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Characterization of the Enzymatic Conversion of Sulfoacetaldehyde and L-Cysteine into Coenzyme M (2-Mercaptoethanesulfonic Acid)†

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ABSTRACT: Sulfoacetaldehyde was shown to be converted enzymatically into coenzyme M by cell-free extracts of methanogenic bacteria. Gas chromatography–mass spectrometry (GC–MS) of the *S*-methyl methyl ester derivative of the coenzyme M isolated from the extracts was used to measure both the extent and position of the deuterium incorporated into coenzyme M from [2,2-$^2H_2$]sulfoacetaldehyde. The conversion of sulfoacetaldehyde into coenzyme M was greatly stimulated by the addition of L-cysteine (20 mM) to the extracts and/or by incubating the extracts under hydrogen, whereas incubation in the presence of sulfide (20 mM) greatly reduced coenzyme M synthesis. Incubation of a cell-free extract from *Methanobacterium formicicum* with [2,2-$^2H_2$]sulfoacetaldehyde and [^{34}S]-L-cysteine (92.6 atom % ^{34}S) led to the production of coenzyme M in which the thiol portion of the molecule contained 90 atom % ^{34}S. [*ethylene*-$^2H_4$]-*S*-(2-Sulfoethyl)cysteine, incubated with this cell-free extract at a concentration of 22 mM, readily cleaved to coenzyme M. On the basis of these observations, it is concluded that sulfoacetaldehyde is converted into coenzyme M by reacting with cysteine to form the thiazolidine adduct [2-(sulfomethyl)thiazolidine-4-carboxylic acid], which undergoes a reductive cleavage of the heterocyclic C(2)–N bond to form *S*-(2-sulfoethyl)cysteine, which, in turn, undergoes a β-elimination to produce coenzyme M.

Coenzyme M (2-mercaptoethanesulfonic acid) is one of several recently described coenzymes involved in the biological production of methane (Escalante-Semerena et al., 1984). The methylation of coenzyme M to *S*-methyl-coenzyme M, which undergoes a subsequent reductive cleavage to methane and coenzyme M, is involved in the production of methane from H_2 and CO_2 (Escalante-Semerena et al., 1984), methanol and

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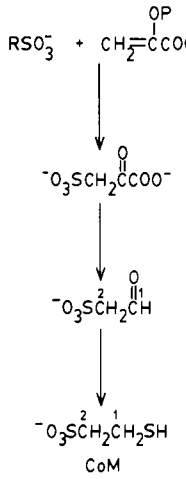

FIGURE 1: Proposed pathway for the biosynthesis of coenzyme M.

methylamines (Shapiro & Wolfe, 1980), and acetate (Lovley et al., 1984; Nelson & Ferry, 1984). Coenzyme M, whose structure was determined in 1974 by Taylor and Wolfe, is unique among coenzymes in that it occurs only in methanogenic bacteria (Balch & Wolfe, 1979). It is also the smallest coenzyme (M_r 142), it contains the highest percentage of sulfur (45%), and it is one of the few sulfonic acids found in nature. Recent work has shown that coenzyme M is most likely biosynthesized by the series of reactions shown in Figure 1 (White, 1985, 1986). The last reaction of this sequence is the conversion of sulfoacetaldehyde into coenzyme M. In this paper, I demonstrate the occurrence of this enzymatic activity in extracts of methanogenic bacteria and present a mechanism for this novel reaction, which represents the first biochemical example of the conversion of an aldehyde to a thiol in a biological system.

Materials and Methods

Materials. [1,1,2,2-$^2H_4$]-2-Bromoethanol (99.0 atom % ^2H) was obtained from Cambridge Isotope Laboratories, Inc., Woburn, MA, and [1,1,2,2-$^2H_4$]-1,2-dibromoethane (99.0 atom % ^2H) was obtained from Merck Sharp & Dohme of Canada. Elemental sulfur (^{32}S, 2.48 atom %; ^{33}S, 2.23 atom %; ^{34}S, 92.59 atom %; and ^{36}S, 1.7 atom %) was obtained from Monsanto Research Corp.'s Mound Facility in Miamisburg, OH. Bromoacetaldehyde dimethyl acetal and sodium bromoethanesulfonate were obtained from Aldrich Chemical Co., Milwaukee, WI.

