleted by ultracentrifugation at 100,000 × *g* for 1 h. Thereafter, the supernatant was concentrated by adsorption chromatography on XAD-4 (24). The corrinoid-containing fractions were combined and concentrated by flash evaporation. For spectrophotometric quantification, an ε_{580} at pH > 9 of 10.2 × 10^3 $M^{-1} \cdot cm^{-1}$ for dicyanocobalamin was used (20).

Chemicals, gases, and radioisotopes. All chemicals were reagent grade and were purchased from Merck AG, Darmstadt, Germany. ATP, 1-iodopropane, methyl-tetrahydrofolate, and hydroxycobalamin were obtained from Sigma Chemical Co., Deisenhofen, Germany SF6847 (3,5-di-*tert*-butyl-4-hydroxybenzylidenemalonitrile) was from Wako Chemicals GmbH, Neuss, Germany. Potassium 2-bromoethanesulfonate was from Fluka, Buchs, Switzerland. $^{22}NaCl$ (carrier free) was from New England Nuclear Corp., Dreieich, Germany. Gases were from Messer Griesheim, Kassel, Germany.

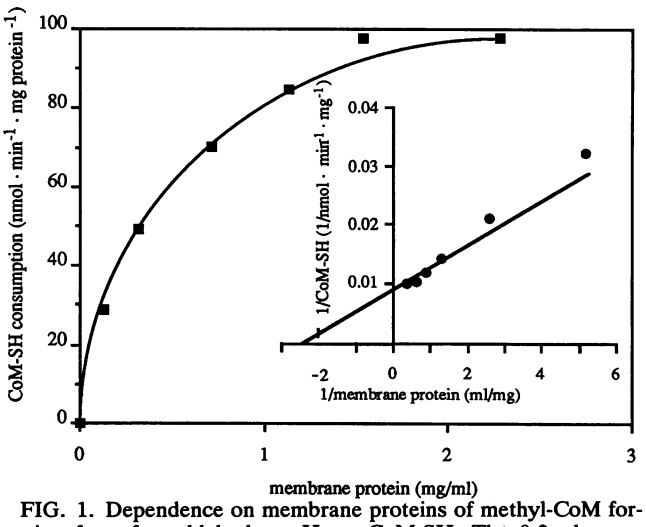

FIG. 1. Dependence on membrane proteins of methyl-CoM formation from formaldehyde + H_2 + CoM-SH. The 0.2-ml assay mixture contained 2.5 mM ATP, 4 mM CoM-SH, 0.94 mg of cytoplasmatic protein, and increasing amounts of washed vesicles from *Methanosarcina* strain Gö1. The reaction was started by the addition of formaldehyde (final concentration, 4 mM).

RESULTS

Distribution of enzymes involved in the formation of methyl-CoM from formaldehyde + H_2 + CoM-SH. In the presence of 2-bromoethanesulfonate, crude vesicle preparations of *Methanosarcina* strain Gö1 converted HCHO + H_2 + CoM-SH to methyl-CoM at a rate of 80 to 100 nmol · min^{-1} · mg of $protein^{-1}$. When these vesicles were separated into washed membranes and cytoplasm, the membranes still catalyzed methyl-CoM formation from formaldehyde + H_2 + CoM-SH; this activity was strictly dependent on H_4MPT and F_{420} in the buffer system, which is in accordance with the involvement of the F_{420}-dependent hydrogenase and the F_{420}-dependent methylene-H_4MPT reductase in this reaction sequence. On the other hand, cytoplasm alone did not catalyze the reaction but required the addition of membranes (Fig. 1).

The locations of the three enzymes involved in this reaction sequence were determined. We found that 98.1 and 83.4% of the F_{420}-dependent hydrogenase and the F_{420}-dependent methylene-H_4MPT reductase, respectively, were located in the cytoplasmic fraction (Table 1). Only the methyltransferase was associated with the membrane fraction. Washing the membranes three times led to an enrichment of the methyltransferase by a factor of 17, from 16 nmol · min^{-1} · mg of $protein^{-1}$ in crude vesicles to 268 nmol · min^{-1} · mg of $protein^{-1}$. The activity of methyl-H_4MPT:CoM-SH methyltransferase was routinely measured with CH_3-H_4F as the methyl group donor, because methyl-

TABLE 1. Distribution of enzymes involved in the formation of methyl-CoM from formaldehyde + H_2 + CoM-SH[a]

