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Preparation of Coenzyme M Analogues and Their Activity in the Methyl Coenzyme M Reductase System of *Methanobacterium thermoautotrophicum*†

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ABSTRACT: A number of 2-(methylthio)ethanesulfonate (methyl-coenzyme M) analogues were synthesized and investigated as substrates for methyl-coenzyme M reductase, an enzyme system found in extracts of *Methanobacterium thermoautotrophicum.* Replacement of the methyl moiety by an ethyl group yielded an analogue which served as a precursor for ethane formation. Propyl-coenzyme M, however, was not converted to propane. Analogues which contained additional methylene carbons such as 3-(methylthio)propanesulfonate or 4-(methylthio)butanesulfonate or analogues modified at the sulfide or sulfonate position, *N*-methyltaurine and 2-(methylthio)ethanol, were inactive. These analogues, in addition to a number of commercially available compounds, also were tested for their ability to inhibit the reduction of methyl-coenzyme M to methane. Bromoethanesulfonate and chloroethanesulfonate proved to be potent inhibitors of the reductase, resulting in 50% inhibition at 7.9×10^{-6} M and 7.5×10^{-5} M. Analogues to coenzyme M which contained modifications to other regions were evaluated also and found to be weak inhibitors of methane biosynthesis.

The cofactor, coenzyme M, was demonstrated by McBride & Wolfe (1971) to be involved in the terminal steps of methane biosynthesis by the methanogenic bacterium, *Methanobacterium* strain M.o.H. The structure of the novel coenzyme was determined by Taylor & Wolfe (1974a) to be 2-mercaptoethanesulfonic acid (HS-CoM, **1**).[1] The methylated form of the cofactor, 2-(methylthio)ethanesulfonic acid (CH_3-S-CoM, **4**), was found to be reductively demethylated by cell extracts to methane with molecular hydrogen as the reductant (Taylor & Wolfe, 1974a). Little is known about the chemical specificity of the methyl-coenzyme M reductase. The dimethylsulfonium analogue of coenzyme M, 2-(dimethylsulfonium)ethanesulfonate (**10**), however, was unable to serve as a substrate for methane formation (Taylor & Wolfe, 1974b). Here we describe the synthesis of a number of coenzyme M analogues which contain modifications to the alkyl sulfide, ethylene carbon, and sulfonate regions. The ability of these analogues to serve as potential substrates or inhibitors for methane formation was tested using the cell-free assay system developed for the thermophilic methane organism *M. thermoautotrophicum* (Gunsalus & Wolfe, 1977).

Experimental Section

Preparation of Analogues. Ammonium 2-mercaptoeth-

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[1] Abbreviations used: CH_3-S-CoM, methyl-coenzyme M or 2-(methylthio)ethanesulfonic acid; HS-CoM, 2-mercaptoethanesulfonic acid; $(S\text{-CoM})_2$, 2,2′-dithiodiethanesulfonic acid; $(CH_3)_2$-S^+-CoM, 2-(dimethylsulfonium)ethanesulfonate; Tes, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid.

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anesulfonate (**1**) was prepared by a modified procedure of Schramm et al. (1955). A mixture of 500 mg of sodium 2-bromoethanesulfonate monohydrate (2.18 mmol, Aldrich Chemical Co.) and 167 mg of thiourea (2.18 mmol) was refluxed for 5 h in 2 mL of 95% EtOH. The reaction mixture was cooled to −20 °C and the precipitate was collected by filtration. The precipitate was refluxed for 2 h in 0.7 mL of concentrated NH_4OH and then flash evaporated to dryness. The residue was taken up in H_2O and applied to a Sephadex SP-C25 column (NH_4^+, 1.2 × 18 cm) equilibrated with H_2O, and **1** was eluted with H_2O; a sample of each fraction was spotted with Ellman reagent (Ellman, 1959), and fractions containing **1** were pooled and flash evaporated to dryness. The residue was taken up in 3 mL of H_2O, and acetone was added to cause precipitation. The precipitate was removed by filtration and discarded. The filtrate was flash evaporated to dryness, and 154 mg of **1** (44%) was crystallized from MeOH–diethyl ether. Anal. Calcd for $C_2H_9NO_3S_2$: C, 15.09; H, 5.70; N, 8.80; S, 40.27. Found: C, 15.11; H, 5.47; N, 8.51; S, 40.27.

