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Catalytic properties of the heterodisulfide reductase involved in the final step of methanogenesis

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Reduction of the heterodisulfide of coenzyme M (H-S-CoM) and 7-mercaptoheptanoyl(L)threonine phosphate (H-S-HTP) is a partial reaction in methanogenesis. The CoM-S-S-HTP reductase mediating this reaction has thus far not been studied. We report here that the enzyme from *Methanobacterium thermoautotrophicum* and *Methanosarcina barkeri* catalyzes the reduction of CoM-S-S-HTP with reduced viologen dyes and, in the reverse direction, the oxidation of H-S-CoM plus H-S-HTP to the heterodisulfide by methylene blue. The CoM-S-S-HTP reductase from *M. thermoautotrophicum* (strain Marburg) was partially purified (30-fold) to a specific activity of 10 μmol·min^{-1}·mg protein^{-1}. The enzyme was highly substrate specific: e.g. neither the heterodisulfide derived from 6-mercaptohexanoylthreonine phosphate nor the homodisulfide of H-SCoM or of HSHTP was reduced. The D-enantiomer of CoM-S-S-HTP was, however, converted at 35% of the specific rate of the L-form. Apparent K_m and apparent V_{max} values for substrates and products were determined.

Methanogenic bacteria; Methanogenesis; Coenzyme M; Mercaptoheptanoylthreonine phosphate, 7-; Disulfide reductase; (*Methanobacterium thermoautotrophicum, Methanosarcina barkeri*)

1. INTRODUCTION

The final step in methanogenesis is the reduction of methyl-coenzyme M (CH_3-S-CoM) by 7-mercaptoheptanoyl (L) threonine phosphate (H-S-HTP) to methane and CoM-S-S-HTP [1,2]:

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

This reaction is catalyzed by methyl-coenzyme M reductase, a multi-subunit nickel enzyme, that has been extensively characterized [1,3]. The products formed indicate that methanogenic bacteria must possess a CoM-S-S-HTP reductase for regeneration of the reduced coenzymes. Indeed, it was recently shown that cell extracts of *Methanobacterium thermoautotrophicum* (strain Marburg) contain an enzyme system that catalyzes reduction of the heterodisulfide with H_2, albeit at very low specific activity [4]:

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

The enzyme system mediating this reaction has not yet been resolved. It is probably composed of a hydrogenase, an electron carrier, and a CoM-S-S-HTP reductase. In this paper, an assay for the heterodisulfide reductase and some properties of this enzyme are reported.

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Abbreviations: H-S-CoM or coenzyme M, 2-mercaptoethanesulfonate; H-S-HTP, 7-mercaptoheptanoyl (L) threonine phosphate; CH_3-S-CoM or methyl-coenzyme M, 2-(methylthio)ethanesulfonate; CoM-S-S-HTP, heterodisulfide of H-S-CoM and H-S-HTP

2. MATERIALS AND METHODS

M. thermoautotrophicum (strain Marburg) (DSM 2133) and *Ms. barkeri* (strain Fusaro) (DSM 804) were from the Deutsche

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Sammlung von Mirkoorganismen (Braunschweig) for both species of bacteria, conditions for growth, harvesting, and preparation of cell extracts were as described [2,5].

For purification of CoM-S-S-HTP reductase, a cell extract (5 ml, 160000 × *g* supernatant) was applied to a DEAE-Sepharose fast-flow column (2.6 × 11 cm) (Pharmacia, Freiburg) previously equilibrated with 50 mM Tris-HCl, pH 7.6 (1.5 ml/min). The column was washed with 40 ml of this buffer, followed by 100 ml of 0.2 M NaCl, 100 ml of 0.3 M NaCl and 100 ml of 0.36 M NaCl in 50 mM Tris-HCl, pH 7.6. Active fractions were pooled and concentrated by ultrafiltration with Centricon 30 microconcentrators (cut-off *Mr* 30000) (Amicon, Witten, FRG) and applied to a Mono Q column (10 mm × 10 cm) (Pharmacia) previously equilibrated with 50 mM Tris-HCl, pH 7.6, containing 0.3 M NaCl. The column was washed with 16 ml of the above buffer and protein eluted with a linear gradient of NaCl (0.3–0.6 M, 100 ml) at a rate of 4 ml/min. Fractions containing the disulfide reductase were concentrated and applied to a Superose 6 column (1.6 cm × 50 cm) (Pharmacia), previously equilibrated with 50 mM Tris-HCl, pH 7.6, containing 0.2 M NaCl and eluted with the same buffer (0.4 ml/min). All of these purification steps were performed in an anaerobic chamber (Coy, Ann Arbor, MI).

