JOURNAL OF BACTERIOLOGY, May 1983, p. 640–649
0021-9193/83/050640-10$02.00/0

Vol. 154, No. 2

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

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Received 29 November 1982/Accepted 28 February 1983

The methylreductase of *Methanobacterium bryantii* required ATP for activity. There was sufficient ATP synthesis in extracts to account for the observed activity. Hexokinase inhibited the methylreductase by competing for endogenously synthesized ATP. The uncoupler, carbonyl cyanide p-trifluoromethyoxyphenyl hydrazone, inhibited only at concentrations greater than 0.5 mM, and detergents and non-halogenated membrane-permeable ions did not inhibit. Thus, membrane proton gradients are not important in activation. In addition, maximal activation was obtained with less than 0.25 mM ATP, was inhibited by β,γ-imido ATP, and was strongly temperature dependent. The activated state was very unstable, having a half-life of 5 to 15 min. After gel filtration at 5°C, the methylreductase retained partial activity for a short time in the absence of ATP. These observations indicate that activation involves the modification of a protein or protein-bound cofactor of the methylreductase system.

The terminal step in the reduction of CO_2 to methane by methanogenic bacteria involves the reduction of methyl coenzyme M by hydrogen to methane and coenzyme M–2-mercaptoethanesulfonate (12, 25). In crude extracts and partially purified enzyme systems, this reaction requires ATP (7, 8, 26, 27). The ATP requirement is anomalous because in vivo the methylreductase yields ATP (18), presumably by coupling with a chemiosmotic membrane and a membrane-bound ATPase (4, 5, 14, 22, 23). Furthermore, the role of ATP is not known.

Before the elucidation of the methylreductase system, Robertson and Wolfe (17) showed that ATP was required in catalytic amounts for the reduction of CO_2 to CH_4 by cell-free extracts. In addition, ATP was only required early in the reaction, and significant ATP synthesis did not occur during methanogenesis (17). Subsequent investigations confirmed some of these results with the methylreductase system (7). In contradiction to these results, ATP was not required with a preparation containing a membrane-bound methylreductase (20, 21). Thus, it was proposed that a transmembrane gradient is the actual activator of methane synthesis (20). The studies described herein were undertaken to clarify the role of ATP in the methylreductase of *Methanobacterium bryantii*. In addition, the activation of the methylreductase was further characterized.

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MATERIALS AND METHODS

Abbreviations. HS-CoM, Coenzyme M–2-mercaptoethansulfonate; CH_3-S-CoM, methyl coenzyme M or 2-(methylthio)ethanesulfonate; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate; G6P, glucose-6-phosphate; FCCP, carbonyl cyanide-p-trifluoromethyoxyphenyl hydrazone; CCCP, carbonyl cyanide-*m*-chlorophenyl hydrazone; PNP, β,γ-imido ATP.

Materials. All reagents were analytical grade or better. Biochemical reagents and inhibitors were obtained from Sigma Chemical Co. (St. Louis, Mo.). CH_3-S-CoM was synthesized as described elsewhere (19) except that sodium–2-mercaptoethanesulfonate (Pierce Chemical Co., Rockford, Ill.) was substituted for the ammonium salt, and the reaction was run for 36 h at 25°C.

Preparation of extracts. *M. bryantii* strain M.o.H. was grown in a 12-liter fermentor (New Brunswick Scientific Co., New Brunswick, N.J.) under a H_2 + CO_2 (80:20) atmosphere at a flow rate of 400 ml/min and a temperature of 37°C. The agitation was initially set at 100 rpm but was gradually increased to 300 rpm by the end of day 2 of growth. Medium 1 of Balch et al. (1) supplemented with 1.25 g of NH_4Cl per liter was used. The inoculum size was 1.5%. After 5 days, the specific rate of methane synthesis had attained a maximum of 300 μmol min^{-1} g^{-1} of cell dry weight, and the cell yield was greater than 0.45 g (dry weight) per liter. At this time, the cells were harvested in a Sharples continuous centrifuge under an atmosphere of CO_2. The cell paste was transferred to a flask under N_2 and suspended in an equal volume of anaerobic buffer saturated with H_2 gas and containing 50 mM K–TES (pH 7.1), 0.2 M sucrose, 1 mM cysteine, and 1 mM dithiothreitol (buffer A). The anaerobic buffer was prepared at least 12 h before use. On occasion, 1 mg of

resazurin per liter was included and shown to be fully reduced within that time. In addition, 0.1% toluene was included as a bactericide. The cell suspension was centrifuged at 30,000 × *g* for 15 min, and the pellet was suspended in an equal volume of buffer A. The cell suspension was loaded in a French pressure cell in an anaerobic glove box (Coy Laboratories, Ann Arbor, Mich.), and the cells were disrupted at 110 MPa. The effluent from the French pressure cell was collected in a serum vial containing 2 mg of DNase under an H_2 atmosphere. The cell extract was transferred to 50-ml polypropylene Oak Ridge-style centrifugation bottles (Dupont Co., Biomedical Products Div., Newtown, Conn.) that had been equilibrated in an anaerobic glove box for at least 24 h; centrifugation was for 30 min at 38,000 × *g* at 4°C. The supernatant fluid was decanted. For limited dialysis, the supernatant fluid was dialyzed in tubing with an average pore size of M_r = 3,500 (Arthur H. Thomas Co., Philadelphia, Pa.) for 20 h against at least 40 volumes of anaerobic buffer A at 4°C. For extensive dialysis, tubing with an average pore size of M_r = 20,000 was used. The dialysis tubing was washed with 4 g of disodium EDTA per liter and 5 g of $NaHCO_3$ per liter at 100°C for 1 h. It was then rinsed for several days with at least five changes of glass-distilled water. Tubing was made anaerobic by adding it to buffer A and sparging with N_2 for 1 h. After dialysis, 3-ml portions of the extract were transferred to 15-ml serum bottles, stoppered with butyl-rubber aluminum-seal tube stoppers (Belco Glass Co., Vineland, N.J.), flushed, and pressurized to 1 atm (ca. 101.3 kPa) with H_2 gas. The extracts were stored up to 3 weeks at −20°C without loss of activity.

