Biosynthesis of Coenzyme M (2-Mercaptoethanesulfonic Acid)†

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ABSTRACT: ^2H- and ^{13}C-labeled precursors were used to establish the pathway for the biosynthesis of coenzyme M (2-mercaptoethanesulfonic acid) in methanogenic bacteria. The extent and position of the label incorporated into the coenzyme were measured from the mass spectrum of the methyl ester derivative of methyl coenzyme M that was prepared from the coenzyme M present in the cells. In the three different strains of methanogenic bacteria examined, growth in the presence of [2,2,2-$^2H_3$]acetate led to the production of coenzyme M with up to two deuteriums on the C-1 carbon. The extent of labeling of this carbon was the same as that calculated for the phosphoenolpyruvate present in the cells. [1,2-$^{13}C_2$]Acetate was found to be incorporated into the coenzyme as a unit. DL-[3,3-$^2H_2$]Sulfolactic acid and $H^{34}SO_3^-$ were both found to be incorporated, whereas [1,1,2,2-$^2H_4$]taurine, [2,2-$^2H_2$]sulfoacetic acid, L-[3,3-$^2H_2$]cysteic acid, DL-[3,3-$^2H_2$]cysteine, [1,1,2,2-$^2H_4$]isethionate, and $^{34}SO_4^{2-}$ were found not to be incorporated. On the basis of this and additional information presented in this paper, it is concluded that the first committed step for coenzyme M biosynthesis in methanogenic bacteria is the reaction of either free or bound sulfite with phosphoenolpyruvate to form sulfopyruvate either directly or indirectly via a two-electron oxidation of sulfolactate. Decarboxylation of the resulting sulfopyruvate produces sulfoacetaldehyde which is then converted into coenzyme M.

The involvement of coenzyme M (2-mercaptoethanesulfonic acid) in the terminal steps of methane biosynthesis was first demonstrated in 1971 by McBride and Wolfe. Its structure was determined in 1974 by Taylor and Wolfe, and along with a whole new series of recently described coenzymes, coenzyme M has been shown to serve as an essential cofactor for the conversion of CO_2 to methane in methanogenic bacteria (Escalante-Semerena et al., 1984). Among coenzymes, it is unique in that it occurs only in methanogenic bacteria (Balch & Wolfe, 1979), it is the smallest (M_r 142), and it contains the highest percentage of sulfur (45%). It is also one of the few sulfonic acids found in nature. In this paper, I report on the first work on the biosynthesis of this unique coenzyme.

Materials and Methods

Bacterial Strains. Rumen isolate 10-16B, as described by Lovley et al. (1984a), was used in most of the work described herein. It is a methanogenic bacteria with a coccobacillus morphology similar to that of *Methanobrevibacter ruminantium*, but unlike this organism, it is able to grow rapidly (μ = 0.24 h^{-1}) in a complex medium at 39 °C, and it has simple nutritional requirements. Cultures of *Methanobacterium formicicum* were supplied by James G. Ferry. *Methanosarcina* strain TM-1 was supplied by Steven H. Zinder. This organism is unable to grow on H_2/CO_2, and in the absence of methanol and methylamines, it produces methane solely from acetate (Zindler & Mah, 1979).

Labeled Compounds. Bromo[$^2H_4$]ethanol (99.0 atom % ^2H) and [1,2-$^{13}C_2$]acetate (99.0 atom % ^{13}C) were obtained from Cambridge Isotope Lab., Inc. in Woburn, MA. Elemental $^{34}S_8$ (^{32}S, 2.48 atom %; ^{33}S, 2.23 atom %; ^{34}S, 92.59 atom %; ^{36}S, 1.7 atom %) and ^{18}O-enriched water (2.8 atom % ^{16}O, 1.8 atom % ^{17}O, and 95.4 atom % ^{34}S) were obtained from Monsanto Research Corp. Mound Facility in Miamisburg, OH. Sodium sulfate (90 atom % ^{34}S) was obtained from Prochem/Isotopes U.S. Services, Inc., Summit, NJ. 1,2-Dibromo[1,1,2,2-$^2H_4$]ethane (99.0 atom % ^2H) was obtained from Merck Sharp & Dohme of Canada and [$^4H_4$]acetic acid (98 atom % ^2H) was obtained from Sigma Chemical Co.

Growth of Bacterial Strains. Methanobacterium formicicum and bacterial strain 10-16B were grown in 2-L bottles pressurized to 30 psi with H_2/CO_2 (80/20) on 500 mL of the minimal salts medium containing 2.8 mM cysteine and 2.08 mM sulfide as described by Lovley et al. (1984a). The design of the bottles was similar to that described by Balch & Wolfe (1976). The bottles were shaken on their sides at 150 rpm at 39 °C. The acetate in the medium was 62.5 mM and was labeled or unlabeled as indicated in the tables. Precursors were added to the medium before autoclaving at the concentrations indicated. An ~10% (v/v) inoculum of cells grown to A_{660} > 0.5 was used to start the 500 mL of minimal medium. This inoculum was grown on a medium of the same composition as used in each experiment. After the completion of 48–72 h of growth, the cells were harvested by centrifugation. *Methanosarcina* strain TM-1 was grown by Derek R. Lovley as described by Lovley et al. (1984b).

