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Activation of the Methylreductase System from *Methanobacterium bryantii* by Corrins

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Corrins activated the methylreductase system from *Methanobacterium bryantii* three- to fivefold in extracts resolved from low-molecular-weight factors. Corrins did not substitute for ATP and component B, which were also required for maximal activity. The concentration of diaquacobinamides required for one-half maximal activity was 1 μM. The concentrations of cyanocobalamin, methylcobalamin, Coα-(5-hydroxybenzimidazoyl)-Coβ-cyanocobamide, and 5′-deoxyadenosylcobinamide required for one-half maximal activity were between 4 and 7 μM. Deoxyadenosylcobalamin was nearly inactive. Activation was independent of thiols, coenzyme M, and ATP. Activation was also observed after partial purification of the methylreductase system by agarose column chromatography. Corrins were required in catalytic concentrations, methylcobalamin was not required, and methanogenesis was enzymatic. Corrin activation of the methylreductase is a novel effect on methanogenesis. However, the physiological significance of the corrin activation is uncertain.

After the discovery by Blaylock and Stadtman in 1963 that the methyl group of the methylated corrin methylcobalamin (MeCbl) could be reduced to methane by extracts of *Methanosarcina barkeri* (3), MeCbl was found to serve equally well as a substrate for methanogenesis in extracts of other methanogens, and it was considered likely that MeCbl was a general methyl carrier in methanogenesis (39). However, with the discovery of 2-mercaptoethanesulfonic acid (coenzyme M; HS-CoM) it was soon established that this coenzyme was required for conversion of the methyl group of MeCbl to methane and that 2-(methylthio)ethanesulfonic acid (methylcoenzyme M; CH_3-S-CoM) was an intermediate (26). A MeCbl:HS-CoM methyltransferase was purified 100-fold from *Methanobacterium bryantii* by Taylor and Wolfe in 1974 (27). Despite much effort in the last 10 years, no evidence has been obtained for the role of a corrin in the methylreductase system of nonmethylotrophic methanogens, and the only substrate for this system so far identified is CH_3-S-CoM. This reaction is CH_3-S-CoM + H_2 → CH_4 + HS-CoM. It requires at least four proteins, A1, A2, A3, and C, as well as the cofactors ATP, Mg^{2+}, flavin adenine dinucleotide, and component B.

Only two proteins, component A2 (24) and the nickel tetrapyrrole-containing protein, component C (6, 8, 19), of this complex, multienzyme system have been purified to homogeneity. Component A1 has hydrogenase activity, and A3 is highly oxygen sensitive (16); A1 and A3 are resolved protein fractions that contain many proteins. It has not been established which of these proteins participate in the methylreductase system. Component B is a heat-stable cofactor of unknown structure and function (12).

Recently the enzymology of the conversion of methanol to methane has been examined in *Methanosarcina* spp. by van der Meijden et al. (30). One of the enzymes (designated MT_1) was shown to be a corrin-containing protein. The firmly bound cobamide moiety accepted a methyl group from methanol. A second enzyme had the properties of the MeCbl:HS-CoM methyltransferase of *Methanobacterium bryantii* (27); this enzyme (MT_2) transferred a methyl moiety from the bound cobamide to HS-CoM but also could transfer the methyl group from soluble MeCbl (30). ATP was required to activate MT_1 under reductive conditions, and in the absence of ATP the corrinoid enzyme was quickly inactivated (32).

A role for the MeCbl:HS-CoM methyltransferase in methanogenesis by *Methanobacterium* strains grown on hydrogen and carbon dioxide has not been found, nor has evidence for a corrin been found in the methylreductase system. Nevertheless, when cell extracts of *M. bryantii* were desalted by G-25 Sephadex chromatography, 80% of the methylreductase activity was lost. Addition of ATP (37) and component B did not restore activity. As shown here, activation by corrins restored activity to the levels found in crude extracts. Because corrins also activated the more highly purified methylreductase system from *M. thermoautotrophicum* (16), corrin activation may be a general phenomenon of methylreductases from methanogens. This report further characterizes the corrin activation of the methylreductase system from *M. bryantii*. We have studied this activation in crude extracts, since the methylreductase system is largely undefined enzymatically. A preliminary report of this work has been presented (W. B. Whitman and R. S. Wolfe, Fed. Proc. 41:1152, 1982).

MATERIALS AND METHODS

Preparation of extracts. Growth of *M. bryantii* M.o.H. and anaerobic preparation of "crude" cell extracts were described previously (37). Sephadex G-25-treated extracts were prepared from crude extracts that had been stored at −20°C under H_2 for 3 to 5 days. Crude extracts were thawed, and 20 ml of extract was passed through an anaerobic Sephadex G-25 medium column (25 by 2.5 cm) at a flow rate of 20 ml/h. The column was prepared and equilibrated with buffer A at 5°C as described previously (37). Buffer A contained 50 mM potassium-*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate (TES) (pH 7.1), 0.2 M sucrose, 1 mM cysteine hydrochloride, 1 mM dithiothreitol, and 0.1% toluene under an atmosphere of H_2 gas. Fractions containing

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protein were pooled and stored at −20°C under H_2 until used. Extracts retained full activity for 3 to 4 weeks under these conditions.

