Nonaromatic Products from Anoxic Conversion of Benzoyl-CoA with Benzoyl-CoA Reductase and Cyclohexa-1,5-diene-1-carbonyl-CoA Hydratase

Received for publication, March 6, 2000, and in revised form, April 13, 2000
Published, JBC Papers in Press, April 13, 2000, DOI 10.1074/jbc.M001833200

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The enzymes benzoyl-CoA reductase and cyclohex-1,5-diene-1-carbonyl-CoA hydratase catalyzing the first steps of benzoyl-CoA conversion under anoxic conditions were purified from the denitrifying bacterium, Thauera aromatica. Reaction products obtained with [ring-13C6]benzoyl-CoA and [ring-14C]benzoyl-CoA as substrates were analyzed by high pressure liquid chromatography and by NMR spectroscopy. The main product obtained with titanium(III) citrate or with reduced [8Fe-8S]-ferredoxin from T. aromatica as electron donors was identified as cyclohexa-1,5-diene-1-carbonyl-CoA. The cyclic diene was converted into 6-hydroxycyclohex-1-ene-1-carbonyl-CoA by the hydratase. Assay mixtures containing reductase, hydratase, and sodium dithionite or a mixture of sulfite and titanium(III) citrate as reducing agent afforded cyclohex-2-ene-1-carbonyl-CoA and 6-hydroxycyclohex-2-ene-1-carbonyl-CoA. The potential required for the first electron transfer to the model compound 3-ethyl-thiobenzoate yielding a radical anion was determined by cyclic voltammetry as −1.9 V versus a standard hydrogen electrode. The energetics of enzymatic ring reduction of benzoyl-CoA are discussed.

Traditionally, the biodegradation of aromatic compounds has been considered as a domain of aerobic metabolism. More recently, an increasing number of bacteria has been shown to metabolize aromatic compounds under anoxic conditions. Benzoyl-CoA (Fig. 1, compound 1) has been identified as a central intermediate in anoxic degradation of many aromatic substrates (for review see Refs. 1-4).

Benzoyl-CoA reductase has been purified from the denitrifying bacterium Thauera aromatica (5). The 160-kDa protein consists of four different subunits specified by the bcrCBAD genes. The enzyme contains a minimum of two Fe-S clusters (5, 6) and is only active under strictly anoxic conditions (5). The reduction of benzoyl-CoA is coupled to the hydrosysis of two molecules of ATP/molecule of benzoyl-CoA. The natural electron donor for the enzyme-catalyzed reduction of benzoyl-CoA is a [8Fe-8S]-ferredoxin with a midpoint potential of −450 mV (versus a standard hydrogen electrode) (7). Alternatively, titanium(III) citrate, dithionite, or reduced methyl viologen can serve as artificial reductants. Some CoA-thioesters of fluoro-, amino-, and hydroxybenzoate can be reduced by benzoyl-CoA reductase albeit at low rates. Moreover, low molecular mass compounds such as hydroxylamine and azide are also reduced. All these reactions strictly depend on the hydrolysis of ATP (5).

In analogy to the Birch reduction of aromatic compounds by metallic sodium in liquid ammonia, the enzyme-catalyzed reduction of benzoyl-CoA has been proposed to proceed by two one-electron transfer reactions, each of which is followed by a protonation step (8, 9). The crucial step is the first electron transfer to the aromatic ring yielding a radical anion. EPR studies indicated that the hydrolysis of ATP is responsible for conformational changes in the vicinity of [4Fe-4S] clusters, most probably resulting in a lowered reduction potential of the clusters (6).

[ring-13C6]Cyclohexa-1,5-diene-1-carbonyl-CoA was identified in a product mixture obtained after incubation of a crude extract preparation from T. aromatica containing [ring-13C6]benzoyl-CoA, coenzyme A, ATP, Mg2+, and titanium(III) citrate (8). Cyclohexa-1,5-diene-1-carbonyl-CoA (Fig. 1, compound 2) was proposed tentatively to be the committed product of benzoyl-CoA reductase. Furthermore, an enzyme (Dch protein; subsequently designated as 1,5-dieneyl-CoA hydratase) catalyzing the formation of 6-hydroxycyclohex-1-ene-1-carbonyl-CoA (compound 3) by addition of water to the cyclic 1,5-diene (compound 2) has been purified and characterized (10).