In some cases, extracts were desalted on a Sephadex G-25 column (25 by 2.5 cm) at 5°C. After centrifugation at 38,000 × *g*, extracts were frozen 3 to 5 days at −20°C before gel filtration. A 20-ml portion was flushed with H_2 and thawed. The column was loaded and run at 20 ml/min in buffer A under strictly anaerobic conditions (8). Fractions containing protein were pooled and stored at −20°C until used.

Methylreductase assay. Bottles containing frozen extract were flushed with H_2, repressurized to 1 atm (ca. 101.3 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 $MgCl_2$, and 50 nmol of disodium ATP) were combined in 2.6-ml calibrated serum vials and taken to dryness in vacuo during passage through the air lock of the anaerobic glove box. Once inside, the vials were stoppered with butyl rubber aluminum seal stoppers (West Co., Inc., Phoenixville, Pa.), crimp sealed, and transferred to a gassing manifold on ice. The vials were flushed with H_2 at a flow rate of 40 ml/min for 5 min, and 0.2 ml of extract was added with a 1-ml Glaspak syringe (Becton, Dickinson & Co., Rutherford, N.J.) equipped with a 23-gauge needle. Additional anaerobic solutions, including hexokinase and in some cases ATP, were then added. 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 a more precise addition of the extracts, the extract was dispensed in an anaerobic glove box with a micropipette. In this case, ATP was always added while flushing the vials on ice.

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; quantitation was determined by measuring the peak height. The velocity and lag in each assay were calculated from the least-squares fit to the linear portion of the time course. The velocity of the reaction was equal to the slope and the lag was equal to the intercept in the abscissa. All experiments were conducted with extracts prepared from at least two different cultures. The activity of the methylreductase is given in nanomoles of CH_4 produced per minute per milligram of protein.

Hexokinase trap. An ammonium sulfate suspension of hexokinase (Sigma Chemical Co., St. Louis, Mo.) was dialyzed against 10 mM Tris-chloride (pH 7.5) before use. The enzyme solutions were made anaerobic by transferring the solution (typically 0.1 ml, 2 U/μl) through the airlock of the anaerobic glove box in a stoppered vial vented with a 22-gauge needle. The vial was opened inside the hood, and the contents were allowed to equilibrate for 20 min. Unless stated otherwise, 10 U of anaerobic enzyme were added to 0.2 ml of extract containing 2 μmol of glucose, in addition to the components of the methylreductase assay. When G6P was measured, the assay was terminated with 2 volumes of 10% trichloroacetic acid, and the assay mixture was frozen on dry ice. Immediately before assaying for G6P, the extracts were thawed and centrifuged at 10,000 × *g* for 4 min. A 0.1-ml portion of the supernatant fluid was added to 0.9 ml of 0.2 M Tris-chloride buffer (pH 8.5) containing 10 mM $MgCl_2$, 0.4 mM NADP, and 1 U of G6P dehydrogenase. After 30 min at 25°C, the increase in absorbance at 340 nm was determined. The amount of G6P present was calculated from the moles of NADP reduced, using an extinction coefficient of 6.22×10^3 cm^{-1} M^{-1}. A unit of hexokinase and G6P dehydrogenase is that quantity of enzyme which yields 1 μmol of product per min.

ATPase assay. Extracts prepared as described above were extensively dialyzed against 50 mM Tris-acetate buffer (pH 7.5) containing 2 mM disodium EDTA at 4°C. ATPase activity was determined at 38°C in a reaction mixture containing 1 to 2 mg of dialyzed extract, 20 mM Tris-acetate buffer (pH 7.5), 1.2 mM disodium EDTA, 80 mM KCl, 4 mM disodium ATP, and 10 mM $MgCl_2$. After 10 min, 2.5 volumes of 10% trichloroacetic acid was added to terminate the reaction. The inorganic phosphate released was measured (2). The assays were linear with time for at least 15 min.

Protein determination. The extracts were digested in 0.1 M NaOH for 30 min at 90°C before determination of the protein content by the method of Lowry et al. (11). The standard was bovine serum albumin.

