The hyperthermophilic euryarchaeon Methanococcus jannaschii uses coenzyme M (2-mercaptoethanesulfonic acid) as the terminal methyl carrier in methanogenesis. We describe an enzyme from that organism, (2R)-phospho-3-sulfolactate synthase (ComA), that catalyzes the first step in coenzyme M biosynthesis. ComA catalyzes the stereospecific Michael addition of sulfite to phosphoenolpyruvate over a broad range of temperature and pH conditions. Substrate and product analogs moderately inhibited activity. This enzyme has no significant sequence similarity to previously characterized enzymes; however, its Mg²⁺-dependent enzyme reaction mechanism may be analogous to one proposed for enolase. A diverse group of microbes and plants have homologs of ComA that could have been recruited for sulfolactate or sulfolipid biosyntheses.

Each year, archaeal methanogens produce >400 million tons of methane by anaerobically reducing acetate or single carbon compounds (1). Regardless of which carbon substrate these cells use for methanogenesis, all methyl intermediates are transiently bound to the terminal methyl carrier coenzyme M (CoM; 2-mercaptoethanesulfonic acid) (2). Methyl-CoM reductase releases methane from the methylated coenzyme, forming a CoM=S=S-coenzyme B heterodisulfide, which is reduced in a key energy-yielding step that makes methanogenesis a viable physiology (3). Alkene-oxidizing bacteria also use CoM as a cofactor (4).

CoM is unique among coenzymes in having a strong anionic sulfonate group. This sulfonate moiety solubilizes CoM, makes it membrane-impermeable, and provides a handle for enzymes such as methyl-CoM reductase to bind (5). Like glutathione, CoM is a soluble scavenger of reactive chemicals, and its simple chemical synthesis has fostered its therapeutic use in humans as a mucolytic and uroprotective agent during chemotherapy (6).

Sulfonate-containing natural products are believed to be biosynthesized either by oxidizing thiol groups or by adding nucleophilic sulfite at unsaturated carbon bonds (7). Taurine and cysteate are produced from cysteine by the oxidative mechanism (8). The nonenzymatic sulfonation of α,β-unsaturated carbonyl compounds is also well known (9, 10). Yet, only recently have the biosyntheses of sulfoquinovose, sulfolactate, and CoM been demonstrated to occur by the sulfite addition mechanism. Plants and photosynthetic bacteria produce UDP-sulfoquinovose, a sulfolipid head group precursor, using SQDI/SqdB proteins to add sulfite to UDP-glucose (11, 12). This reaction may proceed via a UDP-4-ketoglucone-5-ene intermediate (13). Bacillus subtilis produces sulfolactate during sporulation (14), apparently using homologs of CoM biosynthesis proteins (15).

The proposed pathway for the biosynthesis of coenzyme M from phosphoenolpyruvate (P-enolpyruvate) was deduced by analysis of labeling patterns of CoM purified from methanogens grown on stable isotope-labeled acetates (Fig. 1) (15, 16). Incubation of P-enolpyruvate and bisulfite with cell extracts of Methanobacterium formicicum produced sulfolactate, sulfoacrylate, and sulfoacetaldehyde, establishing the role of these sulfo-derivatized intermediates in the pathway (17). Recently, three of the enzymes involved in this pathway (ComB, ComC, and ComDE) have been identified and characterized in a hyperthermopholine marine methanogen, Methanococcus jannaschii (18–20). In this work, we describe the first dedicated enzyme required for CoM biosynthesis in M. jannaschii, (2R)-phospho-3-sulfolactate synthase (ComA). This novel enzyme catalyzes the stereospecific Michael addition of sulfite to P-enolpyruvate, forming 1,2-phospho-3-sulfolactate (PSL). Analyses of metal cofactor requirements, substrate specificity, the proton exchange reaction, a site-directed mutant, and reaction inhibitors suggest that the CoMA-catalyzed reaction is analogous to those reactions catalyzed by β-elimination enzymes that proceed through an enolate intermediate (21).

**EXPERIMENTAL PROCEDURES**

Materials—All reagents and synthetic precursors were purchased from Sigma unless otherwise specified. (RS)-2-Phosphoxypropionic acid (racemic phosphosulfolate), both enantiomers of 2-phospho氧propionic acid (phosphosulfolactate), and both enantiomers of 2-phospho氧butanediol acid (phosphomalate) were synthesized as described previously (18). Hydroxycarbamoylmethylphosphonic acid (phosphoacetohydroxamic acid) (22) and 2-phosphomethacrylic acid (23) were also prepared as described previously. t-Cysteic acid was prepared by the oxidation of t-cystine with bromine (24).

