Biosynthesis of (7-Mercaptoheptanoyl)threonine Phosphate†

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ABSTRACT: The biosynthesis of (7-mercaptoheptanoyl)threonine phosphate (HS-HTP) has been studied in the methanogenic bacteria *Methanococcus volta* and *Methanosarcina thermophila*. Growth of these cells in medium containing [7,7-$^2H_2$]-7-mercaptoheptanoic acid, [3,4,4,4-*threonine*-$^2H_4$]-*N*-(7-mercaptoheptanoyl)threonine, [7,7-$^2H_2$]-*N*-(7-mercaptoheptanoyl)threonine, or DL-[3,4,4,4-$^2H_4$]threonine led to the generation of labeled HS-HTP containing a portion of the molecules with the same number of deuteriums as the precursor molecule. This result indicated that each of these labeled molecules can serve as a precursor for the biosynthesis of HS-HTP. Cell-free extracts of these methanogens were shown to carry out the ATP-dependent phosphorylation of *N*-(7-mercaptoheptanoyl)threonine to HS-HTP. These observations indicate that the biosynthesis of HS-HTP involves the coupling of mercaptoheptanoic acid with threonine to form (7-mercaptoheptanoyl)threonine, which is then phosphorylated to HS-HTP.

(7-Mercaptoheptanoyl)threonine phosphate (HS-HTP), originally designated as component B before it was fully characterized, was first identified as a low molecular weight, heat-stable cofactor required for the reductive demethylation of methyl coenzyme M in cell extracts of *Methanobacterium thermoautotrophicum* (Gunsalus & Wolfe, 1980). The structure of component B was assigned as the mixed disulfide of (7-mercaptoheptanoyl)threonine phosphate and 2-mercaptoethanol by Noll et al. (1986) and has been confirmed by chemical synthesis (Noll et al., 1987).

The first work on the biosynthesis of HS-HTP was directed at determining the biosynthesis of the 7-mercaptoheptanoic acid moiety of the molecule. This work demonstrated, using cells of *Methanococcus volta* and rumen isolate 10-16B, that it was formed from 7-oxoheptanoic acid (White, 1989a,b). The 7-oxoheptanoic acid was, in turn, found to be generated by the nonoxidative decarboxylation of α-ketosuberate, which was produced by the repeated α-keto acid chain elongation of α-ketoglutarate (White, 1989c). This work, however, gave no information on the pathway used by methanogens for the generation of the intact HS-HTP molecule. In this paper, the results of studies directed toward establishing the specific steps in the biosynthesis of HS-HTP are presented.

MATERIALS AND METHODS

Materials. (7-Methylthio)heptanoic acid and *N*-[7-(methylthio)heptanoyl]threonine were prepared as previously described (Clements et al., 1993). The disulfide of 7-mercaptoheptanoic acid was prepared by the iodine oxidation of 7-mercaptoheptanoic acid as described by Noll et al. (1987). The disulfide of *N*-(7-mercaptoheptanoyl)threonine was prepared as described below for the synthesis of the deuterated molecule. L-Threonine, L-threonine methyl ester hydrochloride, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonate (TES), bovine intestinal mucosa alkaline phosphatase (Sigma product number 7640), ATP, GTP, UTP, CTP, phosphoenolpyruvate, and DNase I were obtained from Sigma Chemical Co. [U-$^2H_4$]Acetaldehyde, anhydrous pyridine, hexamethyldisilazane, and trimethylsilyl chloride were obtained from Aldrich Chemical Co. Thiolyte (monobromobimane, mBBr) was obtained from Calbiochem.

