Anaerobic toluene-catabolic pathway in denitrifying *Thauera aromatica*: activation and β -oxidation of the first intermediate, (R)-(+)-benzylsuccinate

Christina Leutwein and Johann Heider

Author for correspondence: Johann Heider. Tel: +49 761 203 2774. Fax: +49 761 203 2626.
e-mail: heiderj@ruf.uni-freiburg.de

Mikrobiologie, Institut für
Biologie II, Universität
Freiburg, Schänzlestr. 1,
79104 Freiburg, Germany

Anaerobic catabolism of toluene is initiated by addition of the methyl group of toluene to the double bond of a fumarate cosubstrate to yield the first intermediate, benzylsuccinate. This reaction is catalysed by the glycyl-radical enzyme benzylsuccinate synthase, as shown for the denitrifying bacterium *Thauera aromatica*. Benzylsuccinate is further oxidized to benzoyl-CoA, the central intermediate of anaerobic degradation of aromatic compounds. The authors show here by experiments with cell extracts of toluene-grown *T. aromatica* that the pathway of benzylsuccinate oxidation requires activation of the free acid to a CoA-thioester, catalysed by a toluene-induced, reversible succinyl-CoA-dependent CoA-transferase. The product of the CoA-transferase reaction, benzylsuccinyl-CoA, is oxidized to benzoyl-CoA and succinyl-CoA in extracts of toluene-grown cells, adding proof to the proposed anaerobic toluene-catabolic pathway. The stereochemical preferences of the enzymes catalysing formation and activation of benzylsuccinate have been analysed. Benzylsuccinate synthase was found to produce exclusively (R)-(+)-benzylsuccinate, although the proposed reaction mechanism of this enzyme proceeds via radical intermediates. In accordance, the reaction of succinyl-CoA:benzylsuccinate CoA-transferase is also specific for (R)-(+)-benzylsuccinate and does not proceed with the (S)-(–)-enantiomer.

Keywords: anaerobic toluene catabolism, benzylsuccinate, CoA-transferase, stereochemistry, β -oxidation

INTRODUCTION

Until recently, microbial catabolism of hydrocarbons has been considered as an absolutely oxygen-dependent process. Only during the last few years has it been realized that aromatic and aliphatic hydrocarbons are also mineralized in the absence of molecular oxygen. Organisms capable of anaerobic hydrocarbon mineralization and available in pure culture include denitrifying, ferric iron- and sulfate-reducing bacteria. The metabolic pathways involved in the catabolism of some hydrocarbons in the absence of oxygen are currently being investigated (for a review see Heider *et al.*, 1999). The simplest alkylbenzene, toluene, appears to be one of

the most readily degradable hydrocarbons under anaerobic conditions: it is preferentially catabolized from crude oil in anaerobic enrichment cultures (Rabus *et al.*, 1996, 1999). The pathway involved in anaerobic toluene degradation has been identified in toluene-grown denitrifying and sulfate-reducing bacteria (Biegert *et al.*, 1996; Beller & Spormann 1997a, b, 1998; Rabus & Heider, 1998). The first step of anaerobic toluene catabolism is the addition of the methyl group of toluene to the double bond of fumarate to yield the first intermediate, benzylsuccinate (Biegert *et al.*, 1996; Fig. 1). This reaction is unprecedented in biochemistry and is catalysed by benzylsuccinate synthase, a novel glycyl-radical enzyme (Leuthner *et al.*, 1998). The reaction mechanism of benzylsuccinate synthase most probably proceeds analogously to chemical free-radical addition reactions in chemical synthesis ('Giese-reactions'), which proceed under relatively mild conditions and

Abbreviations: DCPIP, dichlorophenolindophenol; PMS, phenazine methosulfate.