Bacterial Strains and Growth Conditions. Methanobacterium formicicum was grown at 39 °C in a 2-L Multigen fermenter (New Brunswick Scientific Co., Inc., Edison, NJ) that was continuously supplied with a gas mixture consisting of H_2/CO_2 (80/20). The medium used was the same as that previously described (Schauer & Ferry, 1982) but without the addition of yeast extract or trypticase. Rumen strain 10-16B (Lovley et al., 1984) was grown in 2-L bottles pressurized to 30 psi with the same H_2/CO_2 mixture as previously described (White, 1985). Cells were removed from the medium by centrifugation under nitrogen at the end of log-phase growth and were washed twice with an anaerobic assay buffer, 50 mM in 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid (TES), 10 mM in $MgCl_2$, and 2 mM in mercaptoethanol.

Preparation and Purification of Cell Extracts. All operations were performed under nitrogen with solutions degassed with nitrogen in order to assure anaerobic conditions throughout the procedure. The cell pellet isolated above was suspended in a volume of assay buffer equivalent to twice its wet weight in grams and the resulting suspension sonicated at 0 °C. The extent of cell lysis was determined by measuring the release of coenzyme F_{420} at 420 nm and was found to be at a maximum after two 30-s, 50-W bursts from a Sonifier cell disruptor with a microtip. After centrifugation for 20 min at 10000*g*, 1–2 mL of the resulting clear, greenish solution (~10–15 mg of protein/mL) was transferred to sealed 5-mL vials containing H_2.

Enzymatic Incubations. Concentrated (50×), degassed solutions of the indicated substrates were added to the above extracts to give the concentrations indicated in Table I; the reactions were then allowed to proceed at 39 °C with shaking under 30 psi of H_2 or N_2. The individual reactions were then terminated at the indicated times (Table I) by the addition of 0.2 mL of 6 M HCl and centrifuged to remove the precipitated proteins.

Analysis of the Deuterium Incorporated into Coenzyme M. The coenzyme M present in the resulting reaction mixtures was isolated and converted into the *S*-methyl methyl ester derivative. The incorporation of deuterium into the coenzyme M molecule was measured by gas chromatography–mass spectrometry (GC–MS) of this derivative as previously described (Lovley et al., 1984; White, 1985). Deuterium incorporation into the coenzyme M could be measured either from the molecular ion of this derivative at m/z 170 or from a more intense fragment ion ($M^+ - 96$) at m/z 74. In either case, the distributions of ^2H reported in Table I were corrected for their natural isotopic abundances as outlined by Biemann (1962).

Calculation of the Total Amount of Coenzyme M Formed. The C-2 deuteriums of [2,2-$^2H_2$]sulfoacetaldehyde exchange with water, with a half-life of ~2 h, under the reaction conditions used. Due to this exchange, the deuterium contained in the sulfoacetaldehyde, which is converted into coenzyme M, decreases as a function of time, resulting in a smaller increase in the amount of deuteriated coenzyme M than would be expected had no exchange occurred. In addition, since the deuteriated sulfoacetaldehyde has two exchangeable deuteriums, there is a shift in label distribution from two deuteriums in a majority of the newly biosynthesized molecules to one deuterium and finally to no deuterium as the exchange proceeds. The molar ratios of molecules containing no, one, or two deuteriums will be given by a^2, $2ab$, and b^2, respectively, where b represents the mole fraction of each site containing one deuterium and a represents the mole fraction of each site containing one hydrogen. Since those coenzyme M molecules, isolated from the incubation mixture that contains either one or two deuteriums, can only originate from the labeled sulfoacetaldehyde, the ratio of the molecules containing one or two deuteriums can be used to calculate the values of a and b. Thus

$$\frac{b^2}{2ab} = \frac{\text{mole fraction of coenzyme M with } ^2H_2}{\text{mole fraction of coenzyme M with } ^2H_1}$$

since $a + b = 1$, then

$$a = \frac{1}{1 + \dfrac{2(\text{mole fraction of coenzyme M with } ^2H_2)}{\text{mole fraction of coenzyme M with } ^2H_1}}$$

The value a^2 then represents the mole fraction of the unlabeled coenzyme M molecules that is derived from the fed [2,2-$^2H_2$]sulfoacetaldehyde after exchange. Thus, the mole fraction of the total coenzyme M, which originates from the unlabeled coenzyme in the extract, will be (total mole fraction of the unlabeled molecules) – a^2. The ratio of the coenzyme M in