Bacterial Strains and Growth Conditions. Methanococcus volta strain P. S. (DSM 1537) (Whitman et al., 1982) and *Methanosarcina thermophila* strain TM-1 (Zinder et al., 1985) were used for most of the work described herein. *Methanococcus volta* was grown as previously described (White, 1985) at 39 °C in 2-L bottles pressurized to 30 psi with H_2/CO_2 (80/20) on 500 mL of the medium described by Whitman et al. (1982). *M. thermophila* cells were grown in the presence of precursors (without shaking) in a 1-L round-bottom flask at 50 °C using the medium described by Sowers et al. (1984). For those experiments where *M. volta* and *M. thermophila* were grown in the presence of different precursors, the precursors were added to the anaerobic medium prior to autoclaving (see Table 1). After autoclaving, the medium was allowed to cool to the growth temperature of the cells before inoculation with a 10–20% volume of cells actively growing on a medium of the same composition but without the added precursor. In all cases, the cells were harvested from the medium by centrifugation.

M. thermophila used for the generation of the cell extracts was grown in Dr. James G. Ferry's laboratory, as previously described (Sowers et al., 1984). Frozen cells of *Methanobacterium thermoautotrophicum* were kindly supplied by Dr. Ralph S. Wolfe (Urbana, IL).

Preparation and Separation of Cell Extracts. The preparation of cell extracts of *M. volta* and *M. thermoautotrophicum* consisted of sonicating cell suspensions under nitrogen in an anaerobic buffer (pH 7.5) consisting of 50 mM *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonate (TES), 10 mM $MgCl_2$, and 2 mM mercaptoethanol as previously described (White, 1988). Cell extracts of *M. thermophila* were prepared as described by Nelson and Ferry (1984), except that the breakage buffer contained 50 mM K-TES (pH 6.8), 10 mM $MgCl_2$, 5% (v/v) glycerol, and 0.01 mg of DNase I per milliliter of buffer used. Cell extracts of *M. thermophila* were fractionated anaerobically at 23 °C on a Mono-Q 10/10 anion-exchange column equilibrated with 50 mM K-TES (pH 6.8) containing 10% (v/v) ethylene glycol, as described by Terlesky et al. (1986). Proteins were eluted from the Mono-Q

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Table 1: Incorporation of Deuterated Precursors into HS-HTP by Methanogen[a]

methanogen	added to growth medium (μM)	HS-HTP (%) with $^2H_0$	$^2H_1$	$^2H_2$	$^2H_3$	$^2H_4$
10-16B	$HSC^2H_2(CH_2)_5COOH$ (610)	85	4.1	11[b,c]		
M. thermophila	$HSC^2H_2(CH_2)_5COOH$ (610)	98		2.5[b]		
M. volta	$HSC^2H_2(CH_2)_5CO$-threonine (34)	81	8.6	10[b]		
M. volta	$HSC^2H_2(CH_2)_5CO$-threonine (340)	70	22	7.8[b]		
M. volta	$HSCH_2(CH_2)_5CO$-DL-[3,4,4,4-$^2H_4$]threonine (180)	62				38[d]
M. volta	DL-[3,4,4,4-$^2H_4$]threonine (976)	80				20[d,e]

[a] Each experiment was carried out on cells grown and isolated from 500 mL of growth medium, to which the indicated concentration of precursor was added. [b] Data were obtained from the M^+ m/z 190 molecular ion of the *S*-methyl methyl ester derivative of 7-mercaptoheptanoic acid derived from the HS-HTP present in the cells and are corrected for the natural isotopic distribution of the unlabeled molecule. [c] Data used for this calculation were previously reported by White (1989a). [d] Data were obtained from the m/z 406 ($M^+ - 15$) fragment ion of the $(TMS)_2$ derivative of [7-(methylthio)heptanoyl]threonine derived from HS-HTP by phosphatase cleavage. [e] Data were obtained from the $M^+ - C_4H_7COO$ m/z 266 fragment ion of the *n*-butyl trifluoroacetyl derivative of threonine derived from the HS-HTP by acid hydrolysis.

column using a linear gradient from 0 to 600 mM KCl in the equilibration buffer at a flow rate of 2 mL/min.

