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Anaerobic aniline degradation via reductive deamination of 4-aminobenzoyl-CoA in *Desulfobacterium anilini*

Sylvia Schnell and Bernhard Schink

Lehrstuhl Mikrobiologie I, Eberhard-Karls-Universität, Auf der Morgenstelle 28, W-7400 Tübingen, Federal Republic of Germany

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Abstract. The initial reactions involved in anaerobic aniline degradation by the sulfate-reducing *Desulfobacterium anilini* were studied. Experiments for substrate induction indicated the presence of a common pathway for aniline and 4-aminobenzoate, different from that for degradation of 2-aminobenzoate, 2-hydroxybenzoate, 4-hydroxybenzoate, or phenol. Degradation of aniline by dense cell suspensions depended on CO_2 whereas 4-aminobenzoate degradation did not. If acetyl-CoA oxidation was inhibited by cyanide, benzoate accumulated during degradation of aniline or 4-aminobenzoate, indicating an initial carboxylation of aniline to 4-aminobenzoate, and further degradation via benzoate of both substrates. Extracts of aniline or 4-aminobenzoate-grown cells activated 4-aminobenzoate to 4-aminobenzoyl-CoA in the presence of CoA, ATP and Mg^{2+}. 4-Aminobenzoyl-CoA-synthetase showed a K_m for 4-aminobenzoate lower than 10 μM and an activity of 15.8 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1}. 4-Aminobenzoyl-CoA was reductively deaminated to benzoyl-CoA by cell extracts in the presence of low-potential electron donors such as titanium citrate or cobalt sepulchrate (2.1 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1}). Lower activities for the reductive deamination were measured with NADH or NADPH. Reductive deamination was also indicated by benzoate accumulation during 4-aminobenzoate degradation in cell suspensions under sulfate limitation. The results provide evidence that aniline is degraded via carboxylation to 4-aminobenzoate, which is activated to 4-aminobenzoyl-CoA and further metabolized by reductive deamination to benzoyl-CoA.

Key words: Carboxylation – 4-Aminobenzoate – Benzoate – 4-Aminobenzoyl-CoA synthetase – 4-Aminobenzoyl-CoA reductase

Aniline is synthesized in large amounts to produce dyes, plastics, pesticides and other industrial products (Franck and Stadelhofer 1987). Nitrobenzenes, which are used as solvents, oxidizers and blasting agents, are reduced to aniline derivatives under anaerobic conditions (Hallas and Alexander 1983). Arylamines are highly toxic and cause often severe pollution problems if discharged into natural waters. Several bacteria are known (Reber et al. 1979; Aoki et al. 1985; Wyndham 1986) which decompose aniline in the presence of oxygen, mostly via catechol as intermediate (Backofer et al. 1975; Janke et al. 1988). A denitrifying *Paracoccus* sp. catalyzes anaerobic transformation and incomplete degradation of aniline (Bollag and Russel 1976). The first bacterium known to degrade aniline completely under anaerobic conditions is the sulfate-reducing *Desulfobacterium anilini* (Schnell et al. 1989). The present study elucidates the initial steps in anaerobic aniline degradation by this bacterium.

Materials and methods

Cultivation of Desulfobacterium anilini

Strain Anil (DSM 4660) was grown in bicarbonate-buffered, sulfide-reduced brackish water mineral medium under a N_2/CO_2 atmosphere (90%/10%) in infusion bottles (125 ml) sealed with latex septa. Growth conditions and medium composition have been described previously in detail (Schnell et al. 1989). Aromatic substrates were added successively at 0.5–1.0 mM amounts from filter-sterilized stock solutions stored under an N_2 atmosphere.

Preparation of dense cell suspensions and cell-free extracts

Cells were harvested anaerobically by centrifugation at 3300 *g* for 40 min in the culture bottles in a rotor equipped with rubber adaptors. Cells were washed with medium without substrate and electron acceptor. For experiments with intact cells, pellets were suspended in medium to yield a final protein content of 0.5 mg $\cdot$ ml^{-1}. Cell-free extracts were prepared from similar suspensions in N_2-sparged 50 mM sodium phosphate buffer, pH 7.0, containing 20 mM $MgCl_2 \times 6\ H_2O$ and 2.5 mM 1,4-dithiothreitol. Cells were broken in a cooled French pressure cell gassed with N_2. The crude extract was centrifuged again in N_2-gassed vials at 5000 *g* at 4° C to remove cell debris.