Isolation of Coenzyme M and the Preparation of a Volatile Derivative of Coenzyme M. The cells were removed from the growth medium by centrifugation for 15 min at 1000*g*. (In a typical growth, ~1 g wet weight of cells was isolated from 500 mL of medium.) Three milliliters of water was then added for each gram wet weight of cell pellet and the resulting suspension heated under nitrogen at 100 °C for 1 h. The insoluble material was removed by centrifugation and reextracted for 5 min at 100 °C with 1 mL of water/g of original cell pellet weight. After a second centrifugation, the combined supernatants were concentrated to 1 mL by evaporation with nitrogen gas at 95 °C. One milliliter of methanol, 0.1 mL of 7 M aqueous ammonia, 20 mg of $NaBH_4$, and 10 μL of methyl iodide were added with stirring to the cooled solution (3 °C) in the order given. After 10 min at room temperature, an additional 10 μL of methyl iodide was added. After the alkylation of the thiols had proceeded for 30 min at room temperature, the methanol, ammonia, and excess methyl iodide were removed by evaporation with a stream of nitrogen at 60

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°C. The resulting aqueous solution, acidified with dilute HCl to decompose any excess borohydride, was then diluted with water to 2 mL and applied to a 5 × 60 mm column of Dowex-1 X8-200 Cl^-. The column was washed successively with 3 mL of water, 2 mL of 1 M HCl, and 0.5 mL of 3 M HCl, and the methyl derivative of coenzyme M was eluted off with 3 mL of 3 M HCl. The eluted material was then evaporated to dryness, dissolved in a small volume of water, and passed through a small Dowex 50W-8X H^+ (2 × 5 mm) column. Evaporation of the water gave the free acid which was dissolved in 50 μL of methanol, reacted with excess diazomethane in ether, and evaporated to dryness to give the methyl ester.

The coenzyme M derivative was separated on a 0.3 × 183 cm glass column containing 10% SP-2100 on 100/120 mesh Supelcoport (Supelco, Inc.) programmed from 150 °C at 8 °C/min. Under these conditions, the methyl derivative had a retention time of 1.4 min. Mass spectra were recorded at 70 eV by using a Varian MAT 112 mass spectrometer with a source temperature of 200 °C.

Analysis of Isotopic Distributions of the Cellular Constituents. The insoluble pellet remaining after extraction of the coenzyme M was used to isolate the bound sugars and amino acids present in the cells. The isotopic distributions of the ^2H or ^{13}C in the bound amino acids were determined by gas chromatography–mass spectrometry (GC–MS) of the *N*-(trifluoroacetyl) and *N,O*-(trifluoroacetyl) *n*-butyl ester derivatives of the amino acids and the *S*-methyl-*N*-(trifluoroacetyl) *n*-butyl ester of cysteine. The preparation of these derivatives from the bound amino acids released by the 6 M HCl hydrolysis of the dialyzed pellet previously alkylated with methyl iodide has been described by White (1981). The sugars were assayed by GC–MS of the trifluoroacetyl derivatives of their *n*-butyl glycosides. These derivatives were prepared from the glucose and *N*-acetylglucosamine released from the insoluble pellet by hydrolysis with 1 M HCl at 100 °C for 1 h. After centrifugation to obtain a clear extract, the resulting solution was desalted by passing it through a column of Dowex 50W-X8 H^+ and Dowex-1 X8-200 OH^-. After removal of the water, the sugars were converted to their *n*-butyl glycosides by heating with 3 M HCl in 1-butanol at 100 °C for 1 h. This process also resulted in the deacetylation of the *N*-acetylglucosamine to glucosamine. After removal of the excess 1-butanol by evaporation with nitrogen, the sugars were converted into their tetra(trifluoroacetyl) derivatives with trifluoroacetic anhydride in methylene chloride (1:1). The glucose and glucosamine derivatives had molecular weights of 620 and 619, respectively. They were readily separated by gas chromatography using a 0.3 × 122 cm column packed with 3% SP-2100 on 100/120 mesh Supelcoport programmed from 100 °C at 10 °C/min.

The label incorporated into these bound amino acids and sugars by the growing cells should reflect the average isotopic distribution of the free amino acids and sugars which were present in the cells over the entire growth of the bacteria. This incorporation is expressed as an isotopic distribution and is recorded as the mole percent of the molecular or fragment ions containing a given number of enriched atoms. These observed isotopic distributions or abundances in the derivatives are calculated from the experimentally observed isotopic ion intensities measured from the molecular or fragment ions. The isotopic ion intensities are corrected for the natural abundances of ^{13}C, ^{17}O, ^{18}O, ^{33}S, ^{34}S, and ^{15}N by subtracting the experimentally observed isotopic ion intensities which are determined from unenriched samples. The details of this procedure have been discussed by Biemann (1962).

In order to take into account the isotopic fractionation that occurs during the gas chromatography of the deuterated compounds, six to eight evenly spaced mass spectra were obtained of the desired peak as it eluted from the gas chromatograph. The ion intensity for each major ion of interest in each scan was measured, summed, and normalized to obtain the experimentally observed isotopic ion intensities reported in this paper. The variation in these isotopic ion intensities on repeated analyses of the same compound were always within 1%. Atom percent abundance of sulfur-34 was measured with a standard deviation of less than 1 atom % (White, 1981).

These ion intensity measurements can be taken from any ion in the mass spectra of these derivatives that contains all of the isotopically labeled atoms of interest. However, due to interference from other ions in the GC–MS runs, the lack of retention of all of the label in the fragment, and the low intensity of many of the ions in the mass spectra of the amino acids or sugars, many of these ions were not suitable for accurate intensity measurements. Accurate measurements of the isotopic abundances could, however, be obtained from the m/z 61 ($CH_3SCH_2^+$) ion for the cysteine derivative (White, 1981) and from many of the well-characterized fragmentation ions of the amino acid derivatives (Gelpi et al., 1969). The specific ions used in this work are recorded in the tables. They have the advantage, in many cases, of allowing one to determine both the extent and position of label incorporation. For example, the $M^+ - COOC_4H_9$ fragment in the mass spectrum of alanine grown with [1,2-$^2C_2$]acetate has the same distribution of ^{13}C as the $M^+ - OC_4H_9$ fragment which retains the carboxyl carbon, thereby proving that both the acetate carbons are in the C-2 and C-3 of the alanine.

Label incorporation into glucose and glucosamine was measured from the $M^+ - (CF_3COOH + C_4H_9OCHO)$ ions of their derivatives at 404 and 403, respectively. These ions were used not only because of their high intensity but also because they have specifically lost the C-1 proton and any incorporated deuterium. (Since label from [2,2,2-$^2H_3$]acetate should be incorporated into both the C-6 and C-1 position of the glucose by gluconeogenesis, this ion allows label specifically incorporated into the C-6 of the glucose to be measured.) This mode of fragmentation was confirmed in that the 404 and 403 ions in the mass spectrum of the derivative of [1-$^2H_1$]glucose and [1-$^2H_1$]glucosamine were unchanged.