Assays were performed in 1-ml anaerobic cuvettes closed with a rubber stopper. The gas phase was N_2 at 1.2×10^5 Pa. Additions were made via microsyringes. The reduction of CoM-S-S-HTP with reduced benzyl viologen was determined in a 0.8 ml assay mixture containing 50 mM Tris-HCl (pH 7.6), 2 mM benzyl viologen CoM-S-S-HTP as indicated and 10–50 mU CoM-S-S-HTP reductase. Before initiation of the reaction with either CoM-S-S-HTP or the reductase, dithionite was added until an absorbance at 578 nm of 2.0 [due to the reduction of benzyl viologen ($\epsilon_{578} = 8.6\ cm^{-1} \cdot mM^{-1}$)] was reached. The oxidation of H-S-CoM and H-S-HTP with methylene blue was performed in 0.8 ml assay mixtures containing 50 mM Tris-HCl (pH 7.6), 0.15 mM methylene blue ($\epsilon_{578} = 11.5\ cm^{-1}\ mM\pm^{1}$), 0.5 mM H-S-CoM and H-S-HTP or at the concentrations indicated and 10–50 mU CoM-S-S-HTP reductase. The reaction was started by the addition of either H-S-CoM, H-S-HTP or enzyme, 1 unit enzyme activity (U) = 1 μmol CoM-S-S-HTP reduced or formed per min under the assay conditions. The enzyme from *M. thermoautotrophicum* was tested at 65°C, that from *Ms. barkeri* at 40°C. Protein was determined by the method of Bradford using the Bio-Rad microassay [6].

The heterodisulfides of H-S-CoM and 7-mercaptoheptanoyl (L) threonine phosphate, of H-S-CoM and 7-mercaptoheptanoyl(D)threonine phosphate and of H-S-CoM and 6-mercaptohexanoyl(DL)threonine phosphate were synthesized as in [2]. The compounds were characterized by ^{1}H-NMR and ^{13}C-NMR and from the values for their optical rotation: for L-CoM-S-S-HTP, $[\alpha]_D^{23} = -3.5$ (c = 10.1 mg in 2 ml H_2O); for D-CoM-S-S-HTP, $[\alpha]_D^{23} = 3.5$ (c = 10.1 mg in 2 ml H_2O).

3. RESULTS

Cell extracts of *M. termoautotrophicum* catalyzed the reduction of CoM-S-S-HTP ($E^{\circ\prime} = -210$ mV, assumed to be identical to that of cystine) with H_2 ($E^{\circ\prime} = -414$ mV) at a specific rate of 30 nmol · min^{-1} · mg protein^{-1} [4]. We found that the extracts mediated reduction of the heterodisulfide with reduced benzyl viologen ($E^{\circ\prime} = -360$ mV) or reduced methyl viologen ($E^{\circ\prime} = -446$ mV) at a 10-fold higher specific rate of 0.3 μmol · min^{-1} · mg protein^{-1} (pH 7.6, 65°C). In the following studies heterodisulfide reductase activity was routinely assayed with reduced benzyl viologen which was not oxidized under these experimental conditions via the hydrogenase activity present in the cell extracts.

3.1. *Partial purification of CoM-S-S-HTP reductase activity from* M. thermoautotrophicum

After centrifugation for 30 min at 160000 × *g* more than 90% of the heterodisulfide reductase activity was found in the supernatant. Partial purification was achieved by chromatography on DEAE-Sepharose (fast flow), Mono Q, and Superose 6 (table 1).

Heterodisulfide reductase activity was eluted from the Mono Q column in 2 peaks at 0.48 and 0.54 M NaCl. Peak 1 contained 20% and peak 2 80% of the eluted activity. Only the latter peak was further purified by gel filtration on Superose 6, from which the enzyme activity eluted at an apparent molecular mass of 350–400 kDa.

During purification the CoM-S-S-HTP reductase activity was separated from F420 reducing hydrogenase and from methyl-coenzyme M reductase but not from the methyl viologen reducing hydrogenase. SDS-polyacrylamide gel electrophoresis (12% polyacrylamide gels) revealed the presence of four major protein bands of apparent molecular mass 40, 50, 55 and 66 kDa.

