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Thiosulfate as a metabolic product: the bacterial fermentation of taurine

Abstract Thiosulfate ($S_2O_3^{2-}$) is a natural product that is widely utilized in natural ecosystems as an electron sink or as an electron donor. However, the major biological source(s) of this thiosulfate is unknown. We present the first report that taurine (2-aminoethanesulfonate), the major mammalian solute, is subject to fermentation. This bacterial fermentation was found to be catalyzed by a new isolate, strain GKNTAU, a strictly anaerobic, gram-positive, motile rod that formed subterminal spores. Thiosulfate was a quantitative fermentation product. The other fermentation products were ammonia and acetate, and all could be formed by cell-free extracts.

Key words Taurine · Fermentation · Anaerobic desulfonation · Thiosulfate

Introduction

Naturally occurring, defined organosulfonate compounds with the thermostable C–SO_3^- bond are not numerous [Seitz and Leadbetter 1995; see also Van Loon et al. (1993)], but they occupy important metabolic and physiological niches, and taurine (2-aminoethanesulfonate), with its myriad functions in mammals and many other organisms (Huxtable 1992), is perhaps the best known. The catabolism of organosulfonates, whether of the natural products or of the numerous xenobiotic compounds, was thought to be the preserve of aerobic organisms (Denger et al. 1996; Fuchs et al. 1994), but this view changed when two research groups discovered that sulfonate sulfur could be assimilated by strictly anaerobic bacteria, *Clostridium* spp. (Chien et al. 1995; Denger et al. 1996; Denger and Cook 1997).

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Enzymes responsible for the assimilation of sulfur have low specific activities, and we have been unable to detect enzyme activity in cell-free extracts (Denger et al. 1996; Denger and Cook 1997). Dissimilation of sulfur in the biosphere has a central role in cycling this element; therefore, we explored the dissimilatory cleavage of the C–sulfonate bond in anaerobic bacteria. We found both the anticipated 250-fold increase in specific activity and enzyme reactions in cell-free extracts (Denger et al. 1997; Laue et al. 1997b). The sulfonate moiety was recovered as sulfide (Lie et al. 1996; Laue et al. 1997b) or sulfate (Denger et al. 1997) when the sulfonate was supplied as electron acceptor or electron donor, respectively.

We now report the first organisms, bacteria, known to ferment an organosulfonate, taurine [cf. Seitz and Leadbetter (1995)]. Strain GKNTAU (with the similar strain GRZTAU) was found to represent a novel genus and to yield an initially unidentified fermentation product, thiosulfate, quantitatively from the sulfonate moiety of the substrate.

Materials and methods

Enrichment, isolation and growth of bacteria, and enzyme assays

Enrichment cultures were done in a bicarbonate-buffered, titanium(III)nitrilotriacetate-reduced-salts medium representing fresh water (Denger et al. 1997). Portions (25 ml) of salts medium (pH 7.0) were transferred to 50-ml infusion bottles sealed with butyl rubber septa. Sulfonate was added to a final concentration of 20 mM and the inoculum (5%, v/v) was untreated anoxic sludge. Cultures were incubated at 30°C under an atmosphere of N_2 plus CO_2 (80:20, v/v). Putative enrichments were subject to serial subculture into homologous medium. The pure culture of strain GKNTAU was obtained by the repeated application of the agar shake method (Pfennig 1978). Culture purity was checked microscopically and by growth tests on complex media (Denger et al. 1997). Conditions were confirmed to be anoxic since the resazurin indicator remained colourless. Strain GRZTAU was obtained by similar methods (Laue et al. 1997b). Growth experiments were done as described elsewhere (Denger et al. 1997).

Cells were harvested and disrupted anoxically as described elsewhere (Denger and Cook 1997). The assay for the anoxic enzymic transformation of taurine was based on our previous experi-

ence (Laue et al. 1997b) and on an understanding of the degradative pathway for taurine in *Pseudomonas aeruginosa* (Toyama et al. 1973; Shimamoto and Berk 1979, 1980). Reaction mixtures (initial volume, 2 ml) were examined at room temperature (22–25°C) in anoxic, 6-ml tubes under the standard gas phase and were closed with butyl rubber septa. The reaction mixture contained 200 μmol potassium phosphate buffer (pH 7.5), 10 μmol taurine, 10 μmol pyruvate, 1 μmol NAD^+, 0.2 μmol pyridoxal 5′-phosphate, 0.2 μmol thiamine pyrophosphate, and 0.3 mg protein, with which the reaction was started. Samples were taken at intervals with a gas-tight syringe and were frozen immediately. We could not detect sulfite in the presence of phosphate buffer, and we sometimes used reaction mixtures in which we replaced the phosphate buffer with Tris-HCl (pH 8.0); under these conditions, we could not use the colorimetric determination for ammonia. We also tested potassium phosphate buffer at pH 7.0 and 6.6, and Mops at pH 6.5.