In some experiments, crude extract (210 mg of protein in 10 ml) was passed through an anaerobic agarose column (Bio-Gel A1.5, 3.0 by 37 cm; Bio-Rad Laboratories, Richmond, Calif.) equilibrated in modified buffer A. Modified buffer A contained 20% ethylene glycol instead of sucrose, and toluene was omitted. The resin, column, and end fittings (Pharmacia, Inc., Piscataway, N.J.) were sterilized by immersion overnight in a solution of 0.1% (vol/vol) diethyl pyrocarbonate. Chromatography was performed at a flow rate of 22 ml/h at room temperature. Methylreductase eluted just after the void volume, between 45 and 67 ml. The methylreductase activity was pooled in three fractions: the first 7 ml (agarose fraction I), the subsequent 10 ml (agarose fraction II), and the last 5 ml (agarose fraction III). The protein concentrations in the fractions were 7.2, 7.3, and 8.0 mg/ml for fractions I, II, and III, respectively. Agarose fraction II is also referred to as "agarose-treated extract."

Methylreductase assay. Before the enzymatic assay, bottles containing frozen extract were flushed with H_2 gas, repressurized to 100 kPa, and allowed to thaw at 25°C before being placed in an ice bath. Solutions of the oxygen-stable components of the assay (2 μmol of CH_3-S-CoM, 2 μmol of $MgCl_2$, 10 μl of partially purified component B, 50 nmol of disodium ATP, cobamides) were combined in each 2.6-ml calibrated serum vial and taken to dryness during passage through the airlock of the anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.). Inside the anaerobic chamber, oxygen-labile components (like cob(II)alamin[Cbl^{II}]) were added, and the vials were stoppered with butyl rubber-aluminum seal stoppers (West Co., Inc., Phoenixville, Pa.). The atmosphere of the chamber contained no more than 0.0005% (vol/vol) oxygen, as determined by a couloximeter oxygen sensor (Chemical Sensor Development, Torrance, Calif.). After sealing, the vials were transferred out of the chamber to an ice bath, and each vial was flushed with H_2 at a flow rate of 40 ml/min for 5 min. Then 0.2 ml of extract was added with a 1-ml Glaspak syringe (Becton-Dickinson and Co., Rutherford, N.J.) equipped with a 23-gauge needle. Flushing with H_2 was continued for 5 min, and the assay was initiated by transferring the vials to a 37°C water bath. For more precise addition of extracts, extracts were dispensed in the anaerobic chamber with a micropipet. When extracts were dispensed in the chamber, 10 μl of anaerobic solution containing ATP (or ATP and phosphoenolpyruvate) was added with a syringe while the vials were flushed on ice. For an ATP-generating system, 2 μmol of phosphoenolpyruvate was also included in some assays. Dialyzed extracts similar to the Sephadex-treated extracts generated ATP from phosphoenolpyruvate at a rate of 0.75 nmol of ATP/min per mg of protein, so that the addition of pyruvate kinase was not necessary (37).

Solutions and assays containing corrins were routinely protected from light. Overhead lights were dimmed during the methylreductase assays, and stock solutions were kept in the dark.

To determine the extent of anaerobiosis that was maintained with the type of assay vial used, oxygen inside the vials was measured by gas chromatography with an electron capture detector. Vials were sealed in the anaerobic chamber. After 90 min in air, no increase in oxygen was detected inside the vial. Similarly, vials with stoppers that had been punctured five times with a 22-gauge needle showed no leakage of oxygen into the vial. The limit of detection in these experiments was about 0.002% (vol/vol) oxygen. For comparison, the assay vials used in previous investigations were also tested. Vials sealed with the red-sleeve-type stopper (10) contained 0.04% (vol/vol) oxygen after 40 min. When the red-sleeve stoppers were stored in the anaerobic chamber for 12 h before use (14), no oxygen was detected inside the vials after 40 min in air. However, oxygen was detected after 80 min.

The formation of methane was measured by removing a 10-μl sample from the gas phase and injecting it into a Packard gas chromatograph model 428 furnished with a flame ionization detector and a Porpak Q column. Methane was determined by measuring peak heights. The velocity and lag in each assay were calculated from the least squares fit to the linear portion of the time course (37). One unit of methylreductase activity is 1 nmol of CH_4 produced per min.

