Supplemental information for:
Identification of an Enzyme Catalyzing the Conversion of Sulfoacetaldehyde to 2-Mercaptoethane-sulfonic acid in Methanogens

Robert H. White

Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061, United States

Corresponding author

E-mail: rhwhite@vt.edu. Phone: 540-231-6605. Fax: 540-231-9070.

Materials and Methods

Benzylmercaptan, sodium bisulfite, diethyl 2-bromoacetal, Monobromobimane (mBBr) and *O*-(4-nitrobenzyl)-hydroxylamine·HCl were obtained from Sigma-Aldrich.

Synthesis and Characterization of Sulfoacetaldehyde.

The first synthesis of sulfoacetaldehyde was described in 1971 and involved refluxing a stirred aqueous mixture of "2-bromoacetal" with an aqueous solution containing sodium bisulfite and sodium hydroxide. [1] The product was described as the bisulfite adduct of sulfoacetaldehyde. Subsequent cooling and concentration of the solution produced white crystals, which after two further recrystallizations generated the final product that was also described as the bisulfite addition of sulfoacetaldehyde. No NMR data was presented to support the identification of this product. A more recent report on the synthesis showed that the product was in fact the bisulfite adduct and that this product was quite stable, decomposing only "slowly" to sulfoacetaldehyde. [2] A neutralized solution of sulfoacetaldehyde, however, was shown not to be stable after several weeks in the cold. [2]

We have now analyzed the crystalline product generated as described in the first synthesis by both ^{1}H-NMR and TLC. The results showed that only the first set of crystals contained any organic compounds. The compound detected was in fact the expected chemical product, the diethyl acetal derivative of sulfoacetaldehyde. This was confirmed by ^{1}H NMR (600 MHz, $^{2}H_2O$): δ 5.0 (t, = 5.39 Hz, 1H), 3.645 (m, 4.8, 1.7 Hz, 4H), 3.25 (d, J = 5.30 Hz, 1H), 1.245 (t, J = 7.15 Hz, 3H). This data confirmed the structure and also demonstrated that the $CH_3C\underline{H}_2$ protons are not equivalent, indicating that the CH_2-CH bond of the diethyl acetal derivative of sulfoacetaldehyde does not have free rotation.

TLC analysis of the compound, using a solvent system composed of 5% formic acid in acetonitrile, showed one band with a R_f = 0.38 after spraying with a Mo spray reagent (20 g NH_4MoO_4 dissolved in 25 mL hot H_2SO_4, diluted to 400 ml with water) followed by heating for 5-10 min at ~150 °C. Exposure of the crystals to 1 M HCl for 5 min quantitatively converted this compound to the desired sulfoacetaldehyde, as measured by TLC. In a TLC solvent system composed of acetonitrile−water−formic acid (88%), (19:2:1 vol/vol/vol), the TLC R_f for the starting compound was 0.69 and 0.57 for the product sulfoacetaldehyde. Both spots were made visible on the TLC plate using the Mo spray and no UV absorbing compounds were observed. The hydrolysis product sulfoacetaldehyde was shown to be stable to 1 M HCl at 100 °C for 12

min and overnight at 60 °C in 1 M HCl. Reaction of sulfoacetaldehyde with sulfide under basic conditions in the presence of tris(2-carboxyethyl)phosphine hydrochloride (TCEP) produced HSCoM, further confirming the structure of the sulfoacetaldehyde product (Data not shown).

To chemically confirm the acid hydrolysis product as sulfoacetaldehyde, 8.8 mg of the diethyl acetal derivative of sulfoacetaldehyde was dissolved in 100 μl of 1 M HCl and the solvent evaporated with a stream of nitrogen gas at RT. The residue was dissolved in 1 M sodium acetate, pH 4.0, buffer, 6 mg of *O*-(4-nitrobenzyl)hydroxylamine·HCl was added, and the reaction mixture was adjusted to pH 4.0 with HCl. The reaction was then heated in a sealed vial at 100 °C for 2 hr. TLC analysis of the reaction mixture showed that ~ 80% of the *O*-(4-nitrobenzyl)hydroxylamine had been converted into a new spot with an R_f = 0.53 in the 5% formic acid in acetonitrile solvent system. This new yellow spot was purified by preparative TLC and -ESI-MS showed a mass of 273 for the $(M - H)^-$ ion. The ^{1}H-NMR spectrum was that expected for the *O*-(4-nitrobenzyl)hydroxylamine oxime derivative of sulfoacetaldehyde. ESI ionization of the ^{1}H-NMR sample maintained at RT in ^2H_2O for one month after preparation showed a $(M - H)^-$ ion at m/z = 275 and a $(2M - H + Na)^-$ at 573. Since the calculated mass of the $(M - H)^-$ ion should have been 273, this indicated that the sample mass had increased by two *m/z*. This can be explained by exchange of the methylene protons with the ^2H_2O solvent. This was confirmed by a slow back exchange while in non-deuterated water.