Fig. 1. Proposed pathway of anaerobic toluene catabolism. The electron acceptors used in this study to couple to the three oxidation steps are given in parentheses. Enzymes: (R)-benzylsuccinate synthase (1), succinyl-CoA:(R)-benzylsuccinate CoA-transferase (2), (R)-benzylsuccinyl-CoA dehydrogenase (3), phenylitaconyl-CoA hydratase (4), 3-hydroxyacyl-CoA dehydrogenase (5), benzoylsuccinyl-CoA thiolase (6), succinate dehydrogenase (7).

therefore are particularly useful with substituted or chiral reactants (Giese & Meister, 1977). Further catabolism of benzylsuccinate proceeds via oxidation to benzoyl-CoA, the common and the last aromatic intermediate in the anaerobic mineralization pathways of most aromatic compounds. This pathway has been proposed to proceed via β -oxidation, in analogy to mineralization of fatty acids (Biegert *et al.*, 1996; Fig. 1), but no detailed information has been obtained so far.

In this communication, we present biochemical evidence for the existence of a specific β -oxidation pathway for benzylsuccinate in *Thauera aromatica*. The initial enzyme is identified as a succinyl-CoA:benzylsuccinate CoA-transferase, and the role of benzylsuccinyl-CoA as intermediate in the degradation pathway leading to benzoyl-CoA is shown. We also examine the stereochemical preferences of the enzymes involved in the formation and further metabolism of benzylsuccinate.

METHODS

Growth of bacteria and preparation of cell extracts. *Thauera aromatica* strain K172 (DSMZ 6984; Anders *et al.*, 1995) was grown at 30 °C under denitrifying conditions in mineral salt medium with toluene or benzoate as sole substrate, as described previously (Tschek & Fuchs, 1987; Biegert *et al.*, 1996). Cultures were harvested by flow-through centrifugation in the exponential growth phase; cells were stored frozen in liquid N₂. Frozen cells were suspended in the same volume of basal buffer (10 mM triethanolamine hydrochloride/NaOH pH 7.5; 10%, v/v, glycerol) containing 0.05 mg DNase I (g cells)⁻¹. All procedures were performed at 4 °C under N₂ or

N₂/H₂ (95:5, v/v) atmosphere. Cells were broken by a passage through a French pressure cell at 137 MPa; the lysate was centrifuged at 100 000 g for 60 min. The supernatant was kept frozen at -70 °C until use. Membrane proteins were prepared from washed membranes by differential centrifugation steps, as follows. The cell lysate was centrifuged for 1 h at 20 000 g to remove cell debris and unbroken cells, then the supernatant was centrifuged at 100 000 g for 1 h. The resulting pellet was washed with the original volume of basal buffer and resuspended in basal buffer containing 0.5% (w/v) Triton X-100. The suspension was centrifuged for 1 h at 100 000 g, and the supernatant containing solubilized membrane proteins was stored at -70 °C.

Chemical preparations

Synthesis of succinyl-CoA and benzylsuccinyl-CoA. Succinyl-CoA and benzylsuccinyl-CoA were synthesized from the internal anhydrides. Benzylsuccinic anhydride is not commercially available and was synthesized from benzylsuccinate and acetic anhydride, as follows. Benzylsuccinate (384 μ mol) was dissolved in 800 μ l acetic acid at 80 °C, and an equimolar amount of acetic anhydride was added. The assay was then incubated at 120 °C, until the solvent acetic acid and acetic acid formed from acetic anhydride were completely evaporated. Quantitative formation of yellow-brown benzylsuccinic anhydride was obtained. The preparation of CoA-thioesters from the anhydrides was as described by Schachter & Taggart (1976); the compounds were freeze-dried and stored at -20 °C. ¹³C-NMR analysis of the synthesized benzylsuccinyl-CoA revealed that the preparation consisted of the two isomeric mono-CoA thioesters (60% 2-carboxymethyl-3-phenylpropionyl-CoA and 40% 3-carboxy-4-phenylbutyryl-CoA; data not shown). The two chemically synthesized isomers co-migrated in HPLC analysis under the conditions described, as did biologically formed benzylsuccinyl-CoA.