Identification of tritiated cobalamins after methylreductase assay. The methylreductase assay solution contained 1 ml of G-25 Sephadex-treated extract, 25 μmol of $MgCl_2$, 10 μmol of CH_3-S-CoM, 25 μl of partially purified component B, 250 nmol of disodium ATP, and 15 nmol of tritiated cyanocobalamin (CN-[G-^{3}H]Cbl) (8×10^5 cpm) under an H_2 atmosphere. After 20 min at 37°C, 4 ml of ice-cold methanol was added, the suspension was centrifuged at 10,000 × *g* for 10 min, and the supernatant was saved. The pellet was washed with 2 ml of 80% aqueous methanol. After centrifugation, the supernatants were pooled and passed through an ion exchange column containing 0.5 ml of DEAE-Sephadex and 0.5 ml of carboxymethyl-Sephadex at pH 7.0. Methanol was removed from the effluent under a stream of N_2. All the radiolabel initially added to the methylreductase assay was recovered after ion exchange chromatography. The aqueous solution was then lyophilized and suspended in 35 μl of glass-distilled water. A 7-μl portion was used for high-pressure liquid chromatography analysis (38). The HPLC conditions were as follows. A μBondapak C-18 column (0.39 by 30 cm; Waters Associates, Inc., Milford, Mass.) was equilibrated with 100 mM LiCl in 24% aqueous methanol at a flow rate of 1 ml/min at 23°C. At 2 min after injection of the sample, a 30-min linear gradient was initiated to a final concentration of 100 mM LiCl in 48% aqueous methanol. Fractions (1 ml) were collected. A 0.1-ml portion was used for liquid scintillation counting in 3.0 ml of toluene–triton X-100–2,5-diphenyloxazole (70:30:0.1, vol/vol/wt).

Other methods. The hexokinase trap for ATP and protein determinations were performed as described previously (37).

Materials. All common reagents were analytical grade or better. Biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo. Sephadex G-25 was from Sigma. Agarose A1.5 was from Bio-Rad. CH_3-S-CoM was synthesized as described previously (22), except that sodium 2-mercaptoethanesulfonate (Pierce Chemical Co., Rockford, Ill.) was substituted for the ammonium salt and the reaction time was 36 h at 25°C.

CN-Cbl, MeCbl, and 5′-deoxyadenosylcobalamin (AdoCbl) were obtained from Sigma. Cbl^{II} was prepared from MeCbl by photolysis (42). Aquacobalamin (AqCbl) was prepared from Cbl^{II} by spontaneous oxidation with air. Diaquacobinamide (Aq_2Cbi; factor B) and diaquacobinic acid pentaamide ($Aq_2Cbi[a:g\text{-}(NH_2)_5]$) were prepared by acid hydrolysis of CN-Cbl (34) and purified by DEAE-cellulose chromatography, HPLC, and preparative paper electrophoresis at pH 6.95 (13). Purified samples were recrystallized from acetone and identified spectrally. Purified samples migrated as a single species during paper electrophoresis at pH 2.63, 6.95, and 11.0 (13). Molarity was determined from

the extinction coefficient of the dicyano form which was 3.06 $\times 10^4$ cm^{-1} M^{-1} at 367 nm (5).

Coα-(5-hydroxybenzimidazoyl)-Coβ-cyanocobamide[(5-HOBza)CN-Cba; factor III] and 5'-deoxyadenosylcobinamide (AdoCbi) were purified from whole cells of *M. bryantii* (38). The purified compounds were identified by their absorption spectra in water, 0.1 M HCl, and 0.1 M NaOH (15, 20). Purity was monitored by HPLC. The extinction coefficient of the dicyano forms derived from both compounds was assumed to be 3.06 $\times 10^4$ cm^{-1} M^{-1} at 367 nm (5).

CN-[G-^{3}H]Cbl was obtained from Amersham Corp., Arlington Heights, Ill. The specific activity was 5.4 Ci/mmol. It comigrated as a single peak which was coincident with unlabeled CN-Cbl after HPLC.

