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On the role of *N*-7-mercaptoheptanoyl-*O*-phospho-L-threonine (component B) in the enzymatic reduction of methyl-coenzyme M to methane

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The reduction of methyl-coenzyme M (CH_3SCoM) to methane in methanogenic bacteria is dependent on component B (*N*-7-mercaptoheptanoyl-*O*-phospho-L-threonine, HSHTP). We report here that *S*-methyl-component B (*N*-7-(methylthio)heptanoyl-*O*-phospho-L-threonine, CH_3SHTP) can substitute for neither CH_3SCoM nor HSHTP in the methyl-CoM reductase reaction. Rather, CH_3SHTP proved to be an inhibitor competitive with HSHTP (apparent $K_i = 6\ \mu M$) and noncompetitive with CH_3SCoM. These results make it very unlikely that HSHTP functions as a methyl group carrier. A role for HSHTP as direct electron donor for CH_3SCoM reduction to CH_4 is proposed.

Methanogenic bacteria; Methanogenesis; Methyl-CoM reductase; Component B; *N*-7-Mercaptoheptanoylthreonine phosphate; Coenzyme F430; Vitamin B12; ATP synthesis

1. INTRODUCTION

Methane is formed in methanogenic bacteria from methyl-coenzyme M [2-(methylthio)ethanesulfonate, CH_3SCoM] [1] in a reaction mediated by methyl-CoM reductase [2]. The prosthetic group of this enzyme is coenzyme F430 [3], which is a nickel porphinoid of unique structure [4–10]. Per mol F430 the enzyme also contains non-covalently bound approx. 1 mol coenzyme M (2-mercaptoethanesulfonate, HSCoM) [6,9,11] and an undefined amount of component B [12]. The structure of component B has recently been assigned as *N*-7-mercaptoheptanoyl-*O*-phospho-L-threonine (7-mercaptoheptanoylthreonine phosphate, HSHTP) [13–15].

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Purified methyl-CoM reductase from *Methanobacterium thermoautotrophicum* (strain Marburg) can catalyze the reduction of methyl-CoM to methane with dithiothreitol (DTTred) as electron donor [16]. The reaction is strictly dependent on HSHTP and strongly stimulated (20-fold) by aquocobalamin (B12a), which functions as an electron carrier [14,16] in the system:

$$CH_3SCoM + DTTred \xrightarrow[\text{HSHTP, B12a}]{\text{methyl-CoM reductase}} CH_4 + HSCoM + DTTox$$

The role of HSHTP in methyl-CoM reduction to methane has not yet been elucidated. Principally, HSHTP could function as a methyl group carrier and/or as a redox carrier. In the former case, *N*-7-(methylthio)heptanoyl-*O*-phospho-L-threonine [7-(methylthio)heptanoylthreonine phosphate, CH_3SHTP] should be an intermediate in methanogenesis. We therefore synthesized

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CH_3SHTP and investigated whether this compound can substitute for HSHTP or CH_3SCoM in the methyl-CoM reductase reaction.

2. MATERIALS AND METHODS

Dicyclohexylcarbodiimide (puriss), *N*-hydroxysuccinimide (purum), sodium methanethiolate (pract.), dioxane (puriss, filtered through basic alumina), tetrahydrofuran (purum, distilled over Na/benzophenone), acetonitrile (puriss), triethylamine (puriss), and silica gel 60 (0.040–0.063 mm) were from Fluka (Buchs, Switzerland). 7-Bromoheptanoic acid was from Riedel-de Haen and XAD-2-polystyrene absorbent (0.1–0.2 mm) from Serva (Heidelberg). Thin-layer chromatography (TLC) was performed using kiesel gel 60 TLC plates (0.25 mm) from Merck (Darmstadt).