Bilophila wadsworthia RZATAU (DSM 11045; Laue et al. 1997b) was used as a control organism in some experiments.

Analytical methods and chemicals

Taurine was derivatized and determined by reversed-phase HPLC (Denger et al. 1997). Acetate was determined and tentatively identified by GC (Laue et al. 1997b), and the identity was confirmed in the enzymic determination for acetate (Bergmeyer 1983). Ammonia was determined and tentatively identified by the Berthelot reaction (Gesellschaft Deutscher Chemiker 1996), and the identity was confirmed in the specific reaction of glutamate dehydrogenase (Bergmeyer 1983). Thiosulfate was routinely determined by ion chromatography (Laue et al. 1996) and tentatively identified by cochromatography; the identification was confirmed titrimetrically and turbidimetrically (Gesellschaft Deutscher Chemiker 1996). Sulfite and sulfate were determined by ion chromatography (Laue et al. 1996). Sulfide was determined colorimetrically (Cline 1969). The presence of sulfite reductase (desulfoviridin) in extracts of cells disrupted under air was detected as an absorbance band at 630 nm and by its red fluorescence upon irradiation with UV light at 366 nm (Postgate 1956, 1959). Protein in whole cells was solublized in 0.4 M NaOH (30 min at 80°C), the solution was neutralized to pH 6–7 with phosphoric acid, and the protein content was assayed by the method of Bradford (1976).

A partial sequence of 16S rDNA was determined by the German Culture Collection (DSMZ; Braunschweig, Germany), which also derived the phylogenic information from the sequence data (Denger and Cook 1997). Strain GKNTAU has been deposited with the DSMZ as DSM 11270.

The sources of the chemicals used are given elsewhere (Denger et al. 1997).

Results

We tested for fermentative growth with nine organosulfonates as substrates, of which four were natural products [methane- and ethanesulfonates, taurine, and cysteate (2-amino-3-sulfopropionate)] and five were xenobiotics (1-heptanesulfonate, benzene- and 4-toluenesulfonates, 4-sulfobenzoate, and 2,6-naphthalenedisulfonate). Two independent inocula from the anaerobic digestors of the sewage works in Konstanz (KN) and Radolfzell (RZ) were used for each substrate. Only two cultures, one each with taurine (TAU) and cysteate (CYSA), showed more growth than the corresponding negative control after 3 weeks. These cultures, GKNTAU and GKNCYSA, grew reproducibly when subcultured and were fully grown in 2 days. After five subcultures, we attempted to isolate pure cultures and succeeded with strain GKNTAU. Another organism, strain GRZTAU, with indistinguishable properties, was isolated by a variant of the enrichment procedure (Laue et al. 1997b).

Strain GKNTAU was a highly refractive, gram-positive, motile, spore-forming rod. The subterminal spores caused slight swelling of the vegetative cell. We tentatively attributed this bacterium (2.5–4.5 × 0.75 μm), which was oxidase- and catalase-negative, to the clostridia. Strain GKNTAU was attributed to the *Syntrophomonas* assemblage within the *Clostridium* subdivision of gram-positive bacteria as a presumed novel genus on the basis of a partial 16S rDNA sequence. Sequence similarities between 87.6 and 80.3% were observed with *Desulfitobacterium dehalogenans*, *Desulfotomaculum geothermicum*, *Moorella thermoacetica*, *Moorella thermoautotrophica*, *Desulfotomaculum thermoacidovorans*, *Thermosyntropha lipolytica*, *Syntrophomonas wolfei*, *Sporomusa paucivorans*, *Desulfotomaculum orientis*, *Desulfotomaculum sapomandens*, *Desulfotomaculum ruminis*, *Syntrophospora bryantii*, *Desulfotomaculum australicum*, *Desulfotomaculum nigrificans*, *Syntrophobotulus glycolicus*, *Selenomonas ruminantium*, *Dictyoglomus thermophilum* and *Dictyoglomus* sp. Rt46-B1. The phylogenetic location of strain GKNTAU will be detailed elsewhere.

