Substrate range of benzylsuccinate synthase from *Azoarcus* sp. strain T

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Abstract

Benzylsuccinate synthase, which catalyzes the anaerobic addition of the methyl carbon of toluene to fumarate, has recently been reported in several denitrifying and sulfate-reducing, toluene-degrading bacteria. In substrate range studies with partially purified benzylsuccinate synthase from denitrifying *Azoarcus* sp. strain T, benzylsuccinate analogs were observed as a result of fumarate addition to the following toluene surrogates: xylenes, monofluorotoluenes, benzaldehyde, and 1-methyl-1-cyclohexene (but not 4-methyl-1-cyclohexene or methylcyclohexane). Benzylsuccinate was also observed as a result of toluene addition to maleate, but no products were observed from assays with toluene and either crotonate or trans-glutaconate. Toluene-maleate addition, like toluene-fumarate addition, resulted in highly stereospecific formation of the (+)-benzylsuccinic acid enantiomer [(R)-2-benzyl-3-carboxypropionic acid]. The previously reported finding that the methyl H atom abstracted from toluene is retained in the succinyl moiety of benzylsuccinate was found to apply to several toluene surrogates. The implications of these observations for the mechanism of benzylsuccinate synthase will be discussed. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Benzylsuccinate synthase; Anaerobic; Toluene degradation

1. Introduction

Benzylsuccinate synthase catalyzes the addition of the methyl carbon of toluene to the double bond of fumarate to form benzylsuccinate [1,2]. This reaction, which is unique among known enzymatic reactions with respect to its manner of carbon-carbon bond formation as well as aromatic hydrocarbon activation, has recently been reported as the initial step of anaerobic toluene mineralization in a number of denitrifying [1,2] and sulfate-reducing [3,4] bacteria. Benzylsuccinate synthase from denitrifying *Thauera aromatica* was recently purified and described [5]; however, the specific activity of the purified enzyme was very low [5] and knowledge of the mechanism is still developing. In this study, we used partially purified benzylsuccinate synthase from denitrifying *Azoarcus* sp. strain T to learn more about

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the substrate range, and, by inference, the mechanism of this unique enzyme.

2. Materials and methods

2.1. Chemicals

Toluene and fumarate surrogates used in this study were typically of ≥99% purity and were used as received. The sources of most chemicals have been described elsewhere [2,6,7].

2.2. Cultivation of Azoarcus sp. strain T and preparation of cell extracts

Denitrifying strain T [8] was cultured anaerobically with the growth medium described by Beller and Spormann [2]. Recent 16S rRNA gene sequencing [9] has revealed that strain T belongs to the genus Azoarcus rather than to Pseudomonas, as previously thought. Growth conditions varied in several respects from those used in our previous studies that involved permeabilized cells of toluene-grown strain T [2,6]. For this study, Azoarcus sp. strain T was grown with benzoate and nitrate in a 5.8-l glass reactor (5 l of growth medium) to an OD 600 of 0.4–0.5, and was then induced overnight with toluene and nitrate in the absence of benzoate. Cells were harvested anaerobically by centrifugation [2], resuspended in 3 ml of 20 mM morpholinopropanesulfonic acid (MOPS) buffer [2] that included 8 mg of DNase I, and were then passed three times through a French pressure cell (at 138 MPa) under anaerobic conditions. The resulting cell extract was frozen under anaerobic conditions at −20°C until use.

