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# Structure of component B (7-mercaptoheptanoylthreonine phosphate) of the methylcoenzyme M methylreductase system of *Methanobacterium thermoautotrophicum*

(coenzyme/methanogens)

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ABSTRACT **Component B, the heat-stable low-molecular-weight cofactor required for methane production by dialyzed cell-free extracts of *Methanobacterium thermoautotrophicum*, has been purified to homogeneity and its structure assigned. Results of low-resolution fast-atom-bombardment and field-desorption mass spectrometry indicated a molecular weight of 419, and high-resolution fast-atom-bombardment mass spectrometry agreed with the molecular formula $C_{13}H_{26}NO_8PS_2$. Evidence from fast-atom-bombardment and field-desorption mass spectrometry and 360-MHz $^1H$ NMR in deuterium oxide argued that the compound was isolated as a mixed disulfide with 2-mercaptoethanol; so the proposed elemental formula of the free acid, free thiol would be $C_{11}H_{22}NO_7PS$ (molecular weight, 343). The proposed structure for an active form of the coenzyme is 7-mercaptoheptanoylthreonine phosphate.**

Results of early investigations demonstrated that the production of methane by extracts of *Methanobacterium thermoautotrophicum* required hydrogen, carbon dioxide, ATP, and magnesium(II) (1). Later, methane was shown to be produced from a methylated compound unique to methanogens, 2-(methylthio)ethanesulfonic acid (2). The enzymes responsible for carrying out this reduction, now called the methylcoenzyme M methylreductase (or simply methylreductase) system, do not require $CO_2$ for methane synthesis, although $CO_2$ can be reduced by crude enzyme preparations (3).

A significant advance in our understanding of methanogenesis was made when anoxic chromatographic techniques were used to resolve the methylreductase into three components, A, B, and C (4). Components A and C were found to be proteins. Component C was purified to homogeneity and is an oxygen-stable protein that contains bound factor $F_{430}$, a nickel tetrapyrrole (5, 6). Component A has been fractionated into the following four components: three protein fractions (A1, A2, and A3) and a bound FAD (7). One of these proteins (A2) has been purified to homogeneity (8), but the reactions catalyzed by the proteins are not yet known.

We report here the purification and structural elucidation of the third component, B. This component has been reported to be heat-stable, oxygen-sensitive, and colorless with a molecular weight of approximately 1000 (4). Subsequent work suggested the presence of phosphate, and an aldopentose (9). The present work demonstrates an improved purification of the compound and utilizes NMR and mass spectrometry to establish its elemental composition. The structure of the compound is proposed to be 7-mercaptoheptanoylthreonine phosphate (HS-HT*P*).

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## MATERIALS AND METHODS

**Growth of Cells and Preparation of Enzymes.** *M. thermoautotrophicum* strain ΔH (ATCC 29096) was grown and extracts were prepared as described (10). Crude enzyme preparations were isolated by use of anoxic column chromatographic techniques (11). A column (80.5 × 2.5 cm) of Sephadex G-25 Superfine (Pharmacia) was equilibrated with 20 mM potassium phosphate (pH 7) that contained 10 mM 2-mercaptoethanol. Cell-free extract from 70 g of cells was passed through the column in two 47-ml batches. Fractions of both eluates that contained methylreductase activity were pooled and concentrated to 65 ml by ultrafiltration with a Diaflo PM10 membrane (Amicon). This concentrate was passed through the same column again in two batches. Fractions with methylreductase activity were again pooled and, if necessary, concentrated to a final concentration of approximately 7 mg of protein per ml by ultrafiltration. The enzyme pool [20% (vol/vol) in ethylene glycol] was stored frozen in 5-ml aliquots under a nitrogen atmosphere at −20°C.