Enantiomer separation of racemic benzy succinate. The (R)-(+)- and (S)-(–)-enantiomers of benzy succinate were prepared from the racemate according to Byers & Wolfenden (1973) by stereoselective precipitation with (S)- and (R)-1-phenylethylamine, respectively. The (R)- and (S)-benzy succinate fractions obtained were checked for enantiomer purity by HPLC analysis of the 1-phenylethylamine diamides, as described below [values obtained: 99% for (R)-(+)-benzy succinate, 90% for (S)-(–)-benzy succinate].

Derivatization of benzy succinate with 1-phenylethylamine and HPLC analysis. Benzy succinate (9.6 μ mol) was suspended in 50 μ l dimethylformamide. To this assay, 76.8 μ mol ethyldiisopropylamine and 48 μ mol O-(7-azobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate were added. After 1 min, 48 μ mol (S)-(–)-1-phenylethylamine was added. The mixture was incubated for 1 h at room temperature. To determine the stereospecificity of benzy succinate synthase, ether-extracted [*phenyl*- 14 C]benzy succinate (220 MBq) from an enzymic conversion assay with [*phenyl*- 14 C]toluene and fumarate was mixed with racemic benzy succinate and derivatized to the diamide. HPLC analysis was performed at room temperature with UV detection at 214 nm and simultaneous radioactivity detection by a flow-through detector (Ramona, Raytest). The assay was diluted 1:100 with dimethylformamide, and 15 μ l of the resulting solution was applied to a LiChrospher 100 RPC-18 column. The column was eluted with a gradient of 30–74% acetonitrile in 0.1% (v/v) trifluoroacetate over 30 min with a flow rate of 1 ml min^{–1}. If necessary, the peak fractions were collected and dried in a vacuum concentrator.

Enzymic synthesis of [*phenyl*- 14 C]benzy succinate. Cell extract (2 ml, containing 50 mg protein ml^{–1}) of *T. aromatica* cells grown anaerobically on toluene was incubated anaerobically at 30 °C with 200 μ M [*phenyl*- 14 C]toluene (ARC/Biotrend; specific radioactivity 2 GBq mmol^{–1}) and 10 mM fumarate. The reaction was stopped after 30 min by adding sulfuric acid (5%, w/v, final concentration), and 14 C-labelled benzy succinic acid was extracted with diethyl ether from the assays, as described by Biegert *et al.* (1996).

Enzymic assays and analysis of the substrates and the products

CoA-transferase. In a photometric enzyme assay, succinyl-CoA:benzy succinate CoA-transferase activity was coupled to the reaction of endogenous succinate dehydrogenase of *T. aromatica*, which was supplied by a solubilized membrane preparation. The assay was performed at 30 °C in 100 mM Tris/HCl pH 7.5 under anaerobic conditions. The reaction mixture contained 0.1 mM dichlorophenolindophenol (DCPIP), 0.05 mM phenazine methosulfate (PMS), 0.03 U succinate dehydrogenase (equivalent to 40 μ g solubilized membrane protein), 10–50 μ l 100 000 g supernatant (0.5–2.3 mg protein) and 50 μ M succinyl-CoA. The reaction was started by adding benzy succinate to a final concentration of 600 μ M. The reduction of DCPIP was followed at 600 nm [ϵ = 22 000 M^{–1} cm^{–1} (Dawson *et al.*, 1986)]. The assay required strictly anaerobic conditions because the reoxidation rate of the electron acceptors by traces of introduced oxygen was faster than the enzyme activity to be measured.

