Sulphoacetaldehyde sulpho-lyase (EC 4.4.1.12) from *Desulfonispora thiosulfatigenes*: purification, properties and primary sequence

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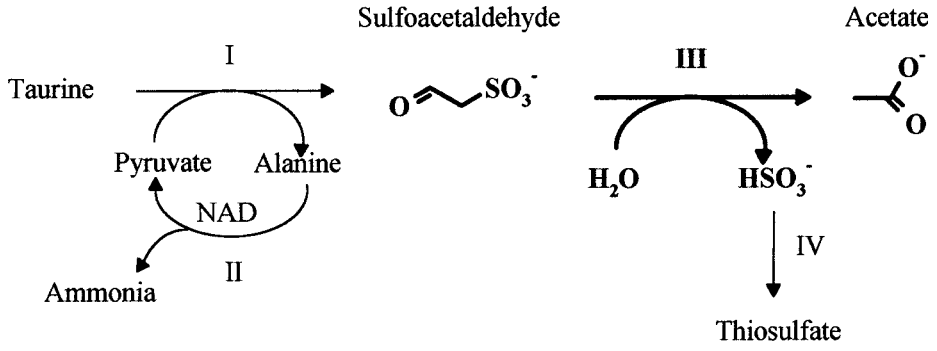
The strictly anaerobic bacterium *Desulfonispora thiosulfatigenes* ferments taurine via sulphoacetaldehyde, which is hydrolysed to acetate and sulphite by sulphoacetaldehyde sulpho-lyase (EC 4.4.1.12). The lyase was expressed at high levels and a two-step, 4.5-fold purification yielded an apparently homogeneous soluble protein, which was presumably a homodimer in its native form; the molecular mass of the subunit was about 61 kDa (by SDS/PAGE). The mass was determined to be 63.8 kDa by matrix-assisted laser-desorption ionization–time-of-flight (MALDI–TOF) MS. The purified enzyme converted 1 mol of sulphoacetaldehyde to 1 mol each of sulphite and acetate, but no requirement for thiamine pyrophosphate (TPP) was detected. The N-terminal and two internal amino acid sequences were determined, which allowed us to generate PCR primers. The gene was amplified and sequenced. The DNA sequence had no significant homologue in the databases searched, whereas the derived amino acid sequence indicated an oxo-acid lyase, revealed a TPP-binding site and gave a derived molecular mass of 63.8 kDa.

Key words: desulphonation, enzyme purification, oxo-acid lyase, taurine fermentation.

INTRODUCTION

Taurine (2-aminoethanesulphonate), at about 1 g/kg of body weight, is the major organic solute in mammals, though no cleavage of the C–SO_3^- bond is known in mammals [1]. Kondo et al. [2,3] established one pathway variant, whereas Shimamoto and Berk [4] showed the pathway that is often presented for aerobic organisms (Scheme 1). The enzyme that cleaves the C–SO_3^--bond, sulphoacetaldehyde sulpho-lyase (EC 4.4.1.12), was purified by Kondo and Ishimoto [5] at a time when protein characterization was quite difficult. So, apart from requirements for thiamine pyrophosphate (TPP) and Mg^{2+}, the stoichiometric formation of acetate and sulphite from sulphoacetaldehyde (confirmed in [4]), and a native molecular mass of 85 kDa, very little is known about the enzyme, apart from that it is widespread [6,7].

For many years, desulphonation reactions were unknown in anaerobic micro-organisms (e.g. [8]), although there is no theoretical objection to the reactions in Scheme 1 occurring in anaerobes. Indeed, precisely this pathway was found or suspected in several anaerobic micro-organisms [9], and the transaminase and the dehydrogenase have been purified from the strictly anaerobic, taurine-reducing bacterium *Bilophila wadsworthia* [10,11]. We have now been able to purify the sulpho-lyase from



Scheme 1 Sulphoacetaldehyde sulpho-lyase in the metabolism of taurine in *D. thiosulfatigenes*

The first interaction of *D. thiosulfatigenes* with taurine is presumably transport across the cell membrane [9]. Work on cell extracts indicates the presence of both a pyruvate-dependent taurine transaminase (I) and an alanine dehydrogenase (II) [12]. The sulphite formed by the sulpho-lyase (III) is apparently subject to reduction by a P582 sulphite reductase [13], whereas the mechanism of formation of thiosulphate (IV) is still unknown.

