A Stable Organic Free Radical in Anaerobic Benzylsuccinate Synthase of *Azooacus* sp. Strain T*

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The novel enzyme benzylsuccinate synthase initiates anaerobic toluene metabolism by catalyzing the addition of toluene to fumarate, forming benzylsuccinate. Based primarily on its sequence similarity to the glycyl radical enzymes, pyruvate formate-lyase and anaerobic ribonucleotide reductase, benzylsuccinate synthase was speculated to be a glycyl radical enzyme. In this report we use EPR spectroscopy to demonstrate for the first time that active benzylsuccinate synthase from the denitrifying bacterium *Azooacus* sp. strain T harbors an oxygen-sensitive stable organic free radical. The EPR signal of the radical was centered at $g = 2.0021$ and was characterized by a major 2-fold splitting of about 1.5 millitesla. The strong similarities between the EPR signals of the benzylsuccinate synthase radical and that of the glycyl radicals of pyruvate formate-lyase and anaerobic ribonucleotide reductase provide evidence that the benzylsuccinate synthase radical is located on a glycine residue, presumably glycine 828 in *Azooacus* sp. strain T benzylsuccinate synthase.

Benzylsuccinate synthase initiates anaerobic toluene mineralization in denitrifying bacteria by catalyzing the addition of toluene to fumarate, forming benzylsuccinate (Fig. 1; Refs. 1 and 2). This toluene fumarate addition reaction may be a general mode for anaerobic toluene metabolism as it has been demonstrated recently in phylogenetically distant bacteria, including several toluene-mineralizing, denitrifying (1, 2), and sulfate-reducing (3, 4) bacteria, as well as a toluene-utilizing phototrophic bacterium (5). Furthermore, this type of fumarate addition reaction may be a general strategy for activating methylenes in the absence of molecular oxygen as it has been shown to be the initial step in anaerobic m-xylene mineralization (6) and has been implicated as the initial step in anaerobic m-cresol mineralization (7). Unlike the initial activation steps in aerobic methylenes metabolism whereby the methylenes is oxidized directly by molecular oxygen as a cosubstrate (8, 9), benzylsuccinate synthase catalyzes a strictly anaerobic, non-redox reaction. Notably, the benzylsuccinate synthase reaction also seems to be a novel biochemical means for forming a new carbon–carbon bond.

Benzylsuccinate synthase, which has been characterized at both the biochemical and molecular level (10–12), is believed to be a heterohexamer ($\alpha_2\beta_2\gamma_2$) composed of three subunits with molecular masses of approximately 98 ($\alpha$ subunit), 86 ($\beta$ subunit), and 6.6 ($\gamma$ subunit) kDa. The predicted amino acid sequence of the PFL- and ARNR-activating enzymes, pyruvate formate-lyase (PFL) and anaerobic ribonucleotide reductase (ARNR), have only one conserved cysteine, corresponding to cysteine 419 of *Escherichia coli* PFL. The conserved amino acids in PFL (13–16) and ARNR (17, 18) are essential for catalysis of their respective reactions. The catalytically active forms of PFL (13–16, 19, 20) and ARNR (17, 18) are essential for catalysis of their respective reactions. The catalytically active forms of PFL (13–16, 19, 20) and ARNR (17, 18, 21, 22) contain a free radical at the conserved glycine residue; this radical is generated enzymatically by a PFL activase and an ARNR activase, respectively. A gene located immediately upstream of the benzylsuccinate synthase structural genes shares strong predicted amino acid sequence similarity to the PFL- and ARNR-activating enzymes, suggesting that benzylsuccinate synthase may be activated in a similar fashion (11, 12). In activated PFL and ARNR, the glycyl radical is assumed to abstract a hydrogen atom from a conserved cysteine residue generating a thiyl radical that initiates substrate transformation (14, 15, 20, 23). Genetic complementation studies in *Thauera aromatica* sp. T1 showed that the conserved glycine and cysteine residues of benzylsuccinate synthase are important for toluene utilization (12). Based on the molecular similarity of benzylsuccinate synthase to PFL and ARNR, we examined active benzylsuccinate synthase for the presence of an organic free radical.