2.3. Partial purification of benzylsuccinate synthase from cell-free extracts of Azoarcus sp. strain T

Chromatographic enrichment of benzylsuccinate synthase from cell-free extracts was performed anaerobically at 20°C with a Pharmacia Biotech FPLC® System (Uppsala, Sweden). The FPLC system was enclosed in an anaerobic glove box (Coy Laboratory Products, Inc., Grass Lake, MI, USA) with an atmosphere of 90% N2 and 10% H2. After centrifugation (14 500 × g, 4°C, 15 min) under anaerobic conditions and dilution with potassium phosphate buffer (pH 6.8) to a final phosphate concentration of 120 mM, crude cell-free extract supernatants (∼22 mg protein ml⁻¹) were applied to an Econo-Pac CHT-II hydroxyapatite column (5 ml bed volume, 20 μm particle diameter; Bio-Rad, Hercules, CA, USA). The chromatographic conditions were as follows: using a flow rate of 1 ml min⁻¹ and a binary eluent system of 10 mM and 500 mM potassium phosphate buffer [pH 6.8; also containing 1 mM dithiothreitol (DTT)], the column was held at an initial phosphate concentration of 120 mM for 1.2 column volumes and protein was then eluted with a linear gradient from 120 to 400 mM phosphate at a rate of 25 mM ml⁻¹. One-ml fractions were collected and assayed for benzylsuccinate synthase activity (as described below). Protein was quantified in chromatographic fractions by the method of Bradford [10]. A second purification step was performed by applying a selected hydroxyapatite fraction to a Superose 6 HR 10/30 gel filtration column (24 ml bed volume, 11–15 μm bead diameter, Pharmacia Biotech). The eluent for gel filtration was 50 mM potassium phosphate (pH 7.0) buffer containing 1 mM DTT and 150 mM KCl, and the flow rate was 0.3 ml min⁻¹. Fractions of 0.5 ml were collected and assayed for benzylsuccinate synthase activity.

2.3.1. In vitro assay for benzylsuccinate synthase activity

The radiological assay used during benzylsuccinate synthase purification was adapted from a GC/MS (gas chromatography-mass spectrometry) assay used in previous studies of benzylsuccinate synthase [2,6]. As described previously [2], kinetic assays (1 ml liquid volume in 5-ml vials) were performed inside an anaerobic glove box at 25°C. Radiological assay mixtures contained MOPS buffer [2] and 290–350 nmol of [ring-U-14C]toluene (specific activity, ∼77 μCi mmol⁻¹), 1–1.5 mM fumarate, and 2 mM DTT as a reductant. Reactions were halted at selected time intervals (e.g., 0, 10, 20 min) by adding 0.1 ml of 2 M NaOH to assay vials. After incubation, the assay vials were removed from the glove box and were amended with an antifoaming agent (Antifoam A, Sigma; ∼100 ppm final concentration). Assay mixtures were then purged with N2 to remove residual [14C]toluene and were combined.
with 10 ml of Ultima Gold XR scintillation liquid (Packard, Downers Grove, IL, USA). The remaining nonvolatile radioactivity (primarily [14C]benzylsuccinate) was measured with a Tri-Carb model 2500 TR/AB liquid scintillation analyzer (Packard). Based on prior GC/MS studies of benzylsuccinate synthase in *Azoarcus* sp. strain T [2,6], benzylsuccinate should have been the only significant product from toluene under the experimental conditions of the radiological assays. This was confirmed by parallel studies comparing the GC/MS and radiological assays under identical assay conditions (e.g., using the same hydroxyapatite fractions as a source of benzylsuccinate synthase activity); in these parallel assays, the absolute benzylsuccinate concentrations determined in 10-min duplicate assays agreed within ~10% for the two detection methods (data not shown).

2.4. *In vitro* substrate range studies

Assays for investigating the substrate range of benzylsuccinate synthase were conducted very similarly to the GC/MS assays described by Beller and Spormann [2,6]. Assay mixtures included 300 nmol toluene or toluene surrogate, 1.2–1.5 μmol fumarate or fumarate surrogate, 2 μmol DTT, and 0.29–0.35 mg protein in 1 ml of 20 mM MOPS buffer [2]. Notable differences from previous studies were that the source of benzylsuccinate synthase activity in the present study was the hydroxyapatite fraction rather than permeabilized cells and that toluene and all toluene surrogates in the present study were amended via methanolic stock solutions (resulting in a final methanol concentration of 0.1% (v/v) in the assays). Analysis of assays involved extraction with diethyl ether after a 60-min incubation, derivatization of ether extracts with diazomethane to convert carboxylic acids to methyl esters, and analysis by GC/MS [2,6]. Authentic GC/MS standards were not available for products other than benzylsuccinate and 3-benzoylpropionate. However, GC/MS identification of putative benzylsuccinate analogs was typically straightforward because of the high degree of homology between the mass spectral fragmentation patterns of the benzylsuccinate analogs and those of the benzylsuccinate and 3-benzoylpropionate standards.

3. Results and discussion

3.1. Partial purification of benzylsuccinate synthase from *Azoarcus* sp. strain T

The two-step purification procedure used to enrich benzylsuccinate synthase activity from *Azoarcus* sp. strain T is summarized in Table 1. The specific activity of the thawed and diluted crude extract (5.9 nmol min⁻¹ mg protein⁻¹; Table 1) constitutes 35–40% of the specific in vivo toluene consumption rate of strain T [2]. A higher specific activity (8.0 nmol min⁻¹ mg protein⁻¹) was observed when fresh (unfrozen) extract was used (data not shown).

