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7-Mercaptoheptanoylthreonine phosphate functions as component B in ATP-independent methane formation from methyl-CoM with reduced cobalamin as electron donor

D. Ankel-Fuchs, R. Böcher, R.K. Thauer, K.M. Noll* and R.S. Wolfe*

Fachbereich Biologie-Mikrobiologie, Philipps Universität, Karl-von-Frisch-Straße, D-3550 Marburg, FRG and **Department of Microbiology, University of Illinois, 407 South Goodwin Avenue, Urbana, IL 61801, USA*

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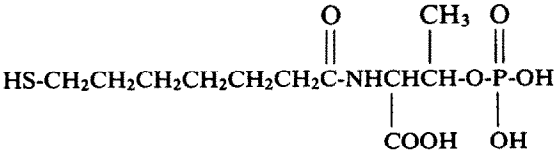
Purified methyl-CoM reductase of *Methanobacterium thermoautotrophicum* (strain Marburg) catalyzed the reduction of methyl-CoM to methane with reduced cobalamin, when either synthetic 7-mercaptoheptanoylthreonine phosphate (HS-HTP) or naturally occurring component B was present. With both compounds the same maximal specific acitivity was obtained and ATP was neither required nor stimulatory. These findings indicate that HS-HTP functions as component B and do not support the idea that HS-HT is only active in an adenosine monophosphorylated form.

Methanogenesis; Methyl-CoM reductase; Component B; 7-Mercaptoheptanoylthreonine phosphate; *(Methanobacterium)*

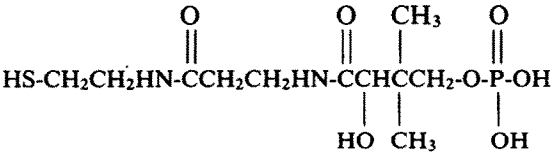
1. INTRODUCTION

Component B is a cofactor required in methanogenesis [1]. Part of component B present in methanogenic bacteria has been reported to be tightly bound to methyl-CoM reductase [2]. Its structure has recently been assigned as 7-mercaptoheptanoylthreonine phosphate (HS-HTP) [3].



HS-HTP

The naturally occurring compound may be larger but breaks down during purification because several elution peaks with component B activity are obtained upon chromatography of boiled cell-free extract. Synthetic and naturally occurring HS-HTP was nevertheless active at low concentrations in promoting methane formation from methyl-CoM and H_2 in cell extracts of *Methanobacterium thermoautotrophicum* (ΔH) [3,4]. Methane formation in the assay system was dependent on ATP. It has therefore been speculated that an adenosine monophosphorylated HS-HTP rather than HS-HTP might be the active form of component B [3]. The similarities of pantheteine 4-phosphate to HS-HTP and of HS-CoA to a possible adenosine monophosphorylated HS-HTP make this speculation attractive.



Pantheteine 4-phosphate

Here, we demonstrate that synthetic DL-HS-HTP can function as component B in the absence of ATP.

Correspondence address: R.K. Thauer, Fachbereich Biologie-Mikrobiologie, Philipps Universität, Karl-von-Frisch-Straße, D-3550 Marburg, FRG

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2. MATERIALS AND METHODS

2.1. *Growth of* Methanobacterium

M. thermoautotrophicum (strain Marburg) (DSM 2133) was grown in 10 l culture volumes at 65°C on H_2 and CO_2 as described [5]. Cells of late logarithmic growth phase (1.6 g dry wt/l) were cooled to less than 10°C, harvested by centrifugation, immediately frozen in liquid N_2 and stored at −55°C until use.

2.2. *Purification of methyl-CoM reductase*

Methyl-CoM reductase of *M. thermoautotrophicum* (strain Marbug) was purified essentially as described by Ankel-Fuchs and Thauer [6] with the following modification. After chromatography on Sephacryl S300, $(NH_4)_2SO_4$ (2 mol/l) rather than KCl (2 mol/l) was added to the fraction containing methyl-CoM reductase. The sample was stirred for 30 min at 0°C, then centrifuged to remove precipitated protein (15 min at 27000 × *g*), and the yellow supernatant was applied to a phenyl-Sepharose CL-4B column (1 × 6 cm) equilibrated with 50 mM potassium phosphate buffer (pH 6.7) containing 2 M $(NH_4)_2SO_4$. Under these conditions methyl-CoM reductase was retained on the column which was washed with 20 ml buffer containing 1 M $(NH_4)_2SO_4$. The enzyme was eluted with buffer containing 0.6 M $(NH_4)_2SO_4$ in 12 ml and immediately desalted by ultrafiltration. The specific activity of methyl-CoM reductase thus obtained was up to 550 nmol · min^{-1} · mg $protein^{-1}$, but activity was rapidly lost (50–70% in 48 h) upon storage at −20°C, on ice, or at 4°C. Protein was determined according to Bradford [7] using chicken egg albumin as standard.