Offprint requests to: S. Schnell

Enzyme assays

Assays were performed in 2 ml vials closed with butyl rubber septa and gassed with N_2/CO_2 or N_2, depending on the buffer used. All additions and sampling were done with gas-tight Unimetrics microliter syringes.

For whole-cell experiments, the reaction mixture (1 ml) contained sulfide-reduced medium buffered with 30 mM bicarbonate, 20 mM sulfate, 0.5–1.0 mM aromatic substrate, 0.25 mM dithionite, and about 0.1 mg cell protein per ml. The CO_2 dependence of substrate degradation was studied in sulfide-reduced medium buffered with 10 mM potassium phosphate, pH 7.2, and prepared under N_2 atmosphere. The CO_2 content was checked in the headspace of strongly shaken medium by a gaschromatograph with thermal conductivity detector (Platen 1989). To remove bicarbonate and free CO_2 from the growth medium, cells were washed twice and gassed with N_2.

Acyl-CoA synthetase: The assay contained 0.5 mM aromatic acid, 0.5 mM CoASH, 1 mM ATP, 20 mM $MgCl_2$, 2.5 mM dithiothreitol, and about 0.17 mg cell protein in N_2-gassed 50 mM sodium phosphate buffer, pH 7.0 in a final volume of 1 ml. In some experiments, acetyl-CoA, succinyl-CoA, ADP, and GTP were used instead of CoASH and ATP.

Two different assays were used to measure 4-aminobenzoyl-CoA reductase activity. *i*) The acyl-CoA synthetase assay was amended with various reductants (0.5 mM NADH, NADPH, reduced methyl viologen, benzyl viologen, titanium citrate, cobalt sepulchrate, anthraquinone 2,6-disulfonate, neutral red, safranin T, bipyridyl, propylviologen sulfonate, FADH, formate, or hydrogen), either right at the beginning or after 2 h when sufficient amounts of 4-aminobenzoyl-CoA were formed. *ii*) Chemically synthesized 4-aminobenzoyl-CoA was added to a reaction mixture with cell extract and reductants. Controls were run with reaction mixtures either lacking cells or cell-free extract, or containing boiled cell extract (5 min at 100°C).

Pyrophosphatase was assayed measuring the formation of orthophosphate (Herbert et al. 1971) from pyrophosphate. The assay was performed after Thebrath et al. (1989).

Synthesis and quantification of benzoyl-CoA

Benzoyl-CoA was synthesized after Merkel et al. (1989), and purified by HPLC. The spectrum of the purified fraction was the same as reported by Webster et al. (1974). Benzoyl-CoA concentration was calculated using the extinction coefficient at 261 nm of 21.1 mM^{-1} cm^{-1} (Webster et al. 1974).

Analyses of substrates and products

Samples were analysed with a Beckman System Gold high pressure liquid chromatograph equipped with a Merck LiChrospher 60 RP-select B column (5 mm × 12.5 cm). The mobile phase contained 100 mM ammonium phosphate buffer, pH 2.6, and methanol. Various gradients were used between 10 and 70% methanol to separate ATP, AMP, CoASH, aromatic acids, and acyl-CoA esters, at 1 ml min^{-1} flow rate. Samples of 20 μl were injected with a Spark Promis I autosampler. A Beckman 167 scanning variable wavelength detector was used at the respective appropriate wavelength for each compound. Data were analysed by a computer program and quantified by comparison with external and internal standards of known composition. Peaks were identified by comparison of retention times and UV-spectra recorded by on-line spectral scans with standard samples.