In contrast to the results obtained with T. aromatica, cyclohexa-2,5-diene-1-carboxylate (Fig. 2, compound 4) and cyclohexa-1,4-diene-1-carboxylate (compound 5) were reported as products of benzoic acid reduction in cell suspensions of the phototrophic bacterium Rhodopseudomonas palustris. The structure assignments were based on gas chromatography and mass spectrometry after hydrolytic treatment of the reaction mixture (11).

The present study was initiated to establish the product of benzoyl-CoA reductase from T. aromatica. For this purpose, benzoyl-CoA labeled with 13C or 14C was incubated with purified benzoyl-CoA reductase using various electron donors. Enzyme products were analyzed by HPLC and two-dimensional homonuclear and heteronuclear NMR spectroscopy. Furthermore, the effect of a thioester residue on the reduction
potential of the aromatic moiety was determined by cyclic voltammetry using S-ethyl-thiobenzoate as a model compound.

EXPERIMENTAL PROCEDURES

Materials—Reagents were obtained from Aldrich, Fluka, Merck, Sigma, Roth, Roche Molecular Biochemicals, and Gerbu. Chromatography materials were obtained from Amersham Pharmacia Biotech, Bio-Rad, and Merck, respectively. [ring-13C6]Benzoic acid was purchased from MS Isolates, and [ring-13C6]benzoate was obtained from American Radiolabeled Chemicals Inc. and Biotrend Chemikalien GmbH.

Bacterial Strains—T. aromatica strain K172 (DSM 6984) was isolated in our laboratory (12, 13) and has been deposited in Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany). Clostridium pasteurianum (ATCC 6013) was obtained from the American Type Culture Collection.

Synthesis of CoA Thioesters—[ring-13C6]Benzyol-CoA was obtained by esterification of benzoic acid with N-hydroxysuccinimide (14). [ring-14C]Benzyol-CoA and cyclohex-1-ene-1-carboxyl-CoA were prepared enzymatically from [ring-13C6]benzoate (specific radioactivity, 2.025 Bq mmol−1) and cyclohex-1-ene-1-carboxylate, respectively, using ccozyme A and enriched benzoate-CoA ligase from T. aromatica as described earlier (15). [ring-13C6]benzyol-CoA and [ring-14C]benzyol-CoA were mixed (600 kBq mmol−1) and co-purified by semi-preparative HPLC as described (10). The yield was 50–80%.

Synthesis of S-Ethyl-thiobenzoate—Treatment of benzoic acid phenyl ester with sodium ethyl mercaptide afforded S-ethyl-thiobenzoate (16). An aliquot (80 μl) of the reaction mixture was dissolved in 920 μl of toluene and purified by preparative thin layer chromatography using silica gel 60 plates (Merck, 2 mm, 20 × 20 cm) with toluene as solvent. A strip of the plate was sprayed with 0.01% fluorescein (m/v) in ethanol, and the bands were visualized under ultraviolet light (254 nm). The product band was extracted with toluene and dried under reduced pressure (1H NMR using CDCl3 as solvent: δ 1.37 (3H, t, CH3), 3.10 (2H, q, CH2), 7.33–8.00 (5H, m, C6H5).

Growth of Bacterial Cells and Preparation of Cell Extract—T. aromatica was grown axenically at 28 °C in a mineral salt medium containing a mixture of 4-hydroxybenzoate and nitrate (ratio 1:3.5) as sole source of carbon and energy (12). Hydroxynitrobenzoate and nitrate were continuously fed at a molar ratio of 1:3.5. Cells were harvested by centrifugation under anaerobic conditions and were stored in liquid nitrogen. Cell extract was prepared as described (17).

Purification of Benzoyl-CoA Reductase—Benzoyl-CoA reductase was partially purified from T. aromatica (wet cell mass, 200 g) under strictly anaerobic conditions in a glove box containing an atmosphere of N2/H2 (95:5, v/v) as described earlier (5). Benzoyl-CoA reductase was further purified by chromatography on a column of Bio Gel P-100 (2 cm × 100 cm) that was equilibrated with 20 mM Tris-HCl, pH 7.5. The column was developed with three bed volumes of equilibration buffer and was then developed with 20 mM Tris-HCl, pH 7.5, containing 500 mM KCl (flow rate, 2 ml min−1). Fractions were assayed for benzoyl-CoA reductase (5) and cyclohexa-1,5-diene-1-carbonyl-CoA hydratase activity (10).