Ammonium 3-mercaptopropanesulfonate (**2**) was prepared in a manner analogous to the preparation of **1.** From a mixture of 358 mg of sodium 3-bromopropanesulfonate (1.59 mmol, Aldrich Chemical Co.) and 121 mg of thiourea (1.59 mmol), 95 mg of **2** (35%) was obtained. Anal. Calcd for $C_3H_{11}NO_3S_2$: C, 20.79; H, 6.41; N, 8.09; S, 37.01. Found: C, 21.26; H, 6.43; N, 7.91; S, 37.26.

Ammonium 4-mercaptobutanesulfonate (**3**) was prepared in a manner analogous to the preparation of **1.** From a mixture of 170 mg of sodium 4-bromobutanesulfonate (0.71 mmol, Aldrich Chemical Co.) and 54 mg of thiourea (0.71 mmol), 25 mg (19%) of **3** was obtained. Anal. Calcd for $C_4H_{13}NO_3S_2$: C, 25.65; H, 7.01; N, 7.48; S, 34.32. Found: C, 26.15; H, 6.86; N, 7.04; S, 33.87.

Ammonium 2-(methylthio)ethanesulfonate (**4**) was prepared by alkylation of **1** (200 mg, 1.26 mmol) with CH_3I (78 μL, 1.26 mmol) in 5 mL of concentrated NH_4OH made anaerobic by sparging with N_2. The reaction was allowed to proceed overnight at 4 °C in the dark and was flash evaporated to dryness, and then **4** (107 mg, 49%) was precipitated from aqueous acetone. Anal. Calcd for $C_3H_{11}NO_3S_2$: C, 20.80; H, 6.40; N, 8.08; S, 37.01. Found: C, 20.63; H, 6.37; N, 7.82; S, 36.33.

Ammonium 3-(methylthio)propanesulfonate (**5**) (74 mg, 68%) was prepared by alkylation of **2** (100 mg, 0.58 mmol) with CH_3I (36 μL, 0.58 mmol) as in preparation of **4.** Anal. Calcd for $C_4H_{13}NO_3S_2$: C, 25.65; H, 7.01; N, 7.48; S, 34.23. Found: C, 25.84; H, 6.99; N, 7.32; S, 34.02.

Ammonium 4-(methylthio)butanesulfonate (**6**) (46 mg, 43%) was prepared by alkylation of **3** (100 mg, 0.53 mmol) with CH_3I (33 μL, 0.53 mmol) as in preparation of **4.** Anal. Calcd for $C_5H_{15}NO_3S_2$: C, 29.89; H, 7.52; N, 6.96; S, 31.85. Found: C, 29.18; H, 7.36; N, 6.96; S, 31.37.

Ammonium 2-(ethylthio)ethanesulfonate (**7**) (171 mg, 72%) was prepared by alkylation of **1** (203 mg, 1.26 mmol) with CH_3CH_2I (104 μL, 1.26 mmol) as in preparation of **4.** Anal. Calcd for $C_4H_{13}NO_3S_2$: C, 25.64; H, 7.01; N, 7.47; S, 34.23. Found: C, 25.75; H, 6.91; N, 7.44; S, 34.21.

Ammonium 2-(propylthio)ethanesulfonate (**8**) (213 mg, 56%) was prepared by alkylation of **1** (300 mg, 1.89 mmol) with $CH_3CH_2CH_2I$ (916 μL, 9.23 mmol) as in preparation of **4** except the reaction was allowed to proceed 4 days. Anal. Calcd for $C_5H_{15}NO_3S_2$: C, 29.83; H, 7.52; N, 6.96; S, 31.85. Found: C, 29.96; H, 7.50; N, 6.82; S, 31.58.