RESULTS

ATP requirement. After extensive dialysis with a membrane of large pore size (M_r = 20,000) or gel filtration, the extracts showed an absolute requirement for ATP for methylreductase activity. However, generally 80% of the total activity was lost. In a typical experiment, the specific activity of the methylreductase after dialysis was 5.6 in the presence of 50 nmol of ATP and less than 0.015 in the absence of ATP

(no activity was observed within 60 min). Furthermore, greater than 1 μmol of methane was synthesized, which indicated that the requirement for ATP was not stoichiometric. However, the balance of the activity before dialysis was not recovered at any concentration of ATP, and the addition of component B, a low-molecular-weight compound of unknown structure that is required for methylreductase activity (8), had no effect. Because a transmembrane gradient has been proposed to be the actual activator instead of ATP (20, 21), a likely explanation for the loss of the majority of the methylreductase in dialyzed extracts seemed to be incomplete activation by a non-physiological ATP-dependent activation system. Therefore, the activation was reinvestigated in undialyzed extracts and in extracts that retained full methylreductase activity after a limited dialysis (pore size, M_r = 3,800).

Undialyzed cell-free extracts of *M. bryantii* did not require ATP for methylreductase activity under a H_2 or H_2+CO_2 (80:20) atmosphere (Fig. 1). However, the addition of 2.5 mM ATP reduced a short lag observed in the absence of ATP. Similar results were obtained for methane

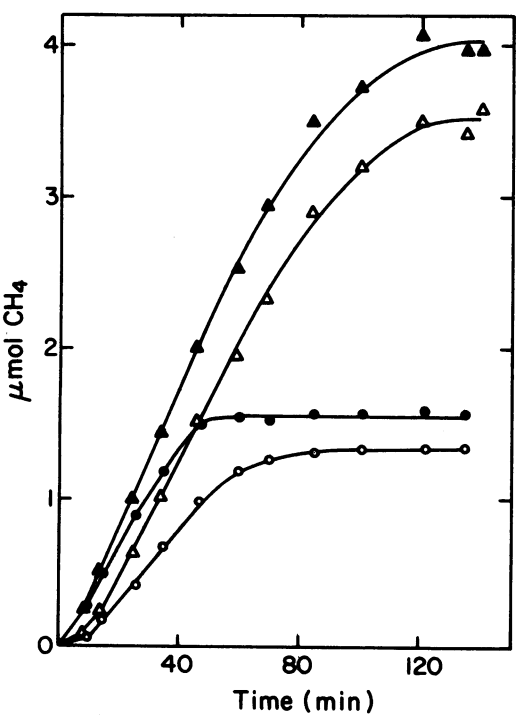

FIG. 1. Methane synthesis by undialyzed extracts in the presence and absence of ATP. Each assay contained 3.6 mg of protein and 1 μmol of CH_3-S-CoM in the presence (▲ and ●) or absence (△ and ○) of 500 nmol of ATP under a H_2 (● and ○) or H_2/CO_2 (▲ and △) atmosphere. In the absence of added CH_3-S-CoM, 510 nmol of CH_4 was formed under H_2,

TABLE 1. Nucleotide substrate for hexokinase in undialyzed extracts

Hexokinase added[a] (U)	Rate of G6P synthesis[b] (μmol/min)	U added/rate of synthesis
2.9×10^{-2}	3.3×10^{-2}[c]	0.88
3.0	1.4×10^{-3}	2,140.00
3.0×10^{-1}	1.1×10^{-3}	270.00
2.9×10^{-2}	6.1×10^{-4}	48.00
9.6×10^{-3}	1.6×10^{-4}	60.00

[a] Hexokinase was added to extracts containing 2.1 mg of protein, 10 mM $MgCl_2$, and 10 mM glucose. One unit of hexokinase is the amount of enzyme needed to form 1 μmol of G6P from ATP and glucose per min at 37°C.

[b] Determined as the G6P formed after 75 min and not corrected for the loss of G6P during the assay.

[c] ATP (5 μmol) was added, and the rate of G6P synthesis was determined between 1 and 8 min.

synthesis from endogenous C-1 units and CO_2 in the absence of CH_3-S-CoM as observed previously (17). One possible interpretation is that sufficient ATP was present in the extracts to activate the methylreductase. However, when the extracts were assayed directly for ATP with hexokinase and G6P dehydrogenase, the ATP concentration was less than or equal to 0.1 mM. Alternatively, ATP may be simultaneously synthesized and degraded so that, although the steady-state concentration is low, ATP is available to activate the methylreductase.

Cell extracts contain ATPase activity (17). Under the conditions used for the methylreductase assay, the level of ATPase was 6 nmol of inorganic phosphate released min^{-1} mg^{-1} of protein, and the apparent K_m for ATP was 0.5 mM. To test for ATP synthesis, we added hexokinase and glucose to the extracts under the conditions used for the methylreductase assay. G6P was synthesized at a linear rate of 0.7 nmol min^{-1} mg^{-1} of protein for 1 h. Therefore, the extracts may, in fact, synthesize substantial amounts of nucleotide triphosphates. This rate was probably an underestimate because G6P was unstable in these extracts: about 23% of 2 μmol of G6P was degraded in 60 min. Attempts to inhibit the breakdown of G6P with 10 mM NaF were unsuccessful. Furthermore, the substitution of glucose by the alternative substrates of hexokinase (fructose, deoxyglucose, or mannose) did not affect the yield of sugar phosphate formed. Thus, these sugar phosphates were also unstable in cell extracts.