Enzyme	Amt of enzymes found in:					
	Crude vesicle preparation		Membranes		Cytoplasm	
	U_{tot}	mU/mg	U_{tot} (%)	mU/mg	U_{tot} (%)	mU/mg
F_{420}-dependent hydrogenase	66.8	108.6	1.2 (1.9)	48.8	60.5 (98.1)	108.1
Methylene-H_4MPT reductase	25.5	41.4	3.8 (16.6)	154.5	19.1 (83.4)	34.1
Methyl-H_4MPT:CoM-SH methyltransferase	9.9	16.1	6.6 (88.0)	268.3	0.9 (12.0)	1.6
Methyl-B_{12}-HBI:CoM-SH methyltransferase	697.6	1,134.3	15.0 (2.3)	609.8	650.8 (97.7)	1,162.8

[a] The enzyme activities were measured with crude vesicle preparation, vesicles that had been washed three times (referred to as membranes), and the supernatant of the first ultracentrifugation (referred to as cytoplasm) as described in Materials and Methods. The concentrations of HCHO, CH_3-H_4F, and CH_3-B_{12} in the assay were 6 mM each, whereas the concentration of H_4MPT was 40 μM.

H_4MPT is not commercially available; the specific activities given represent 60% of those determined with methyl-H_4MPT (2).

Methyl-B_{12}-HBI:CoM-SH methyltransferase was also present at high activity but was found predominantly in the cytoplasmic fraction. It is part of the enzyme system responsible for the conversion of methanol to methyl-CoM, and methanol was used as the growth substrate for *Methanosarcina* strain Gö1.

Sodium dependence of the membrane-bound methyltransferase activity. Recently we presented evidence that in inverted vesicles the methyltransferase of *Methanosarcina* strain Gö1 is associated with a primary sodium ion pump (2), and it was therefore of interest to determine whether the methyltransferase activity of vesicles was sodium dependent. As can be seen from Fig. 2, this activity was strictly dependent on Na^+ in the buffer system. Maximal methyl-CoM formation was obtained at 1 to 2 mM Na^+; the apparent K_m for Na^+ was 0.7 mM. Li^+ but not K^+ could substitute for Na^+, but the maximal rate of methyl-CoM formation was only 50% of the rate observed with Na^+, and the K_m for Li^+ was 5 mM.

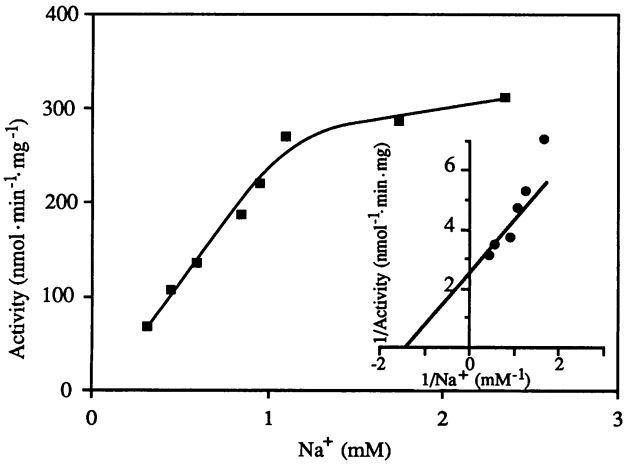

FIG. 2. Sodium dependence of the CH_3-H_4MPT:CoM-SH methyltransferase reaction. Washed vesicle suspensions (2.4 mg of protein per ml) containing 1 mM ATP, 4 mM CoM-SH, and various amounts of NaCl were incubated under an atmosphere of H_2. The sodium concentration in the assay without additional NaCl was 0.35 mM. The reaction was started by the addition of CH_3-H_4F (final concentration, 6 mM).

Participation of a corrinoid protein in the methyltransferase reaction. Corrinoid enzymes mediating methyl transfer reactions are known to be inactivated by 1-iodopropane. This is caused by propylation of the Co(I), which is essential for enzymatic activity; however, the propylated corrinoid can be reactivated by photolysis, which yields Co(I) again (5). An involvement of corrinoids in the methyltransferase reaction as catalyzed by washed vesicles of *Methanosarcina* strain Gö1 is apparent from the experiment depicted in Fig. 3. In the absence of 1-iodopropane, the methyl group was transferred at a rate of 70 nmol · min^{-1} · mg of $protein^{-1}$; this activity was accompanied by a transport of Na^+ at a rate of 4 nmol · min^{-1} · mg of $protein^{-1}$. In the presence of 1-iodopropane, the methyl transfer as well as the simultaneous sodium transport was inhibited. Both activities were restored upon illumination of the sample, indicating that a corrinoid participated in the methyl transfer as well as in the Na^+ transport. The corrinoid content of the washed vesicles was determined and amounted to 0.6 nmol · mg of $protein^{-1}$. The methyltransferase could use not only CoM-SH but also hydroxy-B_{12} as a methyl group acceptor; its methylation was also coupled to Na^+ translocation (Fig. 4).

