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Heike Laue · Karin Denger · Alasdair M. Cook

Fermentation of cysteate by a sulfate-reducing bacterium

Abstract We isolated a strictly anaerobic bacterium, strain GRZCYSA, from a sludge digestor for its ability to ferment cysteate (2-amino-3-sulfopropionate). The organism also fermented the organosulfonates isethionate (2-hydroxyethanesulfonate) and aminomethanesulfonate, but taurine (2-aminoethanesulfonate) was not a substrate. Strain GRZCYSA, a gram-negative, oxidase-negative and catalase-positive vibrio that could reduce sulfate and contained desulfoviridin, was tentatively identified as *Desulfovibrio* sp. Utilization of cysteate as a substrate for fermentative growth led to the formation of four products identified as acetate, ammonia, and equimolar amounts of sulfide and sulfate. The fermentation was in balance. Some reactions involved in this novel process were detected in cell-free extracts in which ammonia and acetate were formed from cysteate.

Key words Anaerobic desulfonation · Desulfonation · Sulfonate fermentation · Sulfide · Sulfate · Sulfate-reducing bacteria · *Desulfovibrio*

Introduction

Cysteate (2-amino-3-sulfopropionate; Fig. 1) is a natural sulfonate that is the precursor of taurine in some tissues (Huxtable 1992) and a component (of weathering) of wool (Budavari 1989). Its degradation by aerobic bacteria (Stapley and Starkey 1970) and fungi (Braun and Fromageot 1962) has been known for some time, but until recently, all desulfonation reactions were believed to be limited to aerobic organisms (Fuchs et al. 1994; Seitz and Leadbetter 1995; Denger et al. 1996). The first reported anaerobic desulfonations of cysteate concerned the assimilation of sulfonate-sulfur (Chien et al. 1995), but these studies were soon followed by work on dissimilative reactions with sulfonates in respirations (Lie et al. 1996; Denger et al. 1997a; Laue et al. 1997); in the course of that work the fermentation of cysteate was discovered (Laue et al. 1997). Research on the dissimilation of sulfonates concentrated on isethionate (Lie et al. 1996) and taurine (Denger et al. 1997a, b; Laue et al. 1997), where different sulfur moieties were recovered in different metabolic situations. Thus, the reduction of sulfonates yielded sulfide (Lie et al. 1996; Laue et al. 1997), the oxidation yielded sulfate (Denger et al. 1997a), and the fermentation yielded thiosulfate (Denger et al. 1997b). Our interest widened to the fermentation of cysteate when it became clear that a new combination of sulfur moieties was formed. We now report the bacterial fermentation of cysteate to equimolar amounts of sulfate and sulfide in conjunction with the formation of acetate and ammonia.

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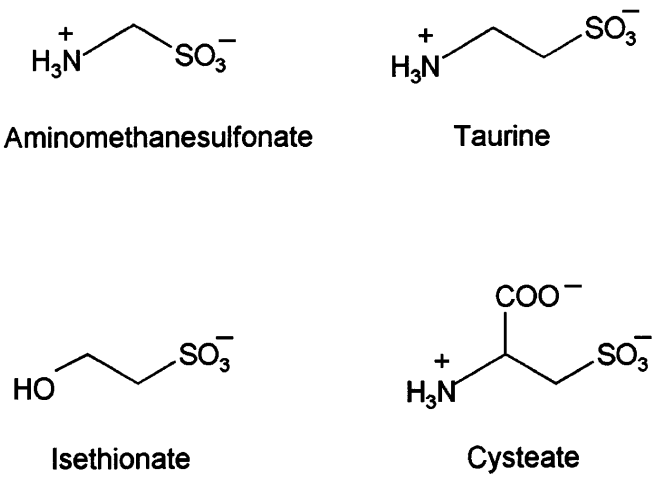

Fig. 1 The structures of aminomethanesulfonate, taurine, isethionate and cysteate

Materials and methods

Organism, growth medium and enzyme assays

Enrichment culture GRZCYSA derived from experiments inoculated with material from the anaerobic digestor of the sewage works in Radolfzell, Germany (Laue et al. 1997). A pure culture, strain GRZCYSA, was obtained from the enrichment culture by repeated application of the agar shake dilution method with selective medium (Pfennig and Trüper 1981); subsequent growth of the culture in liquid medium required the addition of supplements (Laue et al. 1997). Purity of the isolate was checked microscopically and in growth medium supplemented with 0.1% yeast extract, glucose and fructose (20 mM each), and DL-lactate (40 mM). Stock cultures were maintained at 4°C. Strain GRZCYSA has been deposited as DSM 11493 in the Deutsche Sammlung für Mikroorganismen und Zellkulturen (Braunschweig, Germany).

