Vol. 145, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1987 Pages 204-210

THE ROLE OF 7-MERCAPTOHEPTANOYLTHREONINE PHOSPHATE IN THE METHYLCOENZYME M METHYLREDUCTASE SYSTEM FROM *METHANOBACTERIUM THERMOAUTOTROPHICUM*[1]

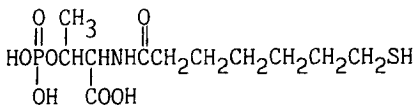
Kenneth M. Noll and Ralph S. Wolfe

Department of Microbiology, University of Illinois,
407 S. Goodwin Ave., Urbana, IL 61810

Received April 13, 1987

The structure of component B of the methylcoenzyme M methylreductase system from *Methanobacterium thermoautotrophicum* was recently found to be 7-mercaptoheptanoylthreonine phosphate (HS-HTP). Three potential roles for this cofactor were considered. First, a methyl thioether derivative of the cofactor was synthesized to investigate its possible role as a methyl donor. This derivative was found to be incapable of acting as a substrate for methanogenesis and proved inhibitory. Secondly, an adenylated form of the cofactor was considered as the potential active form of the coenzyme. This possibility was ruled out based upon collaborative observations with Ankel-Fuchs *et al.* (FEBS Lett., in press) that HS-HTP is required by the methylreductase system even when ATP is not. Finally, HS-HTP was found to act as a reductant in a partially-purified methylreductase preparation that was incubated under nitrogen. The rate of methane production from HS-HTP exceeded that from other thiols or hydrogen.

Methanogenic bacteria have been found to contain a number of novel coenzymes (1). The most recently described cofactor is HS-HTP (2), previously referred to as component B of the methylcoenzyme M methylreductase system (3).



This cofactor is required for *in vitro* methanogenesis from CH_3-S-CoM and hydrogen, along with ATP and the protein components A1, A2, A3, and C (4).

[1]This work was supported by U. S. Public Health Service Grant AI 12277. K. M. N. was a recipient of a Predoctoral U.S. Public Health Service Cell Biology Traineeship GM 07283-11.

ABBREVIATIONS: HS-HTP, 7-mercaptoheptanoylthreonine phosphate; CH_3-S-CoM, 2-(methylthio)ethanesulfonate, methylcoenzyme M; EtOH-SS-HTP, the mixed disulfide of 2-mercaptoethanol and HS-HTP; CH_3-S-HTP, 7-(methylthio)heptanoylthreonine phosphate; DTT, dithiothreitol; HPLC, high-performance liquid chromatography. TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Pipes, piperizine-N,N'-bis(2-ethanesulfonic acid).

0006-291X/87 $1.50

HS-HTP was shown to be tightly bound to component C of the methylreductase complex (5). Since this component also contains the nickel tetrapyrrole, F430, and coenzyme M, it is quite probable that these three cofactors act together at the active site of the enzyme (6, 7). Coenzyme M functions as a carbon carrier in the reduction of the methyl group to methane. F430 may play a role in methyl-activation, electron transfer, or both. The role of HS-HTP has not been reported.

The structure of HS-HTP suggests three possible roles for the cofactor. First, it may function as a methyl-carrying component. It may accept a methyl group from exogenous CH_3-S-CoM and transport it to the catalytic site of the enzyme. Second, since its structure is reminiscent of 4'-phosphopantotheine (and ATP is required for hydrogen-driven methylreductase activity), the active form of HS-HTP may be an adenylated form similar in structure to coenzyme A. Since methanogens have been shown to contain very low levels of pantothenic acid, perhaps they have evolved an analogous coenzyme just as they have done for the folates (8). Finally, HS-HTP may act as a reductant. Thiols such as lipoic acid and glutathione act in this capacity in a variety of enzyme systems. The long aliphatic chain of HS-HTP may serve as an "arm" that can reach into a hydrophobic active site and provide the necessary reducing equivalents for methane formation. Here, these possibilities are addressed to determine the role that component B may play in methanogenesis.

MATERIALS AND METHODS

Growth of Cells and Preparation of Extracts. Cells of *Methanobacterium thermoautotrophicum* strain ΔH were grown and harvested as previously described (2). Cell-free extract was obtained as described (2). Soluble cofactors were removed from extracts by passage twice through a column of Sephadex G25 Superfine (Pharmacia Inc., Piscataway, NY) using 20 mM potassium phosphate (pH 7) in 10 mM 2-mercaptoethanol.