Abbreviations used: *sly*, sulphoacetaldehyde sulpho-lyase gene; MALDI–TOF, matrix-assisted laser-desorption ionization–time-of-flight; TPP, thiamine pyrophosphate.

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the strictly anaerobic, taurine-fermenting bacterium *Desulfonispora thiosulfatigenes* GKNTAU [12,13] and we describe its properties and primary sequence.

EXPERIMENTAL

Materials

The bisulphite addition complex of 2-sulphoacetaldehyde dihydrate [$NaO_3SCH_2CH(OH)SO_3Na \cdot 2H_2O$] was synthesized from 2-bromoacetaldehyde dimethylacetal and sodium metabisulphite in alkaline solution [2]. The product was identified by ^{1}H-NMR and IR spectroscopies, and the spectra were essentially identical with published data [2,14]. The synthetic material was 53 % pure according to elemental analyses; the impurity was mainly sodium sulphate as determined by ion chromatography. The bisulphite adduct was used directly in most experiments, without conversion to the aldehyde (see [15]). We could also generate 2-sulphoacetaldehyde biologically, in a 1 ml reaction mixture containing purified taurine: pyruvate aminotransferase (0.17 mg) from *B. wadsworthia* RZATAU [11], 100 μmol of potassium phosphate buffer, pH 7.8, 5 μmol of $MgCl_2$, 10 μmol of taurine, 10 μmol of pyruvate and 0.2 mmol of pyridoxal 5′-phosphate. 3-Sulphopyruvate was synthesized from 3-bromopyruvate and Na_2SO_3 [16]. The synthetic product, co-chromatographed with authentic material kindly provided by R. H. White (Virginia Polytechnic Institute, Blacksburg, VA, U.S.A.), and this tentative identification was confirmed by matrix-assisted laser-desorption ionization–time-of-flight (MALDI–TOF) MS in the negative ion mode [17], where a peak (m/z) of 167 $[M-H]^-$ indicated a molecular mass of 168 Da ($C_3H_4O_6S$). 3-Sulpholactate was prepared enzymically from 3-sulphopyruvate by the NADH-coupled reaction of malate dehydrogenase (EC 1.1.1.37) [18]. The product was identified by MS (as above), where a peak (m/z) of 169 $[M-H]^-$ was observed, which indicated a molecular mass of 170 Da ($C_3H_6O_6S$).

Analytical methods

Sulphoacetaldehyde or 3-sulphopyruvate was determined as the azine formed by reaction with 2-(diphenylacetyl)indane-1,3-dione-1-hydrazone [19] after separation by HPLC with UV detection (400 nm). Acetate was determined by GC [20]. Sulphite was quantified colorimetrically as the sulphite–fuchsin complex [21] based on use of stock solution A (0.8 M H_2SO_4, 0.08 % fuchsin and 1.6 % formaldehyde), mixed 7:2:1, which was stable for several weeks at 4 °C. The sample (50 μl) was mixed with stock solution A (950 μl) and A_{580} was measured after 5–10 min. The test was linear at 0–2 μmol of sulphite/reaction, as determined with a freshly prepared standard solution of sodium sulphite. Ion chromatography was as described elsewhere [20]. Protein was assayed colorimetrically [21a]. Denatured proteins were separated by SDS/PAGE (10 % gels) and stained with Coomassie Brilliant Blue R250 [22]; molecular masses of bands were determined by comparison with standard proteins (Low Range Marker Proteins, Bio-Rad). The molecular mass of the native protein was estimated by gel filtration [23]; the calibration proteins were from Pharmacia. The molecular mass was determined by dynamic light scattering in a Dynapro instrument (Protein Solutions, Charlottesville, VA, U.S.A.); the conditions were 20 mM Tris/sulphate buffer, pH 7.5, with 200 mM Na_2SO_4 at about 20 °C and the protein concentration was 23 mg/ml. The N-terminal amino acid sequence of a blotted protein was determined after Edman degradation, as indicated previously [24]. The sequences of internal peptides were obtained by MALDI-TOF MS after proteolysis (with lysyl endopeptidase, EC 3.4.21.50) of the separated protein and separation of the peptides by HPLC (Top-Lab Service Facility, München, Germany). MALDI–TOF MS of the native sulpho-lyase was done with the apparatus described elsewhere [17].