2.3. *Assay for methyl-CoM reduction*

All assays were performed in sealed 8 ml serum flasks as in [6]. The gas phase was N_2. The 0.4 ml assay mixture contained: 50 mM potassium phosphate buffer (pH 6.5), 15 mM dithiothreitol, 5.5 mM methyl-CoM, 150 μM OH-cobalamin, 0–50 μg methyl-CoM reductase and synthetic DL-HS-HTP (0–0.6 mM), boiled cell-free extract or purified authentic component B. Methane formation was initiated by increasing the temperature from 22 to 60°C. One unit of methyl-CoM reductase activity is the amount catalyzing the production of 1 μmol $CH_4 \cdot min^{-1}$ at 60°C.

2.4. *Synthesis of DL-HS-HTP*

DL-HS-HTP was synthesized by Noll et al. [8] at the University of Urbana, IL, USA, and by A. Kobelt and A. Pfaltz at the Eidgenössische Technische Hochschule, Zürich. In the latter case the improved method was used [4] and slightly modified (using chloroethyl carbonate for the coupling of $[S(CH_2)_6COOH]_2$ with *O*-phospho-DL-threonine). The thiol was dissolved in 30 mM Tris-HCl (pH 7.8) to a final concentration of 3 mg/ml (8 mM) and stored at −20°C until use. Both preparations gave identical results.

2.5. *Preparation of boiled cell-free extract and purification of naturally occurring component B*

Boiled cell-free extract was prepared from cells of *M. thermoautotrophicum* (strain Marburg) [6].

The purification procedure for naturally occurring component B described here is based on the observation that after complete extraction of coenzyme F430 from *M. thermoautotrophicum* (strain Marburg) with 10 mM $HClO_4$ [9] the residual cell material still contained large amounts of component B. The residue from 200 g cells (wet wt) was suspended in 150 ml H_2O, the pH adjusted to 7.8 with 10 M KOH, and the suspension passed through a French pressure cell (American Instrument Co., Silverspring) once at 60 MPa. After ultracentrifugation at 300000 × *g* for 30 min a very dark brown supernatant was obtained. HCl was added to a final concentration of 30 mM, the solution was stirred at room temperature for 30 min and precipitated material removed by centrifugation (12000 × *g*, 30 min). After neutralization with KOH the light-yellow supernatant was applied to a DEAE-Sephadex A-25 column (30 × 5 cm) equilibrated with 25 mM Tris-HCl (pH 7.5). Component B was eluted with an increasing gradient of 0–1 M NaCl in Tris-HCl buffer. Fractions containing component B were detected from their ability to supplement the methyl-CoM reductase assay. These fractions were pooled, diluted 3-fold with distilled water and applied to a second DEAE-Sephadex A-25 column (5 × 2 cm). Component B was eluted with 150 mM HCl. Fractions containing the component were colorless and showed no

significant absorbance between 210 and 700 nm. They were pooled and lyophilized. A white powder (3 mg) was obtained consisting of HS-HTP as determined by HPLC comparison with synthetic DL-HS-HTP. A solution of component B was prepared in 50 mM potassium phosphate buffer (pH 6.8, 0.5 mg/ml) and stored at −20°C until use.

3. RESULTS

Methane formation from methyl-CoM and H_2 is catalyzed by methyl-CoM reductase (component C) and requires the presence of at least three additional proteins (components A_{1-3}), of FAD, Mg^{2+}, cobalamin and ATP, and of a low-M_r heat-stable compound designated component B [1,10–13]. Recently it has been found that purified methyl-CoM reductase from *M. thermoautotrophicum* (strain Marburg) can mediate the reduction of methyl-CoM to methane with reduced cobalamin as electron donor in the absence of components A_{1-3}, FAD, Mg^{2+} and ATP [6]. Methane formation was, however, still dependent on component B, thus making this system ideal for the study of the essential structural elements of component B. In the following we investigated whether synthetic DL-7-mercaptoheptanoylthreonine phosphate (HS-HTP) can substitute for naturally occurring component B.

3.1. *Kinetics of HS-HTP-dependent methyl-CoM reduction*

Purified methyl-CoM reductase from *M. thermoautotrophicum* (strain Marburg) was found to catalyze the reduction of methyl-CoM to methane with reduced cobalamin (dithiothreitol plus OH-cobalamin) in the presence of synthetic DL-HS-HTP. After a lag period methane formation proceeded linearly with time for 10–20 min (fig.1A). The rate was proportional to the concentration of methyl-CoM reductase over 0–50 μg protein per 400 μl assay volume. At higher protein concentrations the specific activity decreased (not shown). Routinely specific activities of at least 100 nmol methane formed · min^{-1} · mg $protein^{-1}$ were obtained at 60°C and pH 6.5, but with some preparations specific rates of up to 550 nmol · min^{-1} · mg $protein^{-1}$ were observed. Methane formation was strictly dependent on DL-HS-HTP; the concentration dependence was hyperbolic (fig.1B). The half-maximal rate was found at approx. 0.2 mM DL-HS-HTP.