Anoxic, fresh-water, mineral-salts medium (Widdel and Pfennig 1981) with NH_4Cl at 0.5 mM, buffered with bicarbonate (50 mM), reduced with titanium(III) nitrilotriacetate [cf. Denger et al. (1996)], and containing a selenite-tungsten solution, a trace element solution, a seven-vitamin solution, and resazurin (Laue et al. 1997) was prepared by the methods of Widdel and Pfennig (1981). The pH of the medium was adjusted to 7.0–7.1. Cultures (25 ml) were incubated at 30°C under an atmosphere of N_2 plus CO_2 (80:20, v/v) in infusion bottles sealed with butyl rubber septa.

Mass balances were examined in sets of duplicates in 25-ml cultures containing different cysteate concentrations supplemented with 0.05% yeast extract. Growth experiments were done in 100-ml cultures inoculated (10%, v/v) from a homologous preculture. Growth was monitored as the optical density at 580 nm.

Cells were harvested under a nitrogen atmosphere, and cell-free extracts were prepared as described previously (Laue et al. 1997) with the exception that potassium phosphate buffer (100 mM, pH 7.0) was used in place of Tris-HCl. Experiments with cell-free extracts were carried out discontinuously at 30°C under anoxic conditions. The 1-ml reaction mixtures in 6-ml sealed tubes contained anoxic potassium phosphate buffer (approximately 64 μmol, pH 7.0), 5 μmol cysteate, 5 μmol pyruvate or 5 μmol α-ketoglutarate, 0.1 μmol pyridoxal-5′-phosphate, 0.1 μmol thiamine pyrophosphate, 2 μmol $MgCl_2$, 0.5 μmol NAD^+, and up to 1.0 mg of protein, with which the reaction was started. Controls were done without crude extract, or without cysteate or pyruvate. Samples were taken at intervals with gas-tight microlitre syringes and were frozen immediately in liquid N_2.

Desulfoviridin was assayed in oxic extracts of cells as an absorption band in the visible spectrum (at approximately 630 nm) and by fluorescence (Postgate 1956, 1959).

Analytical methods and chemicals

Reversed-phase HPLC with diode array detection was done as described elsewhere (Laue et al. 1996). Cysteate, taurine and alanine were quantified by HPLC after derivatisation with 2,4-dinitrofluorobenzene (Denger et al. 1997a). We occasionally confirmed these data by derivatizing with *o*-phthaldialdehyde (Stipanuk et al. 1987). Acetate was determined by gas chromatography (Laue et al. 1997). The identity of acetate was confirmed by the specific acetyl-CoA synthase assay (Bergmeyer 1983). Formate was determined colorimetrically (Lang and Lang 1972). Ammonia was measured photometrically by the Berthelot reaction (Gesellschaft Deutscher Chemiker 1996) and was confirmed enzymically by the specific reaction of glutamate dehydrogenase (Bergmeyer 1983). Sulfide was determined using the methylene blue method (Cline 1969). The brown precipitation with copper salts (Widdel and Bak 1992) was used to establish the identity of sulfide. Sulfate was detected by a turbidity assay as $BaSO_4$ (Schauder et al. 1986) and was occasionally measured by ion chromatography, which was also used to detect sulfite (Laue et al. 1996). Protein in whole cells was measured by a modification of the Lowry method (Cook and Hütter 1981), whereas protein in crude extracts was assayed by the method of Bradford (1976); bovine serum albumin was used as the standard in each case. Determination of the Gram type was done by the KOH-test (Gerhardt et al. 1994) and by the aminopeptidase assay (Merck, Darmstadt, Germany). Oxidase and catalase tests were carried out following standard methods (Gerhardt et al. 1994).

The sources of the chemicals and gases used (N_2, N_2/CO_2) are given elsewhere (Denger and Cook 1997; Laue et al. 1997).