2.1. *Synthesis of* N-*[7-(methylthio)heptanoyl]*-O-*phospho-L-threonine*

2.1.1. 7-(Methylthio)heptanoic acid

To a cooled (10°C) solution of 7-bromoheptanoic acid (3.0 g, 14.4 mmol) in 12 ml ethanol, 1.5 ml of 10 M aqueous NaOH was added slowly. After addition of $NaSCH_3$ (1.0 g, 14.3 mmol), the suspension was stirred at room temperature under argon. After 6 h the reaction mixture was diluted with 2 ml water to dissolve the precipitated NaBr. The brown solution was then stirred for 14 h at room temperature, acidified with 1 M aqueous HCl (20 ml) and extracted with ether. The organic phase was dried over $MgSO_4$ and concentrated under vacuum. The resulting brown residue was purified by flash column chromatography [17] on silica gel 60 [4 × 15 cm, ether/petroleum ether (30–60°C), 2:3, v/v] to give, after drying at 10^{-2} Torr, 2.1 g (83%) of a viscous, faintly yellow oil. ^{1}H-NMR (80 MHz, $CDCl_3$): 1.2–1.8(m, 8H, $4CH_2$), 2.10(s, 3H, SCH_3), 2.2–2.6(m, 4H, CH_2S/CH_2COO), 10.4(broad s, 1H, COOH) ppm.

2.1.2. *N*-Hydroxysuccinimide ester (cf. [18])

7-(Methylthio)heptanoic acid (735 mg, 4.2 mmol) and *N*-hydroxysuccinimide (503 mg, 4.4 mmol) were dissolved in 25 ml anhydrous dioxane under argon. After addition of dicyclohexylcarbodiimide (858 mg, 4.2 mmol), the reaction mixture was stirred at room temperature for 16 h. The precipitated dicyclohexylurea was removed by filtration and washed with dioxane (3 × 10 ml) followed by isopropanol (2 × 10 ml). The combined filtrates were concentrated under vacuum and the resulting oil was purified by flash column chromatography [17] (silica gel 60, 4 × 18 cm; ether/hexane, 2:1, v/v). The combined fractions containing the pure *N*-hydroxysuccinimide ester ($R_f = 0.25$; silica gel; ether/hexane, 2:1) were concentrated and dried at 10^{-2} Torr to give 812 mg (71%) of a viscous yellowish oil which solidified at 4°C. ^{1}H-NMR (80 MHz, $CDCl_3$): 1.2–2.0(m, 8H, $4CH_2$), 2.09(s, 3H, SCH_3), 2.4–2.8(m, 4H, CH_2S/CH_2COO), 2.82(s, 4H, succinimide) ppm.

2.1.3. *N*-[7-(Methylthio)heptanoyl]-*O*-phospho-L-threonine

Following the procedure of Noll et al. [18], a solution of *O*-phospho-L-threonine [15] (546 mg, 2.74 mmol) and triethylamine (0.76 ml, 5.47 mmol) in 5 ml water was added to a solution of the *N*-hydroxysuccinimide ester (672 mg, 2.46 mmol) in 40 ml tetrahydrofuran. Addition of acetonitrile (14 ml) resulted in a homogeneous reaction mixture which was stirred at room temperature under argon for 36 h. After evaporation of the solvents (30°C, 10^{-3} Torr) the residue was taken up in 25 ml of 1 M HCl and washed with dichloromethane (3 × 10 ml). The aqueous layer was treated under vacuum to remove traces of dichloromethane and applied to a column of XAD-2-polystyrene (2 × 13 cm, equilibrated with 1 M HCl). After washing with 100 ml of 1 M HCl and 100 ml water, the product was eluted using increasing amounts of methanol in water/methanol mixtures (100 ml H_2O/methanol, 4:1; 120 ml H_2O/methanol, 1:1; 80 ml H_2O/methanol, 1:4; 100 ml methanol). The fractions of 100% methanol which, according to TLC analysis (silica gel; *n*-butanol/acetic acid/water, 2:1:1; staining with 0.5% $PdCl_2$ in H_2O or with 2% ninhydrin in *n*-butanol/2 M aqueous acetic acid, 19:1), contained pure CH_3SHTP ($R_f = 0.70$) were combined and concentrated in a vacuum. The residue was dissolved in 2 ml of 2 M aqueous ammonia. The solution was concentrated (10^{-3} Torr, 35°C) to a volume of approx. 1 ml and lyophilized to give 202 mg (20%) CH_3SHTP ammonium salt as a white solid. ^{1}H-NMR (300 MHz, D_2O): 1.29(d, $J = 6$ Hz, 3H, CH_3), 1.30–1.42(m, 4H, $2CH_2$),

