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INHIBITION OF THE METHYLCOENZYME M METHYLREDUCTASE SYSTEM BY NAD^+ AND $NADP^+$ IN CELL-EXTRACTS OF METHANOSARCINA BARKERI

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Summary In cell extracts of Methanosarcina barkeri, the methylcoenzyme M methylreductase system with H_2 as the electron donor was inhibited by NAD^+ and $NADP^+$, but NADH and NADPH had no effect on enzyme activity. NAD^+ (4 and 8 mM) shifted the saturation curve for methylcoenzyme M from hyperbolic (Hill coefficient [n_H] = 1.0; concentration of substrate giving half maximal velocity [K_m] = 0.21 mM) to sigmoidal (n_H = 1.5 and 2.0), increased K_m (K_m = 0.25 and 0.34 mM), and slightly decreased V_{max}. Similarly $NADP^+$ at 4 and 8 mM increased n_H to 1.6 and 1.85 respectively, but the K_m values (0.3 and 0.56 mM) indicated that $NADP^+$ was a more efficient inhibitor than NAD^+.

The soluble enzyme system, methylcoenzyme M methylreductase, is currently accepted as catalysing the terminal step of methanogenesis in which CH_3-S-CoM is reductively demethylated to produce methane and HS-CoM in a reaction requiring ATP, magnesium ions, and a H_2 atmosphere (1-3). In Methanobacterium thermoautotrophicum the methylreductase complex (or system) has been resolved into three components: (i), component A, an oxygen-sensitive protein fraction comprising four subcomponents, having hydrogenase activity and a mass of 500,000 daltons (4); (ii), component B, an oxygen-sensitive, colorless, heat-stable cofactor of unknown structure; and (iii), component C, an acidic protein (5). Component C has recently been identified as the methylcoenzyme M methylreductase protein (6).

Important to the acceptance of the methylreductase step as terminal to the energy-producing pathways of methanogenesis is a knowledge of the enzyme's response to metabolic effectors. With the exception of the studies by Mountfort (7) demonstrating that ATP activation of methylreductase was allosterically

Abbreviations: CH_3-S-CoM, methylcoenzyme M or 2-(methylthio) ethanesulfonic acid; HS-CoM, 2-mercaptoethanesulfonic acid; TES, N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid. BBL, Baltimore Biological Laboratories.

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inhibited by AMP in cell-free extracts of M. barkeri, there have been no attempts to define the possible regulatory mechanisms for the control of the enzyme. This communication describes inhibition of the methylreductase system by oxidised pyridine nucleotides.

METHODS AND MATERIALS

Culture of the organism. Methanosarcina barkeri strain 227 was kindly supplied by R.A. Mah, Division of Environmental and Nutritional Sciences, School of Public Health, University of California. The medium for growth of the methanogen was based on that described by Mah et al. (8) and contained the following components per 100 ml: KH_2PO_4, 30 mg; NH_4Cl, 10 mg; $MgCl_2.6H_2O$, 0.1 mg; yeast extract (BBL, Cockeysville, Md, U.S.A.), 0.2 g; Trypticase (BBL), 0.2 g; and rezazurin; 0.1 mg. The medium was adjusted to pH 6.8, sterilised, and cooled while flushing with 100% N_2. Sterile reducing agent (2 ml of a 1.25% $Na_2S.9H_2O$ - 1.25% cysteine hydrochloride solution) and methanol (2-ml of a 50% [v/v] solution) were added prior to inoculation.

Large scale cultivation of the organism was carried out in 12-litre fermentors and with a 5% inoculum, growth yields of approximately 6 gm wet weight of cells were obtained per litre after eight days incubation. An open gas line was connected to the fermentors at the onset of active gas production to prevent a buildup of pressure in the headspace.

Preparation of cell extracts. Cells were harvested by centrifugation at 7,000xg for 20 min at 2°C in stoppered stainless steel centrifuge tubes that had previously been gassed with hydrogen. Harvested cells were diluted with a equal volume of 50 mM TES buffer (pH 7.0) frozen to -70°C and broken by passage through a Hughes Press. The frozen contents were transferred to a centrifuge tube in an anaerobic glove box (Coy Lab. Prod., Ann Arbor, Mich., U.S.A.) in a 3% H_2/97% N_2 atmosphere. The contents were thawed, placed under a 100% H_2 atmosphere, and centrifuged at 30,000 xg for 30 min at 2°C after addition of 1 mg of DNase (EC 3.1.4.5). The supernatant solution was stored under a H_2 atmosphere at -20°C until used.