3.2. *Effect of O_2 on CoM-S-S-HTP reductase activity*

Purification was routinely performed in an anaerobic chamber. The buffers did not contain a thiol compound or other reducing substances. However, the partially purified enzyme turned out to be insensitive towards O_2. The activity was even somewhat (10–20%) enhanced upon brief (30 min) incubation in the presence of air at 20°C. This stimulatory effect of O_2 was most pronounced in crude cell extracts, where incubation in the presence of air sometimes resulted in a 4-fold increase in specific activity. After longer periods in the presence of air the activity of the partially

Table 1

Partial purification of CoM-S-S-HTP reductase from *M. thermoautotrophicum* (Marburg)

Fraction	Protein (mg)	Activity[a] (μmol · min^{-1})	Specific activity (μmol · min^{-1} · mg^{-1})
Cell extract	250	83	0.3
160000 × *g* supernatant	125	75	0.6
Fractions eluted at 0.36 NaCl from DEAE-Sepharose	50	60	1.2
Fractions eluted at 0.52–0.56 M NaCl from Mono Q	8.6	43	5
Fractions eluted after 56 ml from Superose 6	4	40	10

[a] 1 μmol CoM-S-S-HTP reduced per min equal to 2 μmol reduced benzyl viologen oxidized per min

purified enzyme decreased (50% in 2 h at 20°C). Under anaerobic conditions the activity remained constant.

3.3. *Kinetics of CoM-S-S-HTP reduction*

The partially purified preparations catalyzed the reduction of CoM-S-S-HTP at a specific rate of 10 μmol · min^{-1} · mg protein^{-1} (table 1). The reaction proceeded at a constant rate for several minutes and activity increased linearly with protein. Per mol CoM-S-S-HTP added 2 mol reduced benzyl viologen were oxidize, in agreement with the property of the dye of transfering only one electron (fig.1A). The apparent K_m for reduced benzyl viologen was very low (< 0.05 mM). Interestingly, the activity increased with increasing concentrations of oxidized benzyl viologen present in the assay. Half-maximal rates were obtained at a total concentration of benzyl viologen of 0.4 mM. The apparent K_m for the heterodisulfide was determined to be 0.13 mM (fig.1B).

3.4. *Substrate specificity of CoM-S-S-HTP reductase*

The reductase was found to be highly specific for the heterodisulfide of H-S-CoM and H-S-HTP (L-enantiomer). The homodisulfide of H-S-CoM and that of H-S-HTP were not reduced. This lack of reduction was also observed for the heterodisulfide derived from 6-mercaptohexanoylthreonine phosphate, a homologue differing in chain length by only one methylene group. To our surprise, however, the D-enantiomer of CoM-S-S-HTP was reduced at approx. 35% of the rate observed for the L-form (table 2). Since the disulfide, when present in small amounts, was completely reduced in the assays, this activity was almost certainly not due to contamination of the D-form with the L-form. The apparent K_m for D-CoM-S-S-HTP was 3 mM and thus 30-times higher than that for the L-form. At

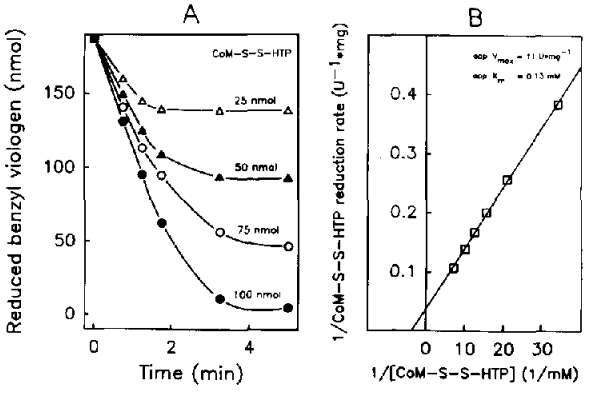

Fig.1. CoM-S-S-HTP reduction by reduced benzyl viologen catalyzed by partially purified CoM-S-S-HTP reductase from *M. thermoautotrophicum* (strain Marburg). (A) Time course (B) Lineweaver Burk plot of $1/v$ 1/[CoM-S-S-HTP].