The mass spectral fragmentation of the coenzyme M derivative can also be used to determine the extent and position of label incorporation. By comparing the extent of label in the m/z 74 fragment ($CH_3SCH{=}CH_2^+\cdot$) of the coenzyme M derivative which has lost a C-2 proton with that in the molecular ion, we can determine if deuterium is incorporated at this carbon. In addition, by measuring the deuterium in the m/z 61 fragment ($CH_3SCH_2^+$), we can independently establish if deuterium was incorporated at C-2. A complete discussion of the fragmentation of this molecule has been presented by Lovley et al. (1984b).

Compound Synthesis. Sodium [^{34}S]sulfite was prepared from [^{34}S]sulfur dioxide which was generated by the O_2 combustion of elemental $^{34}S_8$ in a quartz tube at 900 °C. Iodine titration of the sulfur dioxide absorbed in aqueous base showed that >95% of the sulfur could be recovered as sulfite. [$^2H_4$]Taurine was prepared by the reaction of sodium bromo[$^2H_4$]ethanesulfonate with aqueous ammonia (Marvel & Bailey, 1943). The labeled bromoethanesulfonate was prepared by the reaction of [$^2H_4$]ethylene dibromide with sodium sulfite as described by Marvel & Sparberg (1943). Ammonium [$^2H_4$]isethionate was prepared from bromo[$^2H_4$]ethanol

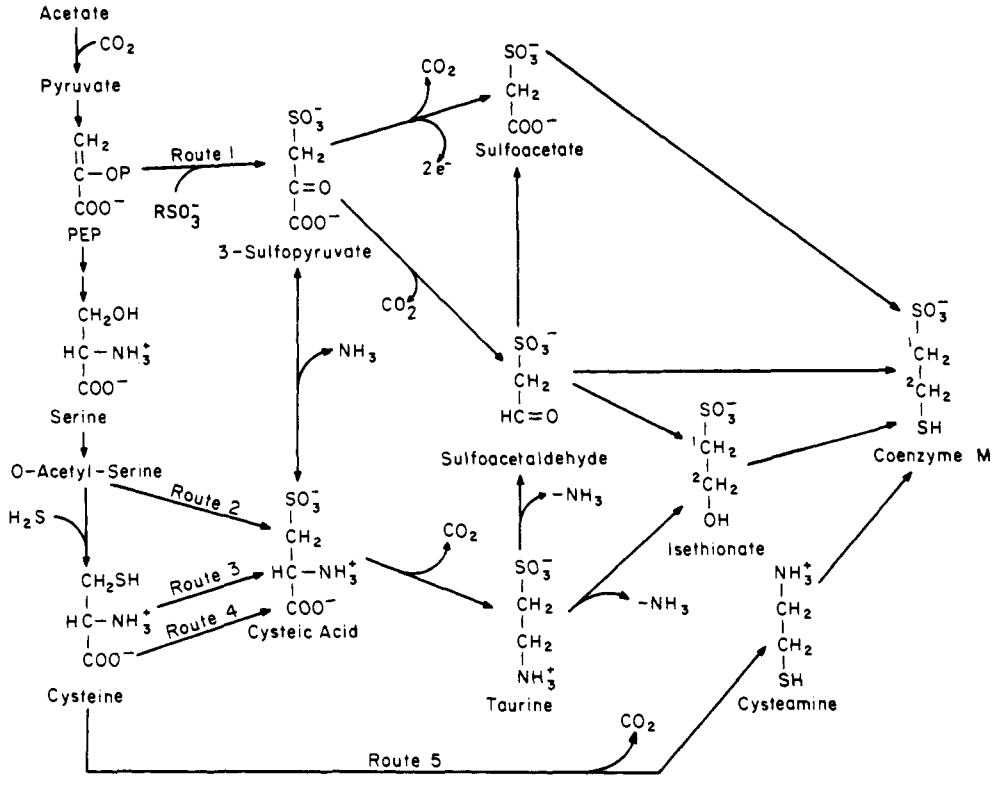

FIGURE 1: Possible pathways for the biosynthesis of coenzyme M. The exact structure of R in RSO_3^- is unknown, but it could be a hydrogen or a bound thiol.

by reacting 1 g of this material with 6 mL of water containing 2 g of sodium sulfite. After the reaction was heated at 70 °C for 4 h, a small amount of water was added to dissolve the excess sulfite. The resulting solution was freed of sodium ions by passing it through a column of Dowex 50W-X8 H^+ and evaporated in vacuo to remove water and volatile acids. The resulting syrup was dissolved in 1 mL of water, brought to neutral pH by aqueous ammonia, and filtered, and the isethionate crystallized as the ammonium salt (0.87 g) by the addition of 5 volumes of ethanol. Mass spectral analysis using both the free acid and the methyl ester established the molecule to contain four deuteriums. DL-Sulfo[3,3-$^2H_2$]lactic acid and L-[*sulfonic*-$^{18}O_2$]sulfolactic acid were prepared by nitrous acid deamination of DL-[3,3-$^2H_2$]cysteic acid and L-[*sulfonic*-$^{18}O_2$]cysteic acid, respectively, as described by Izumi et al. (1971). L-[*sulfonic*-$^{18}O_2$]Cysteic acid was prepared by the oxidation of cystine in ^{18}O-labeled water with bromine (R. H. White, 1984, unpublished experiments). Sulfo[2,2-$^2H_2$]acetic acid was prepared by the reaction of deuterated sulfuric acid with [$^2H_6$]acetic anhydride in [$^2H_4$]acetic acid (Jeffery & Satchell, 1962).