Component B was partially purified from whole cells of *M. bryantii*. In a typical purification, 350 g (wet weight) of cells was suspended in 500 ml of deoxygenated H_2O and heated in a boiling-water bath under a stream of N_2. After the slurry reached a temperature of 90°C, heating was continued for 75 min or until the slurry began to turn black. The slurry was allowed to cool under N_2 and centrifuged for 15 min at 4,200 $\times$ *g* without anaerobic precautions. The supernatant fluid was decanted and taken inside an anaerobic chamber. Sufficient 1 M HCl was added to lower the pH to 5.0. The supernatant fluid was allowed to sit overnight. The copious precipitate which formed was removed by anaerobic centrifugation at 25,000 $\times$ *g* for 30 min. The supernatant fluid was loaded onto an anaerobic DEAE-Sephadex column (5 by 18 cm) equilibrated with 50 mM ammonium acetate (pH 5.0)–1 mM dithiothreitol. After loading, the column was washed at a flow rate of 50 ml/h with 250 ml of the equilibration buffer followed by a 2-liter linear gradient from 50 to 500 mM ammonium acetate (pH 5.0)–1 mM dithiothreitol. The gradient was followed by a 500 mM ammonium acetate (pH 5.0) wash. Component B, which eluted just before the end of the gradient, was detected by assaying for reconstitution of the methylreductase after removal of ammonium acetate, which was inhibitory. Ammonium acetate was removed from 50-μl portions of fractions by drying in vacuo in assay vials before addition of the other components of the methylreductase assay. Fractions containing component B were pooled and concentrated by lyophilization. All subsequent steps were performed aerobically. The resulting syrup was diluted with water and lyophilized; this procedure was repeated to remove residual ammonium acetate. The final syrup, 15 ml, was passed through a G-25 Sephadex superfine column (2.5 by 100 cm) equilibrated with 50 mM ammonium acetate at 5°C; a flow rate of 13 ml/h was used. The fractions containing component B were pooled, lyophilized, and redissolved in water. This lyophilization procedure was repeated three times to remove ammonium acetate. The resulting powder was suspended in water such that three times the concentration of component B required for half-maximal activity in the standard methylreductase assay with 25 μM MeCbl was contained in 10 μl. The solution, 25 ml, was stored at −20°C for over 1 year without loss in activity.

Factor F_{430} was purified from whole cells of *M. bryantii* as described previously (36).

TABLE 1. Cobalamin activation in different preparations of the methylreductase system

Preparation[a]	Methylreductase activity[b]		
	−Cbl	+Cbl	+Cbl/−Cbl ratio
Crude extract	7.7	14.6	1.9
Sephadex-treated extract	2.2	10.7	4.9
Sephadex-treated extract without cysteine and DTT[c]	0.95	5.1	5.4
Agarose fraction I	0.56	2.1	3.8
Agarose fraction II	1.25	8.0	6.4
Agarose fraction III	0.65	3.0	4.6

[a] Preparation of extracts is described in Materials and Methods.

[b] Methylreductase activity (units per milligram of protein) in the presence (+Cbl) or absence (−Cbl) of 25 μM MeCbl.

[c] The crude extract was passed through an anaerobic Sephadex G-25 column as described above except that cysteine and dithiothreitol (DTT) were omitted from buffer A.

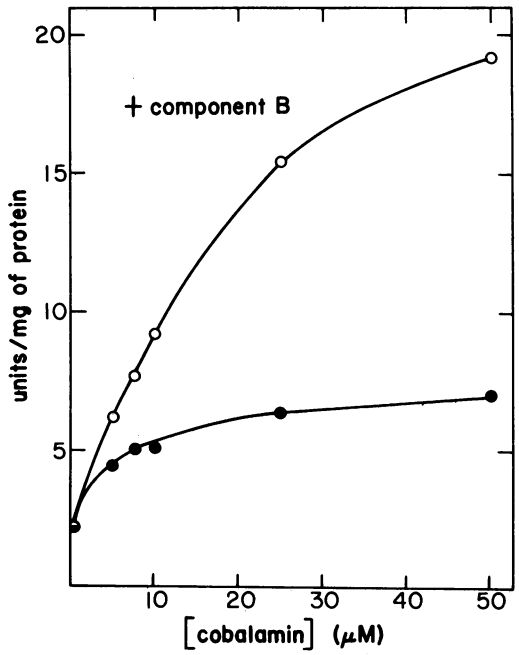

FIG. 1. Saturation of cobalamin activation in the presence and absence of exogeneous component B. Sephadex-treated extract was titrated with CN-Cbl in the presence (○) and absence (●) of five times the concentration of component B required for one-half of the maximal activity in the presence of 25 μM MeCbl. Assays also contained 2 μmol of phosphoenolpyruvate.

RESULTS

Activation by corrins. Low concentrations (25 μM) of MeCbl activate the methylreductase activity in crude extracts of *M. bryantii* twofold (Table 1). Activation was also observed with protein fractions desalted by Sephadex G-25 chromatography or after partial purification by agarose chromatography (Table 1). However, after chromatography the methylreductase activity in the absence of MeCbl was much lower than in the crude extract. Therefore, although the activation by MeCbl was more pronounced after chromatography, MeCbl merely restored activity to the levels observed in crude extracts (Table 1). The difference in activity with MeCbl between the crude and Sephadex-treated extract, about 30%, can be attributed largely to the difference in protein concentrations, which were 26 and 17 mg/ml, respectively (37). This result suggests that the loss in activity after chromatography may be due to the removal of endogenous cobamides from the crude extract.