Purification of Ferredoxin—Ferredoxin was purified from T. aromatica (wet cell mass, 200 g) as described earlier (17). The yield was 60 mg of pure protein.

Assay for Benzoyl-CoA Reductase—Benzoyl-CoA reductase was assayed spectrophotometrically using reduced methyl viologen as electron donor (5) or by HPLC as described earlier (6). The reaction mixture for HPLC analysis contained 150 mM MOPS/KOH, pH 7.3, 10 mM MgCl2, 7.5 mM ATP, 10 mM phosphoenolpyruvate, 0.2 mM [ring-13C6]benzyol-CoA, and 10 mM benzoyl-CoA. A strip of the plate was sprayed with 0.01% fluorescein (m/v) in ethanol, and the bands were visualized under ultraviolet light (254 nm). The product band was extracted with toluene and dried under reduced pressure (1H NMR using CDCl3 as solvent: δ 3.10 (2H, q, CH2), 7.33–8.00 (5H, m, C6H5).
formed as described earlier (8). The duration of the $^1$H spin-lock was 60 ms in the HMQC-TOCSY experiment.

**Cyclic Voltammetry**—The midpoint potential of S-ethyl-thiobenzoate was determined at 25 °C by cyclic voltammetry. All measurements were carried out in super-dry acetonitrile with tetrabutyl ammonium hexafluorophosphate as supporting electrolyte. A three-electrode cell compartment was used throughout. For the electrochemical measurements, a potentiostat model 173 and a programmer model 175 (EG&G, Burlington, Vermont) were used. All potentials were calibrated against a ferrocene/ferroenium couple.

**Miscellaneous Methods**—Polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis were performed as described by Schägger and von Jagow (18). Proteins were visualized by Coomassie Blue staining (19). Protein was determined by the methods of Bradford (20) or Lowry et al. (21) using bovine serum albumin as standard.

**RESULTS**

Benzoyl-CoA reductase was purified from _T. aromatica_ as described earlier (5). The enzyme was enriched 40-fold by four chromatographic steps with a yield of 25% to a specific activity of 0.48 μmol·min$^{-1}$·mg$^{-1}$ (measured with reduced methyl viologen as electron donor (5)) and >95% purity as estimated by SDS gel electrophoresis. This protein fraction contained benzoyl-CoA reductase (7 μmol·min$^{-1}$) and cyclohexa-1,5-diene-1-carbonyl-CoA hydratase (86 μmol·min$^{-1}$) activity. Further purification of benzoyl-CoA reductase was achieved by affinity chromatography on Cibachron Blue (Amersham Pharmacia Biotech). The effluent of the column contained benzoyl-CoA reductase activity (4.5 μmol·min$^{-1}$) and 7% of the initial hydratase activity (6.0 μmol·min$^{-1}$). Using buffer containing 0.5 M KCl, 70% of the initial dienoyl-CoA hydratase activity could be eluted from the column.

A mixture of benzoyl-CoA reductase (7 μmol·min$^{-1}$) and 1,5-dienoyl-CoA hydratase (86 μmol·min$^{-1}$) was incubated with [ring-$^{13}$C]benzoyl-CoA in the presence of reduced ferredoxin or titanium(III) citrate. Reversed phase HPLC of the reaction mixture showed four peaks (1, 2, 3, and F) reflecting at least three radioactive products (Fig. 3A). The compound reflecting peak 3 was apparently more polar than benzoyl-CoA, and product F had the same retention volume as free aromatic carboxylic acids and was probably formed by hydrolytic cleavage of the thioester.

Subsequent experiments were performed with [ring-$^{13}$C]benzoyl-CoA as substrate in an attempt to optimize the sensitivity and selectivity of NMR detection. The reaction mixture was desalted and was analyzed by NMR spectroscopy. Carbon atoms derived from the $^{13}$C-enriched