Assays for methylreductase were performed in calibrated vials as described (7). The reaction mixture consisted of 96 mM K-Pipes (pH 6.3), 4 mM K-ATP, 20 mM $Mg(OAc)_2$, 1.25 mM 2-(methylthio)ethanesulfonic acid, 16.8 mM cyanocobalamin, 0.8 mg of protein (Sephadex G-25-treated extract), component B as desired, and anoxic 20 mM potassium phosphate (pH 7) that contained 10 mM 2-mercaptoethanol to bring the final volume to 200 μl, all concentrations being final concentrations in the assay. The reactions were carried out under a hydrogen atmosphere and were initiated by incubation at 60°C. Methane was measured by gas chromatography as described (7). When column fractions were assayed for the presence of component B, vacuum drying of aliquots was employed to remove inhibitory methanol or ammonium salts carried over from the fractionation procedure. Fractions were considered to contain component B if aliquots from them stimulated methane formation to a rate greater than that of a control that contained no added component B.

**Purification of Component B.** A boiled cell-free extract of *M. thermoautotrophicum* was obtained by boiling 421 g of cells suspended in 840 ml of 20 mM potassium phosphate, pH 7, that contained 10 mM 2-mercaptoethanol for approximately 1 hr while flushing the bottle with oxygen-free nitrogen. After the extract was cooled to room temperature, cell debris was removed by centrifugation for 60 min at 10,000 × *g* at 4°C under aerobic conditions, and the centrifuge bottles were

Abbreviations: SFORD, single-frequency off-resonance decoupled; FAB, fast-atom-bombardment; FD, field-desorption; EI, electron-ionization; MS, mass spectrometry; HS-HT*P*, 7-mercaptoheptanoylthreonine phosphate; $^1HO^2H$, hydrogen deuterium oxide.

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immediately placed in an anoxic chamber (Coy Laboratory Products, Ann Arbor, MI) (11). The supernatant was decanted and acidified to pH 4.8 with anoxic 1 M HCl. After standing at room temperature in the chamber overnight, the precipitate was removed by centrifugation at 45,000 × *g* at 15°C for 25 min under anoxic conditions. The extract was returned to the chamber and applied to an anoxic DEAE-Sephadex A-25 anion-exchange column (14 × 5 cm) equilibrated with 20 mM NaOAc, pH 5, that contained 10 mM 2-mercaptoethanol. After the sample was loaded, the column was washed with the acetate buffer that contained 100 mM NaCl. Component B was eluted with a linear gradient of 100–300 mM NaCl. Fractions were collected inside the anoxic chamber, and those containing component B were pooled and removed from the chamber. Anoxic precautions were no longer employed after this step. This pool of component B was concentrated by lyophilization and desalted by application to a preparative $C_{18}$ reversed-phase column (2.5 × 30 cm, particle size 55–105 μm, Waters Associates) that was equilibrated with distilled water. After the salt was eluted with water, component B was eluted with 80% (vol/vol) methanol in water. The eluate was lyophilized or flash evaporated to dryness and dissolved in water. The solution was injected into a semipreparative Bio-Sil-TSK 540 DEAE-anion-exchange HPLC column (300 × 7.5 mm) (Bio-Rad) equilibrated with 200 mM triethylammonium acetate, pH 5. Fractions of 1 ml were collected, and component B was eluted with a linear gradient of 200–700 mM triethylammonium acetate, pH 5. The column pressure was maintained below 7000 kPa, and the flow rate was 1 ml/min. Waters Associates Model 45 HPLC pumps and Model 660 solvent programmer were used. The eluate was monitored by absorbance at 260 nm using a Varian 2050 variable wavelength detector. Fractions containing component B were pooled, lyophilized, and dissolved in water. The solution was then injected into a semipreparative Waters μBondapak $C_{18}$ reversed-phase HPLC column (300 × 3.9 mm) equilibrated with 30% (vol/vol) methanol in 20 mM triethylammonium acetate, pH 5. Component B was eluted with a linear gradient of 30–50% methanol in the same buffer. The column was maintained below 21,000 kPa, and the flow rate was 1 ml/min. The eluate was monitored by absorbance at 215 nm, and 1-ml fractions were collected. The fractions containing component B were pooled, lyophilized, and dissolved in water. This material was applied to an analytical anion exchange HPLC column of Spherogel-TSK IEX-545DEAE (Altex, Berkeley, CA) (150 × 6 mm) equilibrated with 200 mM triethylammonium acetate, pH 7. Fractions of 1 ml were collected, and component B was eluted with a linear gradient of 200–700 mM triethylammonium acetate, pH 7. The column pressure was maintained below 7000 kPa, and the flow was 1 ml/min. The eluate was monitored by absorbance at 260 nm. The highly purified component B was lyophilized and stored dry at −20°C.