Synthesis of [7,7-$^2H_2$]-7-Mercaptoheptanoic Acid Disulfide. [7,7-$^2H_2$]-7-Mercaptoheptanoic acid was prepared as previously described (White, 1989a) and converted to the disulfide as described by Noll et al. (1987) for the iodine oxidation of 7-mercaptoheptanoic acid.

Synthesis of DL-*[3,4,4,4-$^2H_4$]Threonine.* DL-[3,4,4,4-$^2H_4$]-Threonine was prepared by the condensation of [U-$^2H_4$]-acetaldehyde with (*N*-pyruvylideneglycinato)aquocopper(II) dihydrate, as described for the preparation of the nonlabeled compound (Crout et al., 1990). The DL-[3,4,4,4-$^2H_4$]threonine was separated from the DL-allothreonine, also produced in the reaction, by chromatographic separation of the reaction products on a Dowex 50W-8X H^+ column using an HCl gradient (Wall, 1953). Fractions containing the desired compound were evaporated to dryness, and the resulting labeled DL-threonine was crystallized from ethanol/water.

Synthesis of N-(7-Mercaptoheptanoyl)threonine Disulfide. Samples of the disulfide of *N*-(7-mercaptoheptanoyl)threonine labeled in either the 7-mercaptoheptanoic acid portion or the threonine portion of the molecule were prepared using two different methods. For the sample containing deuterated threonine, DL-[3,4,4,4-$^2H_4$]threonine (20 mg, 0.163 mmol) was placed in 4 mL of methanol, and the solution was saturated with HCl gas. After 5 h at room temperature, the methanol and HCl were evaporated with a stream of nitrogen gas; the resulting threonine methyl ester hydrochloride was dissolved in 2 mL of acetonitrile, to which 70 μL (0.5 mmol) of triethylamine was added. To this mixture was added the dichloride of 7,7′-dithiodiheptanoic acid dissolved in THF. [This diacid dichloride was prepared by refluxing 40 mg (0.129 mmol) of 7,7′-dithiodiheptanoic acid with 0.5 mL of benzene and 0.5 mL of oxalyl chloride for 5 h, followed by evaporation of the solvents with a stream of nitrogen.] After the reaction mixture was stirred overnight, the solvents were evaporated; the residue was dissolved in ethyl acetate, washed with 1 M HCl, and dried over Na_2SO_4. The crude product (51 mg) was purified by column chromatography on silica gel, using methyl acetate as the eluting solvent, to a single homogeneous sample (TLC) of the threonine-labeled dimethyl ester of (7,7′-dithiodiheptanoyl)threonine. After saponification of the sample with 1 M NaOH, reductive methylation of the disulfide, and formation of the bis(trimethylsilyl) derivative, gas chromatography–mass spectrometry (GC–MS) of the resulting sample gave one peak with the same retention time as a known sample of the bis(trimethylsilyl) derivative of *N*-[7-(methylthio)heptanoyl]threonine. The mass spectrum of this peak, however, showed the mass of the molecule to have increased by 4 m/z over that of the unlabeled compound, now showing an $M^+ - 15$ ion at m/z 410 and an $M^+ - 47$ ion at m/z 378.