Using EPR spectroscopy we report here the first experimental evidence that active benzylsuccinate synthase carries a stable organic free radical. Based on the strong spectral similarities between the EPR signal of the benzylsuccinate synthase radical and that of the glycyl radicals of PFL and ARNR, the free radical of benzylsuccinate synthase appears to be located on a glycine residue. Furthermore, we found a direct correlation between the appearance of the free radical and enzyme activity, suggesting that the radical is essential for catalysis.

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1 G. Achong, A. Rodriguez, and A. Spormann, unpublished data.

2 The abbreviations used are: PFL, pyruvate formate-lyase; ARNR, anaerobic ribonucleotide reductase; mT, millitesla.
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**EXPERIMENTAL PROCEDURES**

**Materials**—All the chemicals used in this study, including $^{2}H_{2}O$ (D$_2$O; 99.9 atom %) and [ring-$^{13}$C]-toluene (≥98 atom %; 2.8 mCi/ mmol specific activity), were purchased from either Aldrich or Sigma. The [ring-$^{13}$C]-toluene was diluted to a specific activity of ~42 mCi/ mmol for the experiments.

**Cultivation of Azorarcus sp. Strain T and Preparation of Cell Extracts—**Azorarcus sp. strain T, a denitrifying bacterium capable of anaerobically mineralizing toluene and m-xylene (24), was cultivated under denitrifying conditions in a bicarbonate-buffered mineral salts medium as described previously (1). Batch cultures totaling 10–12 liters were grown in glass reactors sealed with polytetrafluoroethylene Mininert valves (Alltech Associates, Inc., Deerfield, IL). These cultures were incubated at room temperature (25 °C) in an anaerobic glove box (Coy Laboratory Products, Inc., Grass Lake, MI) with an atmosphere of 80% N$_2$, 10% H$_2$, and 10% CO$_2$. Azorarcus sp. strain T was grown exponentially with benzoate (initial concentration 3 mM) and nitrate (initial concentration 2.5 mM) to an A$_{600}$ of 0.25–0.5. Benzoate and nitrate were amended repeatedly to the cultures prior to reaching the desired A$_{600}$. Approximately 20 h prior to harvesting the cells, benzoate (initial concentration 350 mM) was added to the cultures to induce synthesis of benzylsuccinate synthase. During the remaining growth period residual benzoate was consumed completely, and the cells switched to toluene metabolism as indicated by the induction of benzylsuccinate synthase activity (data not shown). Cells were harvested anaerobically by centrifugation (1), washed once in anoxic 120 mM potassium phosphate buffer, pH 6.8, amended with 2 mM dithiothreitol (designated as buffer A), and resuspended in 7–12 ml of buffer A (amended with 12 mg of DNase I and ~6 mC MgCl$_2$). Cells were broken anaerobically by four passages through a French pressure cell at 138 megapascal. Unbroken cells and cell debris were removed by anaerobic centrifugation (27,000 × g, 15 min, 4 °C). The supernatant, defined as the cell extract, was divided into 0.5- and 1-ml fractions that were frozen under anaerobic conditions at ~20 °C until further use.