Ammonium 2,2′-dithiodiethanesulfonate (**9**) and 2-(dimethylsulfonium)ethanesulfonate (**10**) were prepared as described by Taylor & Wolfe (1974a).

Ammonium 2,2′-thiodiethanesulfonate (**11**) was prepared from sodium 2-bromoethanesulfonate (10 g, 44 mmol) and thiourea (3.4 g, 44 mmol). Initially the acid form of **1** was prepared by passing the residue from the NH_4OH reflux step down a Dowex 50 W-X4 column (H^+, 2.5 × 20 cm). The pooled fractions containing acidic **1** were allowed to sit overnight at 22 °C with evolution of H_2S being detected. Upon titration to pH 7.0 with concentrated NH_4OH and flash evaporation to dryness, 8.0 g (64%) of **11** was crystallized from MeOH–diethyl ether. Anal. Calcd for $C_4H_{16}N_2O_6S_3$: C, 16.89; H, 5.68; N, 9.85; S, 33.82. Found: C, 17.15; H, 5.78; N, 9.64; S, 33.52.

The ^1H nuclear magnetic resonance (NMR) spectra were recorded on a Varian HA-100 and were in each case consistent with the assigned structure.

2-Bromoethanesulfonate, 2-chloroethanesulfonate, ethyl-3-(methylthio)propionate, and methyl-3-(methylthio)propionate were purchased from Aldrich Chemical Co.; *N*-methyltaurine and 2-methylthioethanol were from ICN-K & K Laboratories, Inc.; 3-methylthiopropylamine, ethanesulfonic acid, ethanedisulfonic acid, and 2-mercaptoethanol were from Eastman Organic Chemicals; methioninol, isethionic acid, taurine, and cysteic acid were from Sigma Chemical Co.; L- and D-methionine from Nutritional Biochemical Corp.; and *S*-methylcysteine was from Calbiochem.

Preparation of Cell Extracts. Cells of *M. thermoautotrophicum* were grown in a mineral salts medium at 60 °C as previously described by Zeikus & Wolfe (1972). Carbon and energy were supplied to the cultures by constantly bubbling with a H_2 and CO_2 gas mixture (80:20 v/v) at a rate of 200 cm^3 per min. Cells were harvested by continuous Sharples centrifugation after 36 h of growth (late log phase). Harvested cells were suspended in an equal volume of 50 mM Tes buffer [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid], pH 7.0, and were gassed vigorously with H_2 via the Hungate (1969) technique to remove oxygen. Cells were broken by passage of the slurry through a French pressure cell at 12 000 psi, and the eluate was collected under a gentle stream of H_2 in stainless steel centrifuge tubes (2.8 × 10 cm). The broken cell suspension was centrifuged for 30 min at 33 000*g* under an H_2 atmosphere, the resulting supernatant solution was decanted into tubes and regassed with H_2. This extract was then stored at −20 °C until used.

Assay for Alkane Production. Methane production was assayed in small serum-stoppered reaction vials as described by Taylor & Wolfe (1974a). The standard reaction mixture (0.25 mL) contained: Tes buffer, pH 6.0 (when measured at the assay temperature of 60 °C); $MgCl_2$, ATP; and CH_3–S–CoM (**4**); and cell extract as indicated. A hydrogen gas phase was used. The reaction was initiated by transfer of the reaction vials to a shaking water bath at 60 °C. A gas sample (20 μL) was withdrawn by Hamilton syringe and injected into a Packard Model 300 gas chromatograph for methane analysis. Ethane and propane were detected in a similar manner.

Inhibitor Studies. The inhibition of methane formation from CH_3–S–CoM (**4**) by various CoM analogues was measured by following the decrease in the rate of methane production in the standard assay mixture to which the inhibitor had been added. Control reaction mixtures which lacked only the analogue yielded rates of methane formation which are presented as 100% activity; 0.125 μmol of CH_3–S–CoM was used per reaction vial. Cell extract was added immediately before initiation of the reaction.

