Volume 214, number 2, 265–268 FEB 04594 April 1987

The L-form of *N*-7-mercaptoheptanoyl-*O*-phosphothreonine is the enantiomer active as component B in methyl-CoM reduction to methane

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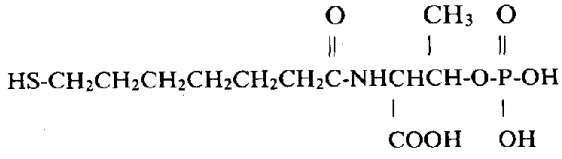
Received 3 February 1987

The reduction of methyl-CoM to methane in methanogenic bacteria is dependent on a low-M_r, heat-stable compound designated component B, the structure of which has recently been assigned as *N*-7-mercaptoheptanoyl-*O*-phosphothreonine. We report here that only the enantiomer derived from *O*-phospho-L-threonine has cofactor activity. *N*-7-Mercaptoheptanoyl-*O*-phospho-D-threonine was neither active nor inhibitory.

Methanogenic bacteria; Methyl-CoM reductase; Component B; 7-Mercaptoheptanoylthreonine phosphate

1. INTRODUCTION

Component B is a cofactor required in methanogenesis [1,2]. Its structure has recently been assigned as *N*-7-mercaptoheptanoyl-*O*-phosphothreonine (7-mercaptoheptanoylthreonine phosphate, HS-HTP) [3,4]. The absolute configuration, however, has not yet been determined.



HS-HTP

The apparent K_m for naturally occurring component B was found to be only half that for HS-HTP which had been chemically synthesized from *O*-phospho-DL-threonine and which was therefore a racemic mixture of D- and L-HS-HTP [5,6]. This suggests that only one of the two enantiomers is active.

To confirm this assumption and to determine the absolute configuration of component B, we synthesized D- and L-HS-HTP and tested the activity of the two enantiomers as cofactor in methyl-CoM reduction to methane as catalyzed by purified methyl-CoM reductase of *Methanobacterium thermoautotrophicum* (strain Marburg) [7].

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2. MATERIALS AND METHODS

D- and L-threonine (puriss., allo-free) and Amberlite IRC-84 ion-exchange resin (20–50 mesh, H^+ form, equilibrated with 2 M aqueous NH_3 and washed with water) were from Fluka (Buchs, Switzerland). Kieselgel 60 thin-layer chromatography (TLC) plates (0.25 mm) were from Merck (Darmstadt); the plates were stained with ninhydrin (2% in *n*-butanol/2 M aqueous

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00145793/87/$3.50

acetic acid, 19:1) or with $PdCl_2$ (0.5% in H_2O).

XAD-2 polystyrene adsorbent (0.1–0.2 mm) was from Serva (Heidelberg) and dithiothreitol (DTT) from Sigma (München).

2.1. *Synthesis of D- and L-HS-HTP*

2.1.1. *O*-Phospho-L- and D-threonine

O-Phospho-L- and D-threonine were prepared by phosphorylation of L- and D-threonine, respectively, with $POCl_3$ following the method of Neuhaus and Korkes [8]. *O*-Phospho-L-threonine: white crystals, m.p. 195°C after decomposition to a brown solid at 184°C; $[\alpha]_D^{23} = -7.5 \pm 0.2°$ (c = 2.4 in H_2O) in accordance with the value of −7.4° (c = 2.6 in H_2O) reported by De Verdier [9]. *O*-Phospho-D-threonine: m.p. 190°C (decomposition at 184°C); $[\alpha]_D^{23} = +7.4 \pm 0.2°$ (c = 2.7 in H_2O).

2.1.2. *N*-7-Mercaptoheptanoyl-*O*-phospho-D-threonine (D-HS-HTP)

The synthesis was carried out according to Noll et al. [6]. In contrast to [6], HS-HTP was not isolated as the free thiol but as the symmetrical disulfide (HTP-S-S-HTP), using the modified work-up procedure described below.