Results

The enrichment culture GRZCYSA was strictly anaerobic (resazurin indicator colourless) and grew reproducibly in 3 days in minimal medium containing 20 mM cysteate. Since the first attempts to isolate a pure culture by the agar shake method yielded colonies that could not be subcultured in liquid medium, we added a combination of supplements (yeast extract, casamino acids, α-lipoic acid, 1,4-naphthoquinone, hemin and putrescine). This allowed us to obtain a pure culture, strain GRZCYSA. Yeast extract alone sufficed as a supplement in the cysteate-minimal liquid medium, and cultures grew to the stationary phase in 3 days. The yeast extract supported background growth, which we wanted to suppress in some experiments. We found that the pure culture could indeed grow without yeast extract, although cultures required 7 days. There was no significant difference in the molar growth yield with and without yeast extract.

Strain GRZCYSA was a strictly anaerobic, gram-negative, motile, vibrioform bacterium (1.5–2.5 × 0.5 μm; Fig. 2). It was oxidase-negative and catalase-positive. Formation of endospores was not observed. Sulfate reduction and the presence of desulfoviridin were detected (see below). We tentatively identified the organism as *Desulfovibrio* sp.

Desulfovibrio sp. strain GRZCYSA was found to utilize two naturally occurring organosulfonates, cysteate and (possibly incompletely) isethionate, for growth in addition to the xenobiotic aminomethanesulfonate. The other sulfonates tested [methanesulfonate, ethanesulfonate, taurine,

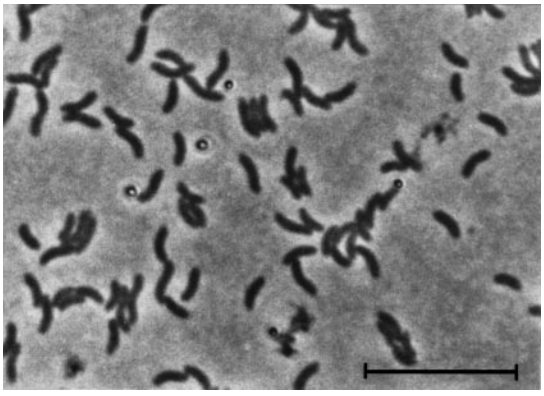

Fig. 2 Phase contrast photomicrograph of *Desulfovibrio* sp. strain GRZCYSA in 20 mM cysteate-salts medium containing 0.05% yeast extract (*bar* 10 μm)