Hydroxyapatite chromatography resulted in a >10-fold enrichment of benzylsuccinate synthase activity relative to the crude cell-free extract (Table 1). A 1-ml fraction eluting between ~200 and 225 mM phosphate contained 36% of the activity and <3% of the protein of the crude cell-free extract applied to the column (Table 1). If one includes the 1-ml fractions eluting immediately before and after the fraction represented in Table 1, the resulting 3-ml fraction (eluting between ~175 and 250 mM phosphate)

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Activity (nmol min⁻¹)</th>
<th>Protein (mg)</th>
<th>Specific activity (nmol min⁻¹ mg protein⁻¹)</th>
<th>Yield (percent)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1.7</td>
<td>220</td>
<td>37.5</td>
<td>5.9</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Hydroxyapatitea</td>
<td>1</td>
<td>79</td>
<td>1.1</td>
<td>72</td>
<td>36</td>
<td>12</td>
</tr>
<tr>
<td>Gel filtrationb</td>
<td>5</td>
<td>1.6</td>
<td>0.48</td>
<td>3.4</td>
<td>0.7</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*a* Fraction eluted between ~200 and 225 mM potassium phosphate buffer (see text).

*b* Fraction eluted between 14.2 and 15.2 ml (see text). The 1-ml hydroxyapatite fraction had to be applied to the gel filtration column in five 0.2-ml aliquots, resulting in five 1-ml gel filtration fractions containing benzylsuccinate synthase activity.
accounted for 67% of the activity and ~7% of the protein of the crude cell-free extract applied to the column (data not shown).

Further purification of benzylsuccinate synthase was attempted by applying 0.2-ml aliquots of the 1-ml hydroxyapatite fraction (i.e., 200–225 mM phosphate) to a gel filtration column. A 1-ml fraction containing benzylsuccinate synthase activity was collected between 14.2 and 15.2 ml. Based on a molecular mass calibration performed under the same chromatographic conditions as used for the extract, the benzylsuccinate synthase retention volume of 14.85 ml corresponded to an apparent native molecular mass of 260 kDa (as compared to 220 kDa reported for benzylsuccinate synthase from T. aromatica; [5]). The molecular mass calibration was performed with aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), thyroglobulin (669 kDa), and blue dextran 2000 (for void volume determination). Unfortunately, gel filtration resulted in a dramatic (>95%) loss of specific activity relative to the hydroxyapatite fraction (Table 1).

An SDS-PAGE gel representing the crude, hydroxyapatite, and gel filtration fractions is presented in Fig. 1. The three most distinct bands in the gel filtration fraction (one band at ~97 kDa and two bands between 6.5 and 14.5 kDa; lane 3 in Fig. 1) correspond well to the three subunits of benzylsuccinate synthase from T. aromatica described by Leuthner et al. [5].

The purification of benzylsuccinate synthase from T. aromatica by Leuthner et al. [5] provides the only basis of comparison for this work. Compared to the results for Azoarcus sp. strain T (Table 1), the three-step purification scheme reported for T. aromatica resulted in a similar yield of benzylsuccinate synthase (1–3% of the total protein) and a similar overall loss of specific activity (~99%)[5]. However, the initial, final, and maximum specific activities observed during purification of benzylsuccinate synthase from Azoarcus sp. strain T were 2–3 orders of magnitude higher than those observed for T. aromatica [5].