The disulfide of *N*-(7-mercaptoheptanoyl)-L-threonine labeled in the 7-mercaptoheptanoic acid portion of the molecule was prepared in the following manner. [7,7,7′,7′-$^2H_4$]-7,7′-Dithiodiheptanoic acid (0.17 g, 0.53 mmol) was dissolved in 1 mL of THF; in the resulting solution were dissolved 180 mg (1.06 mmol) of L-threonine methyl ester hydrochloride and 148 μL (1.06 mmol) of triethylamine. After the removal of the resulting triethylamine hydrochloride salt by filtration, 218 mg (1.06 mmol) of dicyclohexylcarbodiimide (DCC) was added and the mixture was stirred at room temperature for 24 h. The final product was purified by column chromatography, as described above for the purification of the threonine-labeled compound, to give 85 mg of colorless oil that was chromatographically pure (TLC). This oil gave the expected MH^+ at m/z 557 upon direct probe–chemical ionization mass spectroscopy and showed the expected $M^+ - 15$ ion at m/z 687 as the tetrakis(trimethylsilyl) derivative. ^{2}H-NMR of the sample in $CDCl_3$ gave the expected resonances: δ 6.518 (d, 1H, NH, J = 8.85 Hz), 4.601 (dd, 1H, H_α, J = 2.51 and 8.92 Hz), 4.348 (dq, 1H, *H*COH, J = 2.44 and 6.56 Hz), 3.768 (s, 3H, CH_3O), 2.304 (t, 2H, CH_2-CO, J = 7.47 Hz), 1.3–1.8 (m, 8H, CH_2), 1.217 (d, 3H, CH_3, J = 6.41 Hz).

Saponification of these labeled samples with 1 M NaOH gave the free acids that were used in the biosynthetic experiments. The disulfides were reduced to thiols by the cysteine and sulfide present in the growth medium.

Gas Chromatography–Mass Spectrometry (GC–MS) Analysis of the Incorporation of Labeled Molecules into HS-HTP. Several different procedures for the isolation and subsequent measurement of label incorporated into the components of HS-HTP were used during this work. These procedures were developed because of the inability to obtain a suitable derivative for the direct GC–MS analysis of the intact HS-HTP molecule. Each procedure consisted of a specific method for the isolation of a given portion of the HS-HTP molecule and for converting it into a derivative that was suitable for GC–MS analysis of its incorporated label.

For the analysis of label incorporation into the 7-mercaptoheptanoic acid portion of the molecule, the HS-HTP was extracted from the cells with hot water and methylated with methyl iodide (White, 1989a). The resulting *N*-[7-(methylthio)heptanoyl]threonine (CH_3S-HTP) was partially purified by DEAE–Sephadex column chromatography and was hydrolyzed with HCl to release (7-methylthio)heptanoic acid; this was assayed by GC–MS as the methyl ester (White, 1989a). Samples purified in this manner were sufficiently pure for accurate analysis of the label incorporated into the

(7-methylthio)heptanoic acid, but they were not pure enough for an accurate analysis of the label incorporated into the threonine because the sample was still contaminated with other threonine-containing materials. Thus, the partially purified CH_3S-HTP had to be purified further. This was accomplished by concentrating the DEAE–Sephadex column fractions containing the CH_3S-HTP and applying this acidified sample to a C_{18} Sep-Pak column. The C_{18} column was then washed with 2 mL of 0.5% aqueous TFA and 3 mL of 30% methanol in 0.5% aqueous TFA followed by 1 mL of 50% methanol in 0.5% aqueous TFA; the CH_3S-HTP was then eluted with 1.5 mL of 50% methanol in 0.5% aqueous TFA. The resulting sample was purified further by preparative TLC using acetonitrile/water/formic acid (88%) (40/10/5, v/v/v) as the eluting solvent. In this solvent, CH_3S-HTP had an R_f of 0.37. The purified sample was then hydrolyzed with 6 M HCl for 20 h at 100 °C to release the threonine, which was then converted into its *n*-butyl bis(trifluoroacetyl) derivative. The extent of its deuteration was measured by GC–MS using the $M^+ - C_4H_9COO - CF_3COO$ ion at m/z 153 and the $M^+ - C_4H_9COO$ ion at m/z 266 in the 70-eV mass spectrum of this derivative.

The extent of deuteration of the protein-bound threonine in the extracted cell pellets was measured using a procedure specifically designed for this purpose (White, 1981). As in the above case, GC–MS of the *n*-butyl bis(trifluoroacetyl) derivative of the threonine was used for the measurements.