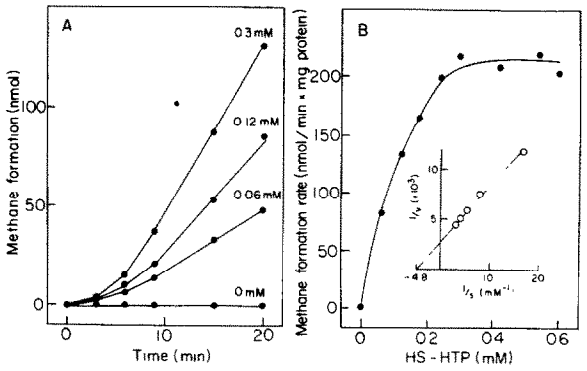

Fig.1. Methane formation from methyl-CoM in the presence of synthetic DL-HS-HTP. The 400 μl assay contained 40 μg purified methyl-CoM reductase from *M. thermoautotrophicum* (strain Marburg). (A) Time course of methane formation for various concentrations of HS-HTP. (B) Dependence of methyl-CoM reductase activity on HS-HTP concentration. (Inset) Reciprocal plot of the same data. An apparent K_m for HS-HTP of 0.21 mM and an apparent V_{max} of 380 nmol · min^{-1} · mg $protein^{-1}$ were obtained.

3.2. *Effect of ATP on HS-HTP-dependent methyl-CoM reduction*

Methane formation in the presence of DL-HS-HTP (0.3 mM) was not dependent on ATP and Mg^{2+}. Several ATP and Mg^{2+} concentrations were tested but only inhibitory effects were observed (table 1).

3.3. *Activity of synthetic DL-HS-HTP as compared to naturally occurring component B*

When the assay system was supplemented with purified component B the same maximal specific activity was reached as with synthetic DL-HS-HTP. With boiled cell-free extract from *M. thermoautotrophicum* (strain Marburg) only 40% of V_{max} was obtained. The kinetics, however, were very similar. The methyl-CoM reductase assay exhibited an apparent K_m for naturally occurring component B of 0.05–0.1 mM (based on the assumption that the preparation of component B was 100% pure HS-HTP with an M_r of 343 [3]). These findings indicate that synthetic DL-HS-HTP

Table 1

Effect of ATP and of Mg^{2+} on methane formation from methyl-CoM in the presence of synthetic 7-mercaptoheptanoylthreonine phosphate (0.3 mM)

[ATP] (mM)	[$MgSO_4$] (mM)	nmol $CH_4 \cdot min^{-1} \cdot mg\ protein^{-1}$
0	0	185
0.1	0	100
0	0.1	127
0.1	0.2	136
1.0	2.0	118
5.0	10.0	91

For conditions see section 2

can completely substitute for naturally occurring component B in methyl-CoM reduction to methane with reduced cobalamin.

4. DISCUSSION

The present results do not support the idea that naturally occurring component B is a modified HS-HTP: synthetic DL-HS-HTP behaved like authentic component B in the methanogenic assay used. This conclusion is drawn from a comparison of maximal specific activities and assumes that a degradation product of component B would be less active.

Since ATP was not present in the assay the data exclude that an adenosine monophosphorylated HS-HTP was formed during incubation. Therefore, at present there is no indication that an adenosine monophosphorylated HS-HTP is the active form of component B in methanogenic bacteria. It should be noted, however, that the data do not exclude this possibility.

Recently an apparent K_m for naturally occurring component B and synthetic DL-HS-HTP of 3 and 6 μM, respectively, has been reported [3,4]. These values were determined in an assay system using cell extract of *M. thermoautotrophicum* (ΔH) and H_2 as electron donor. The maximal specific activities reached were of the order of 1 nmol $\cdot$ $min^{-1} \cdot mg\ protein^{-1}$. Using purified methyl-CoM reductase of *M. thermoautotrophicum* (strain Marburg) and reduced cobalamin as electron donor we found an apparent K_m for naturally occurring component B and for synthetic DL-HS-HTP of approx. 0.05–0.1 and 0.2 mM, respectively, and a maximal specific methyl-CoM reductase activity of 550 nmol $\cdot$ $min^{-1} \cdot mg\ protein^{-1}$. The large differences in the apparent K_m value could be due to different rate-limiting steps in the two assay systems. The higher K_m value for synthetic DL-HS-HTP might be due to the fact that *O*-phospho-DL-threonine was used in its synthesis [4,8]. A racemic mixture of HS-HTP was therefore obtained. It could be that methyl-CoM reductase recognizes only one of the two isomers.

The finding that upon chromatography of boiled cell-free extract several elution peaks with component B activity are obtained (see section 1) still needs an explanation. We have some indications that during purification homo- and heterodisulfides of HS-HTP are formed. These can be separated and are converted to HS-HTP in the assay system by thiols.

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