Reductive activation of the corrinoid-containing methyltransferase. Corrinoid-containing methyltransferases are readily inactivated on cell breakage, and reactivation can be achieved by a reductive activation with low-potential elec-

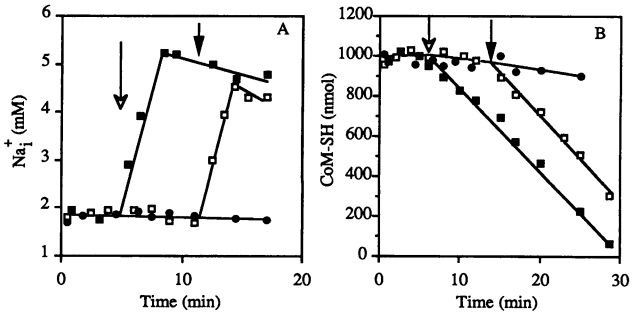

FIG. 3. Effect of 1-iodopropane on $^{22}Na^+$ transport (A) and on CoM-SH methylation (B). We preincubated 550 μl (A) and 200 μl (B) of washed vesicle suspensions (2.4 mg of protein per ml), containing 10 μM SF6847 and 4 mM CoM-SH, for 20 min in the absence (■) or presence (□, ●) of 250 μM 1-iodopropane in the dark. At the time indicated by the open arrow, the reaction was started by addition of methyl-H_4F to a final concentration of 6 mM. One vesicle suspension (□) was illuminated (60-W lamp) at the time indicated by the solid arrow.

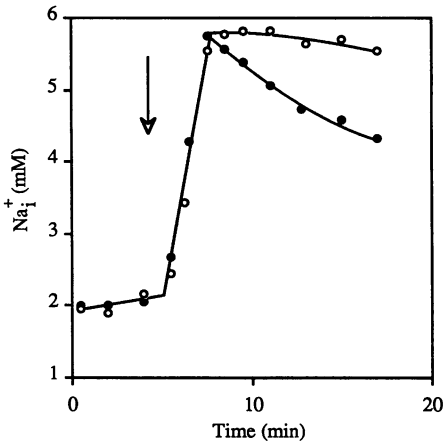

FIG. 4. Hydroxycobalamin-dependent Na^+ transport as catalyzed by inverted vesicles of *Methanosarcina* strain Göl. Washed-vesicle suspensions (2.4 mg of protein per ml) containing 10 μM SF6847 were incubated in the presence of 2.5 mM hydroxycobalamin (●) or 2.5 mM CoM-SH (○). At the time indicated by the open arrow, sodium transport was initiated by addition of methyl-H_4F to a final concentration of 3 mM.

tron donors and/or ATP. In cell extracts of various methanogenic bacteria including *Methanosarcina* strain Göl, the methyltransferase and the methyl-CoM methylreductase show a dependence on ATP. For the latter, such a dependence was not observed with vesicular systems of *Methanosarcina* strain Göl (6); therefore, it was necessary to determine the effect of ATP on the methyltransferase in vesicles of this organism. In the absence of ATP, the methyltransferase exhibited an activity of 60 nmol · min^{-1} · mg of $protein^{-1}$; the addition of ATP led to a stimulation by a factor of 4 (Table 2). The apparent K_m for ATP was 35 μM. Subsequent addition of low-potential electron donors such as Ti(III) or CO stimulated the activity up to 616 nmol · min^{-1} · mg of $protein^{-1}$. In the absence of ATP, Ti(III) or CO did not exhibit a stimulating effect.

DISCUSSION

The sodium-motive reduction of HCHO + H_2 to the level of methanol involves the two enzymes methylene-H_4MPT reductase and methyl-H_4MPT:CoM-SH methyltransferase. The reductase of *Methanosarcina* strain Göl is a soluble protein, which is in agreement with findings on the corresponding enzyme from *Methanosarcina barkeri* and *Methanobacterium thermoautotrophicum* (15, 16, 25). The methyltransferase of *Methanosarcina* strain Göl, however, is a membrane protein, and its activity is Na^+ dependent. Such an Na^+ dependence of methyltransferase activity was not reported for enzyme preparations from *Methanobacterium thermoautotrophicum*, which were extensively studied by Kengen et al. (13). However, these authors solubilized the methyltransferase from membranes of *Methanobacterium thermoautotrophicum*. The purified enzyme is composed of three subunits of 35, 33, and 31 kDa in an αβγ configuration and contains a corrinoid. Our aim was the identification of the primary Na^+ pump, and therefore we studied the methyltransferase in inverted vesicles. Only in such a system could an Na^+ dependence of enzyme activity and a translocation of Na^+ be expected.