Results and Discussion

To determine the specificity of the methyl-coenzyme M

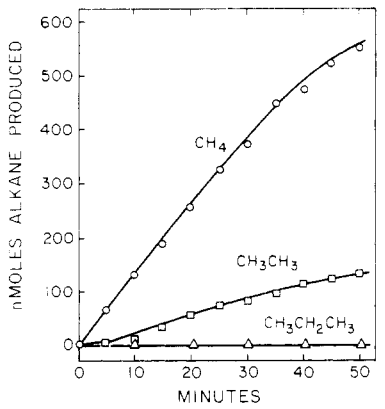

FIGURE 1: Ability of alkyl-coenzyme M analogues to be dealkylated to the corresponding alkane by cell extract. Each reaction vial contained: 30 μmol of Tes buffer; 5 μmol of $MgCl_2$; 1 μmol of ATP; 30 μL of cell extract (1.51 mg of protein); 2.5 μmol of methyl-, ethyl-, or propyl-S-coenzyme M as indicated.

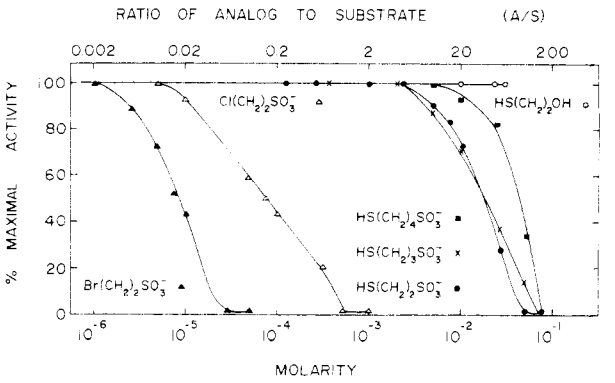

FIGURE 2: Effect of various mercaptan and halogenated analogues of coenzyme M on CH_4 formation from CH_3-S-CoM by cell extract. Each reaction vial contained: 25 μmol of Tes buffer; 5 μmol of $MgCl_2$; 1 μmol of ATP; 0.125 μmol of CH_3-S-CoM; 50 μL of cell extract (2.3 mg of protein); and the indicated concentration of CoM analogue.

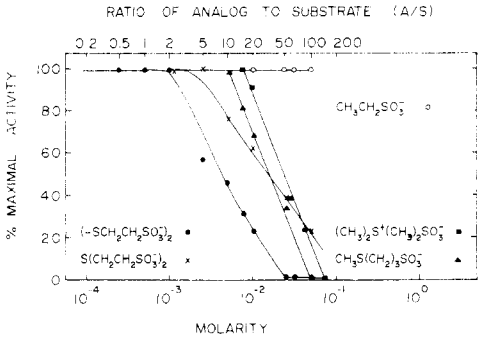

FIGURE 3: Effect of various coenzyme M analogues on methane formation from CH_3-S-CoM by cell extract. Conditions were similar to those of Figure 2.

reductase system for the methylated cofactor, a number of alkylthioether analogues of CoM were prepared which contained modifications to the alkylthio group, to the ethylene-carbon bridge region and to the sulfonate moiety. These compounds were tested for their ability to serve as substrates for reduction leading to alkane formation.

The effect of modifying the cofactor by lengthening the alkyl group of CH_3-S-CoM (**4**) is shown in Figure 1. Ethyl-coenzyme M (**7**) is reduced to ethane by extracts of *M. thermoautotrophicum* at a rate approximately 20% of that obtained from CH_3-S-CoM alone. McBride & Wolfe (1971) demonstrated that ethyl-B_{12} was not reduced to ethane by extracts of *Methanobacterium* strain M.o.H. These results indicated that the methylcobalamin-HS-CoM methyltransferase was not able to transfer the ethyl group from ethyl-B_{12} to HS-CoM (**1**). Similar results were observed for ethyl-B_{12} with extracts of the thermophile, *M. thermoautotrophicum*. Replacement of the methyl moiety by a propyl group to give propyl-CoM (**8**) resulted in an inactive donor; formation of propane was not detectable under a variety of analogue and extract concentrations.