Preliminary experiments established that anoxic conditions for harvesting and disrupting the cells and for enzyme assays [12] were unnecessary; the enzyme was stable under aerobic conditions. A discontinuous assay (1 ml) of the sulpho-lyase was used. Crude extract or column eluate (0.2–0.8 mg of protein) was incubated at 45 °C with 2 mM sulphoacetaldehyde bisulphite adduct in 0.1 M potassium phosphate buffer, pH 7.5, containing 5 mM $MgCl_2$. Portions (50 μl) of the reaction mixture were stopped either by addition to solution A, to determine sulphite, or by acidification with 10 M formic acid (5 μl) to determine acetic acid. The reaction was linear for 2 min. In negative controls with boiled (10 min) cell extracts or boiled enzyme, small amounts of sulphite were produced chemically and the data corrected appropriately. Loss of sulphite due to oxidation to sulphate was negligible during the assay period. Variants of the assay were used to determine stoichiometry of the reaction, kinetic properties of the enzyme and optima. Enzyme activity was quantified in katals, usually in terms of acetate formation.

PCR was done with total DNA [25] from *D. thiosulfatigenes* strain GKNTAU using the Expand Long Template PCR System (Roche), which contains proof-reading activity and degenerate primers (see below). The nucleotide sequence of both strands of the 1.6 kb PCR product was determined by cycle sequencing and primer walking using the Applied Biosystems ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit and an ABI 337 DNA Sequencer, which was operated by GATC (Konstanz, Germany). The fragment contained neither the 5′ nor the 3′ end of the gene. An adaptor-ligated genomic library was constructed (Universal GenomeWalker™ Kit, Clontech). PCR with gene- and adaptor-specific primers (Expand System, Roche) and subsequent sequencing were used to obtain the missing nucleotides. Sequence analysis was done using the DNASTAR Lasergene program package. The National Center for Biotechnology Information BLAST programs were used to search for similarities amongst the new and established sequences [26]. The PROSITE tools of the Swiss Institute of Bioinformatics were used to search for motifs in the SWISS-PROT database.

Organisms, growth conditions and cell disruption

D. thiosulfatigenes^T GKNTAU (DSM 11270, ATCC 700533) was grown anaerobically at 30 °C in bicarbonate-buffered mineral-salts medium containing 40 mM taurine as the sole source of carbon and energy for fermentative growth and titanium(III) nitrilotriacetate to establish a low redox potential [12]. Cells (0.5 g of wet weight/l) were grown in 10 l glass vessels and harvested by centrifugation at 13000 ***g*** and 4 °C for 20 min under air at the end of the exponential growth phase. Pellets were washed twice with 0.1 M potassium phosphate buffer, pH 7.5, containing 5 mM $MgCl_2$; the presence of Mg^{2+} was essential to obtain enzyme activity. Portions of cells were stored at −20 °C until use. Cells (0.8 g) were suspended in chilled 0.1 M potassium phosphate buffer, pH 7.5, containing 5 mM $MgCl_2$, and disrupted by four passages through a chilled French pressure cell (140 MPa). Whole cells and cell debris were removed by centrifugation (5000 ***g***, 10 min, 4 °C). Nucleic acids in this chilled crude extract were precipitated by dropwise addition of streptomycin sulphate over a period of 30 min to an end concentration of 2 % (w/v) and removed by centrifugation (5000 ***g***, 10 min, 4 °C). Ultracentrifugation (220000 ***g***, 30 min, 4 °C) was used to collect membranous material (called the membrane fraction) after two

washing steps in the same buffer, and the supernatant fluid was termed the soluble fraction. If the soluble fraction alone was required, whole cells, debris, nucleic acids and membranous material could be removed in one step by ultracentrifugation.

Comamonas acidovorans SFCD1 [7] and *Acinetobacter* sp. strain ICD [6] were kindly made available by Dr J. Quinn (Queen's University, Belfast, Northern Ireland). They were grown aerobically [6,7] and harvested and disrupted as described above.

Enzyme purification

A two-step purification sufficed. All chromatographic steps were done at room temperature under air. Collected fractions were maintained at 4 °C. The soluble fraction was diluted 1:5 with water and pumped on to an anion-exchange column (Mono Q HR 10/10, Pharmacia) equilibrated with 20 mM Tris/sulphate buffer, pH 7.5. Proteins were eluted with a linear gradient from 0 to 0.5 M Na_2SO_4 in 20 mM Tris/sulphate buffer, pH 7.5. The flow rate was 2 ml/min, and the enzyme eluted at about 200 mM Na_2SO_4.

