Communication

Synthesis of 7-Mercaptoheptanoylthreonine Phosphate and Its Activity in the Methylcoenzyme M Methylreductase System*

(Received for publication, September 2, 1986)

Kenneth M. Noll‡, Mark I. Donnelly§, and Ralph S. Wolfe
From the Department of Microbiology, University of Illinois, Urbana, Illinois 61801

The structure of component B of the methylcoenzyme M methylreductase of Methanobacterium thermoautotrophicum was recently assigned as 7-mercaptoheptanoylthreonine phosphate (HS-HTP) (Noll, K. M., Rinehart, K. L., Jr., Tanner, R. S., and Wolfe, R. S. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4236-4242). We report here the chemical synthesis and biochemical activity of this compound. Thiourea and 7-bromoheptanoic acid were used to synthesize 7,7'-dithiodiheptanoic acid. This disulfide was then condensed with threonine phosphate using N-hydroxysuccinimide and dicyclohexylcarbodiimide. The product was reduced with dithiothreitol to give HS-HTP. It could be oxidized in air in the presence of 2-mercaptoethanol to give the compound as it was isolated from cell extracts. The resulting product was identical to the authentic compound by 1H NMR spectroscopy, mass spectrometry, and coelution using high performance liquid chromatography. The synthetic compound is active in the in vitro methanogenic assay at concentrations comparable to the authentic compound. This confirms the structure of component B as HS-HTP and provides a means to synthesize quantities sufficient for studies of the methylreductase system.

Methane-producing bacteria possess several unique cofactors that are involved in the process of methane formation from hydrogen and carbon dioxide (1). The most extensively studied step of this process is the final reduction to methane of a methylated cofactor (CH3-S-CoM) (2) by the methylcoenzyme M methylreductase system. This is a multienzyme system that has been resolved into a number of components that are involved in the process of methane formation from hydrogen and carbon dioxide (1). The most extensively studied step of this process is the final reduction to methane of a methylated cofactor (CH3-S-CoM) (2) by the methylcoenzyme M methylreductase system. This is a multienzyme system that has been resolved into a number of components that are involved in the process of methane formation from hydrogen and carbon dioxide (1).

EXPERIMENTAL PROCEDURES

HS-HTP Assays—Cells of Methanobacterium thermoautotrophicum strain A8 were grown and extracts were prepared as described previously (4). Assays for HS-HTP were conducted using a cell-free extract stripped of soluble cofactors by passage twice through a column of Sephadex G-25 Superfine resin (Pharmacia P-L Biocemicals). The assay mixture contained all necessary components except HS-HTP. Activity was observed by the measurement of methane formation from the demethylated CH3-S-CoM as previously described (4).

Synthesis of 7,7'-Dithiodiheptanoic Acid—7-Mercaptoheptanoic acid was synthesized by dissolving 3.24 g (42.65 mmol) of thiourea in a stirred solution of 7-bromoheptanoic acid (1.78 g, 8.53 mmol) in 20 ml of ethanol. This mixture was refluxed at 90 °C for 17 h, cooled to room temperature, and 5 ml of a 60% aqueous solution (w/v) of sodium hydroxide was added. The mixture was refluxed for an additional hour, cooled to room temperature, acidified with hydrochloric acid, and extracted with chloroform. The chloroform phase was extracted with 1 M aqueous solution of sodium bicarbonate. The aqueous extract was acidified and extracted with chloroform. The thiol was oxidized to a disulfide by mixing the chloroform phase with an aqueous solution of 10% (w/v) iodine and 20% (w/v) potassium iodide until the brown color persisted. The aqueous phase was removed and the chloroform phase was washed twice with water, dried over anhydrous magnesium sulfate, and concentrated under vacuum. The product was crystallized twice from benzene-pentane to give 588 mg of white crystals (43%).

C14H19O3S2
Calculated: C 52.15 H 8.13 S 19.85
Found: C 52.10 H 7.89 S 19.78

Synthesis of the N-Hydroxysuccinimide Ester—The disulfide product was activated by synthesis of its N-hydroxysuccinimide ester using dicyclohexylcarbodiimide (5). 7,7'-Dithiodiheptanoic acid (200.9 mg, 624 µmol) was dissolved in 6 ml of 1.4-dioxane at room temperature and the solution was stirred while 149.6 mg (1.26 mmol) of N-hydroxysuccinimide was added; when this dissolved, 261.6 mg (1.27 mmol) of dicyclohexylcarbodiimide was added and the solution was stirred 20 h at room temperature. The precipitated dicyclohexylurea was removed by filtration and the filtrate was dried to a clear oil under vacuum. A small amount of 2-propanol was added to the oil, and this solution was dried to a white solid under vacuum. The product was recrystallized twice from 2-propanol to give 201.6 mg (63%) of white crystals.
Synthesis and Activity of HS-HTP

Coupling of the N-Hydroxysuccinimide Ester and Threonine Phosphate—A solution of DL-threonine phosphate (78.0 mg, 392 pmol) in 2.5 ml of water containing 108 µl (775 pmol) of triethylamine was added with stirring to a solution of the N-hydroxysuccinimide ester (100.9 mg, 196 pmol) in 5 ml of tetrahydrofuran; 1 ml of acetonitrile was added to the mixture to achieve a single phase. After 29 h at room temperature, the solvent was removed under vacuum. Inside an anoxic chamber (Coy Scientific Products, Ann Arbor, MI) the contents of the flask were dissolved in 10 ml of deaerated 50 mM ammonium bicarbonate containing 100 mM dithiothreitol. After standing in the chamber for 30 min, this mixture was applied to a 6-ml column of Accell QMA Anion-exchange Medium (Waters Associates, Milford, MA) pre-equilibrated with deaerated 50 mM ammonium bicarbonate. The column was washed with 15 ml of this buffer to remove the dithiothreitol, and HS-HTP was gradient-eluted with successive applications of seven 5-ml aliquots of deaerated buffer, each aliquot containing a 100 mM ammonium bicarbonate increment. Fractions of 5 ml were collected and assayed for HS-HTP. The active fractions were pooled and lyophilized repeatedly to remove the salt. The product, 52.5 mg (37%) of white crystals, was judged as pure by

FIG. 1. Coelution of the authentic and synthetic mixed disulfide of HS-HTP and 2-mercaptoethanol. HPLC conditions were as described under "Experimental Procedures." A Waters analytical (300 × 3.9 mm) µBondapak C18 column was used. A, synthetic compound. B, authentic compound. C, mixture of synthetic and authentic compounds.