A solution of *O*-phospho-D-threonine (86 mg, 432 μmol) and triethylamine (120 μl, 861 μmol) in 0.6 ml water was added with stirring to a solution of 7,7′-dithiobis(succinimido-oxyheptanoate) (77.5 mg, 150 μmol) [6] in 3.9 ml tetrahydrofuran and 0.9 ml acetonitrile. After stirring at room temperature under nitrogen for 36 h, the solvents were removed under vacuum at 30°C. The resulting white residue was dissolved in 1 M HCl (25 ml) and washed with dichloromethane (3 × 8 ml). The aqueous phase was applied to a 2 × 13 cm column of polystyrene XAD-2 (equilibrated with 1 M HCl). The column was washed with 50 ml of 1 M HCl, followed by 80 ml H_2O. The product was eluted applying a methanol gradient (60 ml H_2O/MeOH, 4:1; 100 ml H_2O/MeOH, 1:1; 60 ml H_2O/MeOH, 1:4, 60 ml MeOH). TLC analysis (solvent system: *n*-butanol/acetic acid/H_2O, 2:1:1) of the collected fractions (10 ml each) showed that HTP-S-S-HTP (R_f = 0.35) had been completely separated from *O*-phosphothreonine (R_f = 0.24) and less polar products ($R_f > 0.6$). The combined fractions containing pure HTP-S-S-HTP were concentrated under vacuum to 1/10 of the original volume and transferred onto a column of Amberlite IRC-84 ion-exchange resin (8 g, 1.5 × 9 cm, NH_4^+ form). After elution with H_2O and lyophilization, 23 mg (20%) of a white solid was obtained. The product, (−)-*N,N′*-(7,7′-dithiodiheptanoyl)bis(*O*-phospho-D-threonine) ($[\alpha]_D^{23} = -3.4 \pm 0.5°$, c = 2.3 in H_2O), was indistinguishable by TLC from a sample of racemic HTP-S-S-HTP prepared by the same method and characterized by ^{13}C- and ^1H-NMR-spectroscopy.

2.1.3. *N*-7-Mercaptoheptanoyl-*O*-phospho-L-threonine (L-HS-HTP)

(+)-*N,N′*-(7,7′-dithiodiheptanoyl)bis(*O*-phospho-L-threonine) ($[\alpha]_D^{23} = +4.0 \pm 0.8°$, c = 0.8 in H_2O) was obtained by the same procedure starting from *O*-phospho-L-threonine.

2.2. *Assays for methyl-CoM reduction to methane*

All assays were performed in sealed 8-ml serum flasks as in [4,7]. The gas phase was N_2. The 0.4 ml assay mixture contained: 50 mM potassium phosphate buffer (pH 6.5); 15 mM DTT, 5.5 mM methyl-CoM, 150–300 μM hydroxycobalamin, 0–50 μg methyl-CoM reductase from *M. thermoautotrophicum* (strain Marburg), and synthetic HS-HTP or the disulfide thereof (0–2.2 mM). Methane formation was initiated by increasing the temperature from 22 to 60°C.

3. RESULTS AND DISCUSSION

Recently it was shown that purified methyl-CoM reductase from *M. thermoautotrophicum* (strain Marburg) catalyzes the reduction of methyl-CoM to methane with dithiothreitol in the presence of hydroxycobalamin and a racemic mixture of synthetic D- and L-HS-HTP (apparent K_m = 0.2 mM) [4]. Using the same assay conditions we now tested the D- and L-enantiomers for cofactor activity (fig.1).

Methyl-CoM reduction to methane occurred only in the presence of L-HS-HTP. An apparent K_m of 0.07 mM and a maximal specific activity of 460 nmol·min^{-1}·mg protein^{-1} were found (fig.2A,B). D-HS-HTP showed no significant activity. At very high concentrations (2.2 mM) of the D-enantiomer methane was formed at only very low rates which could be due to contamination of

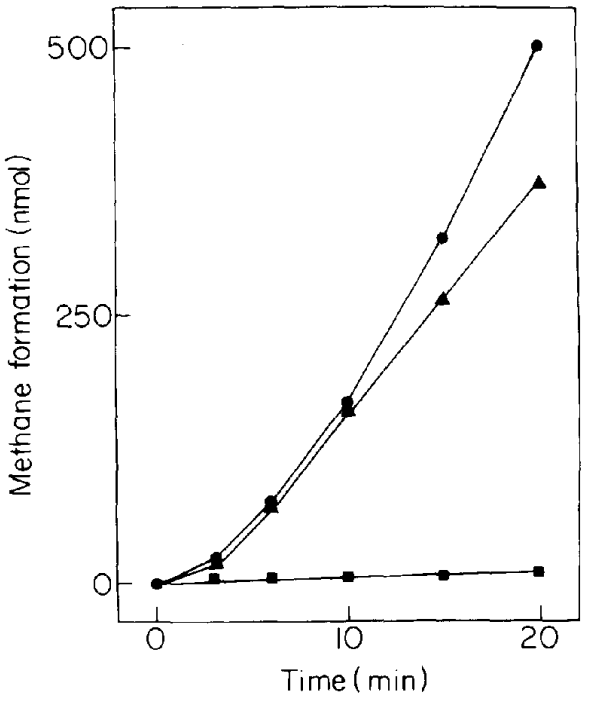

Fig.1. Time course of methane formation from methyl-CoM in the presence of synthetic *N*-7-mercaptoheptanoyl-*O*-phosphothreonine (HS-HTP). The 400 μl assay contained 38 μg purified methyl-CoM reductase from *M. thermoautotrophicum* and 300 μM hydroxycobalamin. HS-HTP was applied in the disulfide form. (●) L-HS-HTP (0.1 mM), (■) D-HS-HTP (0.1 mM), (▲) DL-HS-HTP (0.2 mM).

the D-HS-HTP preparation by low amounts of L-HS-HTP. This interpretation is supported by the finding that D-HS-HTP did not inhibit the action of L-HS-HTP, indicating that D-HS-HTP does not bind to the active site of methyl-CoM reductase (table 1). From these results we conclude that threonine has the L configuration in naturally occurring component B.