**Instrumental Analyses.** NMR spectra were obtained with General Electric QE-300, Nicolet 360-MHz, and Bruker AM 500-MHz Fourier transform spectrometers. All samples were dissolved in 100 atom% deuterium oxide (Sigma) and lyophilized twice before final solubilization in deuterium oxide. For $^1H$ NMR spectroscopy the internal standard used was 3-trimethylsilyltetradeuteriopropionic acid, sodium salt (99 atom% D, KOR, Cambridge, MA), and for $^{13}C$ NMR perdeuteriodioxane (99 atom% D, Merck).

For $^{13}C$ NMR spectra, the observed frequency was 90.546 MHz; the sweep width was 10,000 Hz; the pulse width was 4 μs; the postacquisition delay was 1 s; and the proton 90° pulse was 92 μs for the broad band-decoupled spectrum and 1 ms for the single-frequency off-resonance decoupled (SFORD) spectrum. The decoupler frequency was 360.061 MHz for the broad band-decoupled spectrum and 360.058 MHz for the SFORD spectrum. Broad band-decoupled spectra were obtained with Levitt–Freeman cycle decoupling. The proton 180° pulse was 184 μs.

For the $^1H$ NMR spectrum shown (see Fig. 3), the observed frequency was 500.137 MHz; the sweep width was 6944 Hz; the pulse width was 4 μs; and the postacquisition delay was 200 μs. A Redfield 1331 water suppression sequence was used for the spectrum shown. For the correlated spectroscopy (COSY) spectrum, the observed frequency was 300.151 MHz; the spectral width was 2150 Hz; the postacquisition delay was 1 s; and the pulse width was 6.7 μs.

Mass spectra were obtained on the following instruments: field-desorption mass spectrometry (FDMS) and electron-ionization mass spectrometry (EIMS) on a Finnigan MAT 731 mass spectrometer and fast-atom-bombardment mass spectrometry (FABMS) on VG Analytical 7070E and ZAB SE mass spectrometers. FAB mass spectra were obtained from samples dissolved in water and suspended in a thioglycerol or "magic bullet" (dithioerythritol/dithiothreitol) matrix (12).

**Miscellaneous.** Phosphate was determined by the method of Ames and Dubin (13). Protein was measured by the method of Bradford (14).

## RESULTS

Results of previous studies indicated that component B activity in crude cell extracts was lost upon air exposure (4) but was more stable in partially purified preparations (9). Aerobic anion-exchange chromatography of crude extracts resulted in the formation of two active pools of component B (9). To avoid these problems, the first anion-exchange chromatographic step was performed under anoxic conditions. All subsequent steps could be performed without anoxic precautions with no significant loss of cofactor activity (data not shown). During elution from both anion-exchange and reversed-phase HPLC columns, the compound purified by the method described here gave a single, symmetrical peak by either refractive index or UV absorption (260 nm or 215 nm) that migrated with component B activity. In some preparations, a second fraction from either an anion-exchange or a reversed-phase column contained component B activity. The nature of this second peak has not yet been determined. The data presented here are from preparations that yielded a single fraction of active material. From 1 kg (wet weight) of cells approximately 9 mg of component B could be obtained.