Crude cell extract (3 ml) was dialysed in tubing with average pore size, M_r = 3500 (Arthur H. Thomas Co., Philadelphia, Pa, U.S.A.) at 2°C against 1.5 litres of deoxygenated 50 mM TES buffer (pH 7.0) containing 10 mM $MgCl_2$ and 3.3 mM dithiothreitol. During dialysis (24 h) the buffer was sparged with hydrogen for the first 4 h and stirred under a hydrogen atmosphere for the remaining 20 h. Transfer of cell extract to and from the dialysis chamber was carried out in the anaerobic glove box. Dialysed cell extract was quick frozen using an ethanol-dry ice mixture and stored under H_2 at -20°C until required.

Methyl CoM methylreductase assay. Methylreductase was assayed in 2 ml reaction vials sealed with butyl rubber septum stoppers. The standard reaction mixture (0.25 ml) contained 20 µmol of potassium TES buffer (pH 6.0), 5 µmol of $MgCl_2$, 1 µmol of ATP and 1 µmol of CH_3-S-CoM. Dialysed cell extract was added as indicated in the protocol of each experiment. The gas atmosphere was hydrogen. The reaction was initiated by addition of CH_3-S-CoM and vials were incubated in a water bath at 37°C for 40 min. Gas samples (50 µl) were withdrawn at appropriate intervals using a 50 µl gas sampling syringe (Precision Sampling Co., Baton Rouge, LA., U.S.A.) and methane was determined at 72°C on a Carle model 5900 gas chromatograph equipped with a flame ionisation detector. Rates were determined from the linear portion of the CH_4 production curve. In most experiments methane production was linear after an initial lag of 3-5 min. In cases where low concentrations of CH_3-S-CoM were used, methane production often declined towards the end of the 40 min time-course due to exhaustion of substrate.

Spectrophotometric assay of pyridine nucleotide-linked hydrogenase. In examining the effects of oxidised pyridine nucleotides on the methylreductase system, pyridine nucleotide-linked hydrogenase was assayed to determine loss of NAD^+ or $NADP^+$ due to the formation of NADH or NADPH. The assay mixture (2.0 ml) in sealed 3.5 ml anaerobic cuvettes contained: TES buffer (pH 6.0), 160 µmol; $MgCl_2$, 40 µmol; ATP, 8 µmol; $NADP^+$ or NAD^+, 8 to 16 µmol; and dialysed cell-free extract. The gas atmosphere was 100% H_2. The reaction was started with addition of NAD^+ or $NADP^+$ and cuvettes were incubated at 37°C for 30 min. Enzyme activity was assayed by following the change in absorption at 340 nm using a Beckman ACTA CII spectrophotometer. Reaction mixtures with argon gas were used as controls.

Protein determination. Protein was determined by the method of Gornall et al. (9) using bovine serum albumin as a standard.

Chemicals. DNase, NAD^+, $NADP^+$, NADH, NADPH and TES were obtained from Sigma Chemical Co., St. Louis, Mo, U.S.A. Coenzyme M was obtained from Merck-Schuchardt Co., Munich, and CH_3-S-CoM was kindly supplied from Professor R.S. Wolfe's laboratory, Department of Microbiology, University of Illinois, U.S.A.

RESULTS

Summary of optimal conditions. In all assays dialysed crude cell extract was used because undialysed extract actively produced methane in the absence of added CH_3-S-CoM. Dialysis removed methanogenic precursors present in the extract eliminating endogenous methane production. Near optimal rates of methane production occurred with an ATP concentration of 1 mM, and increasing ATP to 4 mM resulted in only a slight increase in activity. The amount of CH_3-S-CoM required for optimal activity was 4 mM, and for $MgCl_2$, 20 to 30 mM. The pH optimum for the methylreductase system was in the range 5.5 to 6.0. No temperature optimum was determined, and assays were carried out at 37°C as in previous studies (7,10). Hydrogen was the electron donor and no activity was detected when hydrogen or ATP was omitted from the reaction mixture. Under standard conditions the rate of methane production was in the range 1.9 to 2.2 $nmol.min^{-1}.(mg\ cell\ extract\ protein)^{-1}$.