Assays for methane formation were performed as described (2). The standard assay mixture contained 96 mM Pipes buffer (pH 6.3), 4 mM ATP, 20 mM $Mg(OAc)_2$, and other components as indicated. The final volume was 0.2 ml.

Preparation of Crude Methylreductase. A column of Whatman DE52 DEAE cellulose (2.7x4.2 cm) was equilibrated inside an anoxic chamber (Coy Laboratory Products, Ann Arbor, MI) in 50 mM TES (pH 7) with 10 mM 2-mercaptoethanol. Cell-free extract (323 mg protein) was applied to the column and then washed successively with 20 ml of the equilibration buffer and 40 ml of this buffer containing 100 mM potassium chloride. Finally, crude methylreductase was eluted from the column with 40 ml of this buffer containing 320 mM potassium

chloride. The golden brown protein that eluted with this step was collected and desalted by repeated ultrafiltration with a Diaflo PM 30 membrane (Amicon Corp., Danvers, MA). Ultrafiltration was continued until the eluate had the same conductivity as the equilibrating buffer and no cofactors were detectable in the eluate by UV spectroscopy. This enzyme preparation was stored at -20°C under nitrogen.

Synthesis of CH_3-S-HTP. HS-HTP was synthesized as described previously (9), and 3.8 µmol of this compound was dissolved in 1 ml of concentrated ammonium hydroxide in a 5-ml serum bottle containing a stirring bar. The bottle was flushed with nitrogen for ten minutes and wrapped in aluminum foil. To this was added 0.5 µl (32.2 µmol) methyliodide. This solution was stirred overnight. In dim light, the contents of the bottle were dried under vacuum. No free thiol was detectable in the reaction product using a modification of Ellman's assay (10, 11). The sample had been dissolved in water for the thiol assay, so the water was removed by flash evaporation. Ethanol was added, and flash evaporation was repeated to remove traces of water. The product was then washed three times with absolute ethanol until the washes were free of halide as measured by a nitrous acid spot test (12). The product was purified by reversed-phase HPLC using the same conditions used to purify HS-HTP (9). CH_3-S-HTP migrated in the same position as HS-HTP. A large quantity of the homodisulfide of HS-HTP was also present. The yield of the methylated cofactor was 24% as judged by phosphate and thiol assays. ^1H NMR of the product was performed using a Nicolet 360-MHz Fourier transform spectometer as described (2).

Miscellaneous. Dithiothreitol, HS-HTP, and the thiols were added to reaction mixtures inside an anoxic chamber from solutions prepared using water that had been sparged with nitrogen. Thiols were determined by a modification of the method of Ellman (10). HS-HTP was quantitatively determined by both the Ellman assay and the phosphate assay of Ames and Dubin (13).

Chemicals. The thiols used were purchased from Sigma Chemical Co. (St. Louis, MO). Methyliodide was purchased from Aldrich Chemical Co. (Milwaukee, WI).

RESULTS

The first of the three potential roles tested concerned HS-HTP as a methyl-group carrier. CH_3-S-HTP gave a ^1H NMR spectrum similar to that of HS-HTP (Figure 1), a major difference being the singlet at 2.1 ppm, the signal expected from a CH_3-S- unit (14). The triplet at 2.55 ppm is that expected for methylene protons adjacent to a thiol or thioether. When added to dialyzed cell extract, CH_3-S-HTP failed to serve as a methyl donor (Table 1). Even in the presence of CH_3-S-CoM, this methyl group was not reduced to methane. The compound inhibited methanogenesis from CH_3-S-CoM. It appears unlikely, then, that HS-HTP acts as a methyl carrier during reductive demethylation of CH_3-S-CoM.

The second hypothesis considered was that of an adenylated derivative of HS-HTP being the active form of the cofactor. Previous studies had shown that ATP was required in a substoichiometric amount to allow *in vitro*