Stereo-specific Syntheses of Sulfolactic Acid—The scheme used to synthesize sulfolactic acid enantiomers was modified from that described previously (25), in which we used formaldehyde to form 2-chloro-3-sulfopropionic acid. To a solution of t- or l-cysteic acid (5 mmol, 0.93 g) dissolved in 6.25 ml of 3.3 M trifluoroacetic acid, isomylmethanol (15.6 mmol, 2.1 ml) was added dropwise with stirring at room temper-
Phosphosulfolactate Synthase from M. jannaschii

Phosphosulfolactate Synthase (ComA) produces (2R)-phospho-3-sulfolactate, which phosphosulfolactate phosphatase (ComB) hydrolyzes to form (R)-sulfolactate (l-sulfolactate) (18). An NAD+-dependent dehydrogenase (ComC) oxidizes (R)-sulfolactate to form sulfonylpyruvate (19). Sulfonylpyruvate decarboxylase (ComDE) produces sulfoacetaldelyde (20), which is reductively thiolated to yield coenzyme M.

![Diagram of Phosphosulfolactate Synthase](http://www.jbc.org/)

The samples. Samples were separated on a compound comprising the first peak has been previously reported (17). Mallinckrodt Chemical Works) dissolved in 1 ml of water, and the deviation from the expected ratio of 1.0. The second corresponding to the eluted as a single peak, but the dimethyl derivatives were separated showed the presence of only the S-isomer, whereas the (R)-sulfolactate had an enantiomeric ratio of 83:17 R-isomer-S-isomer.

Analysis of Sulfolactates—Sulfolactate samples were converted into volatile methyl derivatives for gas chromatography (17). Samples (10–100 μg) were dissolved in 100 μl of water and passed through a Dowex AG 50-H⁺ (200–400 mesh) column (1 × 4 mm; Bio-Rad) to form the free acids. The effluent was evaporated to dryness with a stream of nitrogen gas and then dissolved in 50 μl of methanol. An ether solution of diazomethane was added until the yellow color of the diazomethane persisted for 1 min, and the sample was evaporated to dryness as described above and then dissolved in 20 μl of methylene chloride. Gas chromatography—mass spectrometry (GC-MS) analyses were performed using a VG–70–70E HF GC-MS apparatus operating at 70 eV and equipped with an HP-5 column (0.52 mm × 30 m) programmed from 70 to 280 °C at 10 °C/min. Under these conditions, two different sulfolactate derivatives were detected. The first peak to elute was the dimethyl ester of O-methyl sulfolactate (M⁺ = 212 m/z, with a base peak at M⁺ − 59 = 153 m/z). The second was the dimethyl ester of sulfolactate (M⁺ = 198 m/z, with a base peak at M⁺ − 59 = 139 m/z) and a strong fragment at M⁺ − 59 − 32 = 107 m/z). The mass spectrum of the compound comprising the first peak has been previously reported (17).

GC-MS analysis of the resulting mixture of methyl ester derivatives on a chiral column was used to establish the stereochemical purity of the samples. Samples were separated on a β-DEX 120 column (0.25 mm × 30 m × 0.25-μm film thickness; Supelco Inc.) programmed from 75 to 230 °C at 3 °C/min. On this column, the trimethyl derivatives eluted as a single peak, but the dimethyl derivatives were separated into two peaks, with the first corresponding to the S-isomer and the second corresponding to the R-isomer. Analysis of a racemic mixture of the two isomers gave two peaks with a ratio of the first to the second of 0.74. Analytical data were corrected to account for this unexplained deviation from the expected ratio of 1.0.

Syntheses of ComA Inhibitor Candidates—3-Sulfo-2-sulfomethylpro-pionic acid was synthesized by the addition of sulfite to 2-bromomethylacrylic acid. 2-Bromomethylacrylic acid (1 mmol, 165 mg) was suspended in 1 ml of water and mixed with sodium sulfite (3 mmol, 378 mg; Mallinkrodt Chemical Works) dissolved in 1 ml of water, and the resulting clear colorless solution was heated at 110 °C for 12 h in a sealed vial. 1H NMR spectroscopy was performed using a 500-MHz Jeol Eclipse 500 NMR spectrometer at the Virginia Polytechnic Institute NMR Facility. 1H NMR analysis of a portion of the solution, after replacement of the water with 2H₂O, showed a quantitative production of the 3-sulfo-2-sulfomethylpropionate with resonances δ 3.26 ppm (4H, m, H-3, and H-3’) and 3.12 ppm (1H, m, H-2). The product concentration was determined from the ratios of the sample peaks to the standard [2,2,3,3-2H₄]trimethylhydroxypropionate (TSP) standard peak (δ = 0). The product was converted to the acid form by passage through a Dowex AG 50W-X8-H⁺ column (Bio-Rad) and evaporation of the solution to dryness.

2-Phosphonomethylacrylic acid (23) at a concentration of 0.18 mM was heated for 1 h at 110 °C with a 3 mM excess of sodium sulfite and, after cooling, passed though a Dowex AG 50-H⁺ column. 1H NMR analysis established that the 2-phosphonomethylacrylate was quantitatively converted into 2-phosphonomethyl-3-sulfolactopro-pionic acid: resonances were δ 3.56 (1H, dd, J₁₋₂ = 2.1 Hz, J₂₋₃ = 14.1 Hz, H-3), 3.22 (1H, dd, J₁₋₂ = 11.6 Hz, J₂₋₃ = 14.1 Hz, H-3’), 2.88 (1H, m, J₁₋₂ = 2.1 Hz, J₂₋₃ = 2.7 Hz, J₃₋₄ = 11.6 Hz, J₃₋₄ = 11.6 Hz, H-2), 1.71 (1H, dd, J₁₋₂ = 2.7 Hz, J₂₋₃ = 14.8 Hz, J₃₋₄ = 11.6 Hz, H-α), and 1.53 (1H, ddd, J₁₋₂ = 11.6 Hz, J₂₋₃ = 14.8 Hz, J₃₋₄ = 14.3 Hz, H-α).