MeCbl did not substitute for other components in the

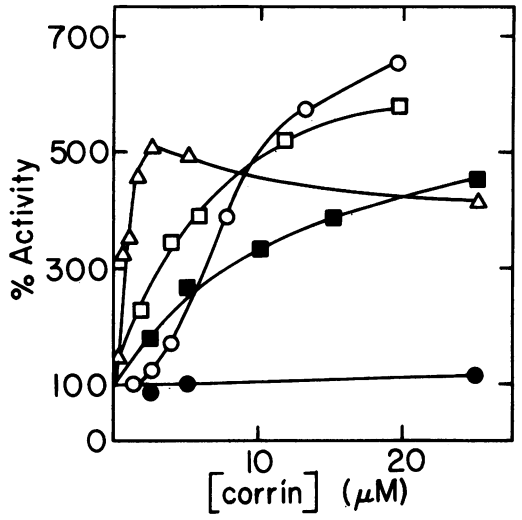

FIG. 2. Specificity of the corrin activation of the methylreductase. "% Activity" is equal to 100 × (activity plus corrin)/(activity minus corrin). Assays contained Sephadex-treated extract, other components of the methylreductase assay, and 2 μmol of phosphoenolpyruvate. Symbols: ■, CN-Cbl; ●, AdoCbl; □, (5-HOBza)CN-Cba; ○, AdoCbi; △, Aq_2Cbi.

methylreductase assay. In the Sephadex-treated extract, ATP was absolutely required for activity in the presence (data not shown) or absence of MeCbl (37). In the absence of component B, extracts retained about 60% of their activity. In itself, this result is equivocal. Partial activity could be due to either contaminating component B or the partial substitution of MeCbl for component B. To distinguish between these possibilities, extracts were titrated with CN-Cbl in the presence or absence of high concentrations of component B. Maximal activity was obtained only in the presence of both component B and CN-Cbl (Fig. 1). Furthermore, saturation of cobalamin activation was obtained in both the presence and absence of exogenous component B. In the absence of exogenous component B, saturation occurred at 2.5-fold lower methylreductase activity. Therefore, CN-Cbl did not replace the requirement for component B.

Corrin specificity. Free cobalt and factor F_{430} did not substitute for cobalamin. At concentrations upto 50 μM, the salts $CoCl_2$, $CuCl_2$, $CaCl_2$, $FeSO_4$, $MnCl_2$, $NiCl_2$, and $ZnCl_2$ produced no activation of the methylreductase (data not shown). Similarly, 25 μM factor F-430, the nickel tetrapyrrole which is tightly bound to component C of the methylreductase (8), did not substitute for cobalamin (data not shown).

The specificity of the activation with respect to the cobalamin was determined. MeCbl, CN-Cbl, Cbl^{II}, and AqCbl at concentrations of 25 μM all activated Sephadex-treated extracts five- to sevenfold (data not shown). In contrast, little activation was observed with AdoCbl. In no experiment was more than twofold activation observed, and activation was generally much less (Fig. 2). These results suggested that substitution of the β-ligand (with the exception of AdoCbl) had no effect on activation.

Although the extent of activation varied no more than twofold for cobalamins, cobinamides, and (5-HOBza)CN-Cba, the vitamin form of the cobamide found in methanogens (Fig. 2; Table 2), the concentrations of corrins required for activation varied greatly. Although these results were obtained with Sephadex-treated extracts, a similar pattern was also observed with agarose-treated extracts. In agarose-treated extracts, the average binding constants (K_{app}, V_{max}) from two experiments were as follows: AqCbl, 30 μM, 19 U/mg; Aq_2Cbi, 3 μM, 49 U/mg; AdoCbi, 23 μM, 19 U/mg; and (5-HOBza)CN-Cba, 19 μM, 19 U/mg. AdoCbl gave less than 50% activation in agarose-treated extracts (data not shown).

Agarose chromatography changed the overall kinetics of the methylreductase system, but the pattern of specificity of corrin activation was unchanged. For example, K_{app} for Aq_2Cbi was 3- to 10-fold lower than K_{app} for the other corrins in each extract, and V_{max} for Aq_2Cbi was about 2-fold higher than V_{max} for other corrins in each extract. The differences between the two extracts were not unexpected, considering that the methylreductase system was very sensitive to dilution (37) and may contain different ratios of the protein components after agarose chromatography.

Several conclusions about the corrin specificity may be drawn from these results. While the absence of an axial ligand or the presence of cyanide, water, or a methyl group were nearly equivalent, the substitution of a nucleoside decreased V_{max} and increased K_{app}. Thus, the order of reactivity was as follows: no nucleoside substitutions (Aq_2Cbi, $Aq_2Cbi[a:g\text{-}(NH_2)_5]$) > one nucleoside substitution [MeCbl, CN-Cbl, AqCbl, (5-HOBza)CN-Cba, AdoCbi] ≫ two nucleoside substitutions (AdoCbl). However, the nature of the nucleoside, dimethylbenzimidazoyl in the cobalamins, 5-hydroxybenzimidazoyl in (5-HOBza)CN-Cba, or deoxyadenosyl in AdoCbi, was not important. In addition, because Aq_2Cbi and $Aq_2Cbi[a:g\text{-}(NH_2)_5]$ yielded nearly identical activation, small modifications of the corrin side chains had little effect.