In the experiments discussed above and elsewhere (17), hexokinase has always been used in great excess. Hexokinase has a broad nucleotide specificity. Because of the number of novel compounds found in methanogens (1), the phosphate donor to hexokinase was in question. To narrow the range of possible substrates, the rate

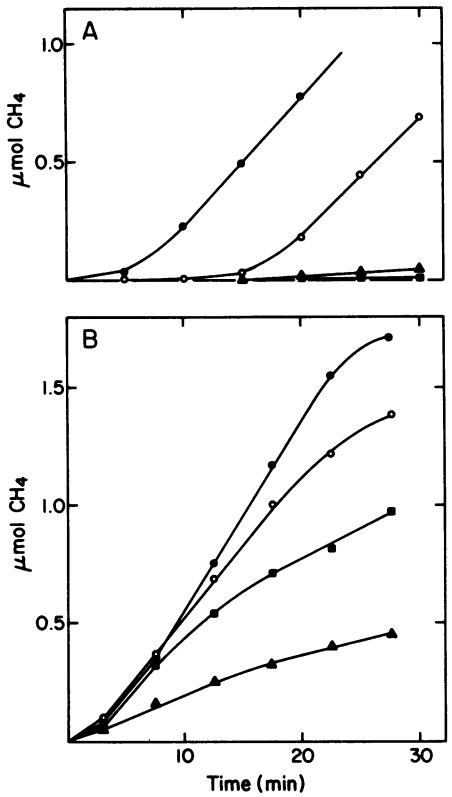

FIG. 2. Preincubation of the methylreductase with ATP or inhibitors. (A) Without preincubation. The assays contained 50 nmol of ATP (●), no additions (○), 1 mM FCCP (▲), or hexokinase (■). (B) Preincubated for 27.5 min at 37°C before the initiation of the assay by the addition of CH_3-S-CoM; ATP or inhibitors were also added at that time [symbols as in (A)]. The dialyzed extract contained 7.3 mg of protein.

of G6P synthesis in extracts was compared with the units of enzyme added (Table 1). When the units of hexokinase added to cell extracts was varied 300-fold, the rate of G6P synthesis varied 10-fold. At low concentrations of hexokinase, the ratio of the two was relatively constant at about 50. To determine whether the inhibitors of the hexokinase were present in cell extracts, the rate of G6P synthesis was also determined in the presence of a saturating concentration of ATP (Table 1). Under this condition, the rate of G6P synthesis was close to the units of hexokinase added. Thus, no potent inhibitors of the hexokinase were present. However, the presence of weak competitive inhibitors would not be detected by this procedure. From the ratio of units added to the rate of G6P synthesized from the endogenous substrate, all known substrates of hexokinase can be eliminated except ATP, dATP, and ITP (3). Therefore, it seems likely that ATP is, in fact, the endogenous substrate of hexokinase.

After the limited dialysis of the extracts, the quantity of G6P formed in the presence of hexokinase was reduced to 8 nmol h^{-1} mg^{-1} of protein. Furthermore, the lag observed in the methylreductase assay in the absence of exogenous ATP was increased (Fig. 2). As in the undialyzed extracts, the addition of ATP greatly reduced the lag. When hexokinase or the inhibitor FCCP was added to extracts, all methylreductase activity was lost (Fig. 2). However, when the extract was preincubated at 37°C before the addition of CH_3-S-CoM, the lag observed in the absence of ATP was abolished. Similarly, when hexokinase or FCCP was added after the preincubation, the inhibition was greatly reduced (Fig. 2).

The inhibition by hexokinase and FCCP was investigated in greater detail. When glucose was omitted, no inhibition was observed in the presence of hexokinase. Thus, inhibition was depen-

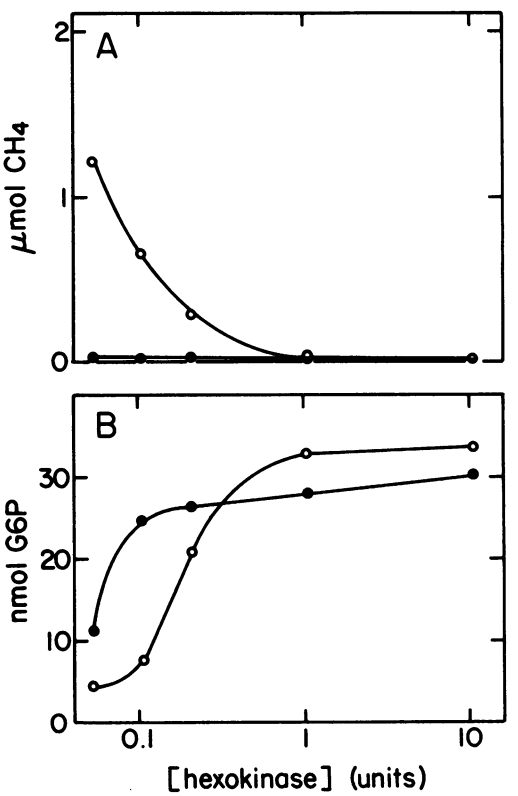

FIG. 3. Simultaneous titration of G6P synthesis and methylreductase with hexokinase. (A) Methane formed after 60 min; without hexokinase, 1.7 μmol of methane was found. (B) G6P formed after 60 min. The reaction mixtures contained 4.1 mg of dialyzed protein and 10 mM glucose in the presence (●) and absence (○) of 1 mM FCCP.