Samples from the anion-exchange column were subject to gel-filtration chromatography (Superose 12 HR 10/30, Pharmacia) at about 20 °C with 50 mM Tris/sulphate buffer, pH 7.5, containing 150 mM Na_2SO_4 as the eluent at a flow rate of 0.4 ml/min. Typically, protein at 1.7 mg/ml (200 μl) was loaded on to the column.

RESULTS

Sulphoacetaldehyde sulpho-lyase as the major protein in *D. thiosulfatigenes*

Crude extract of *D. thiosulfatigenes* was separated into a membrane fraction, which contained negligible sulpho-lyase activity, and a soluble fraction, which contained enzyme activity (21 mkat/kg of protein; Table 1). The proteins in the soluble fraction were dominated by one band, which was shown to be the sulpho-lyase in the purification steps (Figure 1). A purification factor of 4.6 was attained (Table 1), which indicates that the sulpho-lyase represents some 22% of soluble protein, and corresponds to the electropherogram (Figure 1).

Catalytic properties of the sulpho-lyase

The enzyme catalysed the conversion of 1 mol of 2-sulphoacetaldehyde bisulphite adduct to 1 mol of acetate and 2 mol of sulphite, which were identified thoroughly elsewhere [12]. When 1 mol of 2-sulphoacetaldehyde was generated enzymically from taurine, the reaction products in the presence of the sulpho-lyase were 1 mol each of acetate and sulphite. We thus presume that the reaction *in vivo* is indeed the conversion of 1 mol of sulphoacetaldehyde to 1 mol of acetate and 1 mol of sulphite, as shown in Scheme 1.

Table 1 Purification of sulphoacetaldehyde sulpho-lyase

Step	Activity (μkat)	Protein (mg)	Specific activity (mkat/kg)	Purification factor	Yield (%)
Crude extract	0.81	38.0	21	1.0	100
Soluble fraction	0.80	27.0	29	1.4	98
Anion-exchange chromatography	0.69	7.4	93	4.4	85
Gel-filtration chromatography	0.46	4.7	97	4.6	56

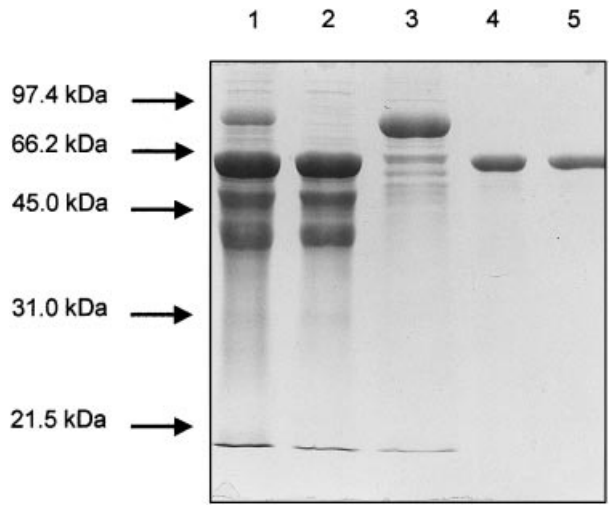

Figure 1 Purification of sulphoacetaldehyde sulpho-lyase from *D. thiosulfatigenes* monitored by SDS/PAGE

Proteins from different stages of the purification were separated by SDS/PAGE and stained with Coomassie Brilliant Blue. Lane 1, crude extract (40 μg); lane 2, soluble fraction (40 μg); lane 3, membrane fraction (40 μg); lane 4, anion-exchange chromatography (5 μg); lane 5, gel filtration (5 μg). Molecular-mass standards: 97.4 kDa, phosphorylase *b*; 66.2 kDa BSA; 45.0 kDa, ovalbumin; 31.0 kDa, carbonic anhydrase; 21.5 kDa, trypsin inhibitor.

We could assay enzyme activity in phosphate buffer only; Hepes, Mops, triethanolamine or Tris/HCl, each at 100 mM and pH 7.5, and supplemented with 5 mM $MgCl_2$, did not support activity. The highest activity was observed at pH 7.8, where the buffer capacity was minimal, so we chose to work at pH 7.5. Another reason for the lower pH value was the instability of the substrate at higher pH values.