Assay for $^{14}CO_2$-carboxyl exchange

$^{14}CO_2$-carboxyl exchange of 4-aminobenzoate was assayed according to Tschech and Fuchs (1989), under strictly anaerobic conditions. The mixture contained 100 mM Tris-HCl buffer, pH 6.9, 2 mM dithioerythritol, 5 mM $NaHCO_3$, 2.5 mM 4-aminobenzoate or 4-hydroxybenzoate, 0.5 mM $MnCl_2$, 20 mM potassium phosphate, and 200 μl cell extract (100 mg protein). The reaction was started after 10 min incubation at 30°C with 1.5×10^4 Bq $NaH^{14}CO_3$. Samples (200 μl) were withdrawn with syringes, stopped with 20 μl 3 M perchloric acid, and centrifuged. 150 μl of the supernatant was added to 150 μl 1 M $KHCO_3$ solution, acidified with 50 μl 10 N formic acid, and gassed for 15 min with N_2/CO_2 (80%/20%). The remaining acid-stable radioactivity was determined by liquid scintillation counting.

Protein assay

Protein was determined by the method of Bradford (1976) with bovine serum albumin as standard.

Results

Induction experiments

Kinetics of substrate degradation were studied with dense cell suspensions grown with various substrates. Cells grown with 4-aminobenzoate immediately degraded 4-aminobenzoate (19.3 $nmol \cdot min^{-1} \cdot mg^{-1}$) and aniline (13.5 $nmol \cdot min^{-1} \cdot mg^{-1}$) at comparably high initial rates. 2-Aminobenzoate, 2-hydroxybenzoate, 4-hydroxybenzoate, and phenol were degraded only after lag times of 4–7 h (Fig. 1). Cells grown with 2-aminobenzoate degraded only 2-aminobenzoate immediately whereas aniline, 4-aminobenzoate, and phenol degradation was significantly delayed. Phenol-grown cells immediately degraded phenol, 2-aminobenzoate, 2-hydroxybenzoate, and 4-hydroxybenzoate; aniline and 4-aminobenzoate were utilized very slowly. Cells grown with aniline degraded aniline, phenol, 2-aminobenzoate, 4-aminobenzoate, 2-hydroxybenzoate and 4-hydroxybenzoate at nearly identical initial rates.

CO_2 dependence of aniline degradation

Degradation of aniline, phenol, and 4-aminobenzoate was observed in CO_2-free medium and for comparison with 30 mM bicarbonate added. Degradation of aniline and phenol was significantly enhanced in the presence of CO_2, whereas 4-aminobenzoate degradation was independent of CO_2 (Fig. 2).

Aniline and 4-aminobenzoate degradation in sulfate-limited or cyanide-inhibited cell suspensions

Degradation of aniline and 4-aminobenzoate was examined in dense cell suspensions washed twice in sulfate-free medium, and incubated without sulfate or with 1 mM sulfate added. In the absence of sulfate, only 0.25 mM aniline was degraded, and no aromatic product was formed. With 1 mM sulfate added, 0.57 mM aniline disappeared. Cells incubated with 1 mM 4-aminobenzoate

