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Desulfonation of aliphatic sulfonates by *Pseudomonas aeruginosa* PAO

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Abstract

Pseudomonas aeruginosa PAO1 used a broad range of alkanesulfonic acids as sole sulfur source for growth, with molar growth yields of 2.2 to 2.9 kg protein per mol sulfur. 4-Phenylbutane-1-sulfonate was desulfonated in vivo to yield 4-phenyl-1-butyric acid quantitatively as the sole product, suggesting that the desulfonation mechanism is the same as when alkanesulfonates serve as a carbon source for growth. This contrasts with aromatic sulfonate utilization in other organisms, where different desulfonation reactions are used to provide carbon and sulfur. Desulfonation of alkanesulfonates to provide sulfur was repressed by sulfate or thiocyanate, and derepressed in their absence. The alkanesulfonatase system is hence controlled as part of the sulfate starvation-induced stimulon.

Keywords: Pseudomonas aeruginosa; Aliphatic sulfonates; Desulfonation; Sulfate limitation

1. Introduction

Organosulfonic acids are widespread in nature, both as arylsulfonate xenobiotics (e.g. linear alkylbenzene sulfonate detergents, sulfonated dyestuffs) and as natural alkanesulfonates such as taurine (2-aminethanesulfonate), sulfonolipids or methanesulfonate. The alkanesulfonates play an important role in the biogeochemical sulfur cycle; much of the sulfur in forest-floor litter is present not as inorganic sulfate (circa 5%) but in the form of sulfonates (> 40%) [1], and methanesulfonate is the major photochemical oxidation product of dimethylsulfide in the atmosphere, and is deposited in rain and snow [2]. Whereas the biochemistry of aromatic desulfonation by bacteria has been studied in detail in recent years [3], less is known about how alkanesulfonates are degraded by microorganisms.

Bacteria able to degrade either synthetic alkanesulfonates (C_4–C_{12}) [4] or taurine [5] as a source of carbon and energy have long been known. Recent studies have also shown that the sulfur in natural alkanesulfonates such as taurine, isethionate and cysteate can also be utilized specifically by a broad range of microorganisms [6–8]. Studies with *E. coli* mutants defective in cysteine biosynthesis showed that uptake of these sulfonates does not involve the sulfate uptake system, and that assimilation of sulfonate sulfur proceeds via cleavage to yield sulfite, and subsequent incorporation to cysteine [8]. Sulfite is also the initial sulfur product from aromatic sulfonates in both the carbon and sulfur cycles, where dioxygenases and monooxygenases are involved re-

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spectively [3]. The biochemistry of alkanesulfonate desulfonation in the sulfur cycle has not yet been elucidated.

During growth in the absence of sulfate or cysteine, several bacterial species (including *Pseudomonas aeruginosa*) produce a set of extra proteins, components of the sulfate starvation-induced stimulon. These proteins have been suggested to play a role in scavenging of alternative sulfur sources such as organosulfates and organosulfonates [9]. The fact that utilization of sulfonates is repressed by addition of sulfate [10] suggests that the desulfonation systems involved are regulated as part of the sulfate starvation-induced stimulon [9]. We have now examined the physiology of alkanesulfonate utilization as sulfur source by a genetically and physiologically well characterized pseudomonad, *Pseudomonas aeruginosa* PAO1.

2. Materials and methods

2.1. Bacterial strains, growth media and growth conditions

Pseudomonas aeruginosa PAO1 [11] was routinely grown in a sulfur-free succinate minimal medium described previously [9]. Sulfur-containing compounds required for growth (100 to 500 μM) were sterilized by filtration of stock solutions (0.2 μm filters) and added as described in the text. The cultures were grown aerobically at 37°C, either with shaking (180 rpm) on an orbital shaker or in vigorously stirred flasks with a continuous air supply [12]. Growth was monitored by measuring optical density at 650 nm, and cultures were harvested when they reached the stationary phase.

2.2. Chemicals

4-Phenylbutane-1-sulfonic acid, toluene-α-sulfonic acid, 1,3-dioxo-2-isoindoline-ethanesulfonic acid, *N*-phenyltaurine, 4-(3-methyl-4-nitrophenoxy)-1-butanesulfonic acid and *n*-hexyl sulfate were supplied by Aldrich (Steinheim, Germany). All other sulfur-containing compounds, and components of growth media, were obtained from Fluka (Buchs, Switzerland).

2.3. Analytical methods

Metabolites of arylalkanesulfonic acids were analysed by reversed-phase high-performance liquid chromatography (HPLC), using a Nucleosil-C_{18} column (250 by 4.6 mm; particle size 7 μm). The mobile phase was 10 mM potassium phosphate buffer (pH 2.2 for α-toluenesulfonic acid and its desulfonation products, pH 6.7 for all other compounds) containing appropriate amounts of methanol (0–80% (vol/vol)). Eluted compounds were detected spectrophotometrically at 200, 210 nm, or 248 nm. For identification purposes, desulfonation products were extracted from stationary-phase culture supernatants (200 ml) after acidification to pH 1.5 with HCl, using an Extract-Clean C_{18} column (1 g sorbent; Alltech) and elution with methanol. The product from 4-phenylbutane-1-sulfonic acid was further purified by reversed-phase HPLC as described above, and analysed by electron impact mass spectrometry (Fisons VG Tribrid; EI + mode; 70 eV).