It should be pointed out that the methyl-H_4MPT:CoM-SH methyltransferase studied here is different from the methyltransferases of methylotrophic methanogens that catalyze the transfer of the methyl groups from methanol or trimethylamine to CoM-SH (19, 27). The latter are soluble proteins not present in obligate hydrogenotrophic methanogens such as *Methanobacterium thermoautotrophicum*. The methyl-H_4MPT:CoM-SH methyltransferase is essential for methanogenesis from all substrates except methanol + H_2. In addition to *Methanosarcina* strain Göl, indirect experimental evidence for an Na^+ dependence of methyl group transfer was also obtained for *Methanobacterium thermoautotrophicum* and *Methanosarcina barkeri* Fusaro in that methanogenesis from formaldehyde + H_2 is coupled to Na^+ translocation in these organisms (11, 18).

TABLE 2. Reductive activation of the methyl-H_4MPT:CoM-SH methyltransferase[a]

Addition	Activity (mU/mg)
None	60.0
1 mM ATP	267.2
20% CO	63.3
50 μM Ti(III)	58.7
1 mM ATP + 20% CO	616.0
1 mM ATP + 50 μM Ti(III)	560.0

[a] The methyl-H_4MPT:CoM-SH methyltransferase activities were determined by using vesicles that had been washed three times as specified in Materials and Methods.

In previous studies the enzyme was assayed indirectly by monitoring the conversion of HCHO + CoM-SH + H_2 to methyl-CoM in the presence of H_4MPT and F_{420} (2, 13). In

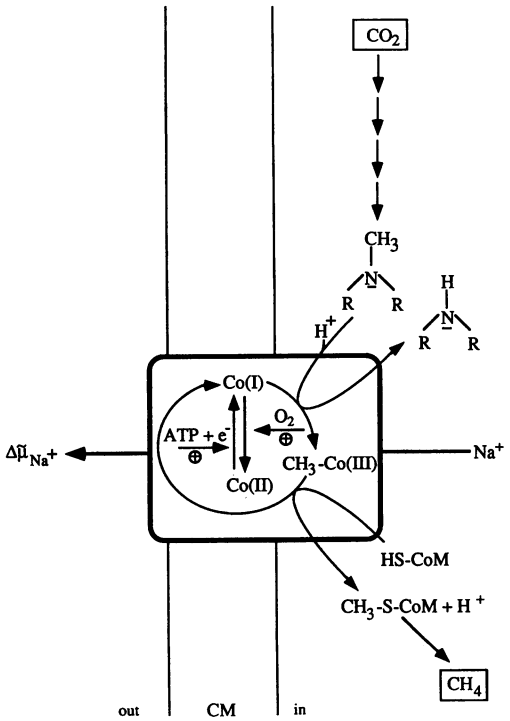

FIG. 5. Tentative scheme of the corrinoid-dependent, sodium-motive methyl-H_4MPT:CoM-SH methyltransferase reaction involved in methanogenesis from H_2 + CO_2. In the structural formulas, only N^5 of H_4MPT is indicated; R denotes the rest of the molecule. CM, cell membrane. $\Delta\mu_{Na^+}$, transmembrane electrochemical gradient of Na^+.

the present study a direct assay was used. The disappearance of CoM-SH with methyl-H_4MPT or methyl-H_4F as a methyl group donor serves as a measure of methyltransferase activity. Since methyl-H_4MPT can be replaced by methyl-H_4F (60% of enzyme activity), the laborious synthesis of methyl-H_4MPT is not necessary.

It is an important observation that the methyl transfer from methyl-H_4MPT to both CoM-SH and hydroxy-B_{12} is coupled to Na^+ translocation. This allows the assumption that the transfer reaction from methyl-H_4MPT to the membrane-bound corrinoid is the actual exergonic step promoting Na^+ extrusion. The reaction mechanism may be similar to the one proposed for methionine synthase, in which methyl group transfer from methyl-H_4F to the acceptor homocysteine proceeds with the intermediate formation of a methylated enzyme (1). A hypothetical model for the methyltransferase reaction is shown in Fig. 5. The reaction is initiated by a nucleophilic attack of a Co(I) species of the corrinoid; from the methyl-Co(III)-corrinoid the methyl group is then transferred to CoM-SH (or to hydroxycobalamin). The redox potential of the Co(II)/Co(I) couple is in the range of −500 mV, and the Co(I) present in methyltransferases is subject to autooxidation to Co(II). For the methionine synthase, the equilibrium is shifted by the exergonic methylation of Co(I) with *S*-adenosylmethionine. In methanogenic bacteria Co(I) is generated from Co(II) by low-potential electron donors such as CO in a reaction requiring ATP. Further progress in understanding the mechanism of methyl group transfer and of Na^+ translocation must await the purification of the enzyme components and the reconstitution of the system into liposomes.

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