The purified material reconstituted methylreductase activity in crude extract preparations as shown in Fig. 1*a*. One-half

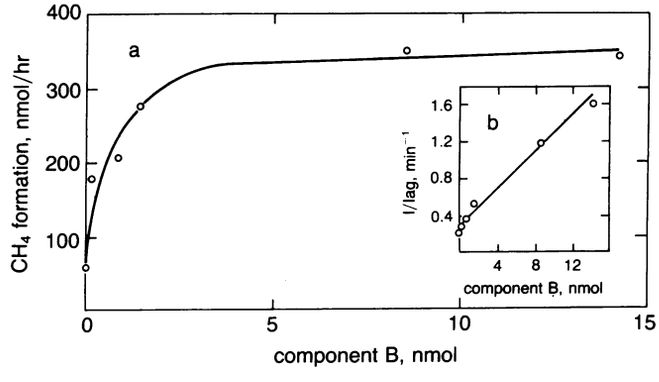



FIG. 1. Dependence of the 2-(methylthio)ethanesulfonic acid methylreductase system on component B. (*a*) Effect of component B on the velocity of the methylreductase reaction. Assay constituents in a total volume of 200 μl were as described except 5.25 mg of protein was used. Component B was quantitatively determined using the phosphate assay (13). (*b*) Effect of component B on the lag.

the maximal rate of methane formation was obtained with approximately 3 $\mu$M component B. Addition of various amounts of component B did not appear to cause inhibition of methanogenesis but did cause a decrease in the lag time (Fig. 1*b*). The lag is defined as the length of time before a linear rate of methanogenesis was achieved. The enzyme preparation used here retained the ability to produce methane from 2-(methylthio)ethanesulfonic acid without the addition of component B. However, the lag before the onset of methanogenesis was long, and the rate at which methane was produced was low compared with those vials with component B added. The extent of this background rate of methanogenesis varied from batch to batch of enzyme. Component B appeared to be rather tightly bound to an enzyme(s), and removal of this background component B activity was difficult.

Component B was found to contain phosphate, and a solution of the triethylammonium salt of the compound (1.03 mg/ml) was found to be 1.8 mM in organic phosphate. If the compound contained only one phosphate group, this would suggest a molecular weight of about 575.

The $^{13}C$ NMR SFORD spectrum of component B is shown in Fig. 2*A*, and the broad band-decoupled spectrum is in Fig. 2*B*. The two strong signals at 47.5 ppm and 9.1 ppm (triplet and quartet in the off-resonance spectrum) are due to the triethylammonium cation in this salt. The signal-to-noise ratio is very low since only a small amount of sample was available, but in this region the molecule appears to have 10 aliphatic carbon atoms (exclusive of the triethylammonium carbons). No signals were visible downfield of 80 ppm, but the low S/N ratio hindered observation of carbonyl carbons, and both amide (1640 $cm^{-1}$) and carboxylate (1610 and 1400 $cm^{-1}$) IR absorption were found (spectrum not shown). The SFORD spectrum indicated mainly triplets for aliphatic $-CH_2-$ groups (including an $-OCH_2-$ group at 60.2 ppm) but also a deshielded doublet for a $-\underset{|}{C}H-X$ group at 73.4 ppm and a quartet for a $-CH_3$ group at 19.3 ppm.

The sample provided a clear $^1H$ NMR spectrum as shown in Fig. 3. No signals appeared downfield of the $^1HO^2H$ signal.

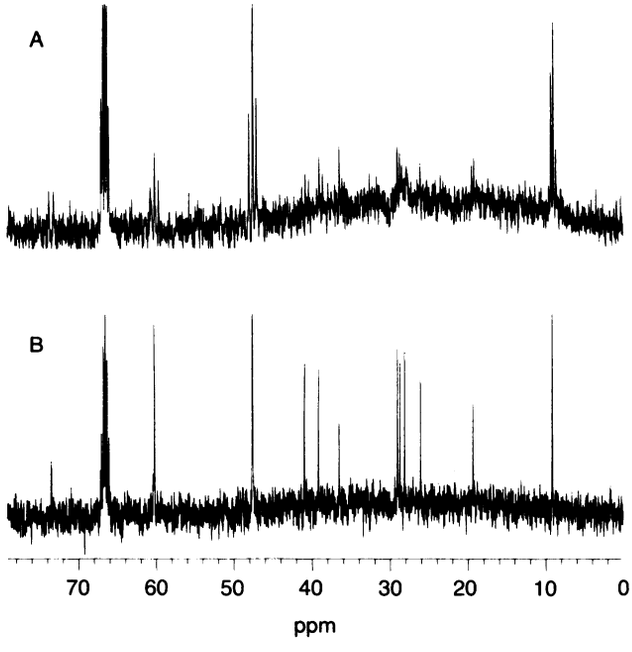



FIG. 2. $^{13}C$ NMR spectra of the triethylammonium salt of component B: solvent $^2H_2O$; chemical shifts relative to perdeuteriodioxane (66.5 ppm). (*A*) SFORD spectrum. (*B*) Broad band-decoupled spectrum.