The sulfate content of all sulfur sources was checked by ion chromatography before use, using a Durasep A-1 anion exchange column (Alltech), with a GA-1 guard column. Ions were eluted isocratically with 4 mM NaOH/5% (v/v) methanol, and were detected by conductimetry after chemical suppression. All sulfur sources contained $< 0.5\%$ (mol/mol) inorganic sulfate.

Total protein in cell suspensions was determined by a modified Lowry method [13] using bovine serum albumin as standard. All glassware used in sulfate-limited experiments was washed with 3 M HCl and rinsed thoroughly with distilled water before use.

3. Results and discussion

3.1. Utilization of alkanesulfonates as sulfur sources by P. aeruginosa PAO1

P. aeruginosa can utilize a broad range of sulfur sources for growth. Although it cannot desulfonate aromatic sulfonates or thiophenols, it can degrade a broad range of aromatic sulfate esters [14], aliphatic sulfate esters and sulfonates, and other sulfur-containing compounds [15]. Desulfonation of simple

alkanesulfonates has been difficult to examine in the past, because they lack a chromophore to facilitate their detection, and are used at low concentrations (30–100 μM) when the sulfur in the compound is utilized specifically. We have therefore chosen to analyse a series of alkanesulfonates that carry an aromatic ring, including 4-phenyl-1-butanesulfonate, toluene-α-sulfonate, *N*-phenyltaurine, 1,3-dioxo-2-isoindoline-1-ethanesulfonate, and 4-(3-methyl-4-nitrophenoxy)-1-butanesulfonate. The molar growth yields obtained for *P. aeruginosa* with these compounds (2.2–2.8 kg protein/mol S) and with simple aliphatic sulfonates such as taurine or pentane-1-sulfonate (2.8 kg/mol S) were found to be very similar to the growth yields obtained when other sulfur sources such as sulfate, cysteine, or methionine were utilized for growth (2.6–2.9 kg/mol S), and these values also corresponded well to values previously obtained with *Pseudomonas putida* grown with aromatic sulfonates [16]. We therefore concluded that the sulfur contained in aliphatic sulfonates was assimilated essentially quantitatively into cell material by the cells during growth. After identification of the desulfonation product (see below) it was possible to complete quantification of the desulfonation mass balance, since > 90% of the desulfonated carbon moiety was also released during the desulfonation reaction (Fig. 1). This organic product was not subsequently further degraded, and was not utilized as a carbon source by *P. aeruginosa* in the absence of succinate either (not shown).

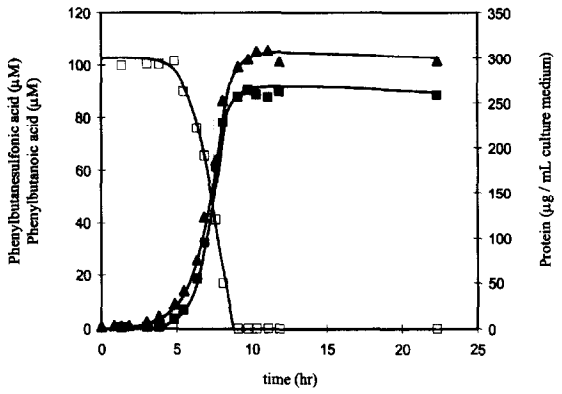

Fig. 1. Growth of *P. aeruginosa* PAO1 in succinate-salts medium with 4-phenylbutanesulfonate as sole sulfur source. The cells were grown aerobically at 37°C, and samples were removed periodically for analysis of total protein content (▲) by a modified Lowry assay, and of 4-phenylbutanesulfonate (□) and 4-phenylbutyric acid (■) by HPLC.

3.2. *Identification of the products of alkanesulfonate desulfonation by P. aeruginosa PAO1*