1.54–1.70(m, 4H, $2CH_2$), 2.09(s, 3H, SCH_3), 2.28–2.44(m, 2H, CH_2CO), 2.53(t, $J = 7$ Hz, 2H, CH_2S), 4.13(broadened d, $J = 4$ Hz, 1H, CHCOO), 4.57–4.68(m, 1H, CH-OPO_3^{2-}), 4.71(s, HDO) ppm. ^{13}C-NMR (75 MHz, broad-band-decoupled, D_2O): 16.7(CH_3S), 21.1(CH_3CH), 27.8/30.2/30.4/30.7($CH_2CH_2CH_2CH_2$), 35.8/38.2 (CH_2CO/CH_2S), 62.8(d, $J_{CP} = 7$ Hz, CH-NH), 74.9(d, $J_{CP} = 5$ Hz, CH-OPO_3^{2-}), 179.3/179.6 (COO, CONH) ppm. FAB-MS (negative ion, glycerol matrix): 358(7), 357(16), 356(100) *m/e*(%).

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

All assays were performed in sealed 8-ml serum flasks as in [14,16]. The gas phase was N_2 at 2×10^5 Pa. The 0.4 ml standard assay mixture contained: 50 mM potassium phosphate buffer, pH 6.9; 15 mM dithiothreitol; 5.5 mM CH_3SCoM; 0.3 mM aquocobalamin; 15–25 μg methyl-CoM reductase from *M. thermoautotrophicum* (strain Marburg); and 0.4 mM DL-HSHTP synthesized in the disulfide form [15]. The reaction was initiated by increasing the temperature from 22°C (room temperature) to 65°C. Gas samples were withdrawn at intervals of 3 min and analyzed for methane as in [16].

Table 1

Methane formation catalyzed by purified methyl-CoM reductase from *Methanobacterium thermoautotrophicum* (strain Marburg)

Conditions	nmol CH_4 formed after 20 min	
Complete	210	500[a]
– Enzyme	< 1	
– CH_3SCoM	< 1	
– HSHTP	< 1	
– Aquocobalamin	10	
– CH_3SCoM + CH_3SHTP (5 mM)	< 1	
– CH_3SCoM + HSCoM (5 mM) + CH_3SHTP (5 mM)	< 1	< 1[a]
– HSHTP + CH_3SHTP (0.2 mM)	< 1	< 1[a] < 1[b]
+ CH_3SHTP (0.15 mM)	30	

[a] 180 μg methyl-CoM reductase per 0.4 ml assay
[b] + 5 mM CH_3SHTP
The assays were performed in 8-ml serum flasks with N_2 as gas phase. The 0.4 ml assay mixture contained: 5.5 mM CH_3SCoM, 0.4 mM DL-HSHTP, 0.3 mM aquocobalamin, 15 mM dithiothreitol, methyl-CoM reductase, 16 μg protein, 50 mM potassium phosphate buffer (pH 6.9)

3. RESULTS

The preparations of methyl-CoM reductase from *M. thermoautotrophicum* (strain Marburg) used in the following experiments were more than 90% pure as judged from SDS-polyacrylamide gel electrophoresis. Under the assay conditions described in table 1 the enzyme catalyzed the reduction of methyl-CoM (app. K_m 2 mM) to methane with dithiothreitol (app. K_m 4 mM) with a specific activity of 0.5 μmol · min^{-1} · mg protein^{-1}. Methane formation was strictly dependent on the presence of HSHTP (app. K_m 0.1 mM DL-HSHTP) and was stimulated over 20-fold by cob(III)alamin (app. K_m 0.04 mM) which was reduced to cob(II)alamin and cob(I)alamin under the experimental conditions [16]. ATP and Mg^{2+} were not required and did not stimulate the reaction. After a lag period of 3–6 min methane formation proceeded linearly for at least 20 min, and the reaction rate was proportional to the protein concentration in the range 0–100 μg methyl-CoM reductase protein per 0.4 ml assay.