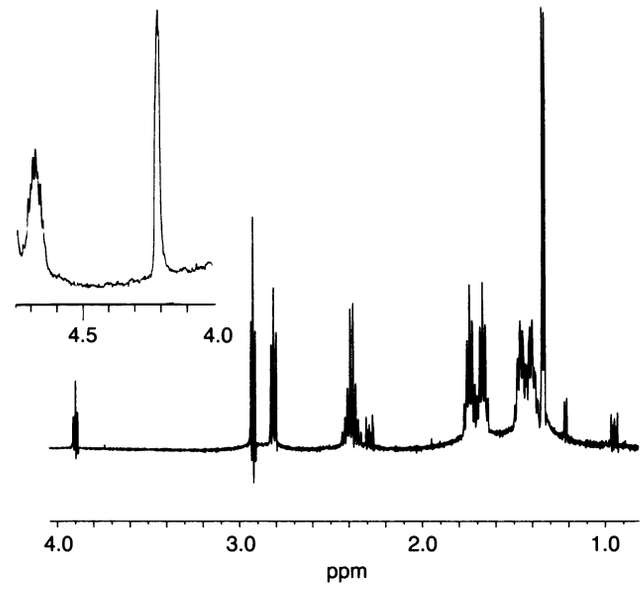
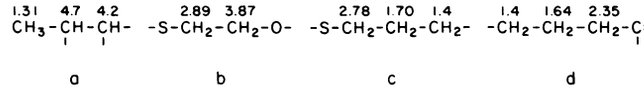



FIG. 3. Resolution-enhanced $^1H$ NMR spectrum (500-MHz) of the ammonium salt of component B: solvent $^2H_2O$; chemical shifts relative to 3-trimethylsilyltetradeuteriopropionic acid (0.0 ppm). The inset shows the 4.7 and 4.2 ppm signals as obtained at 360 MHz. The signal at 4.7 ppm was lost during suppression of the $^1HO^2H$ signal while obtaining the 500-MHz spectrum. The triplet at 3.87 ppm appears smaller in relation to other signals due to the resolution enhancement. Unenhanced spectra appeared as in Fig. 4.

The results of two-dimensional homonuclear decoupling (Fig. 4), manual irradiation of signals, and integration of peak areas (Table 1) demonstrate important connectivities and multiplicities summarized as units a, b, c, and d.




The multiplet at 4.7 ppm corresponds to one proton coupled to doublets at 4.2 ppm and 1.31 ppm, integrating for one proton and three (or four) protons, respectively. Each doublet collapsed to a singlet upon irradiation of the multiplet, and the multiplet was simplified when either doublet was irradiated. Neither of the doublets was coupled to any other signal. This indicates the unit a.

The two triplets centered at 3.87 ppm and 2.89 ppm correspond to two protons each, coupled to one another. Each collapsed to a singlet upon irradiation of the other. The chemical shifts strongly suggest attachment to oxygen (3.87 ppm) and sulfur (2.89 ppm), and they in fact correspond closely to those of 2-hydroxyethyl disulfide (3.82 and 2.85 ppm) (17), arguing unit b. The triplet at 2.78 ppm was simplified to a singlet upon irradiation of the multiplet centered at 1.70 ppm, and half this multiplet was simplified upon irradiation of the 2.78 ppm triplet. Irradiation of the 1.70 multiplet caused a change in the multiplet (actually two 2H multiplets) near 1.4 ppm, arguing unit c, where the sulfur attachment is assigned from the chemical shift (2.78 ppm) in analogy to unit b.