Enzyme assays for HPLC analysis contained 0.9 ml 100 mM Tris/HCl pH 7.5, 600 μ M benzy succinate and 30 μ l dialysed 100 000 g supernatant (45 mg protein ml^{–1}). The reaction was started by adding succinyl-CoA (200 μ M final concentration) and incubated at 30 °C under aerobic or anaerobic conditions. Samples of 0.3–1 ml were taken at different times. The reaction

was stopped by adding formic acid (10%, v/v, final concentration) and the precipitated proteins were removed by centrifugation (20 000 g, 4 °C, 10 min). The reverse reaction was performed as mentioned above, except that benzy succinate in the assay mixture was substituted by 2 mM succinate and the reaction was started with 600 μ M benzy succinyl-CoA. The supernatants were analysed by HPLC at room temperature with UV detection at 260 nm. Samples of 50 μ l or CoA-thioester standards were applied on the column. The following systems were used: (a) a LiChrospher 100 RPC-18 (5 μ m) column (Merck) was eluted with a gradient of 1–20% (v/v) acetonitrile in 50 mM potassium phosphate pH 6.8 over 25 min with a flow rate of 1 ml min^{–1}; (b) a Poros R1 column (10 μ m, Perseptive Biosystems) was eluted with a gradient of 0–50% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetate over 20 min with a flow rate of 5 ml min^{–1}. If necessary, the eluates were collected in fractions of 1 ml and freeze-dried.

Oxidation of benzy succinyl-CoA to benzoyl-CoA. The assay mixture contained 0.9 ml 100 mM Tris/HCl pH 7.5, 2 mM NaNO₃ or 1 mM PMS and 50–100 μ l 100 000 g supernatant (equivalent to 2–4 mg protein). The reaction was started by adding benzy succinyl-CoA (1 mM final concentration). Sampling and HPLC analysis was performed as described above. The assay was varied by using nitrate-reductase-depleted cell extract or by addition of supplements, e.g. 0.5 mM NAD or NADP. Details are described in Results and Discussion.

Nitrate reductase. Nitrate reductase was measured under anaerobic conditions at 30 °C using reduced methyl viologen as electron donor. The assay mixture contained 1 mM methyl viologen, 0.5 mM dithionite, 100 mM Tris/HCl pH 7.5, and 10 μ l of a 1:10 diluted 100 000 g supernatant or solubilized membrane preparation. The reaction was started by adding sodium nitrate (final concentration 2 mM), and the oxidation of methyl viologen was followed at 710 nm [ϵ = 2400 mM^{–1} cm^{–1} (Boll & Fuchs, 1995)].

Other methods. Dialysis of 100 000 g supernatants was performed in dialysis tubes (Visking; 12–14 kDa exclusion limit) against 2 $\times$ 1 litre of basal buffer. Some experiments were performed with 100 000 g supernatants (10 ml portions), which were passaged over a DEAE-Sepharose Column (20 ml; Amersham-Pharmacia) in basal buffer (10 mM triethanolamine hydrochloride/NaOH pH 7.5; 10%, v/v, glycerol; flow rate 1 ml min^{–1}). After elution of the flow-through fractions, the bound proteins were eluted with a step gradient of basal buffer containing 500 mM NaCl, collected and directly used for the assays. Protein was determined according to Bradford (1976) using bovine serum albumin as standard. UV/visible spectra of the CoA-thioesters were recorded by a Perkin Elmer UV/Vis spectrometer Lambda 2S. Electrospray-mass spectrometry of benzy succinate derivatives was performed with a Finnigan model TSQ 700 tandem quadrupole mass spectrometer. Freeze-dried HPLC fractions of the compounds obtained with solvent system (b) were dissolved in 0.2% (v/v) trifluoroacetic acid, 25% (v/v) acetonitrile and injected into the mass spectrometer.

RESULTS AND DISCUSSION

Activity of succinyl-CoA:benzy succinate CoA-transferase

Extracts of toluene-grown cells of *T. aromatica* were assayed for an enzyme that activates benzy succinate to the corresponding CoA-thioester. No ATP-dependent benzy succinate CoA ligase activity was measurable in

Table 1. Cellular localization and induction pattern of succinate dehydrogenase and succinyl-CoA:benzylsuccinate CoA-transferase in cells grown on benzoate (B) and toluene (T)

Values are given as means of three or more experiments with the corresponding standard deviations. ND, Not determined.