dent upon enzymatic activity. When the extract was titrated with hexokinase, the amount of G6P synthesized decreased at the same concentration of hexokinase at which methylreductase activity was restored (Fig. 3). However, when FCCP was also included, G6P synthesis was only slightly inhibited (Fig. 3). Thus, FCCP did not inhibit ATP synthesis. Furthermore, high concentrations of FCCP were required for the inhibition of the methylreductase. At 0.1 mM FCCP, the lag observed in the absence of ATP was lengthened from 15 to 20 min, but the eventual rate of methylreductase activity was only slightly reduced. The complete inhibition of the methylreductase was only obtained with 0.5 mM FCCP. The inhibition by FCCP could also be partially reversed by the addition of ATP (data not shown). In the presence of 1 mM FCCP, 100 nmol of ATP completely restored the activity of the methylreductase. However, the lag in the assay was about double that observed with 100 nmol of ATP in the absence of FCCP. Thus, at high concentrations FCCP appears to selectively inhibit the activation and not the velocity of the methylreductase. The effects of hexokinase and FCCP appear to be entirely consistent with the conclusion that ATP, and not a transmembrane gradient, is required for activation.

The effects of a number of compounds known to inhibit the formation of transmembrane gradients were also tested. As shown previously (18), halogenated uncouplers (CCCP and pentachlorophenol) and dinitrophenol were potent inhibitors of the methylreductase (data not shown). However, non-halogenated membrane-permeable ions (5 mM triphenylmethylphosphonium bromide and 250 mM sodium acetate) had no effect. In addition, 0.125% deoxycholate, 1 mM valinomycin, and 1 mM *N,N'*-dicyclohexylcarbodiimide also had no effect. Toluene was routinely incorporated into buffers as a bactericide. Toluene disrupts the membranes of many bacteria and eucaryotes (24). The results of control experiments indicated that toluene had no effect on the activation of the methylreductase. Thus, a transmembrane gradient has no role in the activation of the methylreductase.

Characteristics of ATP activation. Because ATP dramatically shortened the lag in the methylreductase assay after limited dialysis (Fig. 2), ATP activation could be determined separately from enzyme activity by following the time course of the methylreductase assay. This method has some limitations because the final rate of the methylreductase was not entirely independent of the activation (Fig. 4). Nevertheless, it was possible to characterize the activation qualitatively at significant levels of methylreductase activity. Very little ATP was required for optimal methylreductase activity (Fig. 4). One half of the maximal rate of the methylreductase or activation (1/lag) was obtained at 25 nmol of ATP (0.125 mM). When greater than 35 nmol of ATP was added, the time course of the assay became curvilinear, so that the maximal velocity of methylreductase was difficult to estimate. Thus, in certain experiments, 100 nmol of ATP appeared to inhibit; inhibition by high concentrations of ATP may have been due to the formation of AMP. In addition to ATPase, these extracts contained adenylate kinase (unpublished data). AMP has been shown to be an inhibitor of the methylreductase from *Methanosarcina barkeri* (15). Furthermore, within the concentration range studied, the rate of activation varied nearly 10-fold, whereas the velocity of the methylreductase varied 3-fold. When extracts were prepared without sucrose (Fig. 4), the concentration of ATP required for maximal activity increased, and no activity was observed at low concentrations of ATP. In addition, the

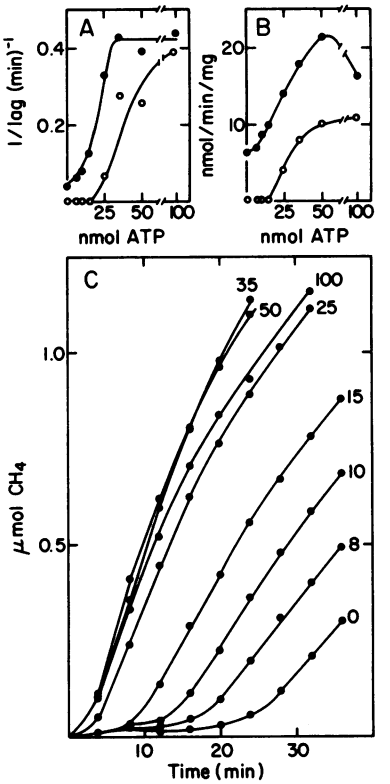

FIG. 4. Titration of the methylreductase with ATP. (A) Effect of ATP on the lag. (B) Effect of ATP on the velocity of the methylreductase. (C) Progress curves of the methylreductase assay where the numbers refer to the nanomoles of ATP added. The assays contained 3.6 mg of dialyzed protein. Symbols: ●, extract dialyzed with sucrose; ○, extract dialyzed without sucrose.

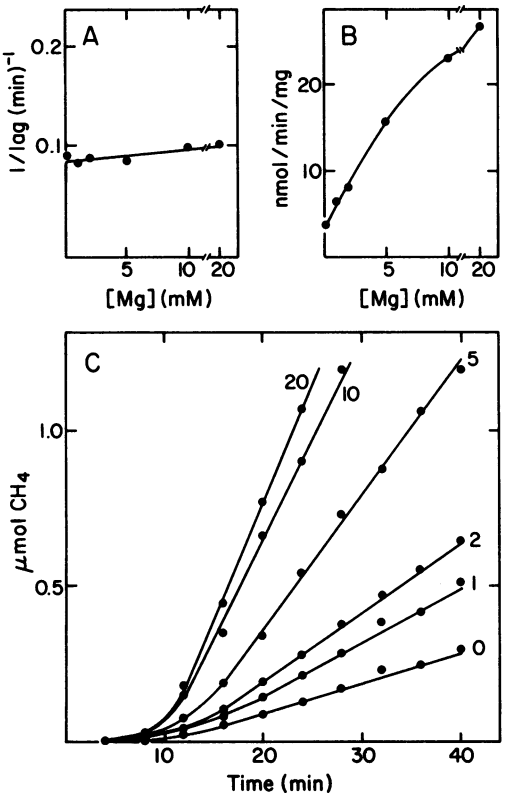

FIG. 5. Titration of methylreductase with magnesium. (A) Effect of magnesium on the lag. (B) Effect of magnesium on the velocity. (C) Progress curves of the methylreductase assay where the numbers refer to millimolar $MgCl_2$. The assays contained 2.8 mg of dialyzed protein.

maximal activity obtained at saturating concentrations of ATP was only one half that found in extracts prepared with sucrose.