Cloning and Overexpression of MJ1681 in *E. coli*.

The MJ1681 gene (Swiss-Prot accession number Q59075) was amplified by PCR from genomic DNA using oligonucleotide primers MJ1681–Fwd (5'-GGTCATATGGAAAAGGAATT-AAAAG-3') and MJ1681-Rev (5-GATGGATC-CTTATATCTTAAACTC- 3'). PCR amplification was performed as described previously [3] using a 55 °C annealing temperature. Purified PCR product of MJ1681 was digested with Nde-1 and BamH1 restriction enzymes and ligated into compatible sites in vector pT7-7. The sequences of the resulting plasmids were verified by DNA sequencing. The plasmids were then transformed into *E. coli* strain BL21-CodonPlus (DE3)-RIL cells (Stratagene). The transformed cells were grown in LB medium supplemented with 100 μg/mL ampicillin at 37 °C with shaking until they reached an OD_{600} of 1.0. The recombinant protein production was induced by addition of lactose to a final concentration of 28 mM. [3] After an additional 2 hours of culture, the cells (200 mL) were harvested by centrifugation (4,000 x *g*, 5 min) and frozen at -20 °C. Induction of the desired protein was confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of total cellular proteins.

The frozen *E. coli* cell pellet (~0.4 g wet wt) containing the desired recombinant protein was suspended in 3 mL of extraction buffer (50 mM *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES), pH 7.0, 10 mM $MgCl_2$). The buffer and cells were mixed and sonicated under argon at 0 °C using a microtip-equipped W-385 Heat-System Ultrasonics, Inc. sonicator over three minutes with alternating 5 second sonication pulses and 5 second pauses. The sonicated material was transferred to sealed argon-flushed vials and stored at -20 °C. After centrifugation, the resulting soluble and insoluble fractions as well as whole cells were assayed for the presence of the desired recombinant protein. The soluble fractions were also tested for the thermal stability of the desired protein by heating the resulting cell extracts for 10 min at 60, 70, and 80 °C, followed by centrifugation (14,000 × *g* for 10 min) to remove the insoluble *E. coli* proteins, which denature and precipitate under these conditions. Portions of the soluble and insoluble pellets were then analyzed by SDS-PAGE and bands that matched the predicted

size of ~41 kd were excised and prepared for analysis by MALDI-MS as previously described [4] for confirmation of MJ1681 expression. Protein concentrations were determined by Bradford analysis. [5]

Measurement of Enzymatic Activity in Sonicated Cell Extracts Expressing MJ1681. Although no detectable amount of MJ1681 protein was found to be present in the soluble portion of the cell extract, this does not prove that a small amount of the protein was not, in fact, present in the soluble fraction. The enzyme could have been at a low level but still high enough to catalyze the desired reaction at a measurable rate. To confirm activity, we tested for the expected enzymatic activity in the soluble cell extracts. Since it had been found that the reaction could proceed without the enzyme, an extract was prepared from *E. coli* containing the plasmid but without the inserted gene to serve as a control. The assay consisted of incubation of 50 μL of each of the different cell extracts (~10 mg/ml protein) under Ar with 2 μL of 1 M NaSH (28 mM), 10 μL of 71 mM sulfoacetaldehyde (~17 mM), 10 μL of 28 mM dithionite (1.8 mM), and 10 μL 10 mM methylviologen (1.4 mM). All samples were prepared in anaerobic extraction buffer except for the NaHS, which was in anaerobic water. All samples were kept under an argon atmosphere. The final concentrations of reactants in the reaction mixtures are shown in parentheses. The cell extracts with substrates were incubated at 37 °C for 1.5 hr under Ar. The reaction mixtures were then acidified with 1 M HCl (pH <2.0) to precipitate the insoluble proteins and centrifuged. The soluble fraction was then adjusted to neutral pH and the HSCoM was converted into the monobromobimane (mBBr) derivative, purified by preparative TLC, and assayed by LC-MS as previously described. [6]

Growth of *E. coli* in the Presence of Sulfoacetaldehyde.