Results and Discussion

On the basis of previously described work related to the metabolism of compounds with structural features similar to those of coenzyme M, there appear to be five possible routes for the biosynthesis of coenzyme M, each originating from a three-carbon metabolite derived from pyruvate (Figure 1). Each route ultimately requires the incorporation of two of the pyruvate methyl protons into either the C-1 or C-2 of the coenzyme M. In route 1, phosphoenolpyruvate (PEP) reacts with sulfite to generate sulfopyruvate which could then be converted into coenzyme M by one of the several possible pathways outlined. One pathway involves the oxidative decarboxylation of 3-sulfopyruvate to sulfoacetate and subsequent reduction of the sulfoacetate to isethionate via sulfoacetaldehyde. Replacement of the hydroxyl group of isethionate by a thiol would then produce coenzyme M. A second possible pathway involves the transamination of the 3-sulfopyruvate to cysteic acid which is then converted into coenzyme M as discussed below for routes 2 and 3.

Routes 2 and 3 have similar chemistries starting with the substitution of the C-3 group of serine, *O*-acetylserine, or cysteine by sulfite to form cysteic acid. Evidence for the occurrence of route 2 in nature comes from the work of Sass & Martin (1972) on an enzyme system in chick liver which converts serine directly into cysteic acid. Evidence for the occurrence of route 3 in nature comes, in part, from the characterization by Tolosa et al. (1968) of a pyridoxal-dependent enzyme present in the yolk sac, cysteine lyase, which catalyzes the formation of cysteic acid from cysteine and sulfite. The cysteic acid produced by either of these routes could be converted into isethionate via either taurine or sulfopyruvate as outlined in Figure 1. Conversion of the hydroxyl group of the isethionate to a thiol would lead to coenzyme M.

A fourth possible route from cysteine to coenzyme M involves the oxidation of cysteine to cysteic acid. This route has been studied in higher organisms and is known to require molecular oxygen (Lombardini et al., 1969). Since molecular oxygen is not present during the growth of methanogens, this oxidation cannot proceed in this manner but could possibly occur with an alternate oxidizing agent. Decarboxylation of cysteine to cystamine and conversion of the amino group of the cystamine to a thiol is another possible route to coenzyme M (route 5).

The determination of the operative pathway was established by using the experimental method referred to by this author as the in vivo stable isotope metabolic labeling technique. Basically, in this method, a microorganism is allowed to grow on an enriched, stable isotope-containing substrate which represents a major part of its total carbon source. During growth, the isotope in this substrate will be incorporated

Table I: Incorporation of Stable Isotope-Labeled Compounds into Coenzyme M by Strain 10-16B

	distribution of ^2H or ^{13}C in							
	$[CH_3SCH{=}CH_2]^{+\cdot}$, m/z 74 ion				$[CH_3SCH_2CH_2SO_3CH_3]^{+\cdot}$, m/z 170 ion			
precursor fed	0	1	2	3	0	1	2	3
[2,2,2-$^2H_3$]acetate[a]	58.1 (100)[b]	18.8 (54.8)	20.9 (48.2)	2.1 (16)	58.8 (100)	17.6 (37.7)	21.1 (46.5)	2.4 (10.6)
[1,2-$^{13}C_2$]acetate[c]	85.0 (100)	0.9 (23.4)	14.0 (21.8)		85.6 (100)	0 (7.8)	14.4 (25.1)	

[a] No label was incorporated into the m/z 61 fragment of the coenzyme M derivative from this compound. [b] The numbers in parentheses are the observed normalized ion intensities. [c] The acetate contained 15.25 atom % of the molecules with two ^{13}C.

Table II: Incorporation of [2,2,2-$^2H_3$]Acetate into Cellular Compounds by Strain 10-16B

	distribution of ^2H					
compound	0	1	2	3	4	ion used for measurement
alanine	58.4 (100)[b]	15.3 (68.2)	14.1 (38.0)	12.2 (31.7)		m/z 140 $M^+ - COOC_4H_9$
	56.4 (100)	16.2 (33.1)	14.6 (27.2)	12.9 (23.9)		m/z 186 $M^+ - C_4H_7$
cysteine[a]	77.8 (100)	10.9 (17.9)	11.4 (21.7)			m/z 61 $CH_3SCH_2^+$
serine	47.2 (100)	14.6 (41.5)	38.2 (87.1)			m/z 298 $M^+ - COOC_4H_9$
phenylalanine	26.5 (100)	35.3 (142.1)	25.1 (107.8)	10.2 (48.3)	2.8 (11.1)	m/z 91 $C_6H_5CH_2^+$
glucosamine	40.1 (100)	23.9 (78.2)	33.0 (96.8)	3.0 (24.6)		m/z 403 $M^+ - (CF_3COOH + C_4H_9OCHO)$
glucose	28.9 (100)	20.8 (90.0)	46.2 (176.1)	4.1 (45.2)		m/z 404 $M^+ - (CF_3COOH + C_4H_9OCHO)$

[a] Measured from the protein-bound cysteine after conversion into its *S*-methyl derivative as described by White (1981). [b] The numbers in parentheses are the observed normalized ion intensities.

unevenly into the different cellular components, including the coenzymes, on the basis of the biosynthetic pathway(s) of each. After growth, a combination of GC–MS and chemical procedures is used to establish both the position and extent of isotope incorporation into the coenzymes and other cellular molecules. By use of this information, conclusions can be reached as to the pathways and precursors used by the cells, in vivo, for the biosynthesis of any given coenzyme.

This approach was used by the author to outline the biosynthetic pathways of thiamin (White, 1978), lipoic acid (White, 1980a), and fatty acids (White, 1980b) in *Escherichia coli*. This basic approach was also used by Csonka (1977) and Csonka & Fraenkel (1977) to determine the in vivo pathways for amino acid and NADPH biosynthesis in *E. coli* using ^3H- and ^{14}C-labeled compounds and by Ekiel et al. (1983, 1984) to determine the biosynthetic pathways in methanogenic bacteria using ^{13}C NMR to determine the extent and position of label incorporation into the biosynthetic products.