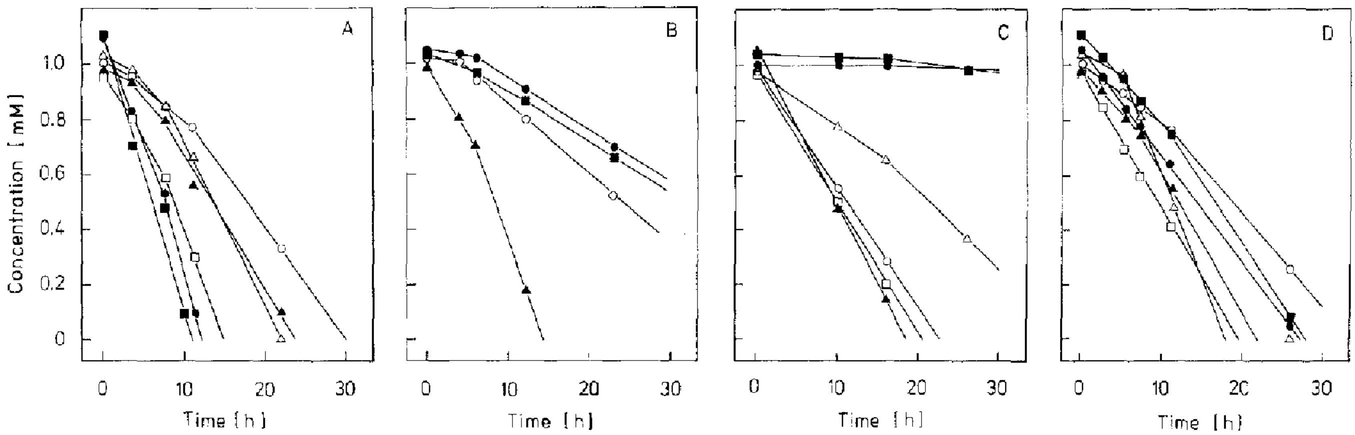

Fig. 1 A – D. Substrate degradation in dense cell suspensions grown with 4-aminobenzoate (**A**), 2-aminobenzoate (**B**), phenol (**C**) and aniline (**D**). ● Aniline, ■ 4-aminobenzoate, ▲ 2-aminobenzoate, ○ phenol, □ 4-hydroxybenzoate, △ 2-hydroxybenzoate

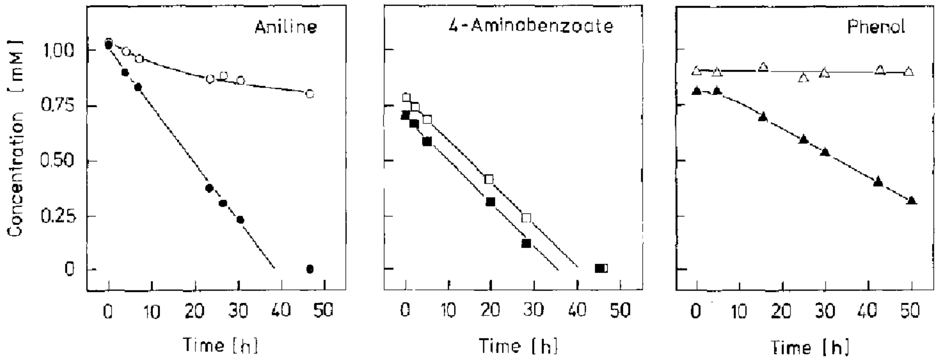

Fig. 2. Degradation of aniline, 4-aminobenzoate, and phenol by dense cell suspensions in the absence of CO_2 and with 30 mM HCO_3^- added. ○, ● Aniline; □, ■ 4-aminobenzoate; △, ▲ phenol; ○, □, △ CO_2-free assays; ●, ■, ▲ assays with 30 mM HCO_3^- added

accumulated 0.22 mM benzoate in the absence of sulfate, while 0.36 mM 4-aminobenzoate disappeared.

In dense cell suspensions, aniline and 4-aminobenzoate degradation were significantly inhibited by cyanide. With 0.2 mM cyanide added, only 0.2 mM aniline was degraded in 30 h, and 80 μM benzoate accumulated. Under the same conditions, also 4-aminobenzoate degradation resulted in benzoate accumulation; 0.49 mM 4-aminobenzoate degraded were converted to 0.46 mM benzoate within 30 h (Fig. 3). Cyanide at higher concentrations (0.2 – 0.6 mM) inhibited aniline and 4-aminobenzoate degradation in a similar manner, but less benzoate accumulated.

Isotope exchange between $^{14}CO_2$ and the carboxylic group of 4-aminobenzoate

In an assay mixture containing 100 mg protein, 5 mM HCO_3^- (labeled and unlabeled) and 2.5 mM 4-aminobenzoate (details see Materials and methods), no significant increase of acid-stable radioactivity was found within 4 h. Addition of inorganic phosphate, $MnCl_2$, or EDTA had no influence. Control experiments with the phenol-degrading *Pseudomonas* sp. strain S100 (Tschech and Fuchs 1989) showed an isotope exchange of 50 nmol $^{14}CO_2$ exchanged per minute and mg protein into the carboxyl group of 4-hydroxybenzoate.