Cloning and Recombinant Expression of the MJ0255 Gene in E. coli—The M. jannaschii gene at locus MJ0255 (encoding the protein submitted to Swiss-Prot Database under Swiss-Prot accession number Q57703 (34)) was amplified by PCR from genomic DNA using a primed nucleotide primers synthesized by Invitrogen. Robby MJ0255

Fwd (5′-GGTGTCATATGAAAGCATTG-3′) introduced an NdeI restriction site at the 5′-end of the amplified DNA, whereas primer MJ0255 Rev (5′-CATGCGATTCTAATACGCTCTC-3′) introduced a BamHI site at the 3′-end. PCRs contained 1X GeneAmp PCR buffer (Applied Biosystems), 1 μM each primer, 200 μM each dNTP, 1 μg of M. jannaschii JAL-1 chromosomal DNA, and 5 units of AmpliTag LD DNA polymerase (Applied Biosystems) in a volume of 100 μl. DNA was amplified during 35 cycles, and each cycle included incubation at 95 °C for 1 min, 45 °C for 2 min, and 72 °C for 3 min. PCR product DNA was purified using a QiAquick spin column (QIAGEN Inc.) and then digested with NdeI and BamHI restriction enzymes (Invitrogen). DNA fragments were ligated into compatible sites in plasmid pET29b (Novagen) or pT7-7 (28) using bacteriophage T4 DNA ligase (Invitrogen). Recombinant plasmids were transformed into Escherichia coli NovaBlue (Novagen) and E. coli BL21-CodonPlus (DE3)-RIL (Stratagene). Sequences of cloned DNA were confirmed by dye terminator cycle sequencing (Virginia Bioinformatics Institute Core Facility).

Transformed E. coli cells were grown in Luria-Bertani/Miller broth (1 liter; Difco) supplemented with 100 μg/mlampicillin. Cultures were shaken at 37 °C until they reached an absorbance at 600 nm of 1.0. Recombinant protein production was then induced with 28 mM lactose. After an additional 4-h incubation with shaking at 37 °C, the cells were harvested by centrifugation (6000 × g, 10 min) and frozen at −20 °C. Purification of the ComA Protein—Recombinant ComA protein was purified by heat treatment and chromatography of soluble cell-free extract. The cell-free extract, containing ComA, was pelleted from the pT7-7-derived plasmid was suspended in 20 ml of buffer A (20 mM bis-Tris-HCl (pH 6.5) and lysed by sonication. Soluble cell-free extract was obtained after centrifugation at 20,000 × g for 10 min at 4 °C. Heat-soluble cell-free extract (17 ml) was sealed in M, 6000–8000 cutoff membranes (Arthur H. Thomas) and dialyzed for 10 h at 4 °C against 1 liter of buffer A. Dialyzed solutions were concentrated in the membranes using polyethylene glycol 8000 and then applied to a MonoQ HR anion-exchange column (10 mm × 8 cm; Amersham Biosciences) equilibrated in buffer A. Pumps attached to the column were controlled by a BioLogic HR workstation (Bio-Rad). Protein bound to the column was washed with 0.33 mM NaCl in buffer A and then eluted with 0.55 mM NaCl in buffer A at a flow rate of 1 ml/min. Fractions (1 ml) were collected automatically, and those containing PSL synthase activity were pooled and dialyzed for 10 h at 4 °C against 1 liter of buffer B (20 mM Tris-HCl (pH 7.6)).