Irradiation of the multiplet at 1.64 ppm (2H) collapsed the multiplet at 2.35 ppm (2H) to a quartet, while irradiation of this multiplet (2.35) simplified the multiplet near 1.64 ppm to a triplet. Irradiation of the multiplet near 1.64 ppm also caused changes in the multiplet near 1.4 ppm, thus assigning unit d, where the terminal methylene at 2.35 is deshielded by unsaturation, presumably a carbonyl group. Since units c and d both contain shielded methylene groups (protons near 1.4 ppm), a reasonable interpretation of these data would be a

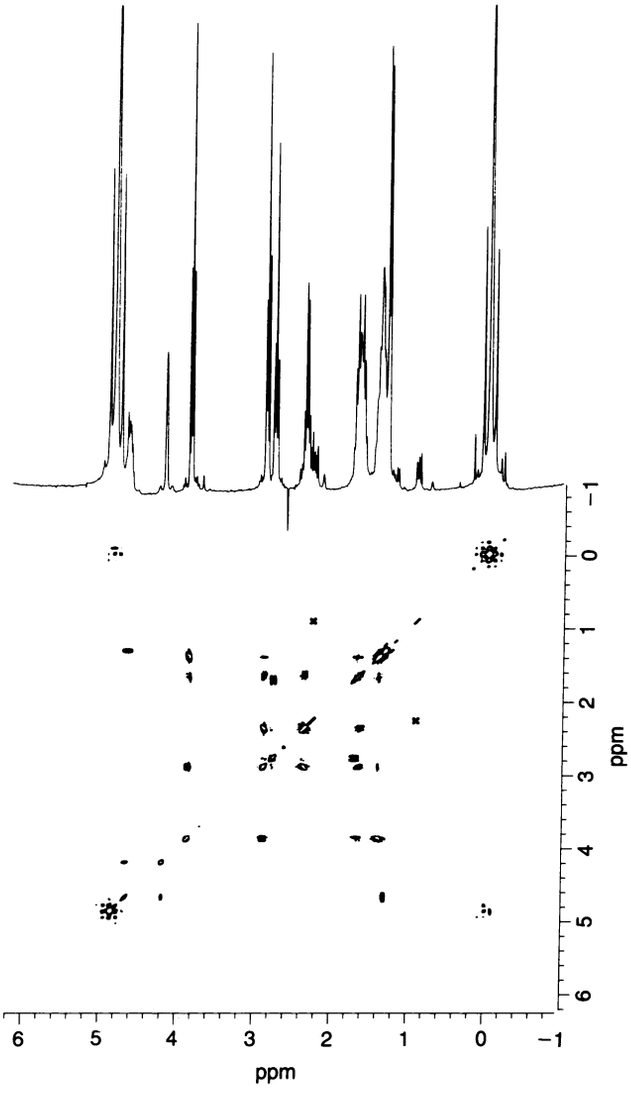
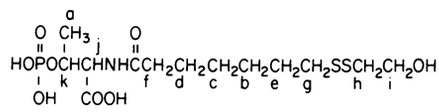



FIG. 4. Contour plot of a 360-MHz $^1$H COSY (correlated spectroscopy) spectrum of component B. For reference, a 360-MHz spectrum is plotted along the horizontal axis.

chain of six methylenes with one terminal methylene (the 2.35 ppm multiplet) in an environment that causes its protons to be nonequivalent, i.e., unit c-d, -S-$CH_2$-$(CH_2)_4$-$CH_2$-C(=)-. In fact, the IR spectrum of component B contains both amide and carboxylate absorption, as noted above. Thus, a 7-mercaptoheptanoyl group can be assigned. The chemical shifts of the protons and carbons in the methylene chain are consistent with those found in long-chain aliphatic acids (16).

The chemical shifts and multiplicities of unit a suggest a threonine in which both the hydroxyl and amino groups are substituted. To account for the amide IR absorption the mercaptoheptanoyl group must acylate the threonine nitrogen (the only nitrogen, cf. below), while the only other group available for deshielding the threonine oxygen is the phosphate. The multiplicity and chemical shift of the 4.7 ppm methine would be consistent with its being bound to the phosphate group that would cause long-range splitting of the proton signal. The chemical shifts reported for *N*-acetylthreonine phosphate (15) are identical to those found in component B. These elements and the proton chemical shifts observed in the $^1$H NMR spectrum can be reconciled by structure **1**, in which a disulfide is formed between the mercaptoheptanoyl and mercaptoethanol groups.