Studies with adenosylcobalamin. Because catalytic amounts of ATP are required for methylreductase activity and the role of ATP is not known (11, 21, 37), it was possible that AdoCbl was made in situ during the activation. This hypothesis would be consistent with the specificity experiments only if a protein-bound cobalamin was the substrate for AdoCbl synthesis and the apoprotein was incapable of binding AdoCbl. This hypothesis was not unreasonable, considering that in cell extracts of *Propionibacterium shermanii* the synthesis of the deoxyadenosyl coenzyme has a substrate specificity remarkably similar to the corrin activation of the methylreductase system (4).

To determine the fate of cobalamins, extracts were incubated with low concentrations of CN-[G-^{3}H]Cbl under the

TABLE 2. Activation of the methylreductase system by corrins

Corrin	K_{app} (μM)[a]	V_{max} (U/mg of protein)
MeCbl	5.2	24
CN-Cbl	7.2[b]	22[b]
Aq_2Cbi	1.2	47
$Aq_2Cbi[a:g\text{-}(NH_2)_5]$	1.3	42
AdoCbi	7.0[c]	ND[c]
(5-HOBza)CN-Cba	4.0	20

[a] Apparent binding constants (K_{app} and V_{max}) where determined by least squares analysis of double reciprocal plots of the data in Fig. 2 and additional data not shown.

[b] Average of two separate experiments.

[c] Because the double reciprocal plots were nonlinear, K_{app} was estimated as the concentration required to obtain one-half the activity found at 20 μM AdoCbi. ND, V_{max} was not determined.

TABLE 3. ATP is not required for activation by cobalamin

Addition[a] at:		Methylreductase activity (U/mg of protein)[b]
Time zero	13 min	
None	None	0.9
MeCbl	None	7.7
Hexokinase	None	0.1
Hexokinase + MeCbl	None	0.0
None	Hexokinase	0.9
None	Hexokinase + MeCbl	5.9
None	MeCbl	7.1

[a] In addition to the components of the methylreductase assay including 50 nmol of ATP, 5 nmol of MeCbl or 18 U of hexokinase was added at the start of the assay (time zero) or 13 min later as indicated.
[b] Methylreductase activity was determined after 20 min.

TABLE 4. Activation of the NADPH-driven methylreductase by cobalamin

Assay conditions	Methylreductase activity[a] for:	
	NADPH-driven reaction	H_2-driven reaction
Complete[b]	6.3	17.6
Without MeCbl	2.6	3.3
Without coenzyme F_{420}	4.3	17.2
Without NADPH	0.4	16.9

[a] In units of methylreductase activity per milligram of protein.
[b] The complete assay mixture contained 0.2 ml of Sephadex-treated extract, 2 μmol of CH_3-S-CoM, 50 nmol of ATP, 5 μmol of $MgCl_2$, component B, 5 nmol of MeCbl, 15 nmol of coenzyme F_{420}, and 500 nmol of NADPH. The NADPH-driven reaction was under a N_2 atmosphere.

conditions of the methylreductase assay, in the presence and absence of ATP. After extraction and enrichment of the cobamide fraction, it was chromatographed by HPLC. Tritiated AdoCbl was not detected (Fig. 3). Because the limits of detection were at least 5% of the total cobalamin, AdoCbl was not formed in appreciable amounts. Thus, the coenzyme form of cobalamin was probably not required for activation. Similarly, only minute amounts of titrated cobalamin comigrated with MeCbl (Fig. 3). Therefore, MeCbl was probably not an important intermediate in activation.

However, AqCbl, which coeluted with CN-Cbl, and an unidentified cobalamin, which eluted before CN-Cbl, were detected (Fig. 3). AqCbl could be formed from the oxidation of Cbl(II) or Cbl(I). Cobalamins also readily react with sulfhydryls, which are abundant in methanogen extracts (1, 18, 20). Possibly, the unidentified cobalamin was a sulfur adduct. Formation of the unidentified cobalamin was not dependent on methylreductase activity, because these extracts were virtually inactive in the absence of ATP (37). However, the unidentified cobalamin was not formed if

