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Methanobacterium thermoautotrophicum contains a soluble enzyme system that specifically catalyzes the reduction of the heterodisulfide of coenzyme M and 7-mercaptoheptanoylthreonine phosphate with H_2

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Cell extracts of *Methanobacterium thermoautotrophicum* (strain Marburg) were found to catalyze the reduction of the heterodisulfide of coenzyme M (CoM-S-H) and 7-mercaptoheptanoylthreonine phosphate (H-S-HTP) with H_2. All the activity was associated with the soluble cell fraction (160000 × *g* supernatant). The enzyme system was purified sevenfold by anion-exchange chromatography. The partially purified system had a specific activity of 100 nmol CoM-S-S-HTP reduced per min and mg protein and exhibited an apparent K_m for CoM-S-S-HTP of below 0.1 mM. The homodisulfides of CoM-S-H, of H-S-HTP, of cysteine, and of glutathione were not reduced. NADPH and NADH could not substitute for H_2 as electron donor.

Methanogenic bacteria; Methane formation; Coenzyme M; 7-Mercaptoheptanoylthreonine phosphate; Component B; Methyl-CoM reductase; Vitamin B_{12}; Hydrogenase

1. INTRODUCTION

It has recently been shown that the final step in bacterial methane formation is the reduction of methyl-CoM (CH_3-S-CoM) with L-7-mercaptoheptanoylthreonine phosphate (H-S-HTP = component B) [1–5].

$$CH_3\text{-S-CoM} + \text{H-S-HTP} \longrightarrow CH_4 + \text{CoM-S-S-HTP} \quad (1)$$

This reaction is catalyzed by methyl-CoM reductase (component C) [3]. In this communication we describe the presence of an enzyme system in *Methanobacterium thermoautotrophicum* (strain Marburg), which specifically catalyzes the reduction of the heterodisulfide CoM-S-S-HTP to the corresponding thiols with H_2 as electron donor.

$$\text{CoM-S-S-HTP} + H_2 \longrightarrow \text{CoM-SH} + \text{H-S-HTP} \quad (2)$$

The soluble enzyme system mediating this reaction is probably identical with component A of the methyl-CoM methyl reductase system [6].

2. MATERIALS AND METHODS

M. thermoautotrophicum (strain Marburg) (DSM 2133) [7] was from the Deutsche Sammlung von Mikroorganismen (Braunschweig, FRG). The bacteria were grown on H_2 and CO_2, and the cells harvested, stored, and extracts prepared as reported previously [3]. The enzyme system catalyzing CoM-S-S-HTP reduction with H_2 was purified by fast-protein liquid chromatography on Mono Q (strongly basic anion-exchange resin from Pharmacia, Freiburg, FRG) as described in section 3 and table 1. All preparations were performed in an anaerobic chamber filled with 95% N_2/5% H_2.

The enzyme assays were performed in 8 ml serum bottles. The reaction was started by increasing the temperature from

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4°C to 68°C. In 3 min intervals 10–25 μl samples were withdrawn with a 25 μl syringe and analyzed for thiols with Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid)). The 10–25 μl samples were injected into a 1 ml cuvette containing 0.1 ml of 1 M Tris-HCl, pH 8.1, at room temperature. Then 0.1 ml of 1 mM Ellman's reagent (solution in 50 mM sodium acetate, pH 5.0) and H_2O (to a final volume of 1 ml) were added, and the absorbance difference against H_2O at 412 nm was determined. The absorbance difference obtained for the sample taken at $t = 0$ was subtracted. The method was calibrated with CoM-S-H (Sigma, Deisenhofen, FRG) as standard.

CoM-S-S-HTP (only the L-form was used) and the homodisulfide of CoM-SH (CoM-S-S-CoM) and of L-H-S-HTP (HTP-S-S-HTP) were synthesized as in [3]. Oxidized glutathione and L-cystine were from Boehringer (Mannheim, FRG) and Merck (Darmstadt, FRG), respectively.

Titanium(III) citrate was prepared from $TiCl_3$. 5 ml of a 15% (1 M) $TiCl_3$ solution in 10% HCl was added to 50 ml of 0.2 M sodium citrate and the mixture neutralized with saturated Na_2CO_3.