Structure **1**

Table 1. $^1$H NMR signals of component B

| Proton* (intensity, multiplicity†) | δ‡ | δ, model§ |
|---|---|---|
| a (3H, d) | 1.31 | 1.31, APT |
| b (2H, m) | 1.37 | 1.29, DD |
| c (2H, m) | 1.44 | 1.31, HA |
| d (2H, m) | 1.64 | 1.60, HA |
| e (2H, m) | 1.70 | 1.68, DD |
| f (2H, s) | 2.35 | 2.21, HA |
| g (2H, t) | 2.78 | 2.70, DD |
| h (2H, t) | 2.89 | 2.85, HED |
| i (2H, t) | 3.87 | 3.82, HED |
| j (1H, d) | 4.2 | 4.24, APT |
| k (1H, m) | 4.7 | 4.69, APT |

*Letters refer to Formula 1.
†s, singlet; d, doublet; m, multiplet; t, triplet.
‡Solvent $^2H_2O$; δ, ppm relative to 3-trimethylsilyltetradeuteriopropionic acid (0.0 ppm).
§Analogous protons for model compounds: APT, *N*-acetylphosphothreonine at p$^2$H 4.0 (15); HA, heptanamide (16); DD, dodecyl disulfide (16); and HED, 2-hydroxyethyl disulfide (17).

The FAB mass spectrum of the molecule is shown in Fig. 5 and high-resolution data are summarized in Table 2. Ions consistent with the loss of 2-mercaptoethanol (or dithiothrei-

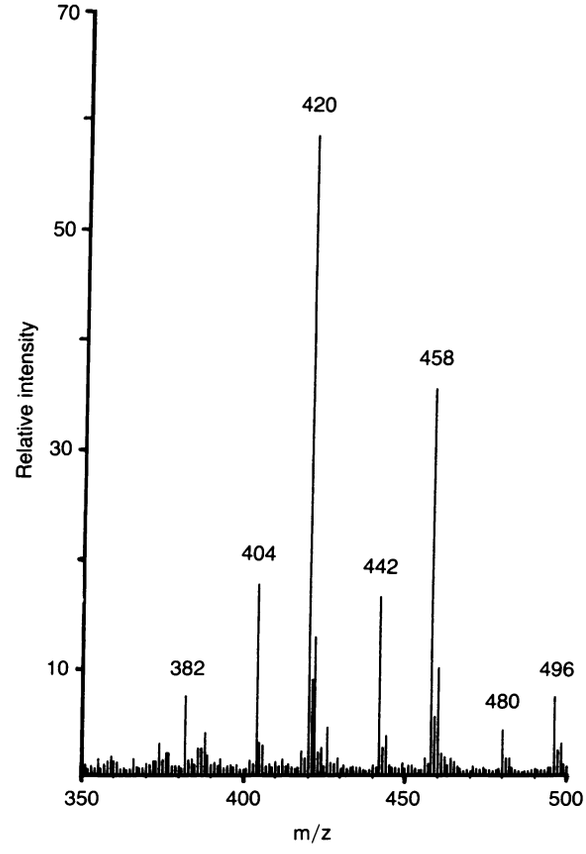



FIG. 5. Positive-ion FABMS of the potassium and sodium salts of component B in a dithiothreitol/dithioerythritol matrix. The acceleration potential was 8 kV, the source temperature was 30°C, and the resolution was 1200.