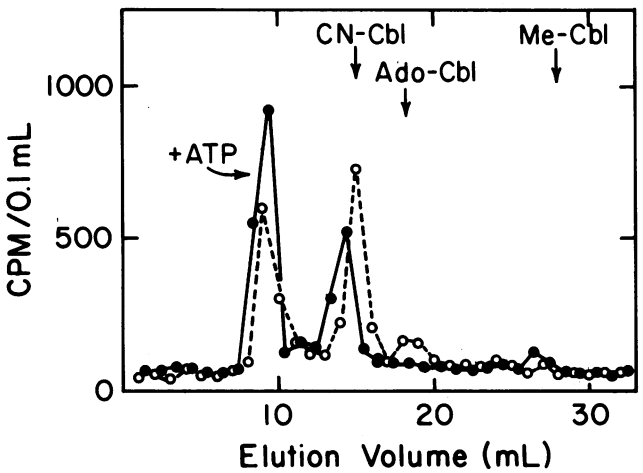

FIG. 3. Recovery of tritium-labeled cobalamins after the methylreductase assay in the presence (●) and absence (○) of 0.25 mM ATP. The assay included 15 μM CN-[G-^{3}H]Cbl (8×10^5 cpm) and Sephadex-treated extract. At the conclusion of the assay, cobamides were extracted and separated by HPLC as described in Materials and Methods. Fractions (1 ml) were collected after HPLC, and the radioactivity was determined by liquid scintillation counting. The elution volumes of standards are indicated by arrows. The background, which was 60 cpm, has not been subtracted. The counting efficiency was 38% as determined by internal standards.

CN-[G-^{3}H]Cbl was added after the reaction was quenched with methanol (data not shown). Thus its formation required active enzyme(s) in the extract.

In addition, activation by MeCbl did not require ATP. After activation of the methylreductase system with ATP, a hexokinase-ATP trap did not prevent further activation by MeCbl (Table 3). As shown previously (21, 37), the hexokinase-ATP trap was inhibitory only when added early in the methylreductase assay. This result confirms the conclusion that the coenzyme form of cobalamin was not required. In addition, other possible ATP-dependent modifications of cobalamin were not required for activation.

Electron donor to methylreductase. In partially purified extracts of *M. thermoautotrophicum*, NADPH can substitute for H_2 as the electron donor for the methylreductase system (7). Therefore, it was possible to determine whether corrin activation was independent of the electron donor. Like the H_2-driven reaction, MeCbl activated the methylreductase when NADPH was the electron donor (Table 4). However, the extent of activation was much lower when NADPH was the reductant. Whether this effect is due to an additional limitation of the methylreductase by NADPH or a requirement for H_2 for full corrin activation is not known.

Both NADPH and coenzyme F_{420} were required for maximal activity in *M. bryantii* (Table 4). In the presence of 500 nmol of NADPH, 501 nmol of CH_4 were formed, while only 79 nmol of CH_4 were formed in the absence of an exogenous electron donor (data not shown). Thus, the ratio of CH_4 formed to NADPH added was 0.84, or close to unity. Furthermore, from 5 μmol of NADPH, less than 45 nmol of H_2 was produced by *M. bryantii* extracts. These results

TABLE 5. Effect of inhibitors on the cobalamin activation

Inhibitor	Methylreductase activity[a]		
	−Cbl	+Cbl	+Cbl/−Cbl ratio
25 μM CN-Cbl	2.1	12.9	6.1
+1.25 mM HS-CoM	1.8	13.4	7.4
+5.0 mM HS-CoM	1.6	11.7	7.3
25 μM MeCbl	1.4	6.0	4.3
+2.5 μM MV	1.2	2.5	2.1
+ 10 μM MV	0.46	0.90	2.0
+ 30 μM MV	0.15	0.23	1.5

[a] Methylreductase activity (units per milligram of protein) in the presence of 25 μM CN-Cbl or 25 μM MeCbl (+Cbl) or their absence (−Cbl).

confirmed that NADPH was an electron donor for the methylreductase of *M. bryantii*, and that reduced coenzyme F_{420}, but not H_2; was an intermediate. Therefore, the NADPH-driven reaction in *M. bryantii* is similar to the reaction in more highly purified extracts of *M. thermoautotrophicum* (7).

Effect of thiols and MV. Because corrins can participate in electron transfer reactions, the effect of reducing agents and electron acceptors on corrin activation was of interest. Corrins readily react with thiols (9, 18, 25), and thiols are also weak inhibitors of the methylreductase system (23). When Sephadex-treated extracts were prepared in the absence of cysteine and dithiothreitol (Table 1), the relative activation by MeCbl was not effected. However, there was a dramatic reduction in methylreductase activity in both the presence and absence of MeCbl. This latter effect was not unexpected, considering the extreme oxygen sensitivity of these extracts.

Coenzyme M, the product of the methylreductase, is also an inhibitor at high concentrations (23). At concentrations less than 5 mM, inhibition was less than 25% (Table 5). Moreover, HS-CoM had no significant effect on activation. Therefore, activation is not due to the reversal of inhibition by free HS-CoM.

MV is an electron acceptor of hydrogenases in methanogens and a potent inhibitor of methane synthesis at low concentrations (11). At low concentrations, MV inhibited the methylreductase in the presence and absence of MeCbl (Table 5). Because the activated methylreductase was more sensitive to MV, MV appeared to specifically reduce the activation. However, these results could be consistent with MV inhibition at an additional rate-limiting step in the methylreductase system as well as a specific inhibition of MeCbl activation.