**Partial Purification of Benzylsuccinate Synthase—**Benzylsuccinate synthase was partially purified from cell extracts of anaerobically grown toluene-induced Azorarcus sp. strain T using a modification of the method described previously (10). Purification steps were conducted anaerobically at ~15 °C in an anaerobic glove box with an atmosphere of 96% N$_2$ and 4% H$_2$. Crude cell extract was passed through a 0.45-μm filter and then applied in batches (35–80 mg of protein) to an Econo-Pac 200 M M potassium phosphate buffer, pH 6.8, ranged from 150 to 400 nmol min$^{-1}$ (mg of protein)$^{-1}$ and that of enriched benzylsuccinate synthase ranged from 150 to 400 nmol min$^{-1}$ (mg of protein)$^{-1}$, depending on the particular fraction. Benzylsuccinate synthase activity typically eluted between 160 and 235 mM phosphate. Attempts to purify benzylsuccinate synthase further by gel filtration resulted in a greater than 95% loss of specific activity relative to the hydroxyapatite-enriched fraction (10). Thus, to retain activity of benzylsuccinate synthase, hydroxyapatite-enriched fractions were used for this study. The hydroxyapatite fractions used in this study contained a greater than 7-fold enrichment of benzylsuccinate synthase and less than 3% of the protein of the cell extract placed on the column. Protein concentrations were determined by the method of Bradford (25) using a commercially available protein-binding dye from Bio-Rad. Bovine serum albumin was used as the standard.

**In Vitro Assay for Benzylsuccinate Synthase—**The activity of benzylsuccinate synthase was measured using a modified version of the radiological assay developed previously (10). Assays were performed in 2.8-ml glass vials sealed with butyl rubber septa in an anaerobic glove box. Assay mixtures (final volume 1 ml) contained 100 mM Tris-HCl buffer, pH 7.8, 300 nmoil of [ring-$^{13}$C]-toluene (specific activity, ~42 mCi/mmol), 10 mM fumarate, and 5 mM dithiothreitol as a reductant. The assay buffer was changed to a Tris-HCl buffer, pH 7.8, because the pH optimum of benzylsuccinate synthase activity was found to be between 7.5 and 8, consistent with the findings of Leutner et al. (11).

Assays were conducted at room temperature (25 °C) in an anaerobic glove box with an atmosphere of 80% N$_2$, 10% H$_2$, and 10% CO$_2$. To prevent a decrease in the pH of the reaction mixture due to the presence of CO$_2$ in the glove box atmosphere, the reaction mixtures were prepared in a glove box with an atmosphere of 90% N$_2$ and 10% H$_2$ and then transferred to the former glove box in a stopped anoxic glass bottle. Assay components were added by syringe through Mininert valves. Reactions were started by the addition of crude cell extract or hydroxyapatite-enriched benzylsuccinate synthase. After incubation for 0, 10, or 12 min on an orbital shaker, the reactions were stopped by the addition of 0.1 ml of 2 m NaOH. The assay vials were removed from the glove box and amended with an antifoaming agent (Antifoam A, Sigma; ~300 ppm final concentration). Assay mixtures were amended with N$_2$ for 20 min to remove any residual [ring-$^{13}$C]-toluene. Ultima Gold XR scintillation liquid (10 ml; Packard Instrument Co.) then was added to the mixtures, and the remaining nonvolatile radioactivity (primarily [ring-$^{13}$C]benzylsuccinate) was measured with a Tri-Carb model 2500 TR/AB liquid scintillation analyzer (Packard Instrument Co.). Benzylsuccinate has been shown to be the only significant product formed from toluene under the experimental conditions of these assays (10).

**Sample Preparations for EPR Spectroscopy—**Hydroxyapatite fractions of catalytically active benzylsuccinate synthase were analyzed by EPR spectroscopy. Although a portion of the hydroxyapatite-enriched benzylsuccinate synthase was tested for activity to ensure the enzyme was catalytically active (see above), the remaining portion of benzylsuccinate synthase analyzed by EPR spectroscopy was not amended with the substrates, toluene and fumarate, and therefore did not catalyze the benzylsuccinate synthase reaction. In an anaerobic glove box (atmosphere of 90% N$_2$ and 10% H$_2$), samples (300 μl) of benzylsuccinate synthase were transferred to EPR tubes. Anaerobic tubing was placed over the end of each EPR tube and clamped closed. The EPR tubes were removed from the glove box immediately frozen in liquid nitrogen and then transferred to liquid nitrogen cryovials (CryoPak Shipper, CP-100, Taylor-Wharton, Theodore, AL) to Amsterdam where they were analyzed by EPR spectroscopy. Three sample sets of benzylsuccinate synthase were prepared and analyzed by EPR spectroscopy: 1) benzylsuccinate synthase, 2) benzylsuccinate synthase exchanged into phosphate-buffered $^{2}H_{2}O$, and 3) benzylsuccinate synthase amended with toluene but not fumarate.