Strain GKNTAU had a narrow substrate range; it was found to utilize only one of the 17 tested sulfonates [the compounds in the enrichments, isethionate (2-hydroxyethanesulfonate), coenzyme M (2-mercaptoethanesulfonate), 2-bromoethanesulfonate, aminomethanesulfonate, 2-aminobenzenesulfonate, *N*-cyclohexanesulfamate, Caps (cyclohexaneaminopropanesulfonate), and Hepes (*N*-2-hydroxyethanepiperazine-*N*′-2-ethanesulfonate)]. None of 20 tested nonsulfonate, fermentable substrates or autotrophic regimes supported growth [methanol, ethanol, glycerol, glucose, fructose, xylose, arabinose, formate plus acetate (2 mM), H_2/CO_2 plus acetate, DL-lactate, pyruvate, succinate, fumarate, DL-malate, ethylamine, ethanolamine, L-alanine, casamino acids (0.1%), and a mixture of lactate,

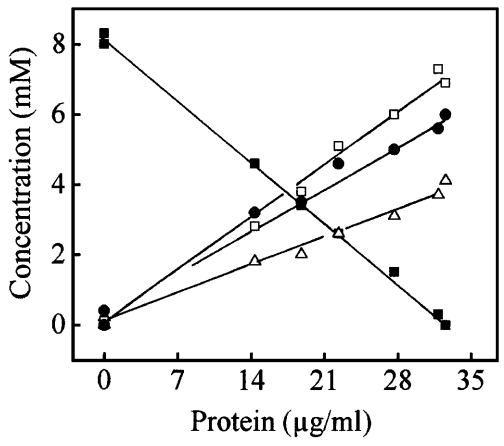

Fig. 1 Utilization of taurine and formation of products as a function of growth of strain GKNTAU in anoxic salts medium. ■ Taurine, □ ammonia, ● acetate and △ thiosulfate

Table 1 Mass balance of the utilization of taurine by strain GKNTAU during growth in taurine-salts medium, with representations of the overall assimilation reactions and the dissimilatory reaction. We used the data of Luria (1960; dry matter is 50% protein, or 50% carbon and 14% nitrogen) to convert the observed protein yield data into material assimilated, which we considered to be two-carbon units (presumably from acetate) and nitrogen (from ammonia). We presumed the assimilated sulfur to be negligible [cf. Luria (1960)]. This led us to generate Eq. 2, since the synthesis of 1 mg of protein required 41.6 μmol of taurine. The electron balance indicated that the reducing equivalents deduced for the oxidation of taurine were accounted for in the end products (acetate and thiosulfate)

Taurine consumed (μmol)	Protein		Assimilation		Products			Balances		
	Formed (mg)	Yield (g protein /mol taurine)	C2 unit (μmol)	Nitrogen (μmol)	Acetate (μmol)	Ammonia (μmol)	Thiosulfate (μmol)	Electron (%)	Nitrogen (%)	Sulfur (%)
407.5	1.63	4.0	67.8	32.6	300	365	205	99	98	101

acetate and ethanol with either sulfate or sulfite as electron acceptor (at 20 mM)].

Strain GKNTAU grew exponentially (μ = 0.09 h^{-1}) when fermenting taurine. Substrate utilization was concomitant with growth (Fig. 1), and a molar growth yield of 4 g protein/mol taurine was observed. Three products were formed concomitantly from taurine during growth (Fig. 1). Ammonia was determined and tentatively identified by the Berthelot reaction, and the identity was confirmed in the specific reaction of glutamate dehydrogenase. Acetate was determined and tentatively identified by GC, and the identity was confirmed in the enzymic determination for acetate. Thiosulfate was routinely determined and tentatively identified by cochromatography in an ion chromatograph; the identification was confirmed titrimetrically and turbidimetrically. We calculate that the products represent mass balances for carbon, nitrogen and sulfur, and for electrons (Table 1 and Discussion).