DISCUSSION

The corrin activation of the methylreductase is unlike previously described effects of corrins on methanogenesis. In early investigations of the mechanism of methane synthesis, MeCbl was shown to be a substrate for methanogenesis in extracts of *M. bryantii* (21) and *Methanosarcina barkeri* (3, 40). However, the methylreductase activation reported here requires catalytic amounts of corrins and not substrate amounts. In addition, MeCbl is not required, and corrins which lack a mobile C-1 group are equally or more effective. Cobamides and cobamide-proteins also have been implicated in CH_3-S-CoM synthesis, especially from methanol (2, 30–32). The activation described here deals solely with CH_3-S-CoM reduction.

Four observations indicate that the methane synthesized during activation is formed enzymatically. (i) ATP and component B, cofactors of the methylreductase system, were either stimulatory or required for methane synthesis during activation. (ii) The rate of methane synthesis was saturated by low concentrations of corrins. This property would not be expected for a chemical reaction. (iii) The activation was independent of thiols. (iv) MeCbl was not formed in appreciable amounts during activation. Although a thiol-dependent, nonenzymatic mechanism of methanogenesis from MeCbl has been reported (9, 25), mechanisms of activation which require chemical methane synthesis, either from exogenous MeCbl or MeCbl synthesized from CH_3-S-CoM during the reaction, are unlikely.

The specificity of the corrin activation is consistent with a direct interaction at the methylreductase system either with or without prior modification of the corrin. The broad specificity and apparent binding constants are within the range observed for cobalamin-dependent enzymes (4, 28, 29). Although AdoCbl is not formed during the activation, reduction or other modifications of the corrins are possible. When the fate of CN-Cbl was followed with tritiated cobalamin, a major product was an unidentified corrin. Because the corrin was formed in the absence of methylreductase activity, its involvement in activation is not certain. It may be a product of an unrelated reaction. Although its structure was not pursued in this study, its formation demonstrates that chemical or enzymatic modifications of the corrins do occur in these extracts.

If enzymatic modification of the corrin is required before activation, purification of the methylreductase system might separate a protein fraction required for activation. No evidence for such proteins was found. Additional proteins were not required for activation in agarose-treated extracts, which contained about one-third of the total protein in crude extracts. Protein fractions collected on either side of the methylreductase peak, agarose fractions I and III, were also activated by corrins. Thus, partial resolution of an "activation protein" and the methylreductase did not occur. Similarly, 25 μM CN-Cbl activates 11-fold the most highly purified methylreductase system prepared to date from *M. thermoautotrophicum* (16). Therefore, a requirement for proteins in addition to the methylreductase system is unlikely.

The methylreductase system requires four proteins, and only two have been purified to homogeneity (8, 24). Therefore, it is premature to propose a specific mechanism of corrin activation. Because the methanogen extracts are highly reducing, reduced corrins could be formed by a number of nonenzymatic reactions (9, 33). Reduced corrins then could participate in electron transfer reactions in the methylreductase system. The inability to obtain maximal activation when NADPH was the electron donor (and H_2 was not formed) and strong inhibition by MV are consistent with this hypothesis. However, neither of these observations provides strong support, because little is known about their respective mechanisms.

The methylreductase activation superficially resembles the corrin-catalyzed reduction of oxygen by thiols previously described by Peel (17, 18). However, a number of important differences appear to preclude the possibility that corrins act as an oxygen-scavenging system in the methylreductase. (i) Although the order of reactivity for cobinamides and cobamides is similar, Aq_2Cbi is active at 100-fold-lower concentrations in thiol oxidation than in activation of the methylreductase. (ii) Removal of thiols from the methylreductase assays has no effect on the activation, whereas the "Peel effect" is thiol dependent. (iii) Thiol oxidation does not show saturation at low concentrations of cobinamides, as demonstrated for activation of the methylreductase. (iv) Thiol oxidation is O_2 dependent, and the methylreductase system was assayed under strictly anaerobic conditions. Nevertheless, corrins could participate in similar electron transfer reactions between reductants and oxidants other than O_2 which may be present in the methylreductase system.

Corrins have many characteristics of a physiological component of the methylreductase. They activate methanogenesis manyfold. They are active at low concentrations. They do not substitute for other known cofactors of the methylreductase system. They are abundant in whole cells of methanogens (20). Nevertheless, corrins are especially reactive, and activation could be an artifact produced on

disruption of the whole cell. In support of this conclusion, the methylreductase activity observed in crude extracts is no more than a fraction of the activity in whole cells (35). Therefore, the methylreductase system may be somewhat altered in vitro. This effect is not surprising, considering the apparent complexity of the system. Unfortunately, it complicates the interpretation of in vitro experiments. In any case, future models of the chemistry of biological methanogenesis must account for the activation by corrins. Because corrins dramatically enhance the methylreductase system in vitro, their addition to enzymatic assays may also expedite the fractionation of the protein components.

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