**Benzylsuccinate Synthase Amended with Toluene—**Portions (300 μl) of the same hydroxyapatite fraction of benzylsuccinate synthase (1.9 mg of protein ml$^{-1}$) was transferred to an EPR tube and analyzed by EPR spectroscopy.

**Deuterium-exchanged Benzylsuccinate Synthase—**A 200 mM potassium phosphate buffer, pH 6.8, was prepared anoxically in 99.9% D$_2$O (referred to as phosphate-buffered D$_2$O). A hydroxyapatite fraction of benzylsuccinate synthase (800 μl of 1.4 mg of protein ml$^{-1}$) was exchanged into the anoxic phosphate-buffered D$_2$O by applying the fraction onto a HiTrap desalting column (5-ml bed volume; Amersham Pharmacia Biotech) and then eluting it with 2.6 ml of the phosphate-buffered D$_2$O. Only the last 1.9 ml of eluted liquid were collected, and a portion of this (300 μl of 0.5 mg of protein ml$^{-1}$) was transferred to an EPR tube and analyzed by EPR spectroscopy.

**EPR Spectroscopy—**EPR spectra at X-band (9 GHz) were obtained with a Bruker ECS 106 EPR spectrometer equipped with an Oxford Instruments ESR 900 helium-flow cryostat with an ITC4 temperature controller. A field modulation frequency of 100 kHz was used.
Benzylsuccinate synthase, as partially purified from extracts of anaerobically grown toluene-induced cells of Azotobacter sp., strain T, was active and catalyzed the addition of toluene to fumarate to form benzylsuccinate at high specific activities (150 to 400 nmol min⁻¹ mg⁻¹). Using EPR spectroscopy, we analyzed a hydroxyapatite fraction of benzylsuccinate synthase (1.8 mg of protein ml⁻¹; 8 μM holoenzyme; spin concentration 2.5 μM). The microwave frequency was measured with an HP 5350B microwave frequency counter. The microwave power incident to the cavity was 9423.4 MHz; microwave power incident to the cavity was calibrated with an AEG magnetic field meter. The magnetic field was calibrated with an AEG magnetic field meter. The microwave frequency was measured with an HP 5350B microwave frequency counter. The microwave power incident to the cavity was 9423.4 MHz; microwave power incident to the cavity was calibrated with an AEG magnetic field meter.

RESULTS AND DISCUSSION

Benzylsuccinate synthase, as partially purified from extracts of anaerobically grown toluene-induced cells of Azotobacter sp., strain T, was active and catalyzed the addition of toluene to fumarate to form benzylsuccinate at high specific activities (150 to 400 nmol min⁻¹ mg⁻¹). Using EPR spectroscopy, we analyzed a hydroxyapatite fraction of benzylsuccinate synthase (1.8 mg of protein ml⁻¹; 8 μM holoenzyme) for the presence of a free radical and observed an EPR signal as shown in Fig. 2A. The EPR signal was centered at g = 2.0021 and was characterized by a 2-fold splitting of about 1.5 mT. The g value of the signal is very close to that of a free radical. Most importantly, the line shape of the EPR signal closely resembles that of the glycy radical of PFL (15, 16) and ARNR (17, 18) from E. coli. The EPR signals of all three enzymes are characterized by a g value between 2.002 and 2.004, as well as a resolved 2-fold splitting of about 1.5 mT (15–18). The simulation in Fig. 2B shows that the hyperfine interactions and linewidth proposed for PFL by Wagner et al. (15) results in an EPR spectrum that is indistinguishable from that of benzylsuccinate synthase. The EPR spectral similarities between benzylsuccinate synthase and the glycy radical enzymes, PFL and ARNR, strongly suggest that the radical observed in benzylsuccinate synthase is also assigned to an exchangeable hydrogen, most likely the hydrogen of the glycy radical. The simulation in Fig. 2D shows that the hyperfine interactions and linewidth proposed for PFL in D₂O indicates an analogous behavior. A rapidly exchangeable hydrogen was found in PFL and was assigned to the α position of the glycy radical (15). Analogous with this assignment, the 2-fold splitting in the EPR spectrum of benzylsuccinate synthase is also assigned to an exchangeable hydrogen, most likely the α hydrogen of the glycy radical. The simulation in Fig. 2D shows that the hyperfine interactions and linewidth proposed for PFL in D₂O (15) results in an EPR spectrum indistinguishable from that of benzylsuccinate synthase in D₂O (Fig. 2C). Contrary to benzylsuccinate synthase and PFL, no such rapidly exchangeable hydrogen was observed for ARNR (17).