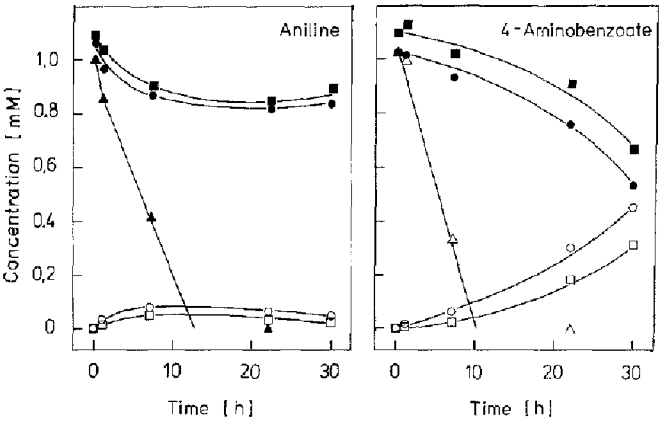

Fig. 3. Degradation of aniline and 4-aminobenzoate by dense cell suspensions inhibited with cyanide. Aniline (▲) and 4-aminobenzoate (△) degradation in assays without cyanide; (●) aniline and 4-aminobenzoate degradation and benzoate (○) accumulation in assays with 0.2 mM cyanide; (■) aniline and 4-aminobenzoate degradation and benzoate (□) accumulation in assays with 0.4 mM cyanide

Synthesis and hydrolysis of 4-aminobenzoyl-CoA

4-Aminobenzoyl-CoA was prepared chemically after Merkel et al. (1989). Formation of 4-aminobenzoyl-CoA from 5 μmol CoASH and 10 μmol 4-aminobenzoic anhydride was followed by HPLC. The reaction was incom-

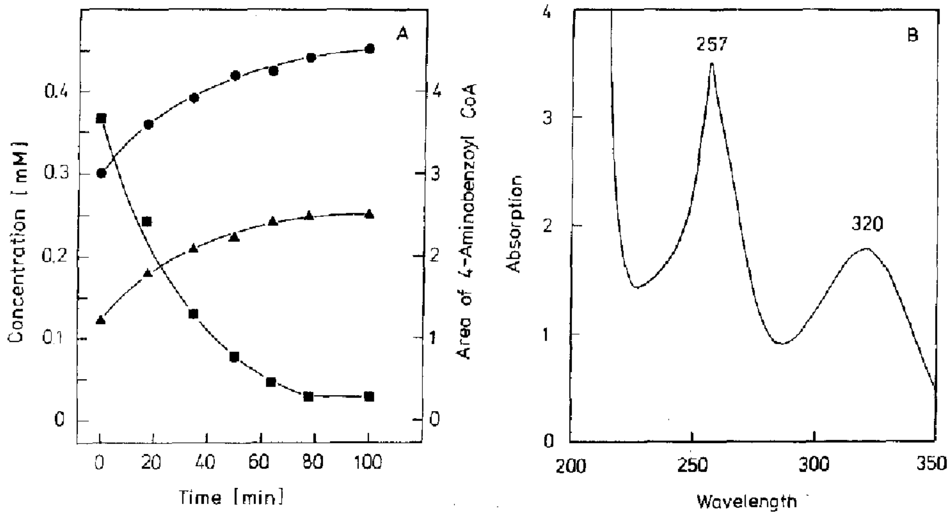

Fig. 4. A Hydrolysis of chemically synthesized 4-aminobenzoyl-CoA with 0.2 M NaOH. 3.39 area units of 4-aminobenzoyl-CoA (■) were converted to 0.15 mM 4-aminobenzoate (●) and 0.13 mM coenzyme A (▲). **B** UV-absorption-spectrum of purified 4-aminobenzoyl-CoA (95 μM) in 100 mM ammonium phosphate buffer pH 2.6/methanol (60/40)