Table I: Incorporation of [2,2-$^2H_2$]Sulfoacetaldehyde into Coenzyme M by Extracts of *M. formicicum*

experiment	distribution of ^2H[d] 0	1	2	ion used for measurement	nmol of CoM/mg of protein
(1) boiled cell extract[a]	100 (100)	0.0 (22.3)	0.0 (5.1)	*m/z* 74	0.0
	100 (100)	0.0 (7.8)	0.0 (8.3)	*m/z* 170	0.0
(2) cell extract incubated under H_2[a]	7.2 (8.8)	15.2 (20.7)	77.6 (100)	*m/z* 74	78.6
(3) cell extract incubated under N_2[a]	15.7 (23.9)	25.1 (43.5)	59.2 (100)	*m/z* 74	36.2
(4) no cysteine or sulfide[b]	52.2 (100)	33.9 (72.8)	13.9 (40.1)	*m/z* 170	11.8
(5) sulfide and no cysteine[b]	45.7 (100)	80.0 (33.0)	21.2 (60.3)	*m/z* 170	11.1
(6) cysteine and no sulfide[b]	12.7 (20.2)	27.6 (45.4)	59.7 (100)	*m/z* 170	51.5
(7) cysteine and sulfide[b]	31.9 (79.8)	33.3 (89.5)	34.8 (100)	*m/z* 170	17.2
(8) 1 h[c]	57.9 (100)	3.0 (27.2)	39.1 (73.6)	*m/z* 74	4.0
(9) 3 h[c]	19.8 (27.8)	12.5 (23.6)	67.7 (100)	*m/z* 74	22.8
(10) 3 h, 4.8 mM NADH[c]	27.5 (41.3)	9.4 (23.3)	63.1 (100)	*m/z* 74	14.6
(11) 3 h, 4.8 mM NADPH[c]	24.8 (38.7)	16.0 (33.6)	59.2 (100)	*m/z* 74	17.4

[a] Experiments 1–3 were run for 6 h and contained 24.2 mM [2,2-$^2H_2$]sulfoacetaldehyde, 20.8 mM cysteine, and 12.2 mg/mL protein. [b] Experiments 4–7 were run for 6 h and contained 35 mM [2,2-$^2H_2$]sulfoacetaldehyde, 13.0 mg/mL protein, and 20 mM cysteine and/or sulfide as indicated. [c] Experiments 8–11 were run for the indicated times and contained 23.5 mM cysteine, 25.7 mM [2,2-$^2H_2$]sulfoacetaldehyde, and 13.6 mg/mL protein. [d] The numbers in parentheses are the observed normalized ion intensities. The observed normalized ion intensity ratios for the ion, ion + 1 *m/z*, and ion + 2 *m/z* for an unlabeled coenzyme M sample were 100, 7.8, and 8.3 for *m/z* M^+ 170 and 100, 22.0, and 5.1 for the *m/z* 74 ion.

the extract at the start of the incubation to that produced by the extract will be given by (mole percent of the molecules with no deuterium) – a^2. Since the coenzyme M present in the extract can be accurately measured (see below), the total amount of coenzyme M produced can be determined.

Measurement of Coenzyme M in Extracts. The amount of coenzyme M in the extracts was measured by isotopic dilution analysis using an internal standard of known $^2H_4$-labeled coenzyme M and GC–MS to determine the ratio of labeled to unlabeled coenzyme. The value found for the *M. formicicum* extracts was 5.42 nmol of coenzyme M/mg of protein (biuret).