A typical procedure used for analysis of the label incorporated into the *N*-(7-mercaptoheptanoyl)threonine (HS-HT) portion of HS-HTP present in cells is as follows. *M. volta* cells (~3 g of wet weight) obtained from growth on medium containing the labeled substrate (500 mL) were isolated and extracted with hot water as previously described (White, 1989a). The resulting extract, containing the cellular HS-HTP, was concentrated to 2 mL, reduced with $NaBH_4$, and methylated with methyl iodide. The resulting CH_3S-HTP was purified using both DEAE–Sephadex and C_{18} reverse-phase column chromatography as previously described (Clements et al., 1993). [Identification of the position of elution of the [7-(methylthio)heptanoyl]threonine phosphate from the DEAE–Sephadex column was facilitated by the addition of a small amount of the fluorescent monobromobimane (mBBr) derivative of coenzyme M (2-mercaptoethanesulfonic acid) to the sample prior to separation. Since this fluorescent compound eluted from the column at the same position as CH_3S-HTP and was easily detected by measuring the fluorescence of the fractions, it served as a tracer to identify the elution position of the CH_3S-HTP.] The resulting purified sample of CH_3S-HTP was dissolved in 50 μL of 0.1 M glycine buffer at pH 10.4 and dephosphorylated by the addition of 1–5 units of bovine intestinal mucosa alkaline phosphatase. After the mixture was incubated for 5 h at 37 °C, the resulting *N*-[7-(methylthio)heptanoyl]threonine was recovered by extraction of the acidified reaction mixture with methylene chloride; it was converted into the bis(trimethylsilyl) derivative by reaction with 20 μL of trimethylsilyl reagent (pyridine/hexamethyldisilazane/trimethylsilyl chloride, 9/3/1, v/v/v). The mixture was separated by GC on a DB-5 30-m capillary column programmed from 100 to 270 °C at 10 °C/min. Under these chromatographic conditions, the compound eluted from the column after 16 min. The label incorporated into *N*-[7-(methylthio)heptanoyl]threonine was measured by GC–MS of the bis(trimethylsilyl) derivative using the $(M^+ - 15)$ ion at m/z 406.

Cell-Free Incubations and Analysis of Thiols Produced. In typical experiments, concentrated anaerobic solutions of the desired substrates and cofactors were added to 0.2–0.5 mL of a cell extract (15–20 mg/mL protein) under hydrogen to produce incubation mixtures containing the desired concentrations of substrates and cofactors. The samples were then incubated at the growth temperature of the organism from which the extract was derived. At specific times, the vials were opened to the air and reacted with 0.5 vol of monobromobimane (mBBr) reagent [50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid, 5 mM diethylenetriaminepentaacetic acid, and 2–3 mM monobromobimane (pH 8.0)] (Fahey & Newton, 1987). After 15 min at room temperature, the fluorescent mBBr adducts of the thiols generated in the reaction mixture were isolated by chromatography on a C_{18} Sep-Pak column and washed with water, and the fluorescent thiol derivatives were eluted with 50% acetonitrile. After evaporation of the solvent, the samples were placed in known volumes of water, and known portions of the samples were purified by preparative TLC. The thiols were quantified fluorimetrically by comparing their fluorescence intensities (excitation λ_{max} = 397 nm, emission λ_{max} = 482 nm) with those of solutions of known samples at known concentrations purified in an analogous manner. The identities of the mBBr derivatives of HS-HTP were further confirmed by cleaving the samples with phosphatase and by demonstrating the generation of the mBBr derivatives of HS-HTP (TLC).