Data obtained by applying these principles to the biosynthesis of coenzyme M are reported in Tables I and II. The coenzyme M isolated from cells of strain 10-16B grown with [2,2,2-$^2H_3$]acetate contained ~18% of the molecules with one ^2H and 21% with two ^2H. This was determined from the observed isotopic incorporations reported for both the m/z 74 and m/z 170 ions. Since both of these ions had the same abundance of deuterium and since the m/z 61 fragment was found to contain no deuterium, all of these deuteriums must reside on C-1 of the coenzyme M. The cysteine in these cells, however, contained only 10.9 and 11.4%, respectively, of the molecules with one and two deuteriums on the C-3 carbon (Table II). Thus, a biosynthetic route originating from cysteine (i.e., routes 3–5, Figure 1) can clearly be eliminated since a much higher amount of deuterium is found in the coenzyme M than is present in the cellular cysteine. [The reduction in the amount of label observed in cysteine from that observed in its biosynthetic precursor serine (Table II) can be explained by the uptake and utilization by the cells of some of the unlabeled cysteine which is present in the medium at a concentration of 2.8 mM.] Further support for the elimination of route 5 comes from the fact that it requires the labeled cysteine C-3 carbon to be incorporated into the C-2 of the coenzyme M, and from the data in Table I, it is clear that no label is found on this carbon. Route 2 from serine can be eliminated since serine (Table II) was found to be much more highly labeled than the coenzyme M (Table I), and pathways originating from carbohydrates can be eliminated since the C-6 of both glucosamine and glucose is more highly labeled than is the C-1 of coenzyme M (Table II).

Thus, only route 1 agrees with the data and is a plausible scheme for the first committed step in the biosynthesis of coenzyme M. In route 1, the deuteriums from [2,2,2-$^2H_3$]-acetate are incorporated only on the C-1 carbon of the coenzyme M (Figure 1) which is the exact labeling pattern one would expect when PEP is formed by the combined action of acetate thiokinase, pyruvate synthase, and PEP synthase with the fed acetate and CO_2. All of these reactions have been described in methanogenic bacteria (Fuchs & Stupperich, 1984). The occurrence of coenzyme M with either no deuterium or one deuterium can be explained by the chemical and/or metabolic exchange of the pyruvate methyl protons during the formation and/or metabolism of pyruvate. That this exchange did not occur from acetate or acetyl coenzyme A was confirmed in that the methyl groups in squalene, which are derived by the cells from the methyl groups of acetate via acetyl coenzyme A (Ekiel et al., 1983), were found to be completely deuterated (data not presented). The exchange of the pyruvate methyl protons can be confirmed by using the label found in alanine, which is derived directly from pyruvate by a transamination reaction with no exchange of the methyl protons, as an indicator of the extent of labeling of the pyruvate methyl group in vivo. It is clear from the alanine data (Table II) that extensive exchange and dilution of label occurred in the pyruvate methyl. The exchange of pyruvate methyl protons has also been observed, although to a lesser extent, in *E. coli* (White, 1978).

The expected labeling pattern of PEP can be calculated from the alanine data if the one proton or deuterium of pyruvate, which must be removed in the generation of PEP, is assumed to be random. This distribution was calculated to be 61.9% $^2H_2$, 20.2% $^2H_1$, and 17.4% $^2H_2$, which is almost identical with the distribution observed in the coenzyme M. This is very strong evidence that either PEP or pyruvate is a precursor of coenzyme M.

The reduction in the amount of deuterium in the pyruvate methyl results in a reduction in the efficiency at which deuteriums can be incorporated from acetate into the other cellular compounds. This decreased efficiency of label incorporation will not be a problem with ^{13}C-labeled acetate. In addition,

Table III: Incorporation of [1,2-$^{13}C_2$]Acetate into Cellular Compounds by Strain 10-16B

compound	distribution of ^{13}C 0	1	2	3	ion used for measurement
alanine	84.5 (100)[b]	0.9 (43.1)	14.6 (20.7)		m/z 140 $M^+ - COOC_4H_9$
	85.0 (100)	0.6 (5.1)	14.4 (16.9)		m/z 186 $M^+ - C_4H_7$
cysteine[a]	100 (92.7)	11.8 (7.3)	4.4 (0)		m/z 61 $CH_3SCH_2^+$
phenylalanine	60.3 (100)	15.0 (33.9)	20.3 (36.9)	4.4 (11.5)	m/z 91 $C_6H_5CH_2^+$
	61.3 (100)	7.6 (27.1)	28.0 (49.2)	3.1 (11.0)	m/z 204 $M^+ - CF_3CONH_2$

[a] Measured from the protein-bound cysteine after conversion into its *S*-methyl derivative as previously described by White (1981). [b] The numbers in parentheses are the observed normalized ion intensities.

by feeding a mixture of unlabeled acetate and acetate containing two ^{13}C, we would be able to determine if the acetate is incorporated into the coenzyme as a unit. The results of the [1,2-$^{13}C_2$]acetate feeding (Table I) confirmed both the increased incorporation of ^{13}C-labeled acetate and the incorporation of acetate as a unit in that about 14% of the coenzyme M contained two ^{13}C. Since the labeled acetate contained 15.25 atom % of the molecules with two ^{13}C, it was possible to calculate from the coenzyme M m/z 74 and 170 isotopic ion intensity data that the acetate was incorporated as a unit into coenzyme M to the extent of 94.0 and 96.6%, respectively. This high incorporation was also confirmed by the alanine data (Table III) which show that 95.7% of the C-2 and C-3 carbons of the alanine are derived from acetate as a unit.