Synthesis and Characterization of [2,2-$^2H_2$]Sulfoacetaldehyde. After 4.22 g (25 mmol) of bromoacetaldehyde dimethyl acetal was combined with 5 mL of 1 M DCl in D_2O, the resulting mixture was heated at 100 °C for 20 min with rapid stirring. (The material becomes a homogeneous solution after ~5 min.) The resulting solution of bromoacetaldehyde was cooled and mixed with 5 mL of D_2O containing 1.9 g of sodium metabisulfite (10 mmol) and 3.78 g of sodium sulfite (30 mmol). The mixture was refluxed for 40 h and then dried in vacuo. Two recrystallizations from 50% EtOH yielded 3.9 g of product, the bisulfite addition complex of [2,2-$^2H_2$]-sulfoacetaldehyde. The product was identical with that described by Kondo et al. (1971). Reduction of the bisulfite addition complex with sodium borohydride in dilute ammonia gave isethionate, which was converted into the free acid by passing the reaction mixture down a Dowex 50W-8X H^+ column and characterized by direct probe mass spectrometry. The mass spectrum displayed a weak ion at M^+ *m/z* 128 (1% of the base peak) and an intense ion at *m/z* 98 ($M^+ - CH_2O$) (base peak) that retained the C-2 deuteriums. Isotopic analysis of the *m/z* 98 ion showed it contained the following distribution of deuterium: 90.6% $^2H_2$; 9.04% $^2H_1$; and 0.22% $^2H_0$.

The proton magnetic resonance spectrum (PMR) (270 MHz, D_2O) of the protonated compound, prepared as above but using HCl and H_2O, showed δ 4.28 (d, 1 H, J = 10.2 Hz, C_1–H), 3.51 (d, 1 H, J = 14.3 Hz, C_2–H), and 3.24 (q, 1 H, J = 10.2 and 14.3 Hz, C_2–H′). Proton-coupled carbon magnetic resonance showed two peaks, a doublet at 85 ppm and a quartet at 54.8 ppm.

A sample of the deuteriated bisulfite addition product was dissolved in D_2O, 2 equiv of DCl was added, and the sample was degassed with N_2 until all the SO_2 was expelled (~30 min). Sodium bicarbonate (2 equiv) was then added and the resulting sample used for the biosynthetic studies.

Synthesis of [$^2H_4$]Isethionate. This synthesis was performed by the treatment of [$^2H_4$]bromoethanol with sodium metabisulfite as previously described (White, 1985).

Synthesis of $^2H_4$-Labeled Coenzyme M. This synthesis was performed by treating sodium bromo[1,1,2,2-$^2H_4$]ethanesulfonic acid (see below) with excess hydrogen sulfite as previously described (Romesser & Balch, 1980).

Synthesis of S-(2-Sulfoethyl)-L-cysteine. After 2.1 g of sodium bromoethanesulfonate (10 mmol) was dissolved in 3 mL of water, a solution of 1.2 g (10 mmol) of cysteine and 1.0 g of $NaHCO_3$ in 5 mL of water was added. The homogeneous reaction mixture was then heated at 90 °C for 12 h, cooled, and passed through a Dowex 50W-X8 H^+ column to remove the sodium ions. Evaporation in vacuo gave a white solid that was recrystallized (3×) from ethanol/water to give 1.8 g of white crystals. This material displayed a molecular ion at *m/z* 353 as the dimethyl trifluoroacetyl ester derivative and gave the expected proton and carbon magnetic resonance spectra. A similar, alternate synthesis of this compound has been described by Niketic et al. (1974). The deuteriated compound was synthesized from sodium [1,1,2,2-$^2H_4$]-bromoethanesulfonate, which was prepared from [1,1,2,2-$^2H_4$]ethylene dibromide as described by Marvel and Sparberg (1943).

Synthesis of [^{34}S]-L-Cysteine. Twenty milligrams of elemental sulfur (3.48 atom % ^{32}S, 2.23 atom % ^{33}S, 92.6 atom % ^{34}S, and 1.7 atom % ^{36}S) was suspended in xylene and reacted with a solution of benzylmagnesium chloride in tetrahydrofuran to produce benzyl [^{34}S]mercaptan, which was reacted with L-β-chloroalanine to produce [^{34}S]benzyl-L-cysteine as described by Wood and Van Middlesworth (1946). The resulting [^{34}S]benzyl-L-cysteine was reduced by sodium in liquid ammonia as described by Wood and du Vigneaud (1939). Measurement by GC–MS of the *S*-methyl *n*-butyl trifluoroacetyl derivative of the synthetic L-cysteine (White, 1981) showed that the isotopic distribution in its sulfur was the same as the distribution in the sulfur used in the synthesis.