RESULTS

Incorporation of Labeled Precursors into HS-HTP by Growing Cells of Methanogens. [7,7-$^2H_2$]-7-Mercaptoheptanoic acid, [7,7-$^2H_2$]-*N*-(7-mercaptoheptanoyl)threonine, [3,4,4,4-*threonine*-$^2H_4$]-*N*-(7-mercaptoheptanoyl)threonine, and DL-[3,4,4,4-$^2H_4$]threonine were each readily incorporated into HS-HTP by growing cells of methanogens (Table 1). Both rumen strain 10-16B and *M. thermophila* were found to readily incorporate deuterated 7-mercaptoheptanoic acid (HS-H) into HS-HTP, but the extents of the incorporations were quite different: 15% for rumen strain 10-16B (the sum of the $^2H_1$ and $^2H_2$ incorporations) and 2.5% for *M. thermophila*. That *M. volta* readily incorporated *N*-(7-mercaptoheptanoyl)threonine (HS-HT) as an intact unit into HS-HTP was confirmed by the detection of molecules of HS-HTP containing the same number of deuteriums as the precursor molecules. A rather surprising observation of both the10-16B and *M. volta* incorporation data was determination of the exchange of one or more of the C-7 deuteriums from the labeled precursors during their incorporation into HS-HTP. [This exchange may have also occurred in the *M. thermophila* experiment, but because of the low incorporation of deuterium measured, it was impossible to determine whether any deuterium exchange had occurred.] In the case of the HS-HT incorporation, this exchange was found to increase as the concentration of [7,7-$^2H_2$]-*N*-(7-mercaptoheptanoyl)-threonine in the medium was increased. Thus, 46% of the total labeled molecules were found to contain only one deuterium at the low level (34 μM in the medium), and 74% of the labeled molecules were found to contain one deuterium at the high levels (340 μM in the medium). Extraction of the cell-free medium after cell harvest showed that all of the labeled HS-HT added to the medium had been removed by the *M. volta* cells.

In the experiment where *M. volta* was grown with the labeled DL-threonine, the protein-bound threonine was found to contain 26% of the molecules having four deuteriums. This was the only labeled threonine species detected.

Table 2: Phosphorylation of *N*-(7-Mercaptoheptanoyl)threonine (HS-HT) to HS-HTP by Cell Extracts and Separated Cell Extracts of *M. thermophila*

	concentration of substrates	nmol of HS-HTP detected	net synthesis (nmol of HS-HTP/mg/h)
M. thermophila[a]	1.24 mM HS-HT	7.3	0
	1.24 mM HS-HT, 8 mM ATP	18	0.6
Mono-Q fraction 9 from *M. thermophila*[b]	0.31 mM HS-HT, 1 mM ATP (1.5 h under hydrogen)		1.2
	0.31 mM HS-HT, 2 mM ATP (1 h under air)		2.2
	0.31 mM HS-HT, 2 mM ATP, 5 mM DTT (1 h under air)		2.3
	0.93 mM HS-HT, 10 mM ATP (0.25 h under hydrogen)		19

[a] To 0.5 mL of a cell extract of *M. thermophila* (15.7 mg/mL protein) in 50 mM TES, 12 mM Mg^{2+}, and 1 mM Mn^{2+} (pH 6.8) buffer were added concentrated solutions of the indicated substrates to give the desired concentrations, and the samples were incubated at 50 °C for 2 h under hydrogen. [b] To 0.2 mL of Mono-Q fraction 9 (0.6 mg/mL protein) were added the substrate, 2 mM Mg^{2+}, and 1 mM Mn^{2+}, and the samples were incubated at 50 °C under the described conditions.

Phosphorylation of N-(7-Mercaptoheptanoyl)threonine (HS-HT) by Cell Extracts of Methanogens. Cell extracts of both *M. volta* and *M. thermophila* were found to catalyze the formation of HS-HTP from HS-HT and ATP, whereas cell extracts of *M. thermoautotrophicum* were found to not carry out this reaction. In the case of *M. volta* (data not shown), this phosphorylation was specific for ATP, in that a mixture of GTP, UTP, and CTP failed to phosphorylate the HS-HT. A small activity for the phosphorylation of HS-HTP by phosphoenolpyruvate (PEP) was observed, likely resulting from the synthesis of ATP from PEP and ADP present in the cell extract. The difficulty, however, with these experiments was the large background level of HS-HTP present in the cell extracts. Thus, in a typical experiment, a cell extract of *M. volta* was found to contain 7.1 nmol of HS-HTP/mg of protein, and this value was increased only to 9 nmol of HS-HTP/mg of protein in the best experiment. This situation was much improved with cell extracts of *M. thermophila*, which were found to contain lower levels of HS-HTP (0.23 nmol/mg of protein) and were also able to produce larger amounts of HS-HTP (Table 2).