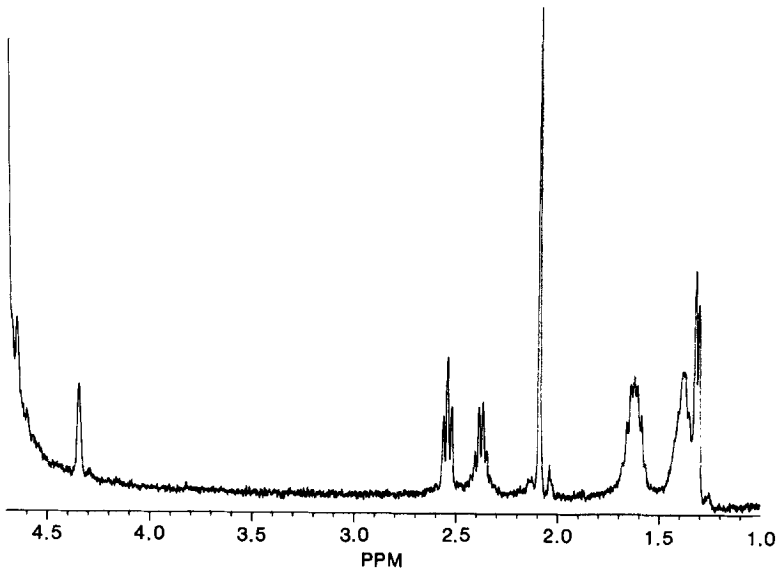


Figure 1. 360-MHz ^1H NMR spectrum of CH_3-S-HTP. Solvent D_2O. Chemical shifts relative to 3-trimethylsilyltetradeuteriopropionic acid, sodium salt (0.0 ppm).

methanogenesis (15). Further, ATP was shown to function as an "activator" of the hydrogen-driven methylreductase; its presence was only required during the first minutes of incubation (16). It may be that during this time HS-HTP is adenylated to give its active form. Recently we provided synthetic HS-HTP (9) for use with a highly purified DTT-driven methylreductase preparation, and rates of methanogenesis of 200 nmol min^{-1} mg^{-1} protein occurred without addition of ATP; addition of ATP was slightly inhibitory (Ankel-Fuchs, D., R. K. Thauer, K. M. Noll, and R. S. Wolfe, FEBS Letters, in press). These results suggest that HS-HTP is fully active in the methylreductase system

Table 1. Methane produced from CH_3-S-HTP by cell extract under hydrogen

Additions			Total Methane
HS-CoM (150 nm)	CH_3-S-HTP (58 nmol)	CH_3-S-CoM (250 nmol)	Formed (nmol)
+	-	-	23
-	+	-	15
+	+	-	4
+	+	+	29
-	+	+	98
+	-	+	255

Assays were carried out under a hydrogen atmosphere using dialyzed cell extract as described in Materials and Methods.

Table 2. The use of thiols as reductants for methanogenesis from CH_3-S-CoM using crude methylreductase

Reductant (nmol)	Methane Formation (nmol min^{-1} mg^{-1} protein)
none	0
HS-CoM (385)	0
cysteine (418)	0
mercaptoethanol (530)	0
glutathione (451)	0
homocysteine (347)	0
DTT (500)/(5000)	0.8/4.5
hydrogen	2.4

The complete reaction mixture contained the standard assay mixture with 0.11 mM EtOH-SS-HTP, 1.25 mM CH_3-S-CoM, and 2.5 mg protein under a nitrogen atmosphere.

without the necessity of adenylation. ATP must play another role in the less purified hydrogen-driven systems.

The final proposal considered here was that of HS-HTP as the source of reducing equivalents. The reduced form of the cofactor was prepared by reduction with DTT while the cofactor was bound to an anion-exchange resin; DTT was then removed, and the cofactor was eluted (9). The crude methylreductase preparation used for these studies could produce methane from CH_3-S-CoM using DTT as a source of reductant (Table 2). The enzyme did not require ATP, magnesium, or hydroxocobalamin; although all were stimulatory. HS-HTP was added as the disulfide, EtOH-SS-HTP, (2) and was required. Other thiols were unable to act as reductants when used in quantities comparable to the amount of DTT used.

Methane could be produced when HS-HTP was used as the sole source of reducing potential (Figure 2). The initial rate of methane formation was faster for HS-HTP than for DTT. The enzyme could also produce methane using hydrogen as an electron donor, although this rate was lower than with HS-HTP. The amount of methane made was not equivalent to the amount of HS-HTP added. When 191 nmol of HS-HTP was added, only 61 nmol of methane was produced. Since a racemic mixture of phosphothreonine was used to synthesize HS-HTP and the enzyme apparently only recognized one isomer, only half the added HS-HTP