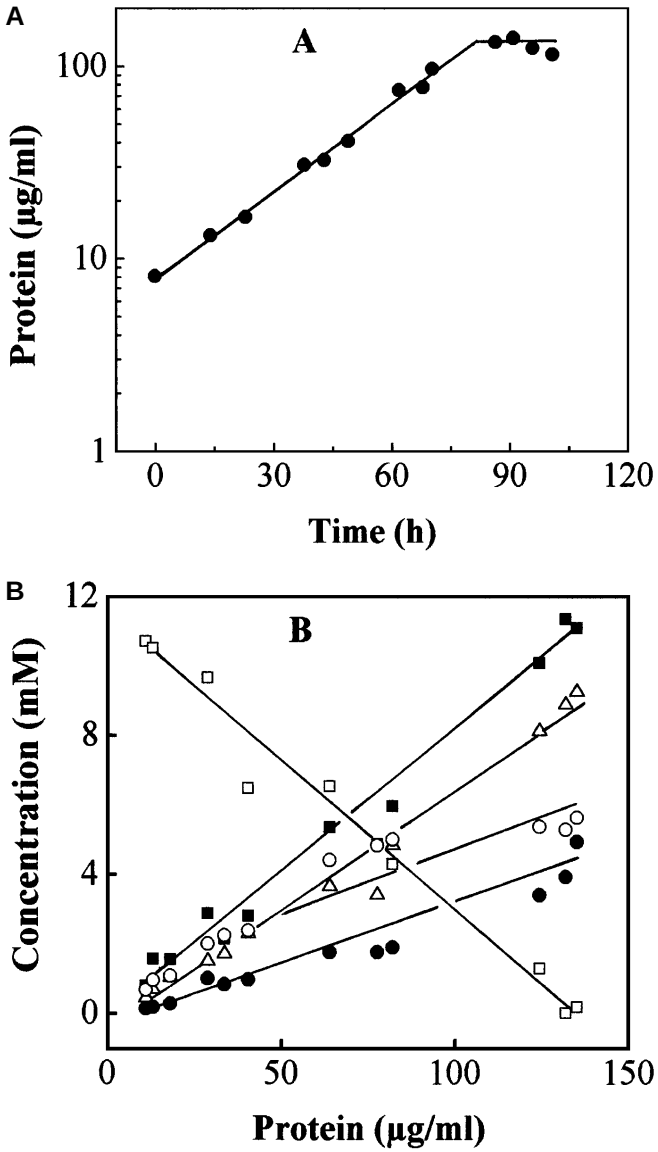

Fig. 3A Growth of strain GRZCYSA with cysteate as the sole source of carbon and energy. **B** Disappearance of cysteate (□) and formation of the products acetate (△), sulfide (●), sulfate (○) and ammonia (■) are shown as a function of protein

coenzyme M (2-mercaptoethanesulfonate), *N*-cyclohexanesulfamate, *N*-2-hydroxyethanepiperazine-*N*′-2-ethanesulfonate (Hepes), and 3-cyclohexaneaminopropanesulfonate (Caps)] were not utilized. The organism also fermented fumarate and pyruvate. Sulfate reduction was detected with lactate or ethanol as the source of electrons and carbon, and with formate or H_2 as the electron source and 2 mM acetate as the carbon source. Neither acetate nor butyrate served as an electron donor. Sulfite and thiosulfate served as electron acceptors with lactate as the source of carbon and electrons. Desulfoviridin was detected in crude extracts of cells from cysteate-minimal-salts medium, from fumarate-minimal salts medium, and from lactate-salts medium containing sulfate.

We detected four metabolic products from the growth of strain GRZCYSA in cysteate-salts medium: acetate, sulfide, sulfate and ammonia. Each was thoroughly identified, as described in Materials and methods.

We have as yet no assay for aminomethanesulfonate, but since we quantitatively recovered ammonia after growth of strain GRZCYSA in approximately 20 mM aminomethanesulfonate-minimal-salts medium, we presume substrate utilization to be quantitative. As with cysteate, the sulfonate moiety was recovered in two products, sulfate (9 mM) and sulfide (7 mM). We detected neither acetate nor formate in medium in which the cells had grown fully.

Strain GRZCYSA grew exponentially ($\mu = 0.03\ h^{-1}$) in cysteate-salts medium (Fig. 3A). The utilisation of cysteate was concomitant with growth, as was the release of products (Fig. 3B). We detected no significant transient intermediates, e.g. taurine. The molar growth yield was 13 g protein/mol cysteate, which, together with the growth rate, allowed us to calculate a specific degradation rate of 0.64 mkat/kg protein. In an independent experiment with a set of different cysteate concentrations in the presence of 0.05% yeast extract, we observed nitrogen and sulfur balances of 105 and 94%, respectively, and an electron balance of 115% (Table 1).

Disappearance of cysteate in reaction mixtures with crude extracts was detected at a significant initial rate (approximately 0.15 mkat/kg protein), though the reaction was incomplete. Very little cysteate disappeared in the absence of pyruvate or α-ketoglutarate (0.04 mkat/kg protein); both pyruvate and α-ketoglutarate supported the reaction. We observed the formation of acetate and ammo-

Table 1 Representative mass balance for the growth of *Desulfovibrio* sp. strain GRZCYSA in cysteate-salts medium containing 0.05% yeast extract. The data represent the mean of results from six cultures grown with different substrate concentrations. Protein was considered to represent 60% of cell dry matter (Mandelstam et al. 1982). The nitrogen content of the cell was taken as 14% (Luria 1960). We considered that 34 µmol of a C2 unit (from the decarboxylation of cysteate) was assimilated to form 1 mg of protein. Assimilation of sulfur from cysteate was considered to be negligible. This gave the following equation for an assimilatory reaction:
$17\ C_2H_3O_2^- + 17\ H^+ \rightarrow 8\ <C_4H_7O_3> + 2\ CO_2 + 6\ H_2O$
The term $<C_4H_7O_3>$ represents a monomer of biomass

Substrate utilized	Products released					Products assimilated		Recovery			Molar growth yield
Cysteate (µmol)	Protein (mg)	Acetate (µmol)	Ammonia (µmol)	Sulfide (µmol)	Sulfate (µmol)	C2-unit (µmol)	Nitrogen (µmol)	Electrons (%)	Nitrogen (%)	Sulfur (%)	(g protein/mol)
319	4.2	276	254	132	164	144	84	115	105	94	13

nia; no taurine was detected. Neither ammonia nor acetate was formed in the absence of cysteate.