Separation of the crude extract of *M. thermophila* on a Mono-Q column gave one fraction (fraction 9, eluting between 32 and 36 mL of buffer from the Mono-Q column) that catalyzed the phosphorylation of HS-HT. The activity of this fraction did not appear to be sensitive to oxygen, in that the amount of HS-HTP produced was not reduced when the reaction was carried out exposed to the air; as expected, the amount of product increased with increasing concentrations of substrates. In all cases, the extent of substrate converted into product was small, being less than 2% of the *N*-(7-mercaptoheptanoyl)threonine substrate added.

DISCUSSION

Considering that growing cells of both rumen strain 10-16B and *M. thermophila* were found to readily incorporate [7,7-$^2H_2$]-7-mercaptoheptanoic acid into HS-HTP (Table 1), it was logical to conclude that this is a general process in methanogens. One could also conclude that the first step in the condensation of subunits leading to the biosynthesis of HS-HTP would be the coupling of 7-mercaptoheptanoic acid with either threonine or threonine phosphate (threonine-P). If the coupling were to occur with threonine-P, this of course would complete the biosynthesis of HS-HTP. On the other hand, if the coupling were to occur with threonine, the biosynthesis would be completed after the phosphorylation of *N*-(7-mercaptoheptanoyl)threonine to HS-HTP. Other possibilities also exist: the HS-H could be coupled to a threonine precursor, and only after the coupling occurred would the group be transformed into the threonine or threonine-P portion of the molecule. Possible choices for this molecule would be one of the intermediates in threonine biosynthesis (i.e., aspartate semialdehyde, homoserine, etc.). The lack of involvement of these molecules in HS-HTP biosynthesis can be tested by simply demonstrating that threonine is incorporated into HS-HTP. This was shown to be the case by demonstrating that the threonine in the HS-HTP isolated from *M. volta* grown with DL-[3,4,4,4-$^2H_4$]threonine (120 μg/mL in the medium) contained 19.8% of the molecules with four deuterium atoms (Table 1). This level of incorporation is close to that found for the cellular threonine, where 26% of the molecules were found to contain four deuterium atoms, and indicated that the cellular threonine pool supplied the threonine used for the biosynthesis of HS-HTP. In addition, since HS-HTP is known to contain L-threonine (Kobelt et al., 1987), it is presumed that only labeled L-threonine was incorporated.

The demonstration that threonine is a precursor of HS-HTP does not, however, prove that 7-mercaptoheptanoic acid is coupled directly to threonine during the biosynthesis. It is possible that the threonine is first phosphorylated to threonine-P, and the resulting threonine-P is then condensed with 7-mercaptoheptanoic acid. One argument against this idea is that threonine-P appears to have never been reported to occur in a free state in a biological system. In addition, experiments (not reported here) showed no ATP-dependent coupling of HS-H to threonine-P in cell-free extracts of methanogens.

The involvement of *N*-(7-mercaptoheptanoyl)threonine in the biosynthesis of HS-HTP by growing *M. volta* cells was confirmed by measuring the incorporation of [7,7-$^2H_2$]-*N*-(7-mercaptoheptanoyl)threonine into HS-HTP (Table 1). As expected, the extent of labeling of HS-HTP was increased as the concentration of HS-HT was increased in the growth medium. An unexpected finding, however, was that many of the labeled molecules contained only *one* deuterium. Furthermore, the percentage of molecules containing one deuterium was found to increase with increasing amounts of HS-HT present in the medium. This loss of a single C-7 deuterium was also observed during the incorporation of [7,7-$^2H_2$]HS-H into HS-HTP by rumen strain 10-16B. Although it is difficult to envision, a reversal of the known biosynthesis pathway to HS-H could be responsible for this loss. Thus, the conversion of portions of the fed precursors back to the 7-oxo compounds and reconversion back to the 7-thiols could explain the generation of molecules containing only one deuterium (White, 1989a).