BL21-CodonPlus (DE3)-RIL cells transformed with the pT7-7 plasmid with and without the *mj*1681 gene were inoculated into LB medium supplemented with 100 μg/mL ampicillin and containing 10 mM sulfoacetaldehyde. The cultures were incubated at 37 °C with shaking at 300 rpm to an OD600 of 0.6-0.7. Then, lactose was added to a 1% concentration and $FeCl_3$ to 120 μM and the cells were then grown for 5 hr at 37 °C.

Assay of HSCoM Produced from Sulfoacetaldehyde by *E. coli* Growing Cells Containing the MJ1681 Plasmid.

The 10 mL cultures, produced as described above, were centrifuged (10,000 × g, 10 min) and the media separated from the pellet. The cell pellets were then extracted with 1 mL of 50% methanol at 90 °C for 10 min and the insoluble material separated by centrifugation (10,000 × g, 5 min). The dry weights of both of the extracted cell pellets was 12.0 mg. The resulting extracts were concentrated by evaporation to ~ 50 μL and the samples assayed for the presence of HSCoM by the formation of the mBBr derivative as previously described. [6]

The resulting media (10 mL) was concentrated by evaporation to ~0.6 ml and 50 μL of 0.1 M borate buffer, pH 9.3, was added followed by 10 μL of 1 M TCEP and the pH adjusted to 9.3 with the addition of NaOH. Then, 1 mg of mBBr in 100 μL of acetonitrile was added. After 1 hr, the sample was acidified with 1 M HCl to pH < 2 and applied to a C18 column (4 x 20 mm) equilibrated with 0.1 M HCl. After application of the sample to the column, it was washed with 1 mL of 0.1 M HCl and the samples were eluted with methanol. After evaporation of the methanol, the sample was dissolved in water and the C18 purification step was repeated. This procedure was required to further remove the large amounts of contaminating compounds

from the media. The HSCoM mBBr derivative in the final sample was then purified by preparative TLC prior to LC-MS analysis.