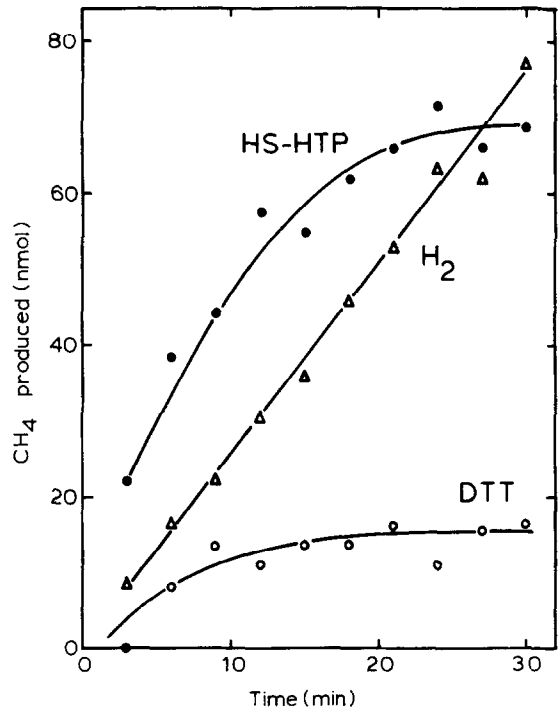


Figure 2. Methane formation from hydrogen, DTT, and HS-HTP. The assay contained the standard reaction mixture with 2 µmol CH_3-S-CoM and 191 nmol HS-HTP or 500 nmol DTT. HS-HTP and DTT were used under a nitrogen atmosphere.

was available as reductant. Therefore, one might have expected 95.5 nmol of methane if the substrates react in a 1:1 stoichiometry. This 64% yield may indicate a different reaction stoichiometry, incomplete utilization of substrate, or the accumulation of an inhibitory end product. In other experiments, yields of up to 81% have been observed. In this regard, it is interesting to note that during the synthesis of HS-HTP, disulfide-containing reaction products have been observed to inhibit methanogenesis (data not shown). The nature of these products has not been determined.

DISCUSSION

Evidence has been presented that HS-HTP acts as a reductant in the methylreductase system. The methyl-carrier role was ruled out, since CH_3-S-HTP was not reductively demethylated to yield methane. The fact that it inhibited methanogenesis from CH_3-S-CoM indicates that it may compete with HS-HTP for the active site of the enzyme. More detailed kinetic analyses will

be necessary to test this possibility. The adenylated form of HS-HTP is not necessary for catalysis.

The role of HS-HTP as a reductant opens interesting areas for the investigation of the mechanism of the methylreductase. DTT, when added, presumably acts as a reductant by reducing $(S\text{-}HTP)_2$ or R-SS-HTP to HS-HTP which then is involved in reduction of the methyl group to methane. This reduction may result in the formation of an inhibitory by-product which may be a disulfide. Under physiological conditions (or *in vitro* under hydrogen) this by-product could be reduced to allow methanogenesis to continue. When HS-HTP is used as the sole reductant, this product may accumulate and inhibit further methane formation.

ACKNOWLEDGMENT

We thank Victor Gabriel for mass culturing of *M. thermoautotrophicum*.

REFERENCES

1. Wolfe, R. S. (1985) Trends Biochem. Sci. 10, 396-399.
2. Noll, K. M., Rinehart, K. L., Jr., Tanner, R. S. and Wolfe, R. S. (1986) Proc. Natl. Acad. Sci. USA 83, 4238-4242.
3. Gunsalus, R. P. and Wolfe, R. S. (1980) J. Biol. Chem. 255, 1891-1895.
4. Nagle, D. P., Jr. and Wolfe, R. S. (1983) Proc. Natl. Acad. Sci. USA 80, 2151-2155.
5. Noll, K. M., and Wolfe, R. S. (1986) Biochem. Biophys. Res. Commun. 138, 889-895.
6. Ellefson, W. L. and Wolfe, R. S. (1981) J. Biol. Chem. 256, 4259-4262.
7. Hausinger, R. P., Orme-Johnson, W. H. and Walsh, C. (1984) Biochemistry 23, 801-804.
8. Leigh, J. A. (1983) Appl. Environ. Microbiol. 45, 800-803.
9. Noll, K. M., Donnelly, M. I., and Wolfe, R. S. (1987) J. Biol. Chem. 262, 513-515.
10. Ellman, G. L. (1958) Arch. Biochem. Biophys. 74, 443-450.
11. Balch, W. E., and Wolfe, R. S. (1979) J. Bacteriol. 137, 256-263.
12. Romesser, J. A., and Balch, W. E. (1980) Methods in Enzymology, 67, pp. 545-552.
13. Ames, B. N., and Dubin, D. T. (1960) J. Biol. Chem. 235, 769-775.
14. Silverstein, R. M., and Bassler, G. C. (1967) Spectrophotometric Identification of Organic Compounds., J. Wiley and Sons, Inc., NY.
15. Roberton, M. A., and Wolfe, R. S. (1969) Biochim. Biophys. Acta 192, 420-429.
16. Whitman, W. B., and Wolfe, R. S. (1983) J. Bacteriol. 154, 40-649.