We investigated whether CH_3SHTP could sub-

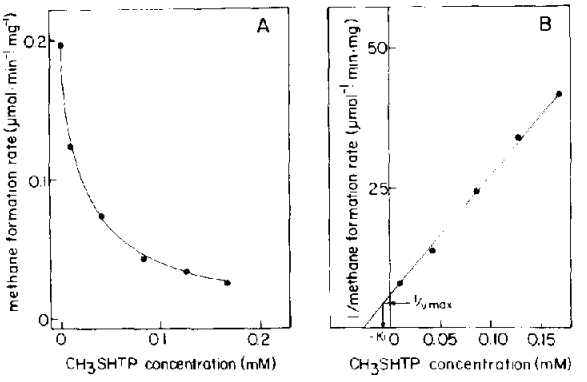

Fig.1. Inhibition of methyl-CoM reduction to methane by CH_3SHTP (A); Dixon plot of the same data (B). For assay conditions see table 1. K_i was determined taking into account that inhibition by CH_3SHTP was competitive with respect to HSHTP (fig.2A). V_{max} was determined from Lineweaver-Burk plots ($1/v$ vs $1/[HSHTP]$) in the absence of CH_3SHTP (not shown).

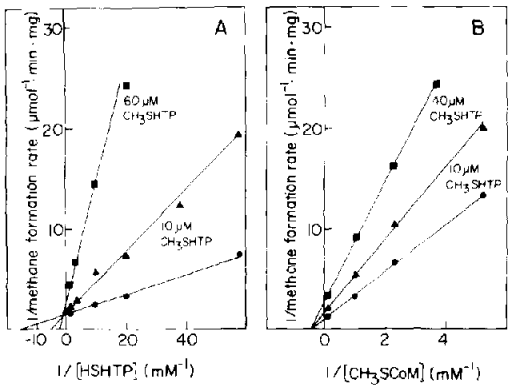

Fig.2. Effect of CH_3SHTP on the apparent K_m and V_{max} values for DL-HSHTP ([CH_3SCoM] = 5.5 mM) (A) and for CH_3SCoM ([DL-HSHTP] = 0.4 mM) (B).

stitute for either CH_3SCoM or HSHTP in the assay. The results are summarized in table 1. Methane was not formed when CH_3SCoM and/or HSHTP were omitted even when very high methyl-CoM reductase concentrations (up to 180 μg per 0.4 ml assay) were employed. When CH_3SHTP was added in addition to CH_3SCoM and HSHTP, then the rate of methane formation was lower than that in the absence of CH_3SHTP. The activity of methyl-CoM reductase decreased with increasing CH_3SHTP concentrations (fig.1A).

In order to determine the type of this inhibition the concentrations of CH_3SCoM, CH_3SHTP and HSHTP in the assay were varied and for each concentration combination the specific activity of methane formation was determined. From Dixon plots ($1/v$ vs CH_3SHTP concentration) an apparent K_i of 6 μM was determined (fig.1B). Lineweaver-Burk plots of $1/v$ vs 1/[HSHTP] at different CH_3SHTP concentrations intersected on the ordinate showing that CH_3SHTP inhibition was competitive with respect to HSHTP (fig.2A). Plots of $1/v$ vs 1/[CH_3SCoM] at different CH_3SHTP concentrations intersected to the left of the ordinate on the abscissa, indicating that CH_3SHTP inhibition was noncompetitive with respect to CH_2SCoM (fig.2B).