We were concerned to prove the biosynthesis of thiosulfate because spontaneous formation of the compound from sulfide and sulfite is known (Piesch 1960). The latter is a slow reaction that is favoured at alkaline pH values (Piesch 1960). We determined that the pH after growth was 6.6–6.8 and, although we could detect traces of sulfite in our medium during growth, we could detect no sulfide. We could detect negligible formation of thiosulfate from sulfide and sulfite in control experiments without cells at pH 6.8. We thus attributed the formation of thiosulfate in cultures of strains GKNTAU and GRZTAU to enzymic reactions.

Cell-free extracts of strain GKNTAU catalyzed the degradation of taurine (Fig. 2). The reaction required the presence of both crude extract and taurine, and was dependent on the presence of pyruvate; 2-ketoglutarate could not replace pyruvate. There was little reaction below pH 7.0; therefore, we routinely used phosphate buffer (pH 7.5) or Tris-HCl (pH 8.0) and incubated at about 25°C since lower rates were found at 30 and 37°C. The specific degradation rate for taurine was approximately 1.4 mkat/kg protein. We could detect formation of alanine and of ammonia when we used the phosphate buffer (Fig. 2). We presumed the initial reaction to be a pyruvate-dependent transamination yielding alanine that was followed by an alanine dehydrogenase yielding ammonia. Acetate was also formed in these reactions (Fig. 2), as

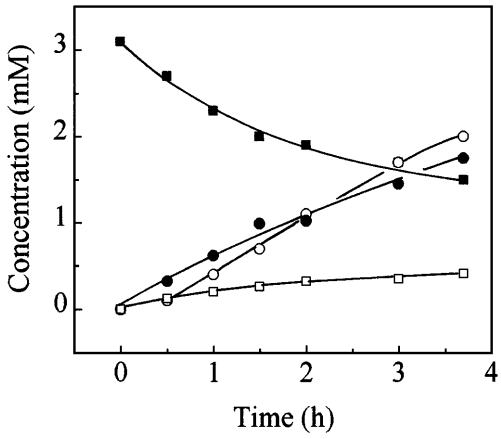

Fig. 2 Transformation of taurine by cell-free extracts from strain GKNTAU. ■ Taurine, ○ alanine, □ ammonia, ● acetate

were traces of sulfite; after overnight incubation, we could detect traces of thiosulfate, but not of sulfide or sulfate. We were unable to detect desulfoviridin in oxic cell extracts; extracts of *Bilophila wadsworthia* RZATAU were used as the positive control [cf. Laue et al. (1997b)].

Discussion

Strain GKNTAU (and strain GRZTAU) grew strictly anaerobically in the presence of a single source of carbon and energy nutrition, taurine. The absence of an external electron acceptor and the internally balanced redox reactions, whereby oxidation of the carbon moiety to acetate and reduction of sulfonate moiety to thiosulfate occur, mark this as a fermentation. This is, thus, the first reported fermentation of a sulfonate [see review by Seitz and Leadbetter (1995)].

This fermentation is the third form of dissimilatory anaerobic metabolism involving organosulfonates and follows two respiratory forms in which isethionate, taurine and cysteate, amongst others, are either reduced in the presence of an electron donor (Lie et al. 1996; Laue et al. 1997b) or are oxidized by a denitrifying bacterium (Denger et al. 1997). The products of this novel reaction have been thoroughly identified and quantified; we be-

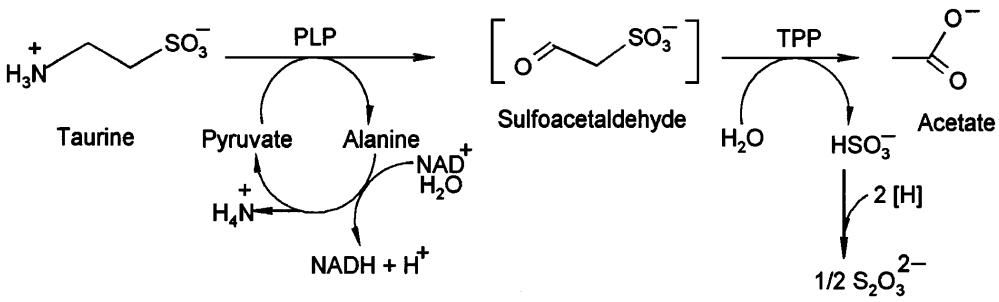

Fig. 3 Working hypothesis for the fermentation of taurine to thiosulfate, acetate and ammonia in strain GKNTAU, created by adding one reaction to the published pathway for taurine degradation in *Pseudomonas* (Shimamoto and Berk 1979, 1980). (*PLP* pyridoxal 5′-phosphate, *TPP* thiamine pyrophosphate)