Dialyzed protein was concentrated using polyethylene glycol 8000 and then applied to a DEAE-Sepharose FF column (16 mm × 5.2 cm; Amersham Biosciences) equilibrated in buffer B. Protein was eluted with a 30-ml linear gradient of 0–1 mM NaCl in buffer B at a flow rate of 0.5 ml/min. Fractions (1.0 ml) were assayed for PSL synthase activity. Fractions containing activity (which elute at −0.35 mM NaCl) were pooled and concentrated in an N₂-pressurized stirred cell with a YM-10 ultrafiltration membrane (Millipore Corp.). Concentrated protein solutions were heated for 20 min at 70 °C.
cooled on ice, and centrifuged for 10 min at 16,000 \( \times g \) at room temperature. The supernatant was applied to a Superose 12HR column (10 mm \( \times 31 \) cm; Amersham Biosciences) equilibrated in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1 mM MgCl\(_2\). Chromatography was performed in this buffer at a flow rate of 0.5 ml/min, and fractions of 0.75 ml were collected. Protein concentrations of fractions containing 0.5 M Mes, and 1 M Tris were adjusted to pH 4.0–8.5 at room temperature using NaOH or HCl. Buffers containing 1 mM Acet, 0.5 mM Tris, and 0.5 mM ethanolamine HCl (Calbiochem) were similarly adjusted to pH 6.0–10.0. Single component buffers containing 0.5 mM glycine/NaOH (pH 10.0), 0.5 mM Caps/NaOH (pH 10–11), 0.5 mM l-arginine/NaOH (pH 11.5–12.0), or 0.5 mM guanidine/NaOH (pH 12) were used to test activity at all pH values. Buffers were prepared in triplicate and reactions were initiated by adding sulfite. Effects of KCl (Fisher), NaCl (Fisher), LiCl (J. T. Baker Inc.), or NH\(_4\)Cl (Fisher) were also tested in standard assays. Divalent cation requirements were tested using Hepes and P-enol-pyruvate reagents by passage through a 7.5 mm \( \times 7.5 \) cm column of Chelex 100-Na\(^+\) (Bio-Rad) (30). ComA was purified on a 0.5 \( \times 6.5 \) cm column containing Chelex 100-100 Na\(^+\) and Henri equation, and kinetic parameters were calculated using the Levensen-Marquardt method of nonlinear least-squares regression (SigmaPlot 2000, SPSS Inc.).

**Phosphosulfolactate Synthase Activity**—One unit of PS synthase activity incorporates 1 \( \mu \)mol/min sulfito into phosphosulfolactate. Standard activity assays included 50 mM Hepes/NaOH (pH 7.5), 5 mM MgCl\(_2\), 1 mM NaHSO\(_3\) (Eastman Kodak Co.), 1 mM Na\(_2\)SO\(_3\), 1 mM P-enolpyruvate, and 1 mM MgCl\(_2\). Protein standards used to calibrate the sizing column were horse spleen apoferritin, potato genase, hen egg white conalbumin, bovine erythrocyte carbonic anhydride, and horse heart cytochrome c. Eluted protein was detected by its absorbance at 280 nm and PSL synthase activity (for wild-type enzyme). Molecular masses and elution volumes of standards were successfully fit to an exponential equation using nonlinear least-squares regression (SigmaPlot 2000, SPSS Inc.).

**Kinetic Analysis of ComA Activity**—For each substrate (P-enolpyruvate, sulfito, and Mg\(^2+\)), initial rates of ComA-catalyzed phosphosulfolactate synthesis were assayed at measured activity concentrations in standard assays initiated by the addition of sulfite to reaction mixtures pre-equilibrated in water baths. Reactions were incubated at temperatures from 23 to 90 °C for 5–10 min and then terminated with alkaline stop solution and cooled on ice. The influence of pH on ComA activity was studied using approximately constant ionic strength buffers prepared from 50 mM Hepes/NaOH (pH 7.5), 5 mM MgCl\(_2\), and 15 \( \mu \)g/ml ComA and were initiated with 1.5 mM NaHSO\(_3\). Assays (120 \( \mu \)l) varying the sulfite concentration contained 10 mM MgCl\(_2\), 2 mM P-enolpyruvate, and 50 mM Hepes/NaOH (pH 8.0) and were initiated with 9 \( \mu \)g/ml ComA. The initial rate at each substrate concentration was measured by calculation of the increase in absorbance at 412 nm recorded at intervals from 0.25 to 5.0 min. Fixed time point assays at varying MgCl\(_2\) concentrations contained 16 \( \mu \)g/ml Chelex-purified ComA, 50 mM Hepes/NaOH (pH 7.6), and 2.6 mM P-enolpyruvate. Reactions were initiated with 1.5 mM sulftite and stopped after 5 min. Initial rate data from low substrate concentrations were successfully fitted to the hyperbolic Michaelis-Menten-Henri equation, and kinetic parameters were calculated using the Levenberg-Marquardt method of nonlinear least-squares regression (SigmaPlot 2000). Initial rate data obtained over the full range of concentrations tested were fitted to a simple hyperbolic equation for substrate inhibition: \( v = V_{max}(K_s + A)^{-1} / (K_s + A) \) (31).

**Analysis of 2-Phosphosulfolactate Deuterium Exchange Activity**—(RS)-Phosphosulfolactate (4.2 \( \mu \)mol) was mixed with 1 \( \mu \)mol of MgCl\(_2\), 1 \( \mu \)mol of ComA (0.75 mg/ml), and 4 \( \mu \)mol of H\(_2\)O. The enzyme solution was added to the (RS)-phosphosulfolactate and incubated at 70 °C for 60 min. Reactions were cooled to room temperature and then analyzed by \(^{1}H\) NMR at 23 °C.

**Stereocchemical Analysis of the ComA Reaction Product**—Phosphosulfolactate was hydrolyzed by bacterial alkaline phosphatase (0.2 units), and then the resulting sulftolate was purified using Dowex AG 1-X2 Cl\(^-\) resin (Bio-Rad) (17). Sulftolate was derivatized and analyzed by chiral GC-MS as described above.