At an optimal concentration of ATP, 20 mM Mg^{2+} was required for maximal activity (Fig. 5). However, the rate of activation did not change from 1 to 20 mM Mg^{2+}. In contrast, the velocity of the methylreductase increased sevenfold over the range of Mg^{2+} concentrations studied. Thus, the activation did not require high concentrations of Mg^{2+}.

Both the rate of activation and the velocity of the methylreductase were dependent on the concentration of extract in the assay (Fig. 6). As the amount of protein in the assay was increased eightfold, the rate of activation increased threefold. In contrast, the specific activity of the methylreductase had a distinct optimum between 4 and 6 mg of protein per assay. A lower specific activity was obtained at lower and higher protein concentrations. A complex dependence of activity on protein concentration is often observed in multicomponent systems, such as the methylreductase (8).

Temperature had a pronounced effect on both the rate of activation and the velocity of the methylreductase. Although the mechanism of neither of these temperature effects is known, Arrhenius plots provide some qualitative information concerning their complexity. Thus, in the presence of exogenous ATP, the activation energy for the methylreductase was 5.3 kcal/mol (ca. 22.2 kJ/mol) (Table 2). For the rate of activation, the temperature effect was much greater. In the presence and absence of exogenous ATP, the activation energy for the activation was 18 and 21 kcal/mol (ca. 75.4 and 87.9 kJ/mol), respectively. Thus, the temperature-dependent step in the activation of the methylreductase may have been the same in the presence and absence of exogenous ATP. In the absence of exogenous ATP, the activation energy of the velocity of the methylreductase increased to 22.7 kcal/mol (ca. 95 kJ/mol). Under this condition, the velocity of the methylreductase may have depended largely upon the extent of activation.

When the extracts were prepared without

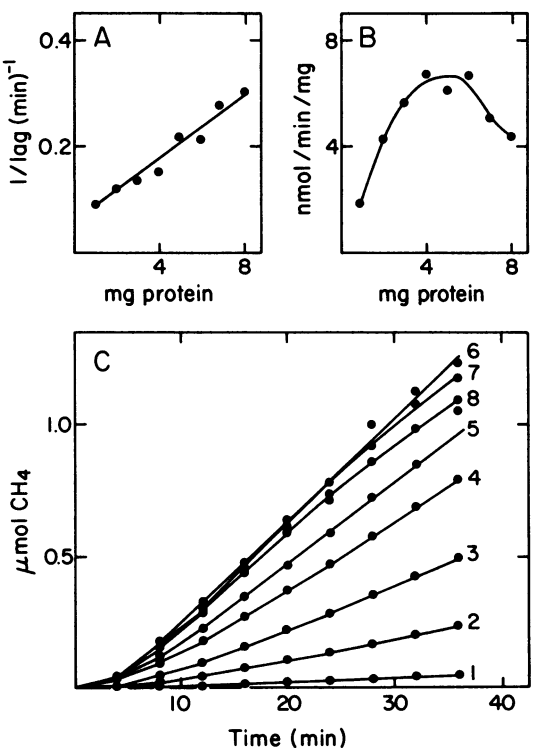

FIG. 6. Effect of the protein concentration on the methylreductase. (A) Effect of protein concentration on the lag. (B) Effect of protein concentration on the velocity. (C) Progress curves of the methylreductase assay where the numbers refer to the milligrams of dialyzed protein in the assay. The volume of the assay was kept constant by the addition of dialysis buffer.

sucrose, the temperature dependence of the velocity of the methylreductase increased even in the presence of exogenous ATP (Table 2). As noted above, sucrose also increased the specific activity of the methylreductase and decreased the amount of ATP required for activation. The cause of these effects is not known. However, it was necessary to add sucrose before cell breakage. Sucrose did not affect the rate of G6P synthesis in the presence of hexokinase. Possible roles of sucrose may be: (i) scavenging free radicals generated upon cell lysis and (ii) altering the osmotic properties of the buffer and thereby stabilizing enzyme tertiary structure.

The stability of the activated state was studied after the activation of the methylreductase in the absence of exogenous ATP. Hexokinase was added to prevent further activation, and the initial velocity of the methylreductase was measured upon the addition of CH_3-S-CoM (Fig. 7). A precipitous drop in the methylreductase activity was observed within several minutes after the addition of hexokinase. The loss in activity followed pseudo-first-order kinetics. The half-life of the activated methylreductase was 4.7 min. In the presence of CH_3-S-CoM (or during catalysis), the activated methylreductase was more stable (the half-life was 37 min).