### Table 2

<table>
<thead>
<tr>
<th>Substrate analog</th>
<th>Producta</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Toluene surrogate</strong></td>
<td></td>
</tr>
<tr>
<td>o-Xylene-d₁₀</td>
<td>(2-methylbenzyl)succinate-d₁₀ ++</td>
</tr>
<tr>
<td>m-Xylene-d₈</td>
<td>(3-methylbenzyl)succinate-d₈ ++</td>
</tr>
<tr>
<td>p-Xylene-d₁₀</td>
<td>(4-methylbenzyl)succinate-d₁₀ ++</td>
</tr>
<tr>
<td>2-Fluorotoluene</td>
<td>(2-fluorobenzyl)succinate ++</td>
</tr>
<tr>
<td>3-Fluorotoluene</td>
<td>(3-fluorobenzyl)succinate ++</td>
</tr>
<tr>
<td>4-Fluorotoluene</td>
<td>(4-fluorobenzyl)succinate ++</td>
</tr>
<tr>
<td>Styrene</td>
<td>–</td>
</tr>
<tr>
<td>1-Methyl-1-cyclohexene</td>
<td>Molecular mass, 240 Da[^d] +</td>
</tr>
<tr>
<td>4-Methyl-1-cyclohexene</td>
<td>–</td>
</tr>
<tr>
<td>Methylcyclohexane</td>
<td>–</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>benzoylsuccinate ++</td>
</tr>
<tr>
<td>3-Benzoylpropionate</td>
<td>–</td>
</tr>
<tr>
<td><strong>Fumarate surrogate</strong></td>
<td></td>
</tr>
<tr>
<td>Maleate</td>
<td>benzylsuccinate ++</td>
</tr>
<tr>
<td>trans-Glutaconate</td>
<td>–</td>
</tr>
<tr>
<td>Crotonate</td>
<td>–</td>
</tr>
</tbody>
</table>

[^a]: Except for benzylsuccinate and 3-benzoylpropionate, products were tentatively identified based on mass spectra because authentic standards were not available (see text).
[^b]: Yield of product relative to yield of benzylsuccinate-d₁₀ in a control assay containing toluene-d₁₀ and fumarate: –, below detection limit (<0.15% of control), +, 1–5% of control, ++, 10–>100% of control. The control ranged from 120 to 210 nmol of benzylsuccinate-d₁₀ with different batches of cell-free extract.
[^c]: No trace of product was observed in derivatized stock solutions of the toluene surrogate.
[^d]: Molecular mass of the derivatized product, which is presumably a dimethyl ester. Thus, the molecular mass of the underivatized product is hypothesized to be 212 Da.
3.2. Substrate range of benzylsuccinate synthase in Azoarcus sp. strain T

In light of the high efficiency of hydroxyapatite chromatography in this study and the large degree of benzylsuccinate synthase deactivation during gel filtration (Table 1), experiments for testing the substrate range of benzylsuccinate synthase employed the hydroxyapatite fraction rather than the gel filtration fraction. Hydroxyapatite fractions of 3 or 4 ml wide were used to maximize the number of assays that could be performed per batch of cell-free extract.

A summary of the toluene and fumarate surrogates tested, the products observed, and the semiquantitative product yields is presented in Table 2. All three xylene and monofluorotoluene isomers were transformed to benzylsuccinate analogs by benzylsuccinate synthase (Table 2). Previous studies with permeabilized cells demonstrated that all three monochlorotoluene isomers were also transformed to the corresponding benzylsuccinate analogs (data not shown). Mass spectra of the methylbenzylsuccinate isomers produced from deuterated o-, m-, and p-xylene (not shown) were entirely consistent with the observation made previously for toluene and o-xylene regarding retention of the abstracted methyl H atom in the succinyl moiety of benzylsuccinate or methylbenzylsuccinate [2]. The mechanistic implications of H atom retention were discussed previously [6].

A minor product (~1% of the control) was observed in assays containing 1-methyl-1-cyclohexene (Table 2). The mass spectrum of the 240-Da derivatized product and a proposed structure consistent with fumarate addition to 1-methyl-1-cyclohexene are shown in Fig. 2. Notably, no product was observed when 4-methyl-1-cyclohexene was included in the assay rather than 1-methyl-1-cyclohexene (Table 2). This observation is consistent with the proposed radical mechanism of benzylsuccinate synthase [5, 6, 11], because the allyl radical intermediate that would be formed by abstraction of an H atom from the methyl carbon of 1-methyl-1-cyclohexene would be much more stable than the corresponding radical produced from 4-methyl-1-cyclohexene. Accordingly, no product was observed for methylcyclohexane (Table 2), which is fully saturated.