3. RESULTS

Cell extract of *M. thermoautotrophicum* catalyzed the reduction of CoM-S-S-HTP with H_2 to CoM-SH and H-S-HTP. Under the assay conditions described in tables 1 and 2 the reaction proceeded linearly with time for 5–10 min and ran to completion within 15–30 min. Per mol heterodisulfide 2 mol thiols were generated. At low protein concentrations (<3 mg/0.5 ml), the specific activity increased with increasing protein indicating that more than one component in the extract was required for activity (see fig.1B).

3.1. *Specific activity*

At cell extract protein concentrations above 3 mg/0.5 ml the specific activity was 25–30 nmol · $min^{-1} \cdot mg^{-1}$ (rate of thiol formation). For comparison, the specific activity of methane formation from CH_3-S-CoM and H-S-HTP in the cell extracts was 5 nmol · $min^{-1} \cdot mg^{-1}$ and that of methane formation from CH_3-S-CoM and H_2 was 45 nmol · $min^{-1} \cdot mg^{-1}$ [1,3,4].

3.2. *Activity with NADPH*

CoM-S-S-HTP reduction in cell extracts was also observed with NADPH but not with NADH as electron donor (table 2). This activity was lost upon partial purification (see below). In this respect it is of interest that *M. thermoautotrophicum* contains an NADPH : coenzyme F420 oxidoreductase [8] and a protein mediating CoM-S-S-CoM reduction with NADPH [9].

Table 1

Partial purification of the enzyme system catalyzing CoM-S-S-HTP reduction with H_2 to CoM-S-H and H-S-HTP in *M. thermoautotrophicum*

Fraction	Protein (mg)	Activity (nmol · min^{-1})	Specific activity (nmol · $min^{-1} \cdot mg^{-1}$)
Cell extract	250	6800	25–30
160000 × *g* supernatant	125	6300	50
Fraction eluted at 0.52 M NaCl from Mono Q[a]	30	6000	200
Ultrafiltration[b]	30	6000	200

[a] This fraction also contained most of the hydrogenase activity tested both with coenzyme F420 and with methyl viologen as electron acceptor. It did not contain methyl-CoM reductase which was eluted from the column only at higher NaCl concentrations [3]

[b] Centricon 30 microconcentrator, cut-off M_r = 30000, from Amicon (Witten, FRG)

The 0.5 ml assay mixture contained: 50 mM Tris-HCl, pH 7.6; 2.1 mM CoM-S-S-HTP; 5 μM aquocobalamin (B12a); 1–5 mg protein. The gas phase was H_2 at 1.2×10^5 Pa; the temperature was 68°C

3.3. *Localization*

The enzyme system mediating CoM-S-S-HTP reduction with H_2 and with NADPH was localized in the soluble cell fraction. More than 95% of the activity was recovered in the supernatant after centrifugation at 160000 × *g* for 30 min (table 1).

3.4. *Partial purification*

For purification the 160000 × *g* supernatant (2.5 ml) was applied to a Mono Q column (10 mm × 10 cm) which was eluted with 16 ml of 0.3 M NaCl and then with a 56 ml of 0.3–0.6 M NaCl gradient (in 50 mM Tris-HCl, pH 7.6). More than 90% of the H_2 : CoM-S-S-HTP oxidoreductase activity present in the supernatant was recovered in the 0.48–0.56 M NaCl fraction (10 ml). This fraction also contained most of the hydrogenase activity (tested both with coenzyme F420 and with methyl viologen as electron acceptors [10]), but not NADPH : CoM-S-S-HTP oxidoreductase activity. The specific activity of CoM-S-S-HTP reduction with H_2 was 200 nmol · $min^{-1} \cdot mg^{-1}$ indicating a sevenfold purification of the enzyme system. The specific activity did not decrease upon repeated ultrafiltration (cut off M_r

Table 2

Cofactor requirement for CoM-S-S-HTP reduction to the thiols CoM-S-H and H-S-HTP by cell extract and by the partially purified enzyme system from *M. thermoautotrophicum*

Conditions	Rate of thiol formation ($nmol \cdot min^{-1} \cdot mg^{-1}$)	
	Cell extract	Partially purified system
Complete	30	200
– CoM-S-S-HTP	<5	<5
– H_2	<5	<5
– H_2 + NADPH (3 mM)	15	<5
– H_2 + NADH (3 mM)	<5	<5
+ ATP (3 mM) + MgCl (24 mM)	30	200
+ FAD (25 μM)	30	200
+ Coenzyme F420 (40 μM)	30	200
– B12a	15	50
– B12a + Ti(III)citrate (1 mM)	nd	50

For assay conditions see table 1. Where indicated the gas phase was N_2 rather than H_2. nd, not determined

30000) (table 1) and did not increase when ATP, Mg^{2+}, FAD and/or coenzyme F420 were added to the assay (table 2). The partially purified enzyme system was stored in 50 mM Tris-HCl, pH 7.6, containing 0.5 M NaCl at 4°C under N_2 as gas phase. Approx. 20% of the activity was lost within 24 h.