Various analogues were tested for their ability to donate a methyl group to the methyl reductase in a standard reaction mixture from which CH_3-S-CoM (**4**) had been omitted. Each was added at a concentration of 4 mM to a separate standard reaction mixture which contained 2.1 mg of crude cell extract protein. The reaction time was 50 min. The control reaction mixture produced 1200 nmol of CH_4 per h from 4 mM CH_3-S-CoM. No CH_4 was detectable in reaction mixtures to which the following types of analogues had been added:

(1) Addition of either one or two methylene carbons to the ethylene bridge between the sulfide and sulfonate moieties: 3-(methylthio)propanesulfonate (**5**); 4-(methylthio)butanesulfonate (**6**).

(2) Addition of a second methyl group to the sulfide moiety: 2-(dimethylsulfonium)ethanesulfonate (**10**). Similar results were obtained with this compound in extracts of *Methanobacterium* strain M.o.H. (Taylor & Wolfe, 1974b).

(3) Replacement of the sulfide moiety: $CH_3NH(CH_2)_2SO_3^-$, *N*-methyltaurine.

(4) Replacement of the sulfonate moiety: $CH_3S(CH_2)_2OH$, 2-(methylthio)ethanol; $CH_3S(CH_2)_2\ COOCH_2CH_3$, ethyl-3-(methylthio)propionate; $CH_3S(CH_2)_2COOCH_3$, methyl-3-(methylthio)propionate (**17**); $CH_3S(CH_2)_3NH_2$, 3-(methylthiopropylamine; $CH_3S(CH_2)_2CHNH_2CH_2OH$, methioninol; $CH_3S(CH_2)_2CHNH_2COO^-$, L-methionine or D-methionine; and $CH_3S(CH_2)CHNH_2COO^-$, *S*-methylcysteine.

The above analogues also were tested for their ability to donate a methyl group to HS-CoM (**1**) which would subsequently be demethylated by the reductase to yield methane. When analogues were incubated at a concentration of either 0.4 or 4.0 mM in the presence of 1 mM HS-CoM, no methane was detected after 1 h. Similar experiments performed with CH_3-S-CoM (1 mM) in place of HS-CoM resulted in normal rates of methane formation. These observations indicated that the analogues were not inhibiting the reductase under the experimental conditions used and that the methylreductase is highly specific; CH_3-S-CoM is the only substrate so far found to yield CH_4.

To better understand the nature of the demethylation reaction leading to methane formation from CH_3-S-CoM, a number of cofactor derivatives and cofactor analogues were screened for their ability to inhibit the methylreductase reaction. Inhibition experiments were carried out by measurement of the decrease in the optimal rate of methane formation from CH_3-S-CoM with increasing levels of analogue. Methane production was linear in all reactions. Typical results of such experiments are shown in Figures 2 and 3. Bromoethanesulfonic acid was found to be the most potent inhibitor of methanogenesis detected to date (Figure 2) causing 50% inhibition at 7.9×10^{-6} M. As this compound is the precursor for organic synthesis of the various CoM derivatives it is essential that no residual bromoethanesulfonate remain in the final preparations. Chloroethanesulfonic acid is a slightly less potent inhibitor of the reductase, requiring higher concen-

trations to cause similar inhibition. In contrast, the observed product of the methylreductase reaction, HS-CoM (**1**), does not cause measurable levels of inhibition of methanogenesis up to a concentration of 10 mM. This would indicate that the rate of methane formation in the routine reductase assay is not influenced by the accumulation of HS-CoM over the time course of the assay.