4. DISCUSSION

The gas-chromatographic method used to determine methane was very sensitive and allowed the detection of less than 1 nmol CH_4 generated per assay mixture. The finding that methane formation was not detectable when CH_3SCoM or HSHTP were substituted by CH_3SHTP thus indicates that free CH_3SHTP was not reduced to CH_4 under the experimental conditions (or only at rates which are not significant for the enzymic mechanism). The conclusion can therefore be drawn that free CH_3SHTP is not an intermediate in CH_3SCoM reduction to methane.

It can be envisaged that CH_3SHTP is in fact formed from CH_3SCoM and HSHTP at the active site of the enzyme but that it does not exchange with the CH_3SHTP added to the enzyme. The latter would then have to bind to an allosteric site of the protein there exerting its inhibitory effect. This possibility is not considered very likely since CH_3SHTP inhibition was competitive with respect to HSHTP, suggesting that CH_3SHTP and HSHTP bind to the same site of the enzyme.

If HSHTP does not act as a methyl carrier in CH_3SCoM reduction to CH_4, then what could its function be? A clue to this question comes from the following findings: (i) we have shown above that CH_3SHTP cannot substitute for HSHTP as cofactor in methyl-CoM reduction, indicating that the free thiol group is required for the function of the molecule; (ii) it has been reported that methyl-CoM reductase contains approx. 1 mol CoM bound per mol coenzyme F430 [9,11,19] and an undefined amount of component B [12]; (iii) evidence has been provided indicating that the CoM bound to methyl-CoM reductase is present in a heterodisulfide form [9,11]. The disulfide has an M_r between 400 and 600 [9]. The heterodisulfide of HSHTP (M_r 340) and HSCoM (M_r 109) has an M_r of 447. These findings suggest that HSHTP reacts

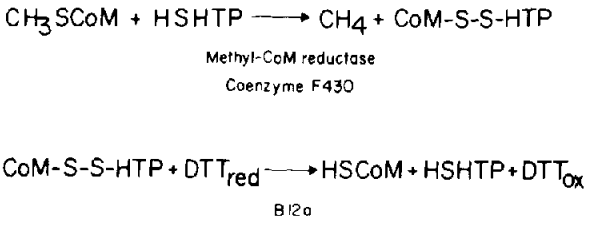

Fig.3. Proposed mechanism for the role of HSHTP (component B) in the enzymatic reduction of CH_3SCoM to CH_4 with DTTred. Reaction 1 is assumed to be catalyzed by methyl-CoM reductase containing coenzyme F430 as prosthetic group. Reaction 2 is possibly nonenzymatic and catalyzed by cobalamin.

with CH_3SCoM to yield CH_4 and CoM-S-S-HTP. If this is indeed the case, then HSHTP would be the direct electron donor for CH_3SCoM reduction to CH_4 and would thus have the function of an electron carrier.

Based on this conclusion we propose a mechanism for the enzymatic reduction of CH_3SCoM to CH_4 (fig.3). The mechanism assumes that the nickel porphinoid coenzyme F430 (as a redox catalyst [20,21]) is involved in the catalysis of CH_4 formation from CH_3SCoM and HSHTP and that the cobalamin functions as an electron mediator [16] in CoM-S-S-HTP reduction to HSCoM and HSHTP with dithiothreitol.

Evidence is available that CH_3SCoM reduction to CH_4 in methanogenic bacteria is coupled with the phosphorylation of ADP via a chemiosmotic mechanism [22]. If the mechanism proposed in fig.3 is correct then the possibility has to be considered that energy conservation is connected to CoM-S-S-HTP reduction rather than to the CH_4-forming reaction proper. In this respect, it is noteworthy that many anaerobic bacteria can live at the expense of sulfur (polysulfide) reduction with H_2 [23] and that a corrinoid protein, which has redox carrier properties, has been detected in the cytoplasmic membrane of methanogenic bacteria [24].

With respect to the role of HSHTP, it is of interest that synthetic *N*-6-mercaptohexanoyl-*O*-phospho-L-threonine and *N*-8-mercaptooctanoyl-*O*-phospho-L-threonine cannot substitute for HSHTP as cofactor in methane formation (unpublished).

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