For a further test of the stability of the activated methylreductase, extracts prepared by column chromatography were activated by ATP and rapidly desalted at 5°C (Table 3). As noted above, the specific activity was less than that obtained after limited dialysis. Nevertheless, the extracts retained activity in the absence of exogenous ATP for 20 min (Table 3). After that time, activity was rapidly lost, with a half-life of 16 min. Thus, the activation by ATP must involve the modification of high-molecular-weight proteins or protein-bound cofactors.

TABLE 2. Effect of temperature on the methylreductase[a]

Condition	Activation energy (kcal/mol)	
	Velocity	1/lag
With 50 nmol of ATP	5.3[b]	18.1[c]
Without added ATP	22.7	21.0
With 50 nmol of ATP, extract dialyzed without sucrose	10.8	15.3

[a] The experimental procedures are described in the text. The activation energies were calculated from the linear least-squares analysis of data not shown.

[b] In kilojoules per mole, the values were 22.2, 95, and 45.2.

[c] In kilojoules per mole, the values were 75.7, 87.9, and 64.1.

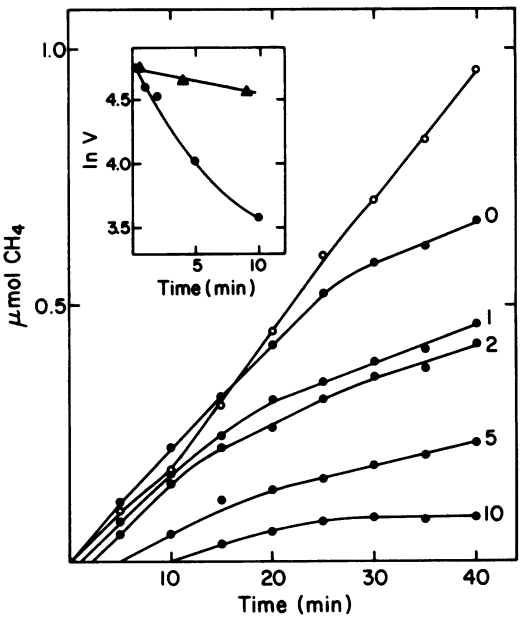

FIG. 7. Inactivation of the methylreductase in the presence of hexokinase. The dialyzed extract, containing 8 mg of protein, was preincubated for 15 min in the absence of ATP before the addition of CH_3-S-CoM (○) or hexokinase (●). CH_3-S-CoM was then added to the assays with hexokinase at 0, 1, 2, 5, and 10 min. Inset. The velocity (V) of the methylreductase versus the time of incubation with hexokinase. The velocities were determined from the initial time points for the incubations without CH_3-S-CoM (●) and between successive time points with CH_3-S-CoM and hexokinase added at 0 min (▲).

ATP analogs. ATP analogs were tested for the inhibition of the activation by ATP. At concentrations of 0.25 mM, neither β,γ-methylene ATP nor α,β-methylene ATP had any effect in the presence or absence of exogenous ATP. However, at a concentration of 0.25 mM, PNP inhibited the velocity of the methylreductase 69% in the absence and 24% in the presence of 0.25 mM (50 nmol) exogenous ATP. When the extract was activated before the addition of PNP, the inhibition was eliminated. Thus, PNP acted at the activation and not during the catalysis of the methylreductase. In the presence of hexokinase sufficient for 83% inhibition of the methylreductase, the further addition of 0.25 mM PNP resulted in 95% total inhibition. Thus, the inhibition by hexokinase and PNP was additive. This result also eliminated the possibility that PNP substituted for ATP in the activation but supported a lower maximal velocity than ATP.

The extracts were titrated with up to 100 nmol of PNP (Fig. 8). In that concentration range, the rate of activation was inhibited sevenfold,

TABLE 3. Stability of the activated methylreductase

Treatment of extracts[a]	Methylreductase activity (nmol min^{-1} mg^{-1} of protein)
a. None	<0.1
b. +0.25 mM ATP	3.9
c. +ATP, desalted	1.5
d. +ATP, desalted, +ATP	1.6

[a] The extract was pretreated by Sephadex G-25 column chromatography as described in the text. The extract (10.1 mg in 0.5 ml) was incubated at 37°C with 12.5 μmol of $MgCl_2$, 125 nmol of ATP, partially purified component B, and 5 μmol of CH_3-S-CoM under H_2. The rate of methane formation was determined between 11 and 14 min (reaction b). In reaction a, the extract was treated as in reaction b except that ATP was omitted. At 15 min, reaction b was cooled on ice, taken into an anaerobic chamber, and passed through an anaerobic Sephadex G-25 column (0.4 by 10.0 cm) at 5°C, and the protein fraction was pooled. A 0.2-ml portion of the protein fraction was combined with 5 μmol of $MgCl_2$, 1 μmol of CH_3-S-CoM, and partially purified component B under H_2, and the methylreductase assay was initiated by warming to 37°C (reaction c). Reaction d was prepared as described for reaction c except that 50 nmol of ATP was added. The time between placing reaction b in the ice bath and the initiation of reactions c and d was 15 min. The rates of the methylreductase in reactions c and d were linear for an additional 20 min, in which time the specific activities were determined.

whereas the velocity of the methylreductase was inhibited threefold. Furthermore, the maximal inhibition of the activation was obtained at 10 nmol of PNP, whereas much higher concentrations were required for the inhibition of the velocity of the methylreductase. The stability of the activated methylreductase in the presence of PNP was also measured. With 0.25 mM PNP, 5 mM PNP, or 26 U of hexokinase, the half-life of the activated methylreductase in the absence of CH_3-S-CoM was 16, 18, or 14 min, respectively. During catalysis in the presence of 0.25 mM PNP, the half-life was 39 min. The inability of high concentrations of PNP to prevent the inactivation of the methylreductase suggests that inactivation was not caused by an enzyme(s) catalyzing the reverse reaction of the activation.