Effect of pyridine nucleotides on activity of the methylreductase complex. In the scan for pyridine nucleotide effects the standard reaction mixture was used, except that the concentration of CH_3-S-CoM was equivalent to its K_m value (0.21 mM). This was in the range where the greatest response to metabolite effectors was expected to occur. The ranges of pyridine nucleotide concentrations chosen were based on the physiological levels found in other anaerobically grown bacteria (11, 12) and the effects on the methylreductase system are summarised

Table 1. Ability of various metabolites to inhibit or stimulate the methylreductase system [a]

Compound	Concentration (mM)	% Inhibition	% Stimulation
NAD^+	4-8	14-50	-
NADH	4-8	0	0
$NADP^+$	4-8	48-74	-
NADPH	4-8	0	0

[a] The reaction mixture and experimental conditions were as described in Materials and Methods. The amount of cell-free extract added to the assay mixture was in the range, 1.8 to 2.0 mg protein, and the gas atmosphere was 100% H_2. In the absence of added effector the mean activity of methylreductase was 1.1 nmol $CH_4.min^{-1}.mg\ (cell\ extract\ protein)^{-1}$.

in Table 1. NAD^+ and $NADP^+$ both inhibited the methylreductase system, but no effect was observed with NADH and NADPH. For all pyridine nucleotide additions pH was monitored at the end of the assays and was always within the range, 5.8 to 6.2.

In the presence of NAD^+ total methane produced (range, 48 to 55 nmol) indicated that methane was formed in approximately stoichiometric amounts from 50 nmol of added CH_3-S-CoM. With $NADP^+$ at 4 and 8 mM, values for total methane were 93% and 85% of the stoichiometric value, respectively.

Characterisation of inhibition by oxidised pyridine nucleotides. Addition of NAD^+ and $NADP^+$ to the reaction system resulted in transformation of the saturation curve for CH_3-S-CoM from hyperbolic to sigmoidal (Figs. 1a, 2a). NAD^+ at 4 and 8 mM increased the Hill coefficient (n_H) from 1.0 to 1.5 and 2.0 respectively (Fig.1b). The concentration of substrate giving half maximal velocity (K_m) increased indicating decreased affinity of the enzyme system for methyl CoM, and V_{max} slightly decreased so that at 8 mM NAD^+, V_{max} was 3.6 nmol $CH_4.min^{-1}$ compared to 4.1 nmol $CH_4.min^{-1}$ in the absence of the effector. Addition of $NADP^+$ at 4 and 8 mM increased n_H to 1.6 and 1.85 respectively (Fig.2b) and the increase in K_m was greater than occurred with the same concentrations of NAD^+. As with NAD^+, there was a decrease in V_{max}. At 8 mM

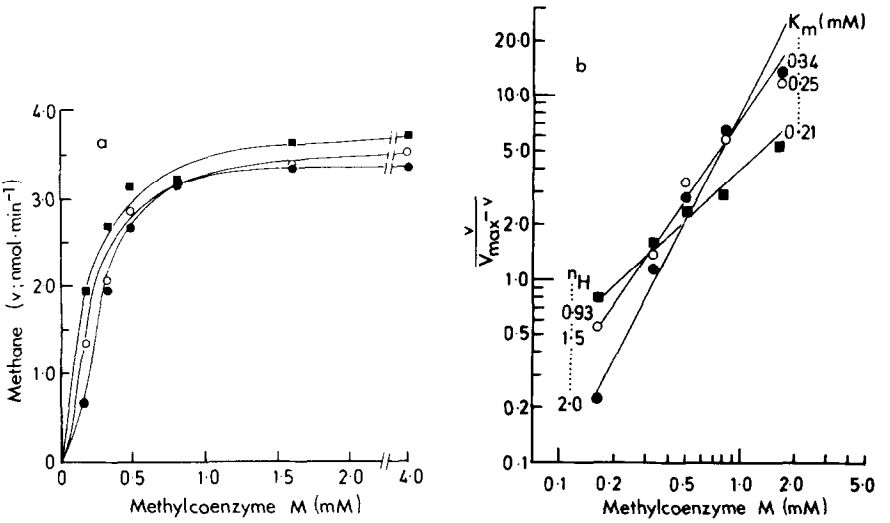

Fig.1. Effect of NAD^+ on the saturation curve for CH_3-S-CoM cleavage by the methylreductase system (a), and (b), Hill plots of the same data. V_{max} values were determined from Lineweaver-Burke plots of 1/v [(nmol of methane/min^{-1})] versus 1/CH_3-S-CoM (mM^{-1}). Each reaction mixture contained 1.65 mg of cell-extract protein. NAD^+ concentrations (mM); ■, 0; ○, 4.0; ●, 8.0.