Mutant studies conducted with PFL from E. coli showed that the hydrogen exchange of the glycy radical of PFL is not spontaneous but involves the active site cysteine 419 (14). Based on this finding, Parast et al. (14) proposed that the α hydrogen of the glycy radical does not exchange directly with the solvent. Instead, the hydrogen of the thiol group of cysteine 419 is proposed to exchange with the solvent, in this case solvent deuterons, forming a deuterated thiol group. The glycy radical is then proposed to abstract the deuterium from the thiol group, generating a transient thyl radical and a chirally deuterated glycy residue. To form a glycy radical with a deuterium in the α position, as observed in the EPR spectra of PFL (15, 16), the thyl radical of cysteine 419 must nonstereospecifically abstract the hydrogen from the chirally deuterated glycy residue (14). As an analog to the findings for PFL, one may postulate that the hydrogen exchange of the glycy radical of benzylsuccinate synthase may not be spontaneous but may involve the conserved cysteine residue. This would suggest that the glycy radical and the conserved cysteine residue in benzylsuccinate synthase are in close proximity to each other in the active site and interact during catalysis.

We also examined whether amending samples of enriched benzylsuccinate synthase with toluene as the sole substrate alters the EPR signal of the benzylsuccinate synthase radical. Control samples included benzylsuccinate synthase alone and benzylsuccinate synthase amended with benzene, a nonmetabolizable surrogate. The intensity and line shapes of the EPR spectra of all three samples were similar to those of the untreated enzyme (data not shown). This suggests that the presence of toluene alone does not quench the radical to any significant degree, or if it does, the quenching is too transient to be observed.

The spectroscopic characteristics of benzylsuccinate syn-
that reported here and the molecular properties discussed previously (10–12, 30) suggest the following reaction mechanism for benzylsuccinate synthase. At the beginning of the enzymatic reaction, active benzylsuccinate synthase harbors an oxygen-sensitive, stable glycyl free radical (presumably at glycine 828 in Azoarcus sp. strain T benzylsuccinate synthase. The glycyl radical abstracts a hydrogen atom from the conserved cysteine residue forming a transient thiyl radical (presumably at cysteine 492 in Azoarcus sp. strain T benzylsuccinate synthase). The thiyl radical then abstracts a hydrogen atom from the toluene methyl group to form a benzylic radical as an intermediate, which then attacks the double bond of fumarate forming a benzylsuccinyl radical. The benzylsuccinate synthase reaction mechanism for benzylsuccinate synthase (14, 20, 31), we presume that the thiyl radical generating the thiyl radical. Based on proposed reaction mechanism for PFL (14, 20, 31), we presume that the thiyl radical abstracts a hydrogen atom from the conserved glycine residue, forming benzylsuccinate and regenerating the glycyl radical. At this point the glycyl radical is competent to undergo another catalytic cycle. Consequently benzylsuccinate synthase can undergo multiple turnovers without reintroduction of its glycyl radical by an activating enzyme, as also indicated by in vitro assays of benzylsuccinate synthase activity conducted in this study: 150 pmol of enriched benzylsuccinate synthase produced 160 nmol of benzylsuccinate in 12 min.

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