**Inhibition of ComA PSL Synthase Activity**—Potential inhibitors of ComA activity were tested in reactions (50 \( \mu \)l) containing 1.2 \( \mu \)mol P-enolpyruvate, 1 \( \mu \)mol NaHSO\(_3\), 50 mM Hepes/NaOH (pH 7.5), 5 mM MgCl\(_2\), and 15 \( \mu \)g/ml ComA. Fixed time point reactions were initiated, incubated, and terminated after 6 min as described for standard assays. To account for interferences with the monobromobimane detection of sulftite, residual sulftolate concentrations in samples were subtracted from those of inhibitor control reactions incubated without ComA. Net specific activities were compared with those of ComA reactions without inhibitor to calculate relative inhibitory activities.

**Site-directed Mutagenesis**—To test the function of a conserved lysine residue in ComA, Lys 137 was replaced with asparagine. The QuikChangeTM site-directed mutagenesis kit (Stratagene) was used according to the manufacturer's instructions with template pMjoComA-His (comA in pET19b). Oligodeoxynucleotide primers were synthesized by Invitrogen: K137N-Fwd (5'-GGTTTACAGAAATGTGGTTAAACT-3') and K137N-Rev (5'-GCTGTTT-
ATGCTTATCTTGACATTTTTACCAACTTCTGGTAAAACCT-5′). DNA sequences were verified by dye terminator sequencing at the University of Iowa DNA Facility.

Identification of ComA Homologs, Sequence Alignment, and Phylogenetic Inference—The translated sequence of the M. jannaschii MJ0255 gene (Swiss-Prot accession number Q57703) was used to query the non-redundant protein data base at the National Center for Biotechnology Information using the BLASTP program (Version 2.2.1) (32) with the BLOSUM62 matrix and default gap costs for existence—11 and extension—1. Homologs were identified in the archaeon Methanobacterium thermoautotrophicum (GenBank™/EBI accession number AA862416.1); in the bacteria Xanthobacter sp. Py2 (GenBank™/EBI accession number CAB12935.1); and in the eukaryotes Arabidopsis thaliana (EMBL accession number CAB79131.1), Lycopersicon esculentum (cDNAs; GenBank™/EBI accession numbers BE436889.1 and AW930285.1); and Glycine max (cDNA; GenBank™/EBI accession number BE346999.1). Additional homologs were identified in incomplete genomic sequences from Ferroplasma acidarmanus, Cytophaga hutchinsonii, and Phanaerochaete chrysosporium (www.jgi.doe.gov); Methanococcus maripaludis (www.genome.washington.edu/Methanococcus); and Aspergillus fumigatus, Entomobola histolytica, and Cestoria burnetii (www.tigr.org).

Amino acid sequences were aligned automatically using the ClustalW program (Version 1.82) (33). From the alignment of 15 protein sequences, 223 positions were deemed to be confidently aligned. These were analyzed by protein maximum likelihood methods using the ProML program (PHYLIP (phylogeny inference package), Version 3.6a2.1) (53) with the JTT amino acid substitution model. Bootstrap proportions were calculated using Seqboot, ProML, and Consense programs from PHYLIP.

RESULTS

Identification, Expression, and Purification of M. jannaschii ComA—The comA gene was identified based on its physical proximity to previously identified CoM biosynthetic genes in M. jannaschii (19, 20, 34) and in Xanthobacter sp. Py2 (35). Homologs of comA are found in complete genome sequences of all organisms known to synthesize CoM or sulfolactate, except for Methanosarcina spp. In E. coli cells that recombinantly expressed the M. jannaschii comA gene, the ComA protein composed 35% of the cells’ total soluble protein. Protein purification by heating (to denature most native proteins and nucleic acids. Table I shows the purification of 58 mg of ComA protein from 7.4 g of recombinant E. coli (wet mass) at a concentration of 54 mg/ml and a specific activity of 2.4 units/mg.

Purified ComA preparations showed a single band on a silver-stained SDS-polyacrylamide gel with an apparent molecular mass of 32,000 Da, close to its predicted molecular mass of 28,370 Da. From a Sepharose 12HR size-exclusion column, ComA eluted with a Stokes radius of 37 Å, corresponding to an apparent molecular mass of 86,700 Da. This elution profile suggests that ComA forms a trimer; a minor peak (<5% total protein) corresponded to a hexameric form of ComA. Diluted in phosphate-buffered saline (pH 7.4), ComA had a single absorption maximum at 276 nm and an extinction coefficient ($\varepsilon_{280}$) of 0.84 ml/mg/cm. The enzyme retained full activity when stored for 2 months at 4 °C in 20 mM Tris-HCl and 1 mM MgCl$_2$ (pH 8.0).