Results and Discussion

It is clear from the data presented in Table I that extracts of *M. formicicum* readily convert sulfoacetaldehyde into coenzyme M. This conversion was unequivocally established for each reaction by the GC–MS identification of deuteriated coenzyme M, which was derived from the deuteriated sulfoacetaldehyde. Furthermore, since the M^+ ion at *m/z* 170 was found to have the same extent of deuteriation as the fragment

ion at m/z 74, which specifically lost a C-2 proton, all of the deuteriums incorporated into the coenzyme M from the labeled sulfoacetaldehyde must have been specifically incorporated into the expected position of the coenzyme, i.e., C-2 (White, 1985).

The fact that sulfoacetaldehyde and L-cysteine failed to produce any coenzyme M when incubated with boiled cell extract (experiment 1) confirmed that the synthesis of coenzyme M is enzymatic. Incubation of sulfoacetaldehyde and L-cysteine in the assay buffer also failed to generate any detectable coenzyme M. Extracts incubated with sulfoacetaldehyde and no added L-cysteine produced a small amount of coenzyme M (experiment 4). This production of coenzyme M was stimulated (4.36×) by the addition of 20 mM L-cysteine to the incubation mixture (experiment 4 as compared to experiment 6). The addition of sulfide to the incubation mixture led to a reduction in the amount of coenzyme M produced both with and without added L-cysteine (experiments 5 and 7). These findings indicate that both sulfoacetaldehyde and cysteine, but not sulfide, were required for the production of coenzyme M. The production of coenzyme M in an extract incubated with L-cysteine and sulfoacetaldehyde was not stimulated by the addition of NADH or NADPH (experiments 10 and 11) but was greatly reduced when the reaction mixture was incubated under nitrogen instead of H_2 (experiment 3), indicating that a reduction not dependent on NAD or NADPH was required for the reaction.

Separation of a cell extract from *M. formicicum* on Sephacryl S-200 (White, 1986) gave a protein fraction that was free of coenzyme M and other small molecules such as coenzyme F_{420}, methanopterin, and NAD and had about the same specific activity as the crude extracts, which further supports the lack of involvement of NAD or other small molecular weight cofactors in the reaction. The possibility that the observed coenzyme M production resulted from intact cells present in the incubation mixture was eliminated since intact cells incubated with labeled sulfoacetaldehyde and cysteine failed to produce labeled coenzyme M.

Results similar to these were obtained when extracts of strain 10-16B were used. However, since these extracts consistently gave lower specific activities, all further work was done with extracts from *M. formicicum*.

Incubation of a cell extract with 32.6 mM [$^2H_4$]isethionate and 21.8 mM L-cysteine for 6 h produced no labeled coenzyme M, demonstrating that isethionate was not an intermediate in the conversion. Incubation in the presence of 22.7 mM [*ethylene*-$^2H_4$]-*S*-(2-sulfoethyl)-L-cysteine for 3 h led to the isolation of coenzyme M with 88% of the molecules containing four deuteriums. Considering the amount of unlabeled coenzyme M present in the extract, this corresponds to the production of 39.6 nmol of coenzyme M/mg of protein.

Incubation of extracts with 24.3 mM [2,2-$^2H_2$]sulfoacetaldehyde and 20.8 mM [^{34}S]-L-cysteine for 6 h led to the isolation of coenzyme M with normalized intensities of the M^+, $M^+ + 1$, $M^+ + 2$, $M^+ + 3$, and $M^+ + 4$ of 100, 36.3, 34.8, 70.1, and 152.0%, respectively. The measurement of coenzyme M molecules that increased by 4 m/z clearly confirms that the coenzyme thiol is derived from the combination of the cysteine sulfur with sulfoacetaldehyde. After the isotopic abundances found in an unenriched sample are corrected for, the mole fraction of the molecules that increased by 0, 1, 2, 3, and 4 mass units was found to be 30.5%, 2.0%, 8.4%, 18.8%, and 40.3%, respectively. Since the molecules that increased by 3 and 4 mass units can only originate from coenzyme M molecules containing one ^{34}S and either one or two ^{2}H, the ratio of the mole fraction of the molecules that increased by 3 and 4 mass units will be the same as the ratio of the number of molecules containing one and two deuteriums. Thus, the values of a and b, which describe the distribution of deuterium in the sulfoacetaldehyde used in the biosynthesis, can be calculated as described above. After a correction was made for the small amount of ^{33}S present in the sample, the values for a and b were calculated to be 0.181 and 0.819, respectively. If the sulfoacetaldehyde containing this deuterium distribution combined only with the labeled cysteine sulfur, then the final coenzyme M molecules would have a distribution of 0.07, 1.1, 3.9, 18.7, and 40.3 for molecules increased by 0, 1, 2, 3, and 4 mass units, respectively. The difference between the observed and calculated abundances of molecules that increased by 2 mass units (i.e., 8.4% − 3.9% = 4.5%) represents the amount of coenzyme M that was produced by using a sulfur source other than labeled cysteine.