The disulfide form of the cofactor, $(S\text{-}CoM)_2$ (**9**), causes noticeable inhibition in the mM range (Figure 3). Extracts of *M. thermoautotrophicum* catalyze the reduction of the disulfide, and CoM was observed to exist in the reduced form in active extracts. The inhibition observed for $(S\text{-}CoM)_2$ may be due to competition between $(S\text{-}CoM)_2$ reductase and methyl-CoM reductase for reducing equivalents from molecular hydrogen or due to the accumulation of HS-CoM.

Results of experiments in which other analogues were tested for their ability to inhibit methane formation from $CH_3\text{-}S\text{-}CoM$ indicated that $CH_3CH_2\text{-}S\text{-}CoM$ (**7**) and $CH_3CH_2CH_2\text{-}S\text{-}CoM$ (**8**) caused only 50% inhibition at the high ratio of analogue to $CH_3\text{-}S\text{-}CoM$ of 17 and 22, respectively. When the following analogues were tested at a ratio of analogue to $CH_3\text{-}S\text{-}CoM$ of 100, no inhibition of methylreductase was detected: $HO(CH_2)_2SO_3^-$; $H_2N(CH_2)_2SO_3^-$; $CH_3NH(CH_2)_2SO_3^-$; $^-O_3S(CH_2)_2SO_3^-$; $CH_3S(CH_2)_2OH$. When $CH_3\text{-}S\text{-}CoM$ was added at the same concentration (5×10^{-2} M), no inhibition in the rate of methane formation was observed.

With the potent inhibitors, $BrCH_2CH_2SO_3^-$ and $ClCH_2CH_2SO_3^-$, inhibition of the methyl reductase is not reversed when the substrate concentration of $CH_3\text{-}S\text{-}CoM$ is increased 100-fold. The precise definition and quantitation of the mode by which these inhibitors act must await resolution and purification of the components involved in methanogenesis.

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Distribution of Ultraviolet-Induced DNA Repair Synthesis in Nuclease Sensitive and Resistant Regions of Human Chromatin†

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ABSTRACT: The distribution of ultraviolet radiation (UV) induced DNA repair synthesis within chromatin was examined in cultured human diploid fibroblasts (IMR-90). Measurement of the time course of repair synthesis yielded two distinct phases: An initial rapid phase (fast repair) which occurs during the first 2–3 h after damage and a slower phase (slow repair) associated with a tenfold decrease in the rate of nucleotide incorporation, which persists for at least 35 h after damage. Staphylococcal nuclease digests of nuclei from cells damaged with UV and labeled during the fast-repair phase revealed a marked preference of fast-repair synthesis for the nuclease-sensitive regions. A new method was developed to analyze the digestion data and showed that approximately 50% of the nucleotides incorporated during the fast-repair phase are located in staphylococcal nuclease-sensitive regions, which comprise about 30% of the genome. Calculations from these data indicate that in the staphylococcal nuclease-sensitive regions the number of newly inserted nucleotides per unit DNA is about twice that of resistant regions. These results were supported by electrophoresis studies which demonstrated a decreased representation of fast-repair synthesis in core particle DNA. In contrast, the distribution within chromatin of nucleotides incorporated during the slow-repair phase was found to be much more homogeneous with about 30% of the repair sites located in 25% of the genome. Digestion studies with DNase I indicated a slight preference of repair synthesis for regions sensitive to this enzyme; however, no marked difference between the distributions of fast- and slow-repair synthesis was observed. This study provides evidence that the structural constraints placed upon DNA in chromatin also place constraints upon UV-induced DNA repair synthesis in human cells.

Although recent studies have greatly increased our understanding of chromatin structure (Hewish and Burgoyne, 1973; Woodcock, 1973; Olins and Olins, 1973, 1974; Sahasrabudde and Van Holde, 1974; Kornberg, 1974; Noll, 1974), to date there have been relatively few studies dealing with the role of chromatin structure in *excision repair* (Ramanathan et al.,

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