DISCUSSION

The evidence that ATP is required for methylreductase activity is as follows. In extensively dialyzed extracts, ATP is absolutely required. Without dialysis or subsequent to limited dialysis, there is sufficient endogenous ATP synthesis to account for the observed activity. Hexokinase inhibits the methylreductase by competing with the methylreductase for ATP. Detergents and membrane permeable ions have no effect. Halogenated uncouplers inhibit, however, FCCP inhibits only at concentrations necessary for the direct modification of proteins and has little effect at concentrations sufficient for its action as an uncoupler. In *Halobacterium* vesicles with a lipid composition similar to methanogen membranes, FCCP collapses proton gradients at a concentration of 10 μM or 50-fold less than the concentration necessary to inhibit the methylreductase (10). Thus, there is little doubt that ATP is required for activation even in very crude extracts. The loss in activity due to dialysis has recently been shown to be due to the removal of a cobamide activator (W. B. Whitman and R. S. Wolfe, Fed. Proc. **41**:1152, 1982).

Surprisingly low concentrations of ATP are required for activation. Because the concentration of ATP required for maximal activation (0.25 mM) is much lower than the ATP concentration in vivo (18), ATP probably does not simply act as an effector. The complicated re-

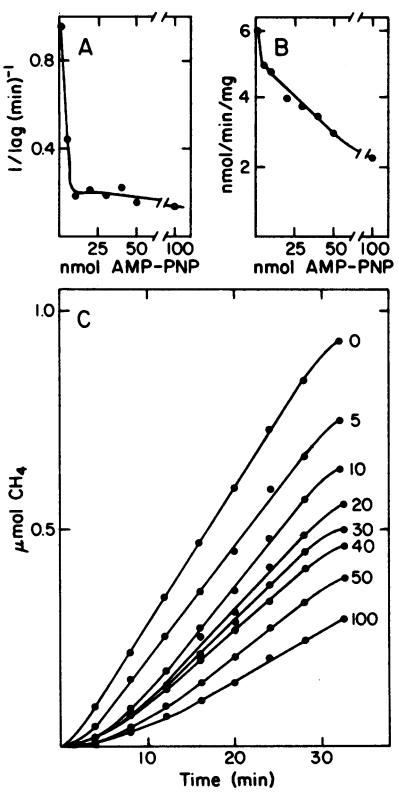

FIG. 8. Titration of the methylreductase with PNP. Each assay contained 5.2 mg of dialyzed extract and no added ATP. PNP was added 10 min before the initiation of the assay with CH_3-S-CoM. (A) Effect of PNP on the lag. (B) Effect of PNP on the velocity. (C) Progress curves of the methylreductase assay where the numbers refer to the nanomoles of PNP.

sponse of activation to the extract concentration and the strong temperature dependence also suggest that activation is complex and probably involves one or more enzymatic steps. In support of this interpretation, the non-hydrolyzable ATP analog PNP also inhibited the activation at low concentrations. Thus, ATP hydrolysis may be necessary for activation.

Previous workers have shown that catalytic amounts of ATP are required by the methylreductase (7, 17). This result was confirmed. In the presence of either hexokinase or PNP, the methylreductase is active for a short time in the absence of further activation by ATP. However, the half-life of the active state was only 5 to 15 min. This result emphasizes the difficulty in determining the chemical nature of the activation in the absence of a means of stabilizing the active factor. Attempts to rapidly extract a low-molecular-weight factor which substituted for ATP were unsuccessful. Thus, the site of activation may be a protein or a protein-bound cofactor. To confirm this hypothesis, we passed activated extracts through an anaerobic desalting column at 5°C. Activity was retained in the high-molecular-weight or protein fraction.

Importantly, the activation was several times more stable in the presence of CH_3-S-CoM than in its absence. Whether this stability was due to catalysis could not be determined due to the long lags and loss in activity after incubations in the absence of H_2. However, this result suggests that ATP activation occurs close to or at the site of CH_3-S-CoM reduction. Similarly, ATP is required for methylreductase activity with either NADPH or H_2 as the electron donor in a partially resolved system from *Methanobacterium thermoautotrophicum* (6). Thus, electron carriers unique to either system cannot be the site of ATP activation.

The methylreductase requires high concentrations of magnesium for maximal activity (7). The requirement for high magnesium was for catalysis and not activation. A requirement for low concentrations of magnesium for activation could not be eliminated because magnesium may contaminate the extracts. In chloroplasts and photosynthetic bacteria, several Calvin cycle enzymes require high concentrations of magnesium for full activity (13). Magnesium is an important physiological effector in chloroplasts (16); by analogy, it may well function as a physiological effector of the methylreductase.

ACKNOWLEDGMENTS

This work was supported by National Science Foundation grant PCM 81-18178 and by U.S. Public Health Service grant AI 12277. W.B.W. was the recipient of Public Health Service postdoctoral fellowship AI05915 from the National Institutes of Health.

We thank V. Gabriel for excellent technical assistance.

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