RESULTS AND DISCUSSION

The synthesis described here yields a product that is active in the methanogenic assay. The activity of the thiol form of HS-HTP had been noted previously (4), but all chemical
characterizations had been performed using the mixed disulfide of HS-HTP and 2-mercaptoethanol, the form that was isolated from cell extracts. This disulfide can be readily obtained by adding an excess of 2-mercaptoethanol to a solution of HS-HTP and drying the solution under vacuum. To compare the authentic and synthetic cofactors, the same form of the coenzyme is required. For the analyses described here, this mixed disulfide was made and the product purified by the HPLC method described under “Experimental Procedures.” The mixed disulfide of the synthetic product was found to co-elute with the mixed disulfide of the authentic cofactor (Fig. 1). When mixed together, the two compounds eluted as a single, uniform peak, thus supporting their identity.

The positive ion of the synthetic mixed disulfide had a mass of 366.0755, as determined by high resolution, fast atom bombardment mass spectrometry while the positive ion of the monosodium salt of HS-HTP has a calculated mass of 366.0755. This confirms the structural formula of C_{11}H_{20}O_{3}NSNa for this ion of the synthetic compound. A fragment ion of mass 246.1169 was also observed from the synthetic compound. This may be assigned to a fragment lacking a phosphate (C_{11}H_{19}O_{3}NS, calculated mass 246.1164).

The 'H NMR spectrum of the synthetic compound was also identical to the authentic compound as shown in Table I. In the thiol form, the triplets at 2.89 and 3.87 ppm are absent and the triplet at 2.78 ppm shifts to 2.56 ppm. This is consistent with the loss of 2-mercaptoethanol and the theoretical chemical shift of a methylene adjacent to a free thiol (6). The multiplets at 1.65 and 1.71 ppm also change and simplify to an apparent sextet (pair of triplets). This is due to the shift upfield of the 1.71-ppm multiplet so that it nearly overlaps the 1.65-ppm multiplet. This corresponds to the expected shift of the methylene protons that are β to the thiol.

The identity of the synthetic product was further confirmed by its biochemical activity. Authentic HS-HTP exhibited one-half the maximal rate of methane formation at a concentration of 3 μM (4). The synthetic mixed disulfide was also active as shown in Fig. 2. One-half maximal activity was found at a concentration of 6 μM. This higher Kₐ may be due to the fact that O-phospho-DL-threonine was used in the synthesis. A racemic mixture of HS-HTP, therefore, was synthesized. The configuration of the authentic compound is unknown.

The effect of added HS-HTP on the length of time before the onset of a linear rate of methane formation (the lag time) was also similar. The addition of increasing amounts of HS-HTP shortened the lag time (Fig. 2). This lag was found to decrease from 18 min (no HS-HTP added) to 7 min (22.5 nmol of HS-HTP added). (Please note that in Ref. 4, Fig. 1b, the ordinate values published are mistakenly a factor of 10 too high. The values reported here for the synthetic compound are comparable.)

The present work outlines a method for the synthesis of HS-HTP using readily available chemicals in a simple synthetic scheme. A more complex synthesis was recently presented (7) starting from diethyl pimelate and using the method of Walton et al. (8) for synthesis of lipoic acid. That synthesis involves seven steps and gave a final yield of 0.4%. The method presented here is a simplification of that method and provides a much better yield. Most importantly, the chemical synthesis of HS-HTP confirms the previously proposed structure. Now, all the known cofactors involved in methanogenesis have been identified. With this synthesis, it will be possible to obtain quantities of this cofactor sufficient for the elucidation of its role in methane formation.

Acknowledgments—We thank K. L. Rinehart for helpful discussions, Victor Gabriel for mass culturing of M. thermoautotrophicum, Michael Ratap for purification assistance, and J. Carter Cook and co-workers for acquisition of mass spectra.

REFERENCES

1. Wolfe, R. S. (1985) Trends Biochem. Sci. 10, 396-399
2. Gunsalus, R. P., and Wolfe, R. S. (1990) J. Biol. Chem. 255, 1881-1895
3. Nagle, D. P., Jr., and Wolfe, R. S. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 2151-2155
4. Noll, K. M., Rinehart, K. L., Jr., Tasner, R. S., and Wolfe, R. S. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4238-4242
5. Anderson, G. W., Zimmerman, J. E., and Callahan, F. M. (1964) J. Am. Chem. Soc. 86, 1839-1842
6. Silverstein, R. M., and Bassler, G. C. (1967) Spectrophotometric Identification of Organic Compounds, J. Wiley & Sons, Inc., New York
7. Noll, K. M., Donnelly, M. L., and Wolfe, R. S. (1986) Fed. Proc. 45, 1543
8. Walton, E., Wagner, A. F., Bachelor, F. W., Peterson, L. H., Holly, F. W., and Folks, K. (1965) J. Am. Chem. Soc. 77, 5144-5149