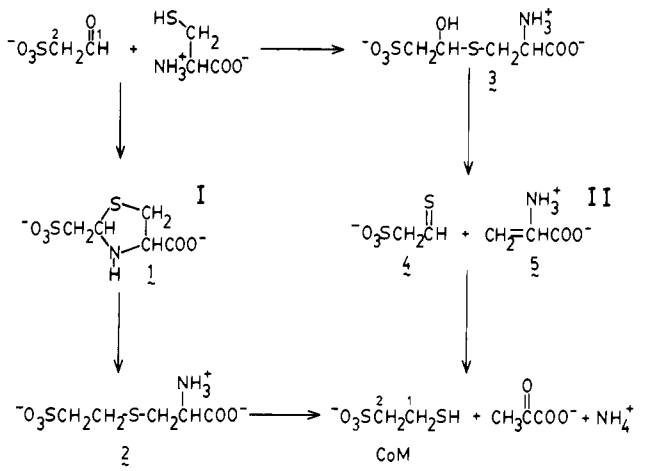

FIGURE 2: Possible routes for the conversion of cysteine and sulfoacetaldehyde into coenzyme M.

Given the above experimental observations, I envisioned three possible mechanisms for the conversion of sulfoacetaldehyde and cysteine into coenzyme M. In the first, the sulfoacetaldehyde would be reduced to isethionate, which would eliminate water to give sulfoethylene. The sulfoethylene would then react with L-cysteine in a Michael-type reaction to form *S*-(sulfoethyl)-L-cysteine, which would subsequently be cleaved to coenzyme M. This reaction sequence, however, was eliminated since the formation of sulfoethylene requires the loss of one of the C-1 protons from isethionate, and coenzyme M is biosynthesized in vivo from acetate with the retention of both of these protons (White, 1985). Also, [$^2H_4$]isethionate is not converted into coenzyme M by the cell-free system.

The two remaining mechanisms for the reaction are shown in Figure 2. In route II, the cysteine thiol reacts with the sulfoacetaldehyde to generate the hemithioacetal addition compound **3**, which undergoes a base-catalyzed elimination of α-aminoacrylic acid to generate the thiolcarbonyl compound **4**. Reduction of this compound would then produce coenzyme M. Although this reaction sequence cannot be eliminated on the basis of available data, both the reactivity and instability of thioaldehyde compounds (Mayer, 1967) and the rapid rate at which **3** would be converted into **1** (Kallen, 1971b) make this route very unlikely.

Route I (Figure 2), in which the cysteine reacts with sulfoacetaldehyde to form the thiazolidine compound **1**, is a more likely route. Reduction of the newly formed C–N bond of this compound would then generate *S*-(sulfoethyl)-L-cysteine (compound **2**, Figure 2). A pyridoxyl-P-assisted, β-elimination

of 2-aminoacrylic acid from this compound in a reaction analogous to that observed in β-cystathionase-catalyzed reactions (Delavier-Klutchko & Flavin, 1965; Guggenheim, 1971) would then give coenzyme M, pyruvate, and ammonia.

In further support of route I, cysteine is known to readily react in dilute aqueous solutions with many different aldehydes including glyoxylate (Rao & Ramakrishnan, 1962; Burns et al., 1984), formaldehyde (Ratner & Clarke, 1937; Kallen, 1971a,b), and pyridoxal derivatives (du Vigneaud et al., 1957) to form stable thiazolidine derivatives.

Reduction of the C(2)–N bond of the thiazolidine would be the next step in the formation of coenzyme M. Several biochemical examples of the reduction of C–N bonds are known, the most analogous example being the Stickland-type reductive cleavage of proline to 5-aminovalerate, which is catalyzed by the proline reductase isolated from *Clostridium sticklandii* (Seto & Stadtman, 1976; Schwartz & Müller, 1979).

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