Table 2. Interpretation of high resolution mass spectrometry data

| Observed positive ion, *m/z* | Theoretical | | |
|---|---|---|---|
| | $M_r$ (Δ, mmu) | Composition | Structure |
| 244.1012* | 244.1010 (0.2) | $C_{11}H_{18}NO_3S$ | 2† − $H_4PO_4$ |
| 366.0782‡ | 366.0755 (2.7) | $C_{11}H_{22}NO_7PSNa$ | 2 + Na |
| 420.0076‡ | 420.0050 (2.6) | $C_{11}H_{21}NO_7PSK_2$ | 2 + $K_2$-H |
| 420.0946§ | 420.0919 (2.7) | $C_{13}H_{27}NO_8PS_2$ | 1 + H |
| 441.9873‡ | 441.9870 (0.3) | $C_{11}H_{20}NO_7PSK_2Na$ | 2 + $K_2Na$-$H_2$ |
| 442.0759‡ | 442.0738 (2.1) | $C_{13}H_{26}NO_8PS_2Na$ | 1 + Na |
| 445.2161‡ | 445.2143 (1.8) | $C_{17}H_{38}N_2O_7PS$ | 2 + TEA |
| 457.9613‡ | 457.9612 (0.1) | $C_{11}H_{20}NO_7PSK_3$ | 2 + $K_3$-$H_2$ |

High resolution data were obtained from *EI, ‡FAB, and §FD mass spectrometry. TEA, triethylammonium cation. †Numbers **1** and **2** refer to structures **1** and **2**.

tol from the FABMS matrix) support the presence of a mixed disulfide of component B and 2-mercaptoethanol. Positive ion FABMS yielded ions derived from both the sodium and triethylammonium salts and both the 2-mercaptoethanol and dithiothreitol adducts. High-resolution FDMS at lower currents gave a strong ion of mass 154.0143, corresponding to the mass of the disulfide of 2-mercaptoethanol (error = 0.0 mmu). The M+2 ions observed in the FAB spectra are relatively large, providing further evidence for the presence of sulfur in the molecule. High-resolution data (Table 2) were obtained agreeing with this mercaptoethanol adduct for both the free acid (FDMS) and sodium salt (FABMS). In addition, high-resolution FABMS data agreed with those calculated for the triethylammonium, potassium, and sodium salts of the free mercaptan. Similarly, high-resolution FABMS data confirm the presence of the phosphothreonine unit.

The structure proposed here has been confirmed by chemical synthesis of component B. Details of this synthesis, comparisons of the natural and synthetic compounds, and evidence for activity of synthetic HS-HT*P* will be presented elsewhere.

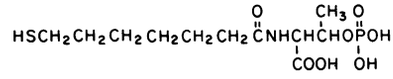
## DISCUSSION

Since its discovery by Gunsalus (4), the purification and structural elucidation of component B of the methylcoenzyme M methylreductase have proven to be challenging problems. The difficulties associated with obtaining crude enzyme preparations that have an absolute requirement for component B suggest that this cofactor is tightly bound to an enzyme(s). Additionally, the occasional presence of two active fractions upon elution from chromatographic columns suggests that the compound may be isolated in different forms, possibly as mixed disulfides, particularly when crude extracts are exposed to air. This exposure also may result in the formation of insoluble or inactive complexes that would explain the previous observations of the oxygen-lability of component B.

The evidence presented here is consistent with the proposed structure (structure **1**). Three different types of mass spectrometry gave ions consistent with a nominal mass of 343 for the compound. A fragment ion at *m/z* 244 was consistent with the loss of a phosphate group. A single phosphate was also detected chemically. The molecular weight as determined by the phosphate assay (575) was close to the molecular weight of the triethylammonium salt of the 2-mercaptoethanol mixed disulfide of component B (520).

The $^1H$ NMR data are also consistent with the proposed structure. In addition, published spectra of model compounds such as *N*-acetylthreonine phosphate, heptanamide, 2-hydroxyethyl disulfide and various alkyl disulfides are in agreement with the assignments made here (15–17).

The mixed disulfide with 2-mercaptoethanol is probably an artifact of purification. The naturally occurring compound may be larger but breaks down during purification. The isolated compound is nevertheless active at low concentration in the *in vitro* methanogenic assay; we propose that the disulfide (structure **1**) is reduced in the assay mixture, the active compound being HS-HT*P*, structure **2**.



Structure **2**

The analogies of pantetheine-4-phosphate to HS-HT*P* and of HS-CoA to a possible adenosine monophosphorylated HS-HT*P* suggest possible roles for HS-HT*P*.

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