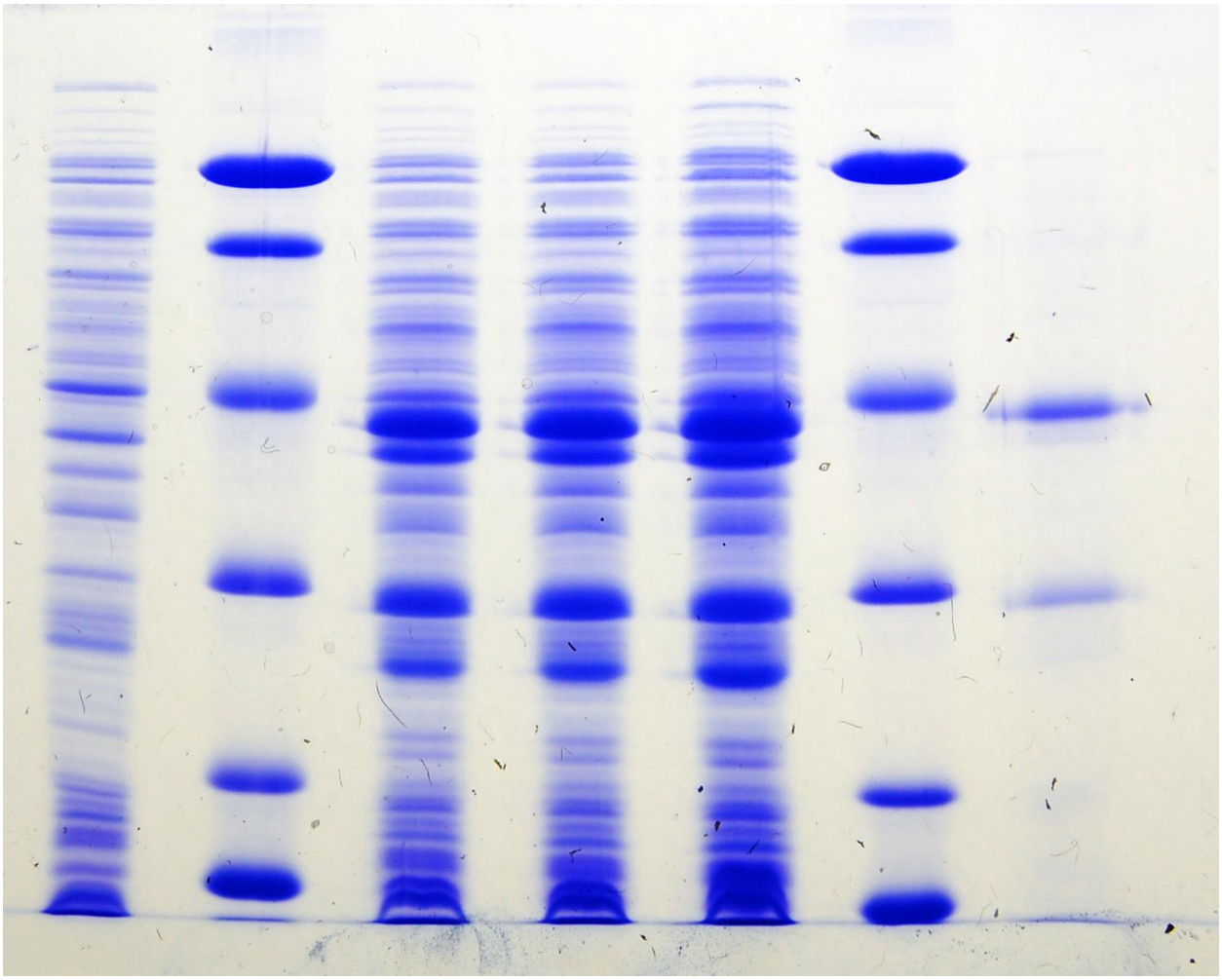

Figure S1. SDS-PAGE analyses of MJ1681 expressions in whole *E. coli* cells. Lane 1 – uninduced *E. coli* cells with no plasmid, lane 2 & 6 – mw protein marker with masses of 97.4, 66.2, 45.0, 31.0, 21.3 and 14.4 kD, lane 3 – induced *E. coli* cells with plasmid containing the *mj*1681 induced with lactose + 0.125 mM Fe (II), lane 4 – induced *E. coli* cells with plasmid containing the *mj*1681 induced with lactose + 0.125 mM Fe(II) + 2 mM 2-mercaptoethanol, lane 5 – induced *E. coli* cells with plasmid containing the *mj*1681 induced with lactose + 0.125 mM Fe + sulfoacetaldehyde; lane 6 – mw protein marker, Lane 7 – 10x diluted cell extract.

[1] Kondo, H., Anada, H., Ohsawa, K., and Ishimoto, M. (1971) Formation of Sulfoacetaldehyde from Taurine in Bacterial Extracts, *J Biochem-Tokyo 69*, 621-&.
[2] Gritzer, R. F., Moffitt, K., Godchaux, W., and Leadbetter, E. R. (2003) Sulfoacetaldehyde bisulfite adduct is a substrate for enzymes presumed to act on sulfoacetaldehyde, *J Microbiol Methods 53*, 423-425.
[3] Graham, D. E., Xu, H., and White, R. H. (2002) Identification of Coenzyme M Biosynthetic Phosphosulfolactate Synthase: a new family of sulfonate-biosynthesizing enzymes, *J. Biol. Chem. 277*, 13421-13429.
[4] Miller, D., O'Brien, K., Xu, H., and White, R. H. (2014) Identification of a 5'-Deoxyadenosine Deaminase in *Methanocaldococcus jannaschii* and its Possible Role in Recycling the Radical SAM Enzyme Reaction Product 5'-Deoxyadenosine, *J. Bacteriol. 196*, 1064-1072.
[5] Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding, *Anal. Biochem. 72*, 248-257.
[6] Allen, K. D., and White, R. H. (2016) Occurrence and biosynthesis of 3-mercaptopropionic acid in *Methanocaldococcus jannaschii*, *FEMS Microbiol. Lett. 363*, 1-7.