Cell fraction	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹] of:			
	Succinate dehydrogenase		CoA transferase	
	B	T	B	T
40000 g supernatant	ND	55 ± 2.5	< 1	15 ± 5
Solubilized 100000 g pellet	120 ± 5	131 ± 5	< 1	< 1
100000 g supernatant	< 1	< 1	< 1	15 ± 5

extracts of toluene-grown cells, as expected from the earlier observation that oxidation of benzylsuccinate to benzoyl-CoA is independent of ATP supplementation (Biegert *et al.*, 1996). Therefore, we assayed for a benzylsuccinate CoA-transferase activity using succinyl-CoA, the most common CoA donor. The benzylsuccinate-dependent conversion of succinyl-CoA to free succinate was monitored in a coupled enzyme assay with succinate dehydrogenase of *T. aromatica* as auxiliary enzyme. Succinate dehydrogenase activity was assayed with the artificial electron acceptors PMS and DCPIP. The enzyme was exclusively localized in washed membrane fractions, and a specific activity of 130 nmol succinate oxidized min⁻¹ (mg protein)⁻¹ was obtained in supernatants of Triton X-100 extracted membranes (Table 1). This supernatant (40 µg protein per test) was used as auxiliary enzyme source for CoA-transferase assays.

Succinyl-CoA:benzylsuccinate CoA-transferase activity was only detected in toluene-grown cells and was localized in the soluble fraction after 100000 g centrifugation (Table 1). The measured specific activity of 15 nmol min⁻¹ (mg protein)⁻¹ is about 40% of that needed to explain the observed growth rate of *T. aromatica* on toluene (0.08 h⁻¹). This discrepancy may be explained by the artificial assay conditions or by side reactions of the coupled enzyme assay, since a direct activity assay yielded a value corresponding to 80% of the activity required for growth (see below). The enzyme was relatively stable against oxygen: 94% activity was recovered in extracts that were exposed to air for 6 h. The complete absence of enzyme activity in control cells of *T. aromatica* grown on benzoate suggests that the CoA-transferase is indeed involved in the toluene catabolic pathway. Succinyl-CoA:benzylsuccinate CoA-transferase probably corresponds to one of several strongly substrate-induced proteins that have been observed in toluene-grown *T. aromatica* cells (Leuthner *et al.*, 1998). Specific activities of the reaction in cell extracts are close to the minimum required for growth, indicating that this is a second rate-limiting step of anaerobic toluene catabolism, in addition to the initial

reaction of benzylsuccinate synthase. This would explain the accumulation of benzylsuccinate as the only detectable intermediate in previous experiments on the degradation of ¹⁴C-labelled toluene in cell extracts (Biegert *et al.*, 1996). Further support for the involvement of a succinyl-CoA-dependent CoA-transferase in the pathway can be derived from experiments with permeabilized cells of another toluene-degrading denitrifying bacterium, which showed enhanced rates of benzylsuccinate oxidation to a phenylitaconate derivative in the presence of succinyl-CoA (Beller & Spormann, 1998).

Product analysis of the succinyl-CoA:benzylsuccinate CoA-transferase reaction and reversibility of the enzyme

The products formed from benzylsuccinate and succinyl-CoA were analysed by HPLC. Succinyl-CoA disappeared at a rate of 49 ± 21 nmol min⁻¹ (mg protein)⁻¹ in extracts of toluene-grown cells, whereas a new CoA-thioester (judged from the UV spectrum of the compound) was formed at a rate of 31 ± 15 nmol min⁻¹ (mg protein)⁻¹ (Fig. 2a). This CoA-thioester was identified as benzylsuccinyl-CoA by electrospray mass spectrometry, yielding a mass of 958.6 Da (expected mass: 958.2 Da). Control extracts of benzoate-grown cells did not catalyse benzylsuccinyl-CoA formation. In order to assay for the reversibility of the succinyl-CoA:benzylsuccinate CoA-transferase reaction, products formed from benzylsuccinyl-CoA and succinate were assayed by HPLC. Benzylsuccinyl-CoA-dependent formation of succinyl-CoA was recorded with extracts of toluene-grown cells (Fig. 2b), but not with benzoate-grown cells (not shown). The calculated initial rates of disappearance of benzylsuccinyl-CoA and formation of succinyl-CoA were very similar and were about twice as fast as the forward direction [73 ± 21 and 70 ± 5 nmol min⁻¹ (mg protein)⁻¹, respectively]. After 1–2 min, the reactions were affected by the presence of thioesterase in the extracts, converting the CoA-thioesters to the free acids. No formation of benzylsuccinyl-CoA was detected when