lieve that such data are mandatory if a new hypothesis is to have valid support, whether the products are common or uncommon. The assimilation of sulfur from organosulfonates by anaerobic bacteria is also a recent discovery and also involves taurine (Chien et al. 1995; Denger et al. 1996; Denger and Cook 1997). Where data are available (Lie et al. 1996; Laue et al. 1997b; this work), it would appear that the same degradative pathway for taurine is involved; this is a pathway – though not the only one (Van der Ploeg et al. 1996; Kondo and Ishimoto 1987) – already known in aerobic bacteria (Toyama et al. 1973; Shimamoto and Berk 1980). We plan to explore whether one set of genes is responsible for the common reactions in these very different bacteria.

Not only the fermentation, but also one of the fermentation products is novel, namely thiosulfate. Thiosulfate is found naturally in anoxic environments (Höll 1979) and is widely used as an electron acceptor or as an electron donor (Jørgensen 1990; Jørgensen and Bak 1991). Despite this importance, no significant biological source of thiosulfate has been reported [see also Fuseler and Cypionka (1996)]. We thus believe that we have detected a potential biological source of thiosulfate, namely the widespread taurine (Huxtable 1992). Taurine could conceivably be the sole significant biological source of thiosulfate, but sulfonates are major components of soil sulfur, reaching 40% in some forests (Autry and Fitzgerald 1990); therefore, it seems possible that other sulfonates could also be fermented to thiosulfate. However, not all fermentations of sulfonates yield thiosulfate; Laue et al. (1997a) have recovered the sulfonate moiety of cysteate as sulfate and sulfide, presumably via sulfite, but have found no thiosulfate. We interpret the latter observations as further support for the absence of significant spontaneous formation, in other words, for the enzymic formation of thiosulfate under our conditions.

We consider the fermentation to be represented by two formal equations, one for dissimilation (Eq. 1) and one for assimilation (Eq. 2). The dissimilatory reaction is exergonic (Eq. 1), and the $\Delta G^{\circ\prime}$ value indicates a maximum energy yield of 1.5–2 mol ATP/mol taurine given a requirement of 60–80 kJ/mol ATP (Thauer et al. 1977). The molar growth yield, approximately 8 g dry wt./mol taurine (Table 1 with legend), thus implies a practical Y_{ATP} of approximately 5 g dry wt./mol, which is reasonable given Y_{ATP}^{max} values of 11.4–14.6 g dry wt./mol (Badziong and Thauer 1978). The material incorporated into biomass (about 17% of taurine carbon) was presumed to have been generated in the assimilatory reaction (Eq. 2) in accordance with the measured products (Fig. 1). These assumptions indicate that there is electron (99%), nitrogen (98%) and sulfur (101%) balance for the fermentation (Table 1).

$$2\ C_2H_7NSO_3 + 1\ H_2O \rightarrow 2\ C_2H_3O_2^- + S_2O_3^{2-} + 2\ NH_4^+ + 2\ H^+ \quad \text{(Eq. 1)}$$

$\Delta G^{\circ\prime}$ = –117.4 kJ/mol of taurine (Thauer et al. 1977)

$$17\ C_2H_7NSO_3 \rightarrow 8\ C_4H_8O_2N + 2\ CO_2 + 8.5\ S_2O_3^{2-} + 9\ NH_4^+ + 8\ H^+ + 5.5\ H_2O \quad \text{(Eq. 2)}$$

We calculated from the growth rate (Fig. 1) and from the growth yield (Table 1) that the specific degradation rate of taurine in growing cells is 6.3 mkat/kg protein. The initial enzyme activity observed, 1.4 mkat/kg protein (Fig. 2), indicates that a significant portion of the activity in the cell-free assay was recovered. The enzyme activities (Fig. 2), the detection of traces of sulfite, and a pathway in aerobes are the basis of our working hypothesis on the degradative pathway (Fig. 3). We have yet to confirm that sulfoacetaldehyde is an intermediate, we do not know how sulfite is reduced to thiosulfate, and we do not know how energy is conserved. Given a published synthesis of sulfoacetaldehyde (Kondo et al. 1971) and our preliminary generation of thiosulfate in enzyme preparations, we hope to be able to answer these questions.

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