We had anticipated a requirement for TPP for enzyme activity [3,5], but it was never necessary to add TPP to any reaction mixture and its addition ($\leqslant$ 10 mM) or preincubation (30 min, 37 °C, 5 mM TPP) did not increase enzyme activity. Denatured enzyme (see below) could not be reactivated in the presence of 1 mM TPP.

The temperature optimum of the enzyme was about 45 °C, which was corrected for instability of the substrate at increased temperatures. A K_m value of 0.22 mM ($\pm$5%, $n = 7$) was determined by Lineweaver–Burk plot and direct linear plots (0.1–5 mM substrate); the value of V_{max} was about 17 mkat/kg of protein. No substrate apart from sulphoacetaldehyde (or its bisulphite adduct) was found; we tested 3-sulphopyruvate, 3-sulpholactate, isethionate (2-hydroxyethanesulphonate), hypotaurine (2-aminoethanesulphinate), 3-aminopropanesulphonate, L-cysteate (2-amino-3-sulphopropionate), taurine, 2-sulphoacetate and acetaldehyde. None of these substances inhibited the turnover of sulphoacetaldehyde.

The enzyme was stable. It could be stored as column eluent at room temperature for many hours without loss; heating at 30, 45 and 55 °C for 2 h caused 0, 50 and 95% loss, respectively. There was no loss over 1 week at 4 °C and frozen material could be thawed several times without loss.

Physical properties of the enzyme

The denatured enzyme gave a single band of 60–62 kDa on SDS/PAGE (Figure 1) and a unique N-terminal amino acid sequence (see below), so there was only one species of subunit. The apparent molecular mass of the native protein was estimated