The labeling pattern of the PEP can also be measured by determining the extent of labeling in the aromatic amino acids which are known to be derived from erythrose-4-P and two PEP. On the basis of work done with *Escherichia coli* by Csonka (1977) and with *Methanospirillum hungatei* GP1 by Ekiel et al. (1983), it is expected that a single proton from the C-3 of one PEP and a single proton from one of the C-4 protons of erythrose-4-P will each be incorporated into one of the two ortho positions of the aromatic ring and that two protons from the other PEP will be incorporated at the C-3 of the aromatic amino acids. Since all of these positions are contained in the m/z 91 tropylium ion derived from the phenylalanine derivative, we can use the deuterium incorporation into this ion to measure the total amount of deuterium incorporated into the phenylalanine from the above labeled precursors. The labeling pattern of this ion should correspond to that calculated from the expanded values of the polynomial

$$(a + b + c)(x + y)(l + m) \quad (1)$$

where a, b, and c are the normalized abundances of deuterium on the C-3 of the PEP, x and y are the normalized abundances of the incorporated single deuterium on the C-4 position of erythrose-4-P, and l and m are the normalized abundances for the incorporated single deuterium on the C-3 of PEP. The a, b, and c values should be exactly the same as those found for coenzyme M (i.e., 0.58, 0.19, and 0.21, respectively) if the coenzyme M is, in fact, derived from PEP. The x and y values can be calculated from the glucosamine data, assuming that the C-4 of erythrose-4-P is labeled to the same extent as the C-6 of glucosamine and assuming that we have a random loss of one of the C-6 protons. From the data in Table II, these values were calculated to be 0.53 and 0.46, respectively, for x and y. Likewise, the l and m values can be generated from the coenzyme M values, assuming the random loss of a single proton. These values were calculated to be $l = 0.69$ and $m = 0.31$. Substitution of these values into eq 1 gave a calculated distribution for the m/z 91 ion of 27.2% $^2H_0$, 33.8% $^2H_1$, 23.5% $^2H_2$, 10.8% $^2H_3$, and 2.1% $^2H_4$. This distribution is almost identical with that observed for the phenylalanine m/z 91 data (Table II) and adds support to the theory that the label in PEP is the same as that found in the coenzyme M.

The extent of incorporation of the $^{13}C_2$-labeled acetate, which is incorporated into the C-2 and C-3 positions of phenylalanine via PEP, can be determined by measuring the difference between the distribution of ^{13}C in the m/z 91 and 204 ions of the phenylalanine derivative. This is possible since one ^{13}C from this $^{13}C_2$-labeled acetate would be incorporated at C-3 of the phenylalanine and would, therefore, be retained in the m/z 91 ion, and the other ^{13}C is incorporated at C-2 so that both ^{13}C from this $^{13}C_2$-labeled acetate would be retained in the m/z 204 ion. This results in a decrease in the number of ions containing two ^{13}C and an increase in the number of ions containing one ^{13}C in the m/z 91 ion as compared to the 204 ion (Table III). If the PEP and sugar used in the biosynthesis of the phenylalanine have the same ^{13}C abundances as the alanine or coenzyme M, then we can calculate that the intensity of the ion + 2 m/z should decrease 12% in going from the m/z 204 to the m/z 91 ion. The value observed (Table III) is 11.2%, indicating that the PEP is labeled, within experimental error, to the same extent as the coenzyme M.

Experiments with *Methanobacterium formicicum* and strain TM-1 were conducted as described above for strain 10-16B. Similar results were obtained with these organisms, further supporting PEP as the most likely precursor for the formation of coenzyme M. In the case of TM-1, however, the label incorporated into the coenzyme M by cells grown with [2,2,2-$^2H_3$]acetate was slightly less than that incorporated into the alanine or PEP, which is probably a result of the exchange of deuteriums from the labeled sulfoacetaldehyde and/or sulfopyruvate intermediate(s) involved in coenzyme M biosynthesis. This exchange is accelerated in slow-growing thermophiles like TM-1, which allows these intermediates to exist for longer times and at higher temperatures in the cells. (The rapid exchange of the protons of sulfoacetaldehyde and sulfopyruvate in water at 23 °C at pH 7.0 has been observed by the author.)

It is possible that another compound with the same label distribution as PEP could be the required intermediate in the first step of coenzyme M biosynthesis. This compound would, of course, go completely undetected by the methods used here. The final proof for the involvement of PEP in coenzyme M biosynthesis must await the isolation of the specific enzymatic reactions involved in the formation of coenzyme M.

Further support for route 1 comes from the observation that when DL-sulfo[3,3-$^2H_2$]lactic acid (3.8 mM), $H^{34}SO_3^-$ (1.7 mM), or $^{34}SO_4^{2-}$ (4.0 mM) is fed to strain 10-16B in the minimal medium at the levels indicated, they are incorporated into coenzyme M to the extent of 3.2%, 3.1%, and 0%, respectively. These results confirm that a three-carbon sulfonic acid, which can be converted into sulfopyruvate by a single oxidation, and sulfite are incorporated into coenzyme M, whereas sulfate is not. Since sulfite is not a normal medium component, both of the sulfurs of the coenzyme M must arise from either the sulfide or cysteine present in the medium.

There are two likely mechanisms for the formation of

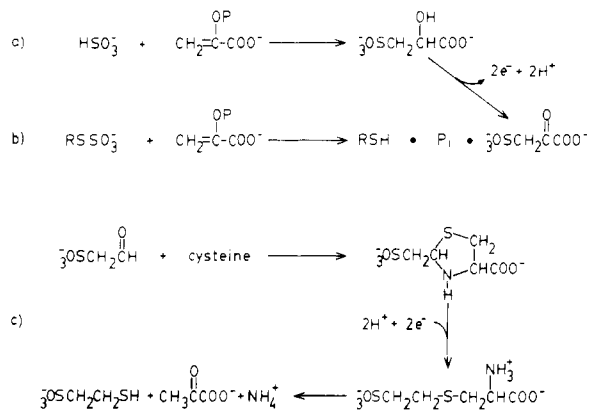

FIGURE 2: Proposed reactions for the incorporation of sulfur into coenzyme M. Both reactions a and b produce sulfopyruvate but in reaction a, a two-electron oxidation of the intermediate, sulfolactic acid, is required in order to produce sulfopyruvate.