The above observations are consistent with HS-HT being taken up by the cells and incorporated as a unit into HS-HTP. It is possible, however, that the threonine could be hydro-

lytically released from the HS-HT, and the resulting HS-H and threonine could be incorporated as separate units into HS-HTP. [Indirect evidence for the possible occurrence of this cleavage was the observation that all of the substrate added to the medium at the start of the experiment was metabolized at the end of the growth.] That the threonine was not hydrolytically separated from the HS-HT was confirmed by demonstrating that 38% of the cellular HS-HTP produced by *M. volta* was derived from the [3,4,4,4-*threonine*-$^2H_4$]-(7,7′-dithiodiheptanoyl)threonine added to the growth medium. Since this incorporation was very close to that observed for the incorporation of the HS-H-labeled HS-HT, this strongly indicates that HS-HT was incorporated as a unit. This follows because it is very unlikely that HS-HT would undergo hydrolysis and be reassembled from pools of threonine and HS-H containing the same percentage of labeled molecules. Thus, these data on the incorporation of the two differently labeled HS-HT molecules confirm the idea that *N*-(7-mercaptoheptanoyl)threonine is taken up by these cells and directly phosphorylated to HS-HTP.

The data presented up to this point indicate that 7-mercaptoheptanoic acid is coupled with threonine, and the resulting (7-mercaptoheptanoyl)threonine is then phosphorylated to HS-HTP. Considering the close structural similarities between biotin, lipoic acid, and 7-mercaptoheptanoic acid, and our present understanding of the involvement of adenylylation reactions in the coupling of biotin (Kasow & Lane, 1962) or lipoic acid (Leach, 1970; Reed et al., 1958) to the amino group of lysine in proteins, it is natural to speculate that this general mechanism of nucleic acid-independent peptide bond formation (Stadtman, 1973) would be involved in HS-HTP biosynthesis. Generally, these reactions require ATP; however, some specific examples involved in the formation of peptide bonds in coenzyme biosynthesis are known (i.e., the formation of the (4′-phosphopantothenyl)cysteine from 4′-phosphopantothenic acid and cysteine in the biosynthesis of coenzyme A in bacteria) that require CTP (Brown, 1959). The involvement of coenzyme A thioesters in the biosynthesis of an amide bond should also be considered; for example, the involvement of acyl-CoA ester in the biosynthesis of *N*-acylsphingosine (Sribney, 1966; Morell & Radin, 1970). The 7-mercaptoheptanoyl-CoA required for this reaction could be formed either from HS-H and ATP via a thiokinase reaction or from HS-H and ATP through the combined actions of an HS-H kinase and a phosphotransacylase. This latter group of enzymes would catalyze the same type of reactions that are involved in the activation of acetate to acetyl-CoA during the disproportionation of acetate to carbon dioxide and methane in species of *Methanosarcina* and *Methanothrix* (Ferry, 1992).

To the author's knowledge, the only previously described enzymes that phosphorylate threonine are the protein–serine/threonine kinases. It will be intriguing to see whether the primary sequence of the enzyme responsible for phosphorylating the threonine in HS-HT has sequence homology with any of these enzymes. Since protein kinases, except for the recently described eukaryotic-like protein kinase in the bacterium *Myxoccus xanthus* (Zhang et al., 1992), appear to exist largely in distinct eukaryote-specific and bacterium-specific genetic families (Cossone, 1988; Hanks et al., 1988), such homology, if it indeed exists, could provide important clues as to the relationship of the archaea to the other phylogenetic domains in nature.

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