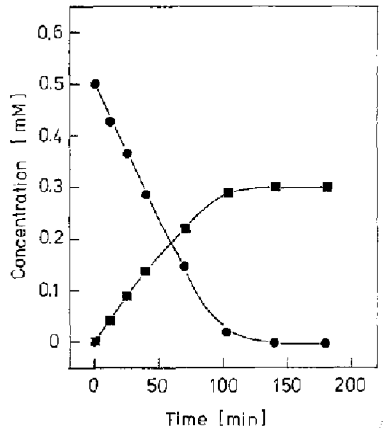

Fig. 5. Time course of 4-aminobenzoate (●) activation to 4-aminobenzoyl-CoA (■) by cell-free extract of *Desulfobacterium anilini*

Table 1. 4-Aminobenzoyl-CoA synthetase activity in extracts of *Desulfobacterium anilini* cells grown with aniline under various conditions. 4AB: 4-aminobenzoate

Conditions	Activity [nmol · min^{-1} · mg^{-1}]
0.05 mM 4AB, 0.25 mM CoASH, 1 mM ATP	11.2
0.1 mM 4AB, 0.5 mM CoASH, 1 mM ATP	13.9
0.25 mM 4AB, 1 mM CoASH, 1 mM ATP	10.5
0.5 mM 4AB, 2 mM CoASH, 2 mM ATP	9.8
1 mM 4AB, 4 mM CoASH, 4 mM ATP	9.8
0.5 mM 4AB, 0.5 mM CoASH	0
0.5 mM 4AB, 1 mM ATP	0
0.5 mM 4AB, 0.5 mM acetyl-CoA	0
0.5 mM 4AB, 0.5 mM succinyl-CoA	0
0.5 mM 4AB, 0.5 mM CoASH, 1 mM ATP, 3 mM AMP	2.3
0.5 mM 4AB, 0.5 mM CoASH, 1 mM GTP	0
3 mM aniline, 30 mM HCO_3^-, 0.5 mM CoASH, 2 mM ATP	0

plete; 3.5 μmol CoASH and 3.6 μmol 4-aminobenzoate were converted to 4-aminobenzoyl-CoA, while 1.5 μmol CoASH and 6.4 μmol 4-aminobenzoate remained. For exact quantification of 4-aminobenzoyl-CoA, an aliquot was hydrolyzed in 0.2 M NaOH. The kinetics of the reaction is shown in Fig. 4A. 0.15 mM 4-Aminobenzoate and 0.13 mM CoASH were formed indicating that 3.39 area units of 4-aminobenzoyl-CoA correspond to 0.14 mM 4-aminobenzoyl-CoA. An UV-spectrum of purified 4-aminobenzoyl-CoA is shown in Fig. 4B.

4-Aminobenzoyl-CoA synthetase

4-Aminobenzoyl-CoA synthetase was determined at an activity of 15.8 nmol · min^{-1} · mg^{-1} in extracts of aniline-grown cells and of 28.3 nmol · min^{-1} · mg^{-1} in extracts of 4-aminobenzoate-grown cells. The kinetics of 4-aminobenzoate activation are shown in Fig. 5. The reaction depended on ATP and CoASH; neither GTP, acetyl-CoA, or succinyl-CoA could replace ATP or CoASH in the assay mixture. 3 mM AMP in the presence of 1 mM ATP inhibited 4-aminobenzoate activation by 90%. No synthetase activity was found with aniline and HCO_3^- as substrate (Table 1). The same activity was measured with cell extract treated with air for 5 min, but no activity remained after boiling for 15 min. With substrate concentrations of 10 μM to 0.5 mM 4-aminobenzoate, the same activities of 4-aminobenzoyl-CoA synthetase were determined indicating that the K_m for 4-aminobenzoate was lower than 10 μM. In extracts of aniline- and 4-aminobenzoate-grown cells, the activity of 4-aminobenzoyl-CoA synthetase was found to be proportional to the amount of extract protein up to 230 μg protein per assay. Cell extracts of aniline-grown cultures also activated 2-hydroxybenzoate, 4-hydroxybenzoate, and benzoate to the corresponding CoA derivatives; low activities were measured with 2-aminobenzoate and 3-hydroxybenzoate, and no activity was found with 3-aminobenzoate. 4-Aminobenzoate-grown cells showed good synthetase activity also for 2-aminobenzoate (Table 2). Pyrophosphatase activity was found at 41 nmol · mg^{-1} · min^{-1}.