sulfopyruvate from PEP (Figure 2). In Figure 2b, the anion of pyruvate, generated during the hydrolysis of PEP, would react with an activated sulfate to generate sulfopyruvate. This reaction would be analogous in many ways to the reaction of the enol form of pyruvate with bicarbonate catalyzed by PEP carboxylase (Utter & Kolenbrander, 1972) and the formation of phosphonopyruvic acid from PEP during the biosynthesis of the phosphonic acids (Horiguchi & Rosenberg, 1978). The activated sulfate could be 3′-phospho-5′-adenylyl sulfate or, more likely, since sulfate was found not to be incorporated, a bound thiosulfonate similar to that described in *Chlorella* by Schmidt et al. (1974). This mechanism has been postulated to be involved in the biosynthesis of the plant sulfolipid component 6-sulfoquinovose (Davies et al., 1966) and in the first step of the sulfoglycolytic pathway proposed by Benson (1963). In Figure 2a, bisulfite addition to the double bond of PEP would generate sulfolactic acid which could then be oxidized to sulfopyruvate. This mechanism is supported by the observed incorporation of labeled sulfolactic acid into coenzyme M. Chemical evidence for this mechanism comes from the ready addition of bisulfite to methyl 6-deoxyhex-5-enopyranosides to give the corresponding hexopyranoside-6-sulfonic acids (Lehmann & Benson, 1964; Lehmann & Weckerle, 1975).

In an attempt to determine the intermediates in the conversion of sulfopyruvate to coenzyme M, cells were grown in the presence of labeled, possible intermediates, and the extent of label incorporation into the coenzyme M was measured. Strain 10-16B was grown on the minimal medium containing either 1.05 mM DL-[3,3-$^2H_2$]cysteic acid, 1.05 mM *L*-[*sulfonic*-$^{18}O_2$]cysteic acid, 2.8 mM [2,2-$^2H_2$]sulfoacetic acid, 1.55 mM [1,1,2,2-$^2H_4$]taurine, or 1.36 mM [1,1,2,2-$^2H_4$]isethionate. In each case, not more than 0.5% of the coenzyme M was found to be derived from the labeled compound. This would indicate either that the compounds were not taken up by the cells or that these compounds are not intermediates in the formation of coenzyme M.

A biosynthetic pathway for coenzyme M, which is consistent with all of the data presented here, would involve the formation of sulfopyruvate from sulfite and PEP as discussed above followed by the nonoxidative decarboxylation of sulfopyruvate to sulfoacetaldehyde by a mechanism similar to that described for pyruvate decarboxylase by Krampitz et al. (1961). With this enzyme, pyruvate is nonoxidatively decarboxylated to give acetaldehyde via a (hydroxyethyl)thiazolium derivative of thiamin pyrophosphate. An analogous reaction with 3-sulfopyruvate would lead to the generation of sulfoacetaldehyde, a compound known to be involved in the bacterial metabolism of isethionate (Kondo et al., 1977) and taurine (Kondo et al., 1973; Shimamoto & Berk, 1980).

The generation of sulfoacetaldehyde from sulfopyruvate by a nonoxidative decarboxylation would be a much more favorable reaction than an oxidative decarboxylation in that the product of a nonoxidative reaction has a more reduced C-2 carbon; i.e., it is biosynthetically closer to coenzyme M. This logic, as well as the lack of any observed incorporation of sulfoacetic acid and the observed cell-free conversion of sulfoacetaldehyde, but not sulfoacetic acid or isethionate, into coenzyme M (R. H. White, 1984, unpublished experiments), strongly favors sulfoacetaldehyde as the intermediate in the biosynthesis of coenzyme M. This unpublished work also demonstrated that the thiol sulfur of coenzyme M originates from cysteine via the thiazolidine intermediate (Figure 2c), which represents a completely new method for the formation of C–S bonds in nature.

Since the methanogenic bacteria represent a very diverse group of microorganisms, the question arises as to the existence of a universal pathway for coenzyme M biosynthesis for all of these different bacteria. The data presented here indicate a common pathway for the three different species of methanogenic bacteria examined. Whether this represents a universal pathway can only be proven by testing a larger, more diverse set of these microorganisms.

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Registry No. AcOH, 64-19-7; SO_3^{2-}, 14265-45-3; coenzyme M, 45127-11-5; sulfolactic acid, 38769-05-0.

REFERENCES

Balch, W. E., & Wolfe, R. S. (1976) *Appl. Environ. Microbiol. 32*, 781–791.

Balch, W. E., & Wolfe, R. S. (1979) *J. Bacteriol. 137*, 256–263.

Benson, A. A. (1963) *Adv. Lipid Res. 1*, 387–394.

Biemann, K. (1962) *Mass Spectrometry*, McGraw-Hill, New York.

Csonka, L. N. (1977) *J. Biol. Chem. 252*, 3392–3398.

Csonka, L. N., & Fraenkel, D. G. (1977) *J. Biol. Chem. 252*, 3382–3391.

Davies, W. H., Mercer, E. I., & Goodwin, T. W. (1966) *Biochem. J. 98*, 369–373.

Ekiel, I., Smith, I. C. P., & Sprott, G. D. (1983) *J. Bacteriol. 156*, 316–326.

Ekiel, I., Smith, I. C. P., & Sprott, G. D. (1984) *Biochemistry 23*, 1683–1687.

Escalante-Semerena, J. C., Leigh, J. A., & Wolfe, R. S. (1984) in *Microbial Growth on C_1 Compounds* (Crawford, R. L., & Hanson, R. S., Eds.) pp 191–198, American Society for Microbiology, Washington, DC.

Fuchs, G., & Stupperich, E. (1984) in *Microbial Growth on C_1 Compounds* (Crawford, R. L., & Hanson, R. S., Eds.) pp 199–202, American Society for Microbiology, Washington, DC.

Gelpi, E., Koenig, W. A., Gilbert, J., & Oró, J. (1969) *J. Chromatogr. Sci. 7*, 604–613.

Horiguchi, M., & Rosenberg, H. (1975) *Biochim. Biophys. Acta 404*, 333–340.