The disulfide of HS-HTP can easily be purified under aerobic conditions and can therefore be prepared in a more homogeneous form than the corresponding reduced (oxygen-sensitive) thiol. Therefore, in the assays the disulfide was routinely applied. It presumably is rapidly reduced to HS-HTP under the assay conditions described (15 mM DTT, pH 6.5, 60°C). Indeed, the disulfide and HS-HTP exhibited the same cofactor activity (not shown). It should be pointed out, however, that

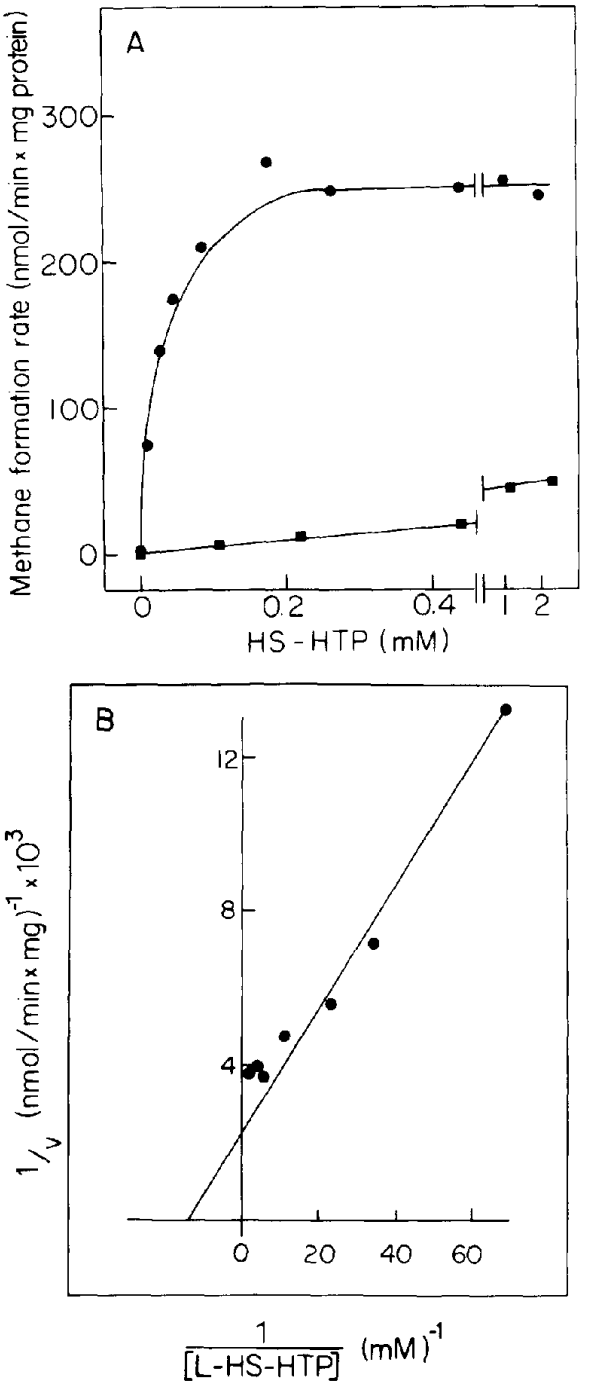

Fig.2. (A) Dependence of methyl-CoM reductase activity on concentration of L- (●) and D-HS-HTP (■). (B) Reciprocal plot of the data obtained for L-HS-HTP. The 400 μl assay contained 23 μg purified methyl-CoM reductase from *M. thermoautotrophicum*, 150 μM hydroxycobalamin and 0–2.2 mM L- or D-HS-HTP (applied in the disulfide form).

Table 1

Effect of D-HS-HTP on L-HS-HTP-dependent methane formation from methyl-CoM (assay conditions as described in the legend to fig.2)

[L-HS-HTP] (μM)	[D-HS-HTP] (μM)	nmol CH_4/min per mg protein
0	0	0
44	0	151
0	110	8
44	110	156
44	220	165
110	0	208
110	110	195

this may not necessarily be the case when the reduction of methyl-CoM to CH_4 with H_2 as electron donor is studied. Besides methyl-CoM reductase reduction with H_2 requires the presence of at least three additional proteins (components A_{1-3}), of FAD, Mg^{2+}, cobalamin, ATP and component B [10–13]. The assay is performed in a buffer containing low concentrations of mercaptoethanol. Under these conditions reduction of HTP-S-S-HTP is expected to be much slower than under the assay conditions for methyl-CoM reduction with DTT.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Deutsche Forschungsgemeinschaft and by the Fonds der Chemischen Industrie.

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