Table 2

Substrate specificity of partially purified CoM-S-S-HTP reductase form *M. thermoautotrophicum* (strain Marburg)

Substrate	Apparent, *V*max (μmol · min^{-1} · mg^{-1})[c]	apparent *K*m (mM)
L-CoM-S-S-HTP	11	0.13
D-CoM-S-S-HTP	3.5	3.0
DL-CoM-S-S-HexTP[a]	<0.01	-
CoM-S-S-CoM	<0.01	-
HTP-S-S-HTP	<0.01	-
Cystine	<0.01	-
G-S-S-B[b]	<0.01	-
ox. lipoamide	<0.01	-

[a] H-S-HexTP, 6-mercaptohexanoylthreonine phosphate
[b] G-S-S-G, oxidized glutathione
[c] Determined by extrapolation from reciprocal plots or (values <0.01) at a substrate concentration of 2 mM

0.2 mM substrate the activity ratio between D- and the L-form was 1:10.

3.5. *Catalysis of the oxidation of H-S-CoM and H-S-HTP*

The partially purified CoM-S-S-HTP reductase was found to catalyze the reduction of methylene blue ($E^{\circ\prime}$ = 33 mV) in the presence of both H-S-CoM and H-S-H-HTP at a specific rate of 5 μmol · min^{-1} · mg^{-1}. Absolutely no activity was observed when only one of the thiol compounds was added or when other thiols were tested (e.g. cysteine, glutathione, or thioglycolate). Half-maximal rates were obtained with 0.25 mM H-S-CoM and < 0.05 mM H-S-HTP, respectively, with the second substrate present at 0.5 mM.

3.6. *CoM-S-S-HTP reductase activity in* Ms. barkeri

Cell extracts (26000 × *g* supernatant) of this organism (grown on acetate) catalyzed the reduction of CoM-S-S-HTP at a specific activity of 0.62 μmol · min^{-1} · mg^{-1} and the oxidation of H-S-CoM plus H-S-HTP with methylene blue at a specific rate of 0.6 μmol · min^{-1} · mg^{-1} (pH 7.6, 40°C).

4. DISCUSSION

It was found that the CoM-S-S-HTP reductase of *M. thermoautotrophicum* copurifies with the viologen dye-reducing hydrogenase. We have obtained preliminary evidence that the two enzymes can be separated by chromatography on Mono Q in the presence of CHAPS (3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate). It thus appears that hydrogenase and CoM-S-S-HTP reductase, catalyzing together reaction 2, form a complex. With respect to the electron carrier possibly involved, it is of interest that the transcription unit for the subunits of the viologen dye-reducing hydrogenase of *M. thermoautotrophicum* (strain ΔH) contains an open reading frame encoding a polyferredoxin [7]. It is not known whether this iron-sulfur protein is involved in CoM-S-S-HTP reduction.

In vivo the reduction by H_2 of methyl-coenzyme M to methane is associated with the conservation of energy via the chemiosmotic mechanism [8]. It should therefore be proposed that in vivo at least one component of the system is membrane-associated. However, after disruption of the cells via French press treatment, all the activities required to reduce methyl-coenzyme M to methane with H_2 were found to be present in the soluble cell fraction. In cell extracts CoM-S-S-HTP reduction with H_2 proceeded at a specific rate of only 30 nmol · min^{-1} · mg^{-1}. On the other hand, in the cell extract the specific activities of hydrogenase and of CoM-S-S-HTP reductase, when tested individually, were at least 10-fold higher and of the order of the rates of in vivo methane formation. These findings indicate that during cell breakage the electron-transport chain between H_2 and CoM-S-S-HTP becomes disconnected.

It has recently been reported that cell extracts of *M. thermoautotrophicum* (strain ΔH) catalyze the reduction of fumarate ($E^{\circ\prime}$ = +33 mV) with both H-S-CoM and H-S-HTP ($E^{\circ\prime}$ = −210 mV) [9]. The soluble enzyme also couples with dyes [10]. It thus had to be considered that the CoM-S-S-HTP reductase activity was a side activity of fumarate reductase. This was excluded by our finding that the partially purified heterodisulfide reductase preparation from *M. thermoautotrophicum* (strain Marburg) did not contain fumarate reductase activity although it was present in the crude extracts. Also, the latter activity could not be found in crude extracts of *Ms. barkeri* which, in contrast to *M. thermoautotrophicum*, does not require this enzyme for the synthesis of cell carbon compounds [11]. However, cell extracts of *Ms. barkeri* did contain a high specific activity of CoM-S-S-HTP reductase.

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