Fig. 2. Kinetics of product formation in enzymic conversion assays with extracts of toluene-grown cells. Products were quantified by HPLC analysis at the time points indicated. Arrows indicate the start points of the reactions. A typical experiment is shown in each case. ●, Benzylsuccinyl-CoA; ▲, succinyl-CoA; ■, benzoyl-CoA. (a) Benzylsuccinyl-CoA synthesis from benzylsuccinate (2 mM) after starting with succinyl-CoA. (b) Reverse reaction of succinyl-CoA:benzylsuccinate CoA-transferase. The reaction contained 2 mM succinate and was started with benzylsuccinyl-CoA. (c) Oxidation of benzylsuccinyl-CoA to benzoyl-CoA in the presence of 1 mM PMS, 0.5 mM NAD and 2 mM NaNO_3 as electron acceptors.

succinyl-CoA was substituted by acetyl-CoA, malonyl-CoA or glutaryl-CoA (data not shown).

Enzymic oxidation of benzylsuccinyl-CoA to benzoyl-CoA

The further metabolism of benzylsuccinyl-CoA was assayed in extracts of toluene- and benzoate-grown cells. Disappearance of benzylsuccinyl-CoA was ob-

served in all assays, due to cleavage of the CoA thioester by thioesterase; however, benzoyl-CoA formation from benzylsuccinyl-CoA was observed only in extracts of toluene-grown cells supplemented with electron acceptors (Fig. 2c). The rate of benzoyl-CoA formation at the expense of nitrate was $4 \pm 2 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$ in untreated extracts. It increased about fourfold in tests supplemented with 1 mM PMS and 0.5 mM NAD (Table 2). The endogenous nitrate reductase activity in the extracts was removed by passage of the extracts through a DEAE-Sepharose column and elution of the bound protein by a step gradient containing 500 mM NaCl in basal buffer. Nitrate reductase activity was found in the flow-through fractions and the benzylsuccinyl-CoA-oxidizing enzymes were recovered in the eluate with a yield of 92% and an enrichment factor of 1.8. No coupling of benzylsuccinyl-CoA oxidation to reduction of nitrate was retained after this step, even if the two fractions were combined (data not shown). The eluate fractions catalysed oxidation of benzylsuccinyl-CoA to benzoyl-CoA if 1 mM PMS was added as electron acceptor, but not with NAD as sole electron acceptor (Table 2). PMS-dependent benzylsuccinyl-CoA oxidation was accelerated sixfold by addition of 0.5 mM NAD to the assay, whereas NADP did not stimulate the reaction (Table 2). The benzylsuccinyl-CoA oxidation rates obtained in NAD-supplemented experiments (in relation to untreated extract) correspond to 40–50% of the rates needed to explain the growth rate of the bacteria on toluene. Since the measured substrate conversion actually represents a series of four individual reactions, this may be regarded as significant match to the predicted pathway. Some implications can also be drawn from the observed electron acceptor dependence of benzylsuccinyl-CoA oxidation. The exact redox potential for oxidation of benzylsuccinyl-CoA to phenylitaconyl-CoA is unknown, but it should be in the range of the fumarate/succinate (+33 mV) or the crotonate/butyrate couple (–24 mV). Therefore, benzylsuccinyl-CoA dehydrogenase should be able to reduce PMS, but not NAD. This probably accounts for the dependence of the overall reaction on a high-potential electron acceptor, e.g. PMS or nitrate. The next oxidation step of the postulated pathway (enzyme 5 in Fig. 1) is the oxidation of a secondary alcohol to a ketone, which occurs at a sufficiently low redox potential to be coupled to reduction of NAD(P). This reaction may account for the observed stimulation of benzylsuccinyl-CoA oxidation to benzoyl-CoA by NAD supplementation.