Phosphosulfolactate Synthase Activity—(R)-Phosphosulfolactate produced by ComA was identified by GC-MS as described below. However, phosphosulfolactate is not readily quantified; therefore, enzymatic activity was determined by measuring residual sulfite substrate in discontinuous assays. Standard reactions were initiated by the addition of sulfite to a mixture of ComA, P-enolpyruvate, buffer, and MgCl$_2$ preincubated at 70 °C. Reactions were stopped with a solution of L-arginine and EDTA (pH 12.8). At alkaline pH, sulfite reacts quantitatively with monobromobimane to form a fluorescent compound that has an excitation maximum at 410 nm and an emission maximum at 480 nm (28, 36). Phosphoenolpyruvate also reacts spontaneously with excess sulfite to form (RS)-phosphosulfolactate (18, 37); however, this non-enzyme reaction was insignificant at the low sulfite concentrations and short incubation times used in the enzymatic assays described here.

The ComA enzyme is metal-activated: it requires several molar equivalents of Mg$^{2+}$/P-enolpyruvate for maximum activity. In the absence of Mg$^{2+}$ or in the presence of a molar excess of EDTA versus Mg$^{2+}$, this enzyme is inactive. To test metal ion requirements, ComA, P-enolpyruvate, and Heps solutions were passed through a Chelex 100 column. The metal-free enzyme had no phosphosulfolactate synthase activity, but activity was restored by the addition of 5 mM MgCl$_2$ (3.3 units/mg for Chelex-treated ComA versus 4.0 units/mg for originally purified ComA). When incubated with 1 mM bisulfite and 1 mM P-enolpyruvate, ComA required 5 mM MgCl$_2$ for maximum activity. No other divalent cation restored full activity at a 5 mM concentration (CaCl$_2$ (25% relative activity), NiCl$_2$ (18%), MnCl$_2$ (15%), and CuCl$_2$ (10%)), whereas CoCl$_2$, Fe(NH$_3$)$_2$(SO$_4$)$_2$, Zn(C$_2$H$_3$O$_2$)$_2$, and BaCl$_2$ supported no activity.

ComA is active over a wide pH range (pH 6–11), with maximum activity at pH 8.5. At alkaline pH, the SO$_3^-$ species (pK$_a$ = 7.2 at 25 °C) rather than bisulfite appears to be the relevant substrate. LiCl was a weak inhibitor of ComA activity (~50% activity at 100 mM LiCl$_2$), but other monovalent cations (K$^+$, Na$^+$ and NH$_4^+$) had no effect on activity at 100 mM concentrations. ComA activity increased with temperature up to 90 °C (the practical limit of the assay). Enzyme heated at 125 °C for 15 min prior to assay at 70 °C showed no activity.

To estimate the efficiency of ComA in synthesizing phosphosulfolactate, initial reaction rates were measured at varying P-enolpyruvate, sulfite, or MgCl$_2$ concentrations, with other substrates present in excess (38). Fig. 2 suggests that high concentrations of each substrate inhibited activity. ComA was also inhibited by phosphosulfolactate; however, concentrations of sulfite converted to PSL in inhibited reactions (measured after 5 min) were <10% of the concentration of racemic phosphosulfolactate (4 mM) required to reduce ComA activity by 50%. Activities measured over the full range of tested P-enolpyruvate concentrations were fit to a simple model for substrate

**TABLE I**

| Purification step       | Volume (ml) | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Yield (%) |
|-------------------------|-------------|--------------------|-----------------------|-------------------------------|----------|
| Soluble extract         | 22          | 579                | 482                   | 0.83                          | 100      |
| Heat treatment          | 17          | 183                | 159                   | 0.87                          | 33       |
| MonoQ pool              | 15          | 125                | 216                   | 1.73                          | 45       |
| DEAE pool               | 3           | 81                 | 159                   | 1.96                          | 33       |
| Superose 12HR pool      | 1           | 58                 | 138                   | 2.37                          | 29       |

*Total protein was measured using the Bio-Rad protein assay.

*Phosphosulfolactate synthase activity was quantified by measuring the monobromobimane derivative of the remaining sulfite substrate.
FIG. 2. Initial rate plots of ComA activity at varying substrate concentrations of phosphoenolpyruvate (A), sulfite (B), or MgCl₂ (C). One unit of phosphosulfolactate synthase activity incorporates 1 μmol/min sulfite into phosphosulfolactate. Initial rate data are fit to a simple model of substrate inhibition.
Phosphosulfolactate Synthase from M. jannaschii

**Fig. 3. Stepwise model for reactions in (R)-phosphosulfolactate formation by ComA.** To form (R)-phosphosulfolactate, P-enolpyruvate must be protonated on the re-face.

Inhibition. The pseudo first-order apparent kinetic parameters for P-enolpyruvate were $V_{\max} = 39$ units/mg, $K_m = 9.8$ mM, and $K_i = 1.9$ mM. When activities measured only at low P-enolpyruvate concentrations (0–3.6 mM) were fit to the Michaelis-Menten-Henri equation, the apparent kinetic parameters were significantly lower: $V_{\max} = 9 \pm 0.6$ units/mg and $K_m = 0.9 \pm 0.2$ mM. Fitting the activities at the full range of tested sulfite concentrations to the inhibition model produced apparent values of $V_{\max} = 33$ units/mg, $K_m = 2$ mM, and $K_i = 3$ mM. Considering only activities at low sulfite concentrations (0–2 mM) produced lower apparent values: $V_{\max} = 18 \pm 2$ units/mg and $K_m = 1 \pm 0.2$ mM. Finally, a model of activities at all tested MgCl$_2$ concentrations produced apparent values of $V_{\max} = 13$ units/mg, $K_m = 4.2$ mM, and $K_i = 1$ mM. At low MgCl$_2$ concentrations (0–5 mM), apparent values were lower: $V_{\max} = 8$ units/mg and $K_m = 2$ mM.