Discussion

Desulfovibrio sp. strain GRZCYSA grows strictly anaerobically in cysteate- (or isethionate- or aminomethanesulfonate-) salts medium. The absence of an external electron acceptor and the internally balanced redox reactions (Table 1) mark this as a fermentation. This novel process differs from the other fermentation of the organosulfonate taurine not only because a different organism is involved, but also because different compounds are utilized and different sulfur-containing products are formed (Denger et al. 1997b).

The fermentation of cysteate yielded acetate and ammonia, equimolar amounts of sulfide and sulfate (Fig. 3), and presumably CO_2. We presume the overall dissimilatory reaction to be:

$$2\ C_3H_6NO_5S^- + 2\ H_2O \rightarrow 2\ C_2H_3O_2^- + 2\ CO_2 + SO_4^{2-} + HS^- + 2\ NH_4^+ + H^+$$

$\Delta G^{0\prime} = -154.0$ kJ/mol cysteate (Thauer et al. 1977; Mavrovouniotis 1991).

During growth, the sulfonate moiety is subject to dismutation to sulfate and sulfide. Disproportionation of sulfite or thiosulfate to these products is known in this genus (Bak and Cypionka 1987), but it is unclear whether strain GRZCYSA can catalyze this dismutation.

The $\Delta G^{0\prime}$ value for the fermentation of cysteate in strain GRZCYSA indicates a maximum energy yield of approximately 2.2 mol ATP/mol cysteate, given a requirement of 70 kJ/mol ATP (Thauer et al. 1977). The molar growth yield, approximately 22 g dry wt./mol cysteate (Table 1), implies a practical Y_{ATP} of approximately 10 g dry wt./mol, which, given the approximations made, is compatible with Y_{ATP}^{max} values of 11.4–14.6 g dry wt./mol (Badziong and Thauer 1978).

The fermentations of organosulfonates by strains GKNTAU (taurine) and GRZCYSA (cysteate) yield different sulfur moieties (thiosulfate and sulfide plus sulfate, respectively), and we wondered what advantages these products give. Therefore, we calculated the $\Delta G^{0\prime}$ values for each fermentation with the hypothetical alternative products and found only minor differences (taurine: –117 kJ/mol and –128 kJ/mol for thiosulfate and sulfate plus sulfide, respectively; cysteate: –143 kJ/mol and –154 kJ/mol, respectively).

The molar growth yield of strain GRZCYSA (13 g protein/mol cysteate) is threefold higher than that of strain GKNTAU (4 g protein/mol taurine) (Denger et al. 1997b). We thus presume that different (rather than overlapping) degradative pathways are involved in the two organisms.

We do not yet know the degradative pathway for cysteate in strain GRZCYSA. A transport system for the highly polar substrate will be required [cf. Biedlingmaier and Schmidt (1986)]. The requirement for α-ketoglutarate or pyruvate suggests a transamination with a subsequent deamination, but whether a decarboxylation precedes the transamination (or vice versa), whether taurine and isethionate are intermediates (as in fungi; Braun and Fromageot 1962) rather than the mammalian reaction yielding taurine (Huxtable 1992), and whether sulfite is the first sulfur moiety formed (Kondo and Ishimoto 1972; Shimamoto and Berk 1980) are still matters of speculation. The role of desulfoviridin in the reaction is presumably in the reductive portion of the fermentation, but is the assumption correct? Are different pathways involved in the degradation of isethionate and, more unusually, of the C1 compound aminomethanesulfonate, or are common elements involved?

We have now found a variety of desulfonation reactions in anaerobic bacteria. These range from a single-reaction type for inert arenesulfonates, which serve as sulfur sources for a variety of anaerobes and whose biochemistry is still unknown (Denger et al. 1996; Denger and Cook 1997), to the possibly common degradative pathway for taurine. This pathway could be identical in some aerobes, in two anaerobic respirations, and in a fermentation, and might just be located in different metabolic surroundings (Denger et al. 1997a, b; Laue et al. 1997). We now suspect that one or more different pathways are involved in the fermentation of cysteate and aminomethanesulfonate in *Desulfovibrio* sp. strain GRZCYSA. The diversity of desulfonation reactions in anaerobic bacteria, phenomena which have long been considered to be artefacts (Denger et al. 1996), is now being established.

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