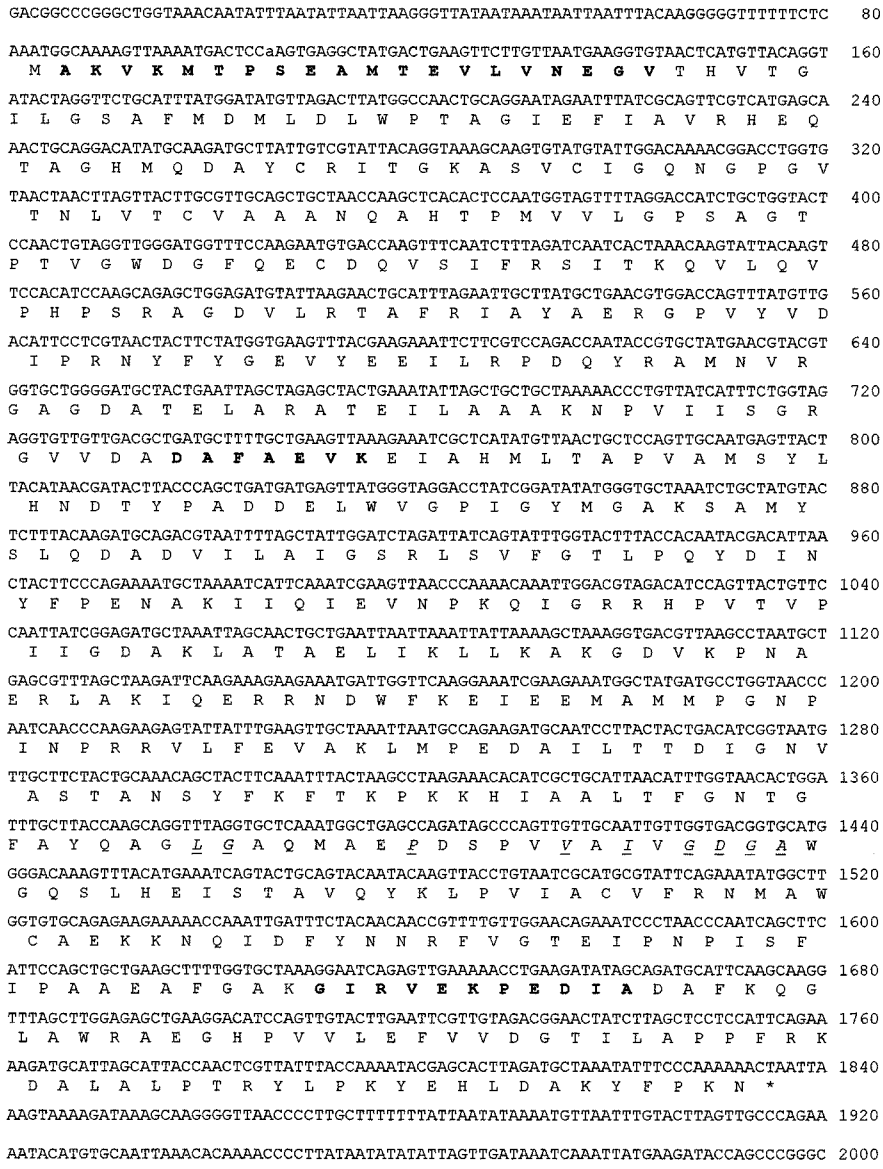

Figure 2 DNA sequence and amino acid sequence of the sulphoacetaldehyde sulpho-lyase from *D. thiosulfatigenes*

The N-terminal and two internal amino-acid sequences, which were determined by chemical methods, are indicated in bold. The conserved amino acids of the putative TPP-binding site are underlined and in italics. The stop codon is marked with an asterisk. Putative promotor elements and termination loop sequences (results not shown) were detected by motif-analysis software in the non-coding sequences upstream and downstream of the *sly* gene.

to be 236 kDa by gel filtration (results not shown), which would indicate a homotetrameric structure. However, dynamic light scattering indicated a native molecular mass of about 125 kDa, so the native protein is presumably a dimer whose structure is far from spherical.

The N-terminal sequence of the enzyme was: AKVKMTPSEAMTEVLVNEGV, which did not show any significant similarity with proteins in the databases. Two internal fragments were obtained, DAFAEVK and GIRVEKPEDIA. The latter was tentatively assumed to be the C-terminus because no terminal K was present, but the conclusion was incorrect (see below).

Degenerate primer pairs for PCR amplification of most of the sulphoacetaldehyde sulpho-lyase (*sly*) gene were deduced from the N-terminal and the supposed C-terminal amino acid sequences of the sulpho-lyase. The successful pair was 5′-GCNAARGTNAARATGACNCC-3′ (N-terminus) and 5′-GCDATRCYTCNGGYTTYTC-3′ (C-terminus). A 1.6 kb fragment was obtained whose derived amino acid sequence included the peptides observed by microsequencing (Figure 2) but lacked the start and stop codons. The GenomeWalker™ Kit was used to complete the gene sequence. The sulpho-lyase comprised 584 amino acids (Figure 2), which corresponds to a calculated molecular mass of 63.8 kDa (allowing for the cleaved N-terminal M), close to the value observed by SDS/PAGE (Figure 1). The value determined by MALDI–TOF MS, 63.8 kDa, was identical with the value deduced from the DNA sequence.

The G+C content of the *sly* gene was established as 39%. This is markedly lower than the 52% G+C content observed for the total DNA of the organism [13]. Our experience with chromosomal genes from *B. wadsworthia* [27] and *Comamonas*

testosteroni (J. Ruff, unpublished work), in contrast, shows close correlation between overall G+C content and that of individual genes.

BLAST searches with the complete DNA sequence did not yield significant similarity with sequences from prokaryotes. Searches with the amino acid sequence in protein databases gave the highest identity (31 %) with the acetolactate synthase (EC 4.1.3.18) large subunit from *Methanobacterium thermoautotrophicum* (accession number AE000905) and from other archaea; an isozyme from *Escherichia coli* had a similarly high identity (30 %). Acetolactate synthases belong to the class of oxo-acid lyases, TPP-dependent enzymes with the TPP-binding site located towards the C-terminus. A putative TPP-binding site in our sequence (Figure 2) fits the PROSITE consensus pattern (L/I/V/M/F)(G/S/A)X_5PX_4(L/I/V/M/F/Y/W)X(L/I/V/M/F)XGD(G/S/A)(G/S/A/C), where the underlining indicates residues in sulphoacetaldehyde sulpho-lyase. A BLAST search with a 23-amino acid sequence including the putative TPP-binding site showed $\leqslant 60$ % identities between acetolactate synthases and many other TPP-requiring enzymes.