When incubated with (RS)-phosphosulfolactate, ComA does not produce sulfite or affect the chemical shift or intensities of the C-3 proton resonances, as observed by $^1$H NMR. Therefore, the phosphosulfolactate synthase reaction is effectively irreversible. In addition, ComA does not have detectable enolase activity when incubated with P-enolpyruvate or d-2-phosphoglycerate in the absence of sulfite (<1% specific activity of yeast enolase).

**Phosphosulfolactate Proton Isotope Exchange**—Abstraction of an α-proton from a carboxylic acid typically proceeds stereospecifically through an enolate (aci-carboxylate) intermediate (39). Proton isotope exchange experiments were performed to test this proposed intermediate in a stereospecific partial reaction of ComA (Fig. 3). ComA$^{His}$ was incubated with (RS)-phosphosulfolactate and MgCl$_2$ in $^2$H$_2$O at 70 °C for 1 h. The $^1$H NMR spectroscopy data in Fig. 4 show that ComA$^{His}$ exchanged 50% of the racemic phosphosulfolactate C-2 protons for deuterons. No exchange of C-3 protons was observed: the intensities of $\delta$-carboxylate intermediates remained unchanged.

**Stereochemistry of the Phosphosulfolactate Product**—The deuterium-exchanged sample was treated with alkaline phosphatase, and the resulting sulfolactate was converted into a dimethyl ester derivative and analyzed by GC-MS with a chiral column. By selective ion monitoring of the m/z 139 and m/z 140 ions in the eluting peaks, the extent of deuterium incorporation into each of the stereoisomers could be measured. The results in Fig. 5 show an equal mixture of the two isomers, with deuterium incorporated only in the R-isomer. The extent of labeling was essentially quantitative considering the amount of non-deuterated water in the sample. A similar analysis of the phosphosulfolactate produced from P-enolpyruvate and sulfite by ComA showed an enantiomeric ratio of 84:16 R-isomer:S-isomer. The presence of (S)-sulfolactate can be attributed to its nonenzymatic formation during the 3-h incubation period used in this experiment.

**Inhibitors of ComA Activity**—In reactions containing 1.2 mM P-enolpyruvate, 1 mM Na$_2$SO$_3$, 5 mM MgCl$_2$, and 26 μM ComA, a number of substrate and product analogs were tested for their ability to inhibit ComA-catalyzed PSL synthesis. Less than 50% relative PSL synthase activity was measured in the presence of 4 mM (RS)-phosphosulfolactate, 3 mM (R)-sulfolactate, 2 mM (R)-phosphomalate, 2 mM (S)-phosphomalate, 200 mM NH$_4$SO$_4$, 200 mM Na$_2$SO$_4$, 10 mM phosphonoacetic acid, 10 mM 2-carboxyethylphosphonate, 3 mM phosphetarylethanol, 5 mM sulfoypyruvate, or 3 mM 2-phosphono-3-sulfooxypropionate. Conversely, ComA retained at least 50% relative activity in the presence of 2 mM 3-bromopropanesulfonic acid, 1 mM CoM, 50 mM NH$_4$SO$_4$, 100 mM NH$_2$Cl, 5 mM NaF (with or without 5 mM NaHPO$_4$), 5 mM BaCl$_2$, 10 mM oxalate, 2 mM Na$_2$SeO$_3$, 10 mM NaN$_2$, 5 mM NaHPO$_4$, 10 mM NaHCO$_3$, 5 mM (S)-phosphosulfolactate, 3 mM (R)-phosphosulfolactate, 5 mM (S)-lactate, 5 mM (R)-lactate, 5 mM (R)-2-phosphoglycerate, 5 mM (R)-glycerate, 5 mM 3-bromopropanesulphonate, 5 mM methyl phosphate, 5 mM sodium acrylate, 5 mM (S)-sulfolactate, 5 mM 2-phosphonomethylcyle, 3 mM phosphonoacetoxyhydroxamate, or 5 mM 3-sulfo-2-sulfoaminoethane.

**Characterization of a K137N Mutant**—Site-directed mutagenesis was used to change the amino acid at position 137 in ComA$^{His}$ from lysine to asparagine. The resulting ComA$^{His}$. K137N mutant had no measurable phosphosulfolactate synthase activity (<4% activity relative to ComA$^{His}$) and did not catalyze the isotope exchange of the C-2 proton of either phosphosulfolactate stereoisomer (Fig. 4). Purified wild-type and mutant ComA$^{His}$ both formed single bands on SDS-polyacrylamide gels corresponding to their expected molecular masses, and both eluted from a size-exclusion column in peaks corresponding to trimeric and hexameric forms.