$NADP^+$, V_{max} was 3.2 nmol.min^{-1} compared to 4 nmol.min^{-1} in the absence of the effector.

To determine whether or not the effects of NAD^+ and $NADP^+$ were due to oxidation of H_2-reduced electron carriers involved in electron transport to methane, activity of pyridine nucleotide-linked hydrogenase was measured. Also,

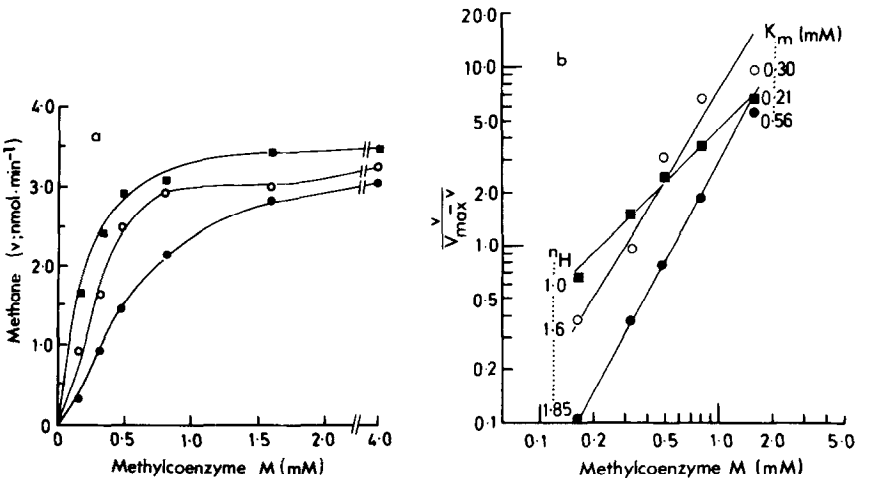

Fig.2. Effect of $NADP^+$ on the saturation curve for CH_3-S-CoM cleavage by the methylreductase system (a), and (b), Hill plots of the same data. V_{max} values were determined from Lineweaver-Burke plots of 1/v [(nmol of methane/min)$^{-1}$] versus 1/CH_3-S-CoM (mM^{-1}). Each reaction mixture contained 1.8 mg of cell-extract protein. $NADP^+$ concentrations (mM); ■, 0; ○, 4.0; ●, 8.0.

Table 2. Comparison of NADH and NADPH with H_2 as electron donors for the methylreductase system of *M. barkeri* [a]

Electron donor	Reaction rate[b] (nmol $CH_4.min^{-1}$)	% activity with H_2 as electron donor
H_2	4.5 ± 0.2	-
NADH[c]	0.8 ± 0.15	17.7
NADPH[c]	1.2 ± 0.03	26.6

[a]The reaction mixture and experimental conditions were as described in Materials and Methods. The concentrations of NADPH and NADH were 4 mM each, and the reaction mixtures all contained 1.9 mg of cell extract protein.

[b]Data represent the mean ± SE for triplicate determinations.

[c]The gas phase was 100% argon.

since both NADPH and NADH were found to act as electron donors for the methylreductase reaction (Table 2) measurement of the reduction of oxidised pyridine nucleotides was made in the absence of CH_3-S-CoM. Under conditions where cell-extract protein was at the same concentrations as for the experiments described in Figs.1 and 2, losses of NAD^+ and $NADP^+$ due to pyridine nucleotide-linked hydrogenase were $\leqslant$0.32 and $\leqslant$0.8 nmol.min^{-1}.ml^{-1} (Table 3).

Table 3. Pyridine nucleotide-linked hydrogenase activity in cell extracts of *M. barkeri* [a]

Pyridine nucleotide	Pyridine nucleotide concentration (mM)	Pyridine nucleotide linked hydrogenase[b] (nmol.min^{-1}.ml^{-1})
NAD^+	4.0	0.20 ± 0.01
	8.0	0.32 ± 0.03
$NADP^+$	4.0	0.52 ± 0.05
	8.0	0.8 ± 0.06

[a]The complete reaction mixture and experimental conditions were as described in Materials and Methods. Cell-extract protein concentration in the reaction mixtures (2 ml) was 6.6 and 7.2 mg.ml^{-1} for the NAD^+ and $NADP^+$ linked hydrogenases respectively. The hydrogen concentration in the gas phase was 100%.