During growth of pseudomonads with aliphatic sulfonates as sole source of carbon and energy, the first product to be released is the carboxylic acid derived from α-hydroxylation, desulfonation to an aldehyde and subsequent oxidation [4]. To determine whether this was also the case when the sulfonate was supplied as a source of sulfur we examined degradation of 4-phenylbutane-1-sulfonate, reasoning that this would best approximate desulfonation of simple *n*-alkanesulfonates. The aromatic product produced from desulfonation was extracted from growth supernatants, and purified by reversed-phase HPLC. Electron-impact mass spectrometry of the resulting compound gave the following spectrum: *m/z*: 164 (M^+), 104 (M^+-CH_3COOH), 91 (M^+-CH_2CH_2COOH) allowing its identification as 4-phenylbutyric acid. This was then confirmed by co-chromatography of the product in growth medium with authentic 4-phenylbutyric acid. When toluene-α-sulfonate was supplied as sole sulfur source the corresponding benzoic acid was identified as product (co-chromatography; not shown), and benzaldehyde could not be detected by HPLC at any point in the growth curve (not shown). Sulfonate disappearance and carboxylate appearance occurred simultaneously, without any lag phase to suggest release of an intermediate compound or the presence of significant intracellular pools of either sulfonate or products (Fig. 1). The carboxylic acid therefore probably arises by oxidation of a putative intermediate aldehyde within the cell, as is known for aromatic sidechain oxidation, where toluenesulfonate monooxygenase is co-induced with its cognate aldehyde dehydrogenase [17]. The sulfur cycle enzyme therefore proceeds by a similar mechanism to that observed under carbon limitation [4]. This contrasts with the situation observed for degradation of aromatic sulfonates, where the carbon cycle enzymes are predominantly dioxygenases, and the sulfur cycle enzymes are monooxygenases [16,18], and for dibenzothiophene desulfurization, where two pathways also exist [19].

3.3. Regulation of desulfonation activity by the sulfur supply to the cell

Aromatic desulfonation activity in *P. putida* S-313 has been shown to be controlled by the sulfur sources present during growth, with the arylsulfonatase system apparently repressed in the presence of sulfate, cysteine or thiocyanate, and derepressed during growth with other sulfur sources [16,18,20]. Regulation of aliphatic sulfonate desulfonation was studied in vivo in *P. aeruginosa* by measuring desulfonation of 4-phenylbutanesulfonate (100 μM) during growth of the cells in the presence of an excess (500 μM) of a second sulfur source (Fig. 2). No desulfonation reaction was observed in the presence of sulfate or thiocyanate, but the alkanesulfonate was converted to its carboxylic acid product in the presence of methionine, aryl- or alkyl-sulfate esters, or cyclamate. Transformation of 4-phenylbutanesulfonate also occurred in the presence of pentanesulfonate, though the rate was reduced, presumably due to competition between the two sulfonates for the same enzyme. The alkanesulfonatase system of *P. aeruginosa* is hence regulated in the sulfur cycle as part of the sulfate starvation-induced stimulon [9], i.e. synthesis of the enzyme is repressed in the presence of preferred sulfur sources, and derepressed in their absence. In several organisms, these preferred sulfur sources have been shown to include sulfate, cysteine, and thiocyanate [6,9,10,21], though the mechanism of assimilation of the last of these is not yet clear. Our data, however, (Fig. 2, and [15]) suggest that the situation may be more complex in *P. aeruginosa* than has been observed in other species, since desulfonation of 4-phenylbutanesulfonate was also observed in the presence of cysteine, though this compound acts to repress arylsulfonate desulfonation in *P. putida*, and also represses synthesis of both arylsulfatase and other sulfate starvation-induced proteins in *P. aeruginosa* [15]. The regulatory pattern observed for desulfonation activity also explains the previously observed loss of carbon-limited desulfonation activity by an isolate which retained the ability to use alkanesulfonates as sulfur source [6]. Two isoenzymes catalyzing the same reaction may well be present in the cell, since cells utilizing sulfonates as source of carbon will necessarily release excess sulfur and repress synthesis of the sulfur cycle enzyme. Loss of the carbon cycle enzyme therefore does not need to involve loss of sulfur-dependent desulfonation, although exact quantification of this will require purification and characterization of the enzymes involved.

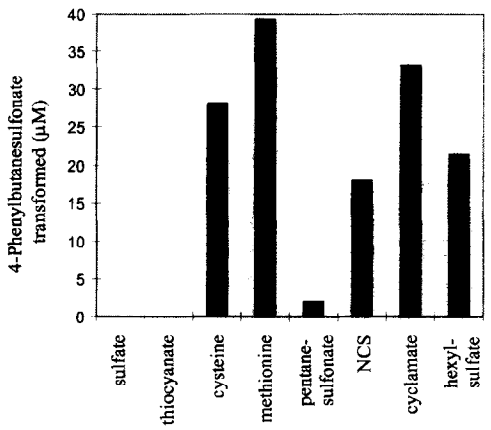

Fig. 2. Desulfonation of 4-phenylbutanesulfonate by *P. aeruginosa* PAO1 during 24 h growth in succinate-salts medium containing 4-phenylbutanesulfonate (100 μM) together with a second sulfur source (500 μM). Desulfonation was quantified for each experiment both as 4-phenylbutanesulfonate disappearance and as production of 4-phenylbutyric acid, and the results are the mean of 2–4 independent experiments. In the absence of a second sulfur source, complete desulfonation was observed (100 μM). NCS – 4-nitrocatechol sulfate.

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