**Sequence Analysis of ComA Homologs and Phylogenetic Inference**—Fig. 6 shows an alignment of the M. jannaschii ComA amino acid sequence with several homologous sequences. Most amino acid positions are not conserved. However, several conserved acidic residues (Asp$^{190}$, Glu$^{103}$, Glu$^{131}$, Glu$^{171}$, and Glu$^{205}$) could function as ligands for Mg$^{2+}$, and two basic residues (Lys$^{13}$ and Lys$^{137}$) may function as general acids to protonate an enolate intermediate (Fig. 3) or to bind sulfite.

The phylogeny of comA homologs, inferred from an alignment of 15 highly diverged sequences, shows two distinct groups (Fig. 7). The first group comprises the archaeal, bacterial, and Entamoeba homologs, which likely function as phosphosulfolactate synthases in the context of sulfolactate or sulfooxyruvate synthesis. Most of the organisms represented in this group have at least one other sulfolactate/CoM biosynthesis gene. Plants and some fungal species contain comA homologs in the second group; these organisms do not have homologs of other CoM biosynthesis genes. Bootstrap re-samplings support the major groupings, suggesting that the plant homolog is widespread and was present early in the evolution of the plant lineage.
DISCUSSION

Phosphosulfolactate synthase is one of the first biosynthetic enzymes demonstrated to use sulfite. Because of its high nucleophilicity and rapid reactivity in vitro with unsaturated carbon atoms in nucleotides (40), fatty acids (41), and aldehydes (42), sulfite has been discounted as a short-lived intermediate in sulfur oxidation or sulfate reduction. Nevertheless, UDP-sulfoquinovose synthase (SQD1) from *A. thaliana* (12).
and now phosphosulfolactate synthase (ComA) from M. jannaschii also add sulfite to unsaturated carbon atoms to form stable sulfonates. A kinetic analysis of SQD1 found the apparent $K_m$ for sulfite to be 10 μM, with a turnover number ($k_{cat}$) of 0.1 min$^{-1}$ (12). By comparison, ComA has a higher apparent $K_m$ (1 μM) and a much higher turnover number (510 min$^{-1}$). ComA is not homologous to SQD1, an NAD$^+$/H-dependent methyl-CoM reductase in vitro (44) and anti-methanogenic agents in vivo (45). To find other inhibitors of methanogenesis and CoM biosynthesis, we screened substrate and product analogs as inhibitors of ComA PSL synthase activity. The most effective ComA inhibitors identified were the reaction product (2-phosphosulfolactate) and a phosphonate analog (2-phosphono-3-sulfopropionate). In the future, even more potent inhibitors could be designed using mechanistic and structural information about the ComA active site.

The catalytic mechanism of ComA likely proceeds through an enolate intermediate (46). Similar to enolase, ComA requires P-enolpyruvate and Mg$^{2+}$, has an essential lysine residue, and can exchange the C-2 proton of a 2-hydroxycarboxylic acid phosphate ester. Further investigations are required to elucidate the structural and mechanistic relationship of ComA to other P-enolpyruvate-utilizing enzymes in the enolase superfamily (47).

Many of the organisms that have homologs of comA also have homologs of other CoM biosynthetic genes. B. subtilis has a gene cluster (yisZ-yitABC) that encodes an adenylylsulfate kinase, a sulfite adenyltransferase, a sulfite-producing 3'-phosphoadenosine-5'-phosphosulfate sulfotransferase, a phosphosulfolactate phosphatase (ComB), and a phosphosulfolactate synthase (ComA), respectively. These enzymes are probably used to synthesize sulfonate from sulfate and P-enolpyruvate during B. subtilis sporulation (14, 48). This organism may also produce sulfonate from cysteic acid (49). The C. burnetii genome contains two unlinked gene clusters (yit-BDC and yitA-yisZ), with the genes in different orientations from B. subtilis. C. hutchinsonii has homologs of comABC, although the genes are unlinked on its chromosome. A related species, Cytophaga johnsonae, produces N-acylcapnine sulfonolipids. Carbon atoms in the capnine head group are derived from CoM.
from L-cysteate, but not from L-cysteine (50, 51). Rather, ComABC proteins in *Cytophaga* spp. could produce sulfoypyruvate, which is then transaminated to form cysteate. Another species, *F. acidarmanus*, a corynarchaeon not known to have CoM, has a yitB-comA cluster and an unlinked comD homolog, which could be used to produce sulfolactate. *A. fumigatus* and white rot fungus could have acquired the plant-type comA homolog by horizontal gene transfer.

Sequence similarity alone does not link any of the CoM biosynthesis genes to universally conserved genes in central metabolic pathways. Yet, mechanistic similarities and conserved structural features join these proteins to large superfamilies of enzymes. Just as catabolic pathways have evolved by stringing together familiar reactions to consume unfamiliar compounds, so have biosynthetic pathways drawn upon a significant unexplored and ancient gene pool (52).

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