Table 2. Acyl-CoA synthetase specificity for various aromatic acids in cell extracts of *Desulfobacterium anilini* grown with aniline or 4-aminobenzoate

Growth substrate	Substrate in activation assay	Activity [nmol · min^{-1} · mg^{-1}]
Aniline	4-aminobenzoate	15.8
	2-aminobenzoate	0.8
	4-hydroxybenzoate	24.2
	2-hydroxybenzoate	5.4
	3-hydroxybenzoate	1.0
	benzoate	18.2
4-Aminobenzoate	4-aminobenzoate	28.3
	2-aminobenzoate	19.9

Reductive deamination of 4-aminobenzoyl-CoA to benzoyl-CoA

The reductive deamination of 4-aminobenzoyl-CoA to benzoyl-CoA was measured in assays in which 4-aminobenzoyl-CoA was synthesized by the cell extract. Consumption of 4-aminobenzoate, and formation of 4-aminobenzoyl-CoA and benzoyl-CoA were followed in the presence of several electron donors (Table 3). Optimal conversion of 4-aminobenzoate to 4-aminobenzoyl-CoA, and reduction to benzoyl-CoA were determined with titanium citrate as electron donor. With NADH, NADPH, or reduced benzyl viologen, only little amounts of benzoyl-CoA were produced. With reduced methyl viologen, cobalt sepulchrate, anthraquinone 2,6-disulfonate, neutral red, safranin T, and bipyridyl, no 4-aminobenzoyl-CoA formation was observed, but benzoyl-CoA increased from 3.8 to 17.7 μM. No reductive deamination was found with propylviologen sulfonate, formate, H_2, or $FADH_2$. 4-Aminobenzoate and 4-aminobenzoyl-CoA were not reduced by any electron donor in the absence

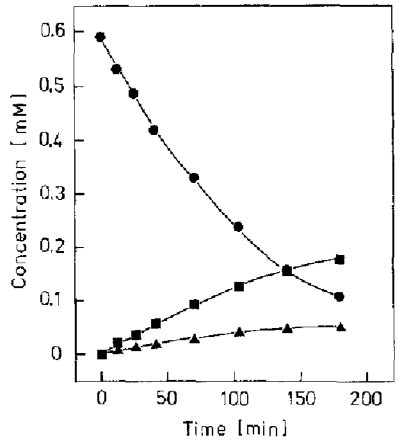

Fig. 6. Kinetics of 4-aminobenzoyl-CoA (■) production from 4-aminobenzoate (●) and coenzyme A, and simultaneous reductive deamination to benzoyl-CoA (▲). The assay mixture contained 0.5 mM 4-aminobenzoate, 0.5 mM CoA, 1 mM ATP, 1 mM titanium citrate and 0.17 mg cell protein in 1 ml

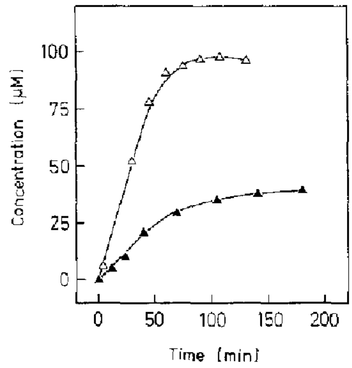

Fig. 7. Benzoyl-CoA formation in an assay with chemically synthesized 4-aminobenzoyl-CoA (△), and in a coupled assay with 4-aminobenzoate activation and simultaneous reductive deamination of the produced 4-aminobenzoyl-CoA (▲) by cell-free extracts with titanium citrate as electron donor