[b]Data represent the mean ± SE for triplicate determinations.

Thus the predicted rates of NADH and NADPH formation in a 0.25 ml reaction system would be $\leqslant$0.08 and $\leqslant$0.2 nmol.min^{-1} respectively.

DISCUSSION

Although the methylreductase system has been resolved into component fractions with component C being the true methylreductase (P. Hartzell and R.S. Wolfe, Abstr. Ann. Meet. Am. Soc. Microbiol. 1983, I 12, p. 141; 6) there are difficulties in studying the effects of metabolites on this protein. Principle among these is that an enzyme system (hydrogenase, or NADPH : F_{420} oxidoreductase) together with other components is required to provide electrons to the methylreductase and a method for studying the effects of metabolites on the methylreductase protein independent of the other components of the system is not yet accessible. The approach used in this study has been to examine the effects of NAD^+ and $NADP^+$ on the methylreductase system before consideration is given to the components which are affected.

The kinetic response of the methylreductase system to NAD^+ and $NADP^+$ suggested that these effectors interacted with the enzyme complex allosterically. The saturation curves for CH_3-S-CoM showed positive co-operativity during inhibition suggesting that the binding of CH_3-S-CoM was facilitated by the binding of other CH_3-S-CoM molecules (13). $NADP^+$ was a more efficient inhibitor than NAD^+ as there was a larger K_m increase resulting from its addition. Oxidation of H_2-reduced carriers involved in electron transport to the site of CH_3-S-CoM reduction by NAD^+ or $NADP^+$ was unlikely to be a cause of inhibition since rates of NADH and NADPH formation were very low [$\leqslant$0.1 nmol.min^{-1}.(mg protein)$^{-1}$ compared to $\geqslant$2 nmol CH_4.min^{-1}.(mg protein)$^{-1}$ for the methylreductase system assayed under standard conditions]. Furthermore, if a significant decrease in electron flow had occurred, then at the higher concentrations of CH_3-S-CoM, a greater inhibition by $NADP^+$ and NAD^+ would have been expected than was observed.

Absence of any effect with NADH and NADPH suggested that these compounds did not act as metabolic effectors. However, both NADPH and NADH were found to substitute for H_2 in the supply of reducing equivalents for CH_3-S-CoM cleavage

(Table 2). Thus, absence of an NADPH or NADH effect must be qualified since either cofactor may have contributed a portion of reducing equivalents along with H_2 although no net increase in activity occurred. Whether NADPH or NADH play an important role in the supply of reducing equivalents to CH_3-S-CoM via various carriers *in vivo* remains to be clarified, but it is known that NADPH and NADPH: F_{420} oxidoreductase can substitute for hydrogenase as the electron donor for the methyl coenzyme M methylreductase protein (component C) in *M. thermoautotrophicum* (6).

Inhibition of the methylreductase system by NAD^+ and $NADP^+$ complements a previous report describing inhibition by AMP (7). Yet it is not obvious why the methylreductase system should be inhibited by oxidised pyridine nucleotides and AMP. In the cell when the levels of oxidised pyridine nucleotides and AMP increase indicating a decrease in the supply of energy and reducing power, stimulation of the energy-producing pathways usually results (14). However, the *in vitro* studies described here and in previous studies (7) indicate that the methylreductase complex responds in the opposite manner. Such observations would appear to be inconsistent with the accepted role of the enzyme complex in the catalysis of the terminal step of methanogenic pathways coupled to energy production. In order to resolve this apparent anomaly it will be important to further elucidate the effects of NAD^+, $NADP^+$ and AMP on the methylreductase reaction. We are now attempting to determine the components of the methylreductase system which interact with these effectors. It is anticipated that these studies together with whole cell investigations relating intracellular adenylate and pyridine nucleotide levels to methanogenesis, should contribute to a clearer understanding of the response to methylreductase to metabolic inhibitors. Consequently the role of the methylreductase reaction in methanogenesis might be better evaluated.

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