3.5. *Kinetics*

The time course of CoM-S-S-HTP reduction with H_2 as catalyzed by the partially purified enzyme system is shown in fig.1A, and the protein dependence in fig.1B. In Tris-HCl buffer the pH optimum was 7.6 (pH at 20°C), in piperazine-*N,N'*-bis(2-ethane sulfonic acid) (Pipes)/KOH buffer it was 6.3. The specific activity was higher in Tris buffer than in Pipes buffer. The temperature dependence showed a Q_{10} of approx. 3. Rapid inactivation was observed at temperatures above 80°C.

3.6. *Specificity for CoM-S-S-HTP*

The partially purified enzyme system was specific for CoM-S-S-HTP as electron acceptor. With the homodisulfides of CoM-SH, H-S-HTP, glutathione and cysteine only background activities were observed (table 3). When the CoM-S-S-HTP concentration was decreased from 2 mM to 0.4 mM the rate of thiol formation remained essentially constant indicating that the apparent K_m for the heterodisulfide is well below 0.1 mM.

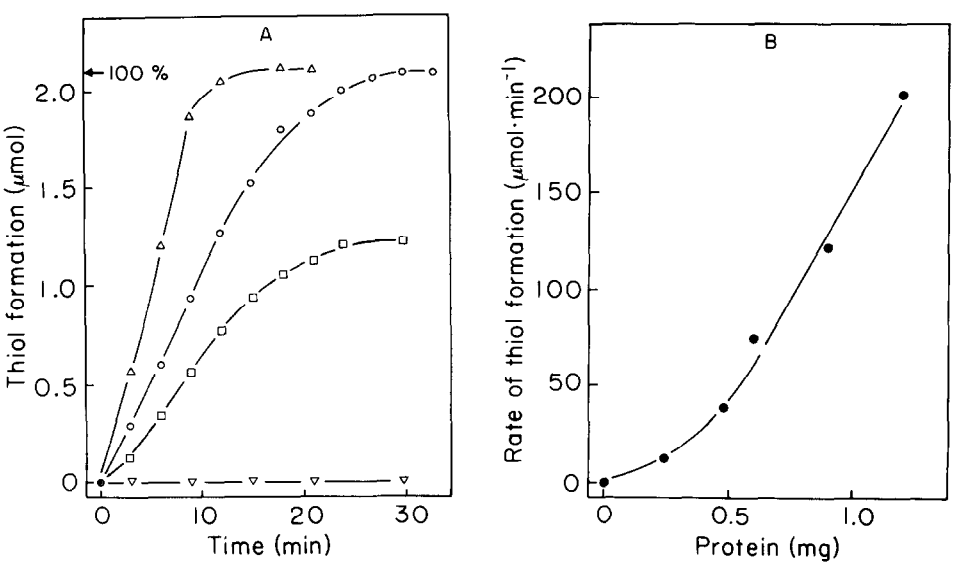

Fig.1. Catalysis of CoM-S-S-HTP reduction with H_2 to the corresponding thiols by the partially purified enzyme system from *Methanobacterium thermoautotrophicum*. (A) Time dependence; (B) protein dependence. For assay conditions see table 1. (△) 1.2 mg protein; (○) 0.9 mg; (□) 0.6 mg; (▽) 0 mg. 100% indicates quantitative reduction of the 1.05 μmol CoM-S-S-HTP present in the assay mixture.

Table 3

Substrate specificity of the partially purified enzyme system from *M. thermoautotrophicum*

Substrate	Rate of thiol formation ($nmol \cdot min^{-1} \cdot mg^{-1}$)	
	+ B12a	− B12a
CoM-S-S-HTP (2 mM)	200	50
CoM-S-S-HTP (0.4 mM)	200	nd
CoM-S-S-CoM (2 mM)	10	10
HTP-S-S-HTP (2 mM)	10	10
G-S-S-G[a] (2 mM)	10	10
Cystine (2 mM)	10	10
Cystine (4 mM)	20	20
Cystine (8 mM)	30	30

[a] Oxidized glutathione

For assay conditions see table 1

An exact determination of the apparent K_m was not possible since at CoM-S-S-HTP concentrations below 0.4 mM the rate of thiol formation could not be determined accurately.