Table 3. Reductive deamination of 4-aminobenzoyl-CoA to benzoyl-CoA with various electron donors by extracts of aniline-grown cells of *Desulfobacterium anilini* in an assay containing 0.5 mM 4-aminobenzoate, 0.5 mM CoASH, and 1 mM ATP. The amount of remaining 4-aminobenzoate (4AB) and of produced 4-aminobenzoyl-CoA and benzoyl-CoA were determined after 120 min. (n.d. = not determined)

Electron donor	Difference between initial and final 4AB concentration [μM]	4-Aminobenzoyl-CoA produced [μM]	Benzoyl-CoA produced [μM]
None	90	100	0
NADH	90	85	1.4
NADPH	120	69	2.6
Methylviologen	290	0	3.8
Benzylviologen	190	26	5.3
Titaniumcitrate	70	20	13.3
Cobalt-sepulchrate	60	0	12.2
Anthraquinone-2,6-disulfonate	60	0	10.2
Neutral red	70	0	10.6
Safranin T	50	0	17.7
Bipyridyl	20	0	9.4
Propylviologen sulfonate	50	43	0
Formate	70	63	0
Hydrogen	100	90	0
FADH	n.d.	n.d.	0

1989). It is remarkable that also the comparably energy-limited sulfate reducer *D. anilini* used a synthetase reaction for 4-aminobenzoate activation; due to the pyrophosphatase activity present, this activation is accomplished at the high expense of two ATP equivalents. One can speculate that this highly exergonic activation reaction with its high substrate affinity maintains an extremly low 4-aminobenzoate concentration and pulls the initial carboxylation reaction; the overall reaction:

$$\text{Aniline} + CO_2 + \text{ATP} + \text{CoASH} \rightarrow \text{4-Aminobenzoyl-CoA} + \text{AMP} + 2\ P_i$$

is estimated (by comparison with the activation of acetate to acetyl-CoA) to be close to equilibrium under standard conditions ($\Delta G'_0 = -3\ \text{kJ} \cdot \text{mol}^{-1}$; Thauer et al. 1977). Such a direct coupling of endergonic carboxylation with exergonic CoA-activation and ATP-hydrolysis would require a 4-aminobenzoate to aniline concentration ratio of about $10^4:1$, and would explain why 4-aminobenzoate could never be detected in our assays. Proof of the carboxylation reaction, e.g. by pulse-chase experiments with radiolabelled aniline, can be obtained only when this reaction becomes measurable in cell-free extract.

After carboxylation to 4-aminobenzoate and activation to 4-aminobenzoate-CoA, the subsequent step in aniline degradation was reductive elimination of the amino group. Reductive elimination reactions were postulated first for fermentative degradation of 3-hydroxybenzoate in defined syntrophic cocultures (Tschech and Schink 1986), and for 2-aminobenzoate (anthranilate) degradation by methanogenic enrichment cultures (Tschech and Schink 1988). Also the fermenting bacterium HQGö1 appears to degrade gentisate via reductive dehydroxylation to benzoate and acetate (Szewzyk and Schink 1989). The only reductive elimination studied so far in cell-free extracts is the dehydroxylation of 4-hydroxybenzoyl-CoA by a denitrifying *Pseudomonas sp.* (Glöckler et al. 1989).

In cell extracts of *D. anilini*, reductive deamination of 4-aminobenzoyl-CoA to benzoyl-CoA was possible with titanium citrate or other low-potential electron donors. The reaction in the coupled assay consisting of 4-aminobenzoate, CoASH, ATP, and titanium citrate was limited by the production of 4-aminobenzoyl-CoA; the activity was low (2.3 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1}) compared to that determined in an assay with 370 μM of chemically synthesized 4-aminobenzoyl-CoA (8.1 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1}). Obviously the affinity of the reductase for 4-aminobenzoyl-CoA and titanium citrate is low. As long as the physiological electron donor of the reductase reaction is unknown, the reaction kinetics cannot be studied in any more detail. Perhaps the enzyme is membrane-bound and hard to provide with electrons in our test assay; this hypothesis could explain why also the measured activity is low in comparison to the calculated in vivo activity of 4-aminobenzoate-grown cells (127 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1} protein).

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