Izumi, Y., Yajima, S., Okubo, K., & Babievsky, K. K. (1971) *Bull. Chem. Soc. Jpn. 44*, 1416–1417.

Jeffery, E. A., & Satchell, D. P. N. (1962) *J. Chem. Soc. 1962*, 1876–1887.

Kondo, H., Kagotani, K., Oshima, M., & Ishimoto, M. (1973) *J. Biochem. (Tokyo) 73*, 1269–1278.
Kondo, H., Nike, H., Takahashi, S., & Ishimoto, M. (1977) *J. Biochem. (Tokyo) 81*, 1911–1916.
Krampitz, L. O., Suzuki, I., & Greull, G. (1961) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 20*, 971–977.
Lehmann, J., & Benson, A. A. (1964) *J. Am. Chem. Soc. 86*, 4469–4472.
Lehmann, J., & Weckerle, W. (1975) *Carbohydr. Res. 42*, 275–295.
Lombardini, J. B., Singer, T. P., & Boyer, P. D. (1969) *J. Biol. Chem. 244*, 1172–1175.
Lovley, D. R., Greening, R. C., & Ferry, J. G. (1984a) *Appl. Environ. Microbiol. 48*, 81–87.
Lovley, D. R., White, R. H., & Ferry, J. G. (1984b) *J. Bacteriol. 160*, 521–525.
Marvel, C. S., & Bailey, C. F. (1943) *Organic Syntheses*, Collect. Vol. II, pp 563–565, Wiley, New York.
Marvel, C. S., & Sparberg, M. S. (1943) *Organic Syntheses*, Collect. Vol. II, pp 558–559, Wiley, New York.
McBride, B. C., & Wolfe, R. S. (1971) *Biochemistry 10*, 2317–2324.
Sass, N. L., & Martin, W. G. (1972) *Proc. Soc. Exp. Biol. Med. 139*, 755–761.
Schmidt, A., Abrams, W. R., & Schiff, J. A. (1974) *Eur. J. Biochem. 47*, 423–434.
Shimamoto, G., & Berk, R. S. (1980) *Biochim. Biophys. Acta 632*, 121–130.
Taylor, C. D., & Wolfe, R. S. (1974) *J. Biol. Chem. 249*, 4879–4885.
Tolosa, E. A., Goryachenkova, E. V., Khomutov, R. M., & Severin, E. S. (1968) in *Pyridoxal Catalysis: Enzymes and Model Systems* (Snell, E. E., Braunstein, A. E., Severin, E. S., & Torchinsky, Yu. M., Eds.) pp 525–535, Interscience Publishers, New York.
Utter, M. F., & Kolenbrander, H. M. (1972) *Enzymes, 3rd Ed. 6*, 117–136.
White, R. H. (1978) *Biochemistry 17*, 3833–3840.
White, R. H. (1980a) *Biochemistry 19*, 9–15.
White, R. H. (1980b) *Biochemistry 19*, 15–19.
White, R. H. (1981) *Anal. Biochem. 114*, 349–354.
Zinder, S. H., & Mah, R. A. (1979) *Appl. Environ. Microbiol. 38*, 996–1008.

Photoaffinity Labeling of Peptide Binding Sites of Prolyl 4-Hydroxylase with *N*-(4-Azido-2-nitrophenyl)glycyl-(Pro-Pro-Gly)$_5$†

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ABSTRACT: The synthesis is described of the photoaffinity label *N*-(4-azido-2-nitrophenyl)glycyl-(Pro-Pro-Gly)$_5$ for the peptide binding site of prolyl 4-hydroxylase. The photoaffinity label is a good substrate and is capable of light-induced inactivation of prolyl 4-hydroxylase activity. Inactivation depends on the concentration of photoaffinity label and is prevented by competition with excess (Pro-Pro-Gly)$_5$. Two moles of photoaffinity label per mole of enzyme is needed for 100% inactivation of enzymic activity. Oxidative decarboxylation of 2-oxoglutarate measured in the absence of added peptide substrate is not affected by labeling. We conclude that the covalently bound nitreno derivative of *N*-(4-azido-2-nitrophenyl)glycyl-(Pro-Pro-Gly)$_5$ acts by preventing the binding of peptide substrate to the catalytic site without interfering with the binding of the other substrates and cofactors 2-oxoglutarate, O_2, Fe^{2+}, and ascorbate. Labeling is specific for the α subunit of the tetrameric $\alpha_2\beta_2$ enzyme. In addition to two catalytic binding sites that are blocked by the photoaffinity label, the enzyme contains binding subsites for peptide substrates, as judged from the capability of photoinactivated enzyme to bind to a poly(L-proline) affinity column. These binding subsites may account for the rapidly increasing affinity for peptide substrates with increasing chain length.

Prolyl 4-hydroxylase [prolyl-glycyl-peptide, 2-oxoglutarate: oxygen oxidoreductase (4-hydroxylating), EC 1.14.11.2] catalyzes the posttranslational modification of certain peptidyl proline residues in nascent pro-α chains of procollagen. This modification results, with slight differences in the various types of collagen, in the formation of approximately 100 hydroxyproline residues per pro-α chain. Hydroxylation of proline residues is essential for the thermal stability of the triple helical structure of the end product collagen (Berg & Prockop, 1973).

The enzyme has as substrates 2-oxoglutarate, molecular oxygen, and a peptide containing one or more -X-Pro-Gly- sequences (X denoting any amino acid but glycine), and it requires ferrous ions and ascorbate. One atom of the oxygen is used for the oxidative decarboxylation of 2-oxoglutarate to succinate (Rhoads & Udenfriend, 1968). The incorporation of the other atom into proline results in the formation of *trans*-4-hydroxyproline. The hydroxylation reaction is most likely via a ferryl complex as the oxygen-transferring intermediate (Siegel, 1979; Hanauske-Abel & Günzler, 1982; de Jong & Kemp, 1984). The reaction sequence resulting in hydroxylation of proline does not require the reducing agent ascorbate. However, in addition to the complete reaction in

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