3.7. *Stimulation by cobalamin*

The standard assay mixture (table 1) contained 5 μM aquocobalamin. When the corrinoid was omitted the H_2:CoM-S-S-HTP oxidoreductase activity decreased by a factor of 2 to 4 (table 2). CoM-S-S-CoM, HTP-S-S-HTP, oxidized glutathione and cystine were reduced at the same relatively low rates in the absence and presence of aquocobalamin.

3.8. *Role of cobalamin*

Cob(I)alamin reacts with organic disulfides to yield cob(III)alamin (B12a) and the corresponding thiols [11]. It had, therefore, to be considered that in the enzyme assay cob(III)alamin is reduced by H_2 to cob(I)alamin and the latter then reduces the heterodisulfide in a non-enzymatic reaction. We tested the reduction of CoM-S-S-HTP and of other disulfides by cob(I)alamin which was regenerated from cob(III)alamin by reduction with titanium(III)citrate. Thiol formation from the different disulfides proceeded at almost identical rates (fig.2). Chemical disulfide reduction by cob(I)alamin thus completely differed from enzymatic disulfide reduction with respect to substrate specificity. This excludes that in the enzyme assay CoM-S-S-HTP is non-enzymatically reduced by cob(I)alamin. The role of cobalamin in heterodisulfide reduction remains to be resolved (see below).

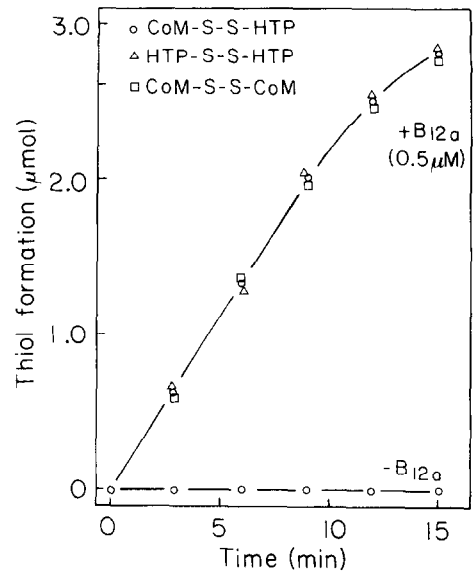

Fig.2. Catalysis of disulfide reduction with titanium(III)citrate to thiols by aquocobalamin (B12a). The 0.5 ml assay mixture contained: 50 mM Tris-HCl, pH 7.6; 3 mM of CoM-S-S-HTP, HTP-S-S-HTP, or CoM-S-S-CoM; aquocobalamin as indicated; and 10 mM titanium(III)citrate. The gas phase was N_2; the temperature was 68°C.

4. DISCUSSION

CoM-S-S-HTP reduction with H_2 is probably catalyzed by at least 2 enzymes, a hydrogenase and a heterodisulfide reductase. This is indicated by the finding that the specific activity of the reaction increased with the protein concentration (fig.1A). The electron carrier between hydrogenase and heterodisulfide reductase is not yet known. A possible candidate is the cobamide-containing protein found in the cytoplasmic membrane of *M. thermoautotrophicum* [12,13].

Cell suspensions of *M. thermoautotrophicum* reduce CO_2 with H_2 at a specific rate of 3–5 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ [3]. The specific activity of methyl-CoM reductase and of H_2:CoM-S-S-HTP oxidoreductase in cell extracts was less than 1% of this value (table 1). In intact cells the proteins involved in methyl-CoM reduction with H_2 are probably organized in methanoreductosomes [14]

which disaggregate when the cells are disrupted. This may be the reason for the low specific activities observed in cell extracts.

Gottschalk and collaborators [15] have shown that in intact cells methyl-CoM reduction to CH_4 with H_2 is coupled with ADP phosphorylation via the chemiosmotic mechanism. It has been suggested that the reduction of the heterodisulfide with H_2 (reaction 2) rather than methane formation from CH_3-S-CoM and H-S-HTP (reaction 1) is associated with energy conservation [2]. The finding of the heterodisulfide reductase in the soluble cell fraction was disappointing in this respect. The enzyme could, however, be a peripheral membrane protein, which looses its contact to the membrane upon cell breakage (see [16]).

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