A major product of assays containing benzaldehyde is strongly considered to be benzoylsuccinate (Table 2). The mass spectrum of the derivatized product (Fig. 3A) has prominent fragment ions at m/z 77 (phenyl) and 105 (benzoyl) that are observed in many aromatic ketones, such as 3-benzoylpropionate methyl ester [7, 12]. Although the molecular ion at m/z 250 is weak in the electron ionization mass spectrum (Fig. 3A), chemical ionization MS analysis using methane as a collision gas confirmed that the
molecular mass was indeed 250 Da (data not shown). Evidence of H atom retention, observed for toluene and xylenes, was also apparent for benzaldehyde: when benzaldehyde-$\alpha^{13}$C, $d_{1}$ was used in assays rather than unlabeled benzaldehyde, the product appeared to have the D atom retained in the succinyl moiety of benzoylsuccinate (Fig. 3B). To illustrate, comparison of Fig. 3A,B reveals that the (M-$OCH_3$)$^+$ ion at m/z 221 and the (M-COOCH$_3$)$^+$ ion at m/z 193 in the labeled product (Fig. 3B) were both 2 amu larger than the corresponding fragments in the unlabeled product (Fig. 3A) as a result of having a $^{13}$C and a D atom. However, the benzoyl fragment in the labeled product ($m/z$ 106; Fig. 3B) is only 1 amu larger than in the unlabeled product (Fig. 3A) because it has a $^{13}$C atom but not a D atom. Thus, the D atom must have been in the succinyl moiety. Despite the consistency of the observed product with known benzylsuccinate synthase characteristics, it is nonetheless possible that the formation of benzoylsuccinate from benzaldehyde and fumurate proceeded by a mechanism other than the proposed radical addition.

A minor product, 3-benzoylpropionate, was also observed in assays containing benzaldehyde (Table 2). Considering that benzoylsuccinate is a β-keto acid, it is plausible that 3-benzoylpropionate is a decarboxylation product that may have formed abiotically during the assay or during the subsequent ether extraction under acidic conditions. Notably, 3-benzoylpropionate was also observed during in vivo experiments involving benzaldehyde and toluene-grown, sulfate-reducing strain PRTOL1 [7]; this species has also been shown to contain benzylsuccinate synthase activity [3]. In strain PRTOL1 suspensions amended with benzaldehyde-$\alpha^{13}$C, $d_{1}$, it was apparent that the D atom was similarly retained in the aliphatic portion of 3-benzoylpropionate [7]. Interpreting the results for strain PRTOL1 in light of the data for Azoarcus sp. strain T, it is plausible that labeled benzoylsuccinate, which retained the D atom from benzaldehyde-$\alpha^{13}$C, $d_{1}$, was decarboxylated to form labeled 3-benzoylpropionate.

Although benzylsuccinate synthase appears to be capable of adding fumarate to a carbonyl carbon atom (e.g., benzaldehyde), it does not appear to catalyze addition to an olefinic carbon atom (e.g., styrene; Table 2).

Of the three fumarate surrogates that were tested, only maleate formed a detectable product, benzylsuccinate (Table 2). Further experimentation with crude cell-free extracts determined that toluene-maleate addition was highly stereospecific, resulting in >98% formation of the (+)-benzylsuccinic acid enantiomer [(R)-2-benzyl-3-carboxypropionic acid]. Stereospecificity was determined by chiral high-performance liquid chromatographic (HPLC) analysis [6]. For this analysis, the solvent extract of a toluene-maleate assay mixture was applied to a chiral HPLC system, and then the chromatographic fractions corresponding to the retention time windows of (+)- and (−)-benzylsuccinate were collected separately, derivatized with diazomethane, and analyzed by GC/MS. Benzylsuccinate was detected only in the (+)-benzylsuccinate fraction. If it were assumed that (−)-benzylsuccinate was present at just below its detection limit, then (−)-benzylsuccinate would constitute less...
than 2% of the total benzylsuccinate produced from toluene and maleate. Notably, other studies with *Azoarcus* sp. strain T have shown that toluene-fumarate addition also results in highly stereospecific formation of (+)-benzylsuccinate [6]. The fact that toluene addition to either fumarate or maleate results in formation of the same benzylsuccinate enantiomer has implications for our understanding of the benzylsuccinate synthase mechanism. If it is assumed that the relative spatial positions of toluene and a C₄-aliphatic acid in the active site are the same regardless of whether the acid is fumarate or maleate, then the acid’s binding to the enzyme in the region of C-1 and C-2 (where C-2 is the site of toluene addition) appears to be much more important than binding in the region of C-4. Since the stereochemistry experiments with maleate were conducted with crude cell-free extracts, it is possible that maleate was converted to fumarate during the assays and that the results actually reflect toluene-fumarate addition. However, if at least 2% of the observed benzylsuccinate was formed by direct toluene-maleate addition, then the conclusion about benzylsuccinate synthase stereospecificity with maleate and the associated mechanistic implications are nonetheless tenable.

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**References**