DISCUSSION

We calculated that the specific activity for the turnover of taurine in growing cells of *D. thiosulfatigenes* GKNTAU is 6.3 mkat/kg of protein [12]. The specific activity of the sulpho-lyase in crude extract (21 mkat/kg of protein, Table 1) is sufficient to explain the activity *in vivo*, so we presume that we have isolated the correct enzyme.

The enzyme is soluble (there was none in the membrane fraction, Figure 1), as is observed in aerobic bacteria [4–7]. It carries out the same reaction determined by Shimamoto and Berk [4] and Kondo and Ishimoto [5], the generation of 1 mol each of acetate and sulphite from sulphoacetaldehyde (Scheme 1). We presume, given a TPP-binding site (Figure 2), that the reaction suggested in [5] is valid. The stabilized TPP-carbanion [28] attacks the carbonyl carbon of the substrate, which undergoes loss of the good leaving group sulphite, rearrangement and hydrolytic attack to release acetate and regenerate the carbanion.

The enzyme in *D. thiosulfatigenes* differs from that already purified [5] and from the separated fractions from *Pseudomonas aeruginosa* [4] in that we observe no requirement for TPP. It is difficult to imagine the reaction (see above) occurring without a TPP cofactor, and the detection of a binding site for TPP led us to speculate that the TPP is either very tightly, but non-covalently, bound, or possibly covalently bound; identical molecular-mass values from MALDI–TOF MS and sequence data indicate the former. Another difference between the two purified enzymes is the molecular mass of the native enzyme: we observed about 125 kDa (236 kDa by gel filtration), whereas Kondo and Ishimoto observed 85 kDa by gel filtration [5]. The molecular mass of the subunit in this paper, 61 kDa (Figure 1) is supported by the genetic (Figure 2) and MS data (63.8 kDa).

We have been unable to detect sulphoacetaldehyde sulpho-lyase activity in extracts of appropriate anaerobic organisms (*Alcaligenes* sp. strain NKNTAU [29] and *B. wadsworthia* RZATAU [20]), or in *C. acidovorans* [7] or *Acinetobacter* sp. [6], with the assay optimized for *D. thiosulfatigenes* [30]; these conditions are effectively those from Kondo and Ishimoto [5]. Indeed, Laue [30] failed to find any gene resembling *sly* by PCR in *B. wadsworthia*. In contrast, the conditions defined by King et al. [6] allowed us to measure activity in at least *Alcaligenes* sp. strain NKNTAU, *C. acidovorans* and *Acinetobacter* sp. (K. Denger, unpublished work). Given these differences between the various sulpho-lyases, we suspect that there are different groups of these lyases.

Kondo and Ishimoto [5] report a purification factor of 14 for their sulpho-lyase, which is inducible. We find a 3-fold higher level of expression (purification factor 4.5, Table 1) in *D. thiosulfatigenes* GKNTAU. Strain GKNTAU is a specialist for taurine metabolism; no other substrate for growth is known among 44 tested [12,13]. So we have, as yet, no alternative substrates with which to explore regulatory mechanisms at the physiological level.

The role of sulphoacetaldehyde sulpho-lyase in the metabolism of *D. thiosulfatigenes* is to produce sulphite, whose conversion to thiosulphate is involved in energy conservation; the acetate is partially channelled to the biosynthesis of cell material, while the larger portion is excreted [12]. In aerobic organisms (and in anaerobic oxidations of sulphonates [29,31]), it is the acetate that is largely involved in energy conservation, the sulphite playing a minor role, being oxidized to sulphate (e.g. [6,29]).

Sequence alignments with *sly* in nucleotide databases gave no significant similarities, which indicates that *sly* is poorly related to known DNA sequences. In contrast, the homologies with amino acid sequences, especially near the TPP-binding site, support our allocating the enzyme to the oxo-acid lyases despite the lack of a requirement for TPP in enzyme assays.

This is the first report of the purification from an anaerobic organism of an enzyme cleaving a C–SO_3^- bond. Natural organosulphonates are widespread in oxic and anoxic ecosystems [9,32,33], and their role, especially that of taurine, in the nutrition of anaerobic mats has recently been detailed [32]. Sulphoacetaldehyde is the point of convergence of several degradative pathways [9], so the enzymes and genes described here and elsewhere [10,11,27] should be the start of an understanding of aspects of these ecosystems at the molecular level.

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