Stereochemical preferences of the enzymes catalysing benzylsuccinate formation and degradation

The diastereomeric diamides of racemic (*R,S*)-benzylsuccinate with (*S*)-1-phenylethylamine separated into two peaks on a C18 HPLC column (Fig. 3). As shown by HPLC runs with diamides from purified benzylsuccinate enantiomers, the earlier-eluting compound is derived from (*R*)-(+)-, the latter from (*S*)-(–)-benzylsuccinate

Table 2. Oxidation of benzylsuccinyl-CoA to benzoyl-CoA with different electron acceptors

Rates were calculated for formation of benzoyl-CoA, as determined by HPLC analysis. Data are shown for extracts of toluene-grown cells; extracts of benzoate-grown cells did not show activities above the detection limit. The values are means of two or more experiments with corresponding standard deviations.

Protein solution	Electron acceptor(s)	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹]
100 000 g supernatant	NO ₃ ⁻	4 ± 2
	NO ₃ ⁻ + PMS + NAD	15 ± 3
Eluate fraction of DEAE-Sephadex column	none	< 1
	PMS	6 ± 1.5
	NAD	< 1
	PMS + NAD	39 ± 4
	PMS + NADP	6 ± 1.5

Fig. 3. Chromatographic separation of the (S)-(-)-phenylethylamine diamides of racemic (R,S)-benzylsuccinate and enzymically produced [phenyl-¹⁴C]benzylsuccinate. Radioactivity is represented as a solid line, A_{214} as a dotted line. The time delay between the UV monitor and the radiodetector is corrected. Retention times of standard compounds: (R)-(+)-benzylsuccinyl diamide, 15.0 min; (S)-(-)-benzylsuccinyl diamide, 15.7 min.

(data not shown). This assay was employed to examine the stereochemistry of benzylsuccinate that had been produced enzymically from ¹⁴C-labelled toluene and fumarate in cell extracts of toluene-grown *T. aromatica* (Biegert *et al.*, 1996). As shown in Fig. 3, the ¹⁴C-labelled diamide of benzylsuccinate and (S)-1-phenylethylamine co-migrated exclusively with the diamide of the (R)-(+)-enantiomer. If any (S)-(-)-benzylsuccinate was formed by the enzyme, it was below the detection limit of the radioactivity monitor. This corresponds to a minimum enantiomer purity of 99% for (R)-(+)-benzylsuccinate. Stereospecific formation of (R)-(+)-benzylsuccinate was also recently reported in a different toluene-degrading denitrifying bacterium (Beller & Spormann, 1998). Thus, the reaction of benzylsuccinate synthase proceeds highly stereospecifically, despite the

probable involvement of radical intermediates in the reaction mechanism (Heider *et al.*, 1999).

The enantiomer specificity of the CoA-transferase catalysing the activation of benzylsuccinate to benzylsuccinyl-CoA was assayed by HPLC analysis. The rates of benzylsuccinyl-CoA formation were 16 ± 5 nmol min⁻¹ (mg protein)⁻¹ with racemic benzylsuccinate (at 0.3 mM), 10 ± 2 nmol min⁻¹ (mg protein)⁻¹ with (R)-(+)-benzylsuccinate (at 0.2 mM), but only 0.9 ± 0.1 nmol min⁻¹ (mg protein)⁻¹ with (S)-(-)-benzylsuccinate (at 0.2 mM). Thus, the toluene-catabolic pathway involves (R)-(+)-enantiomer-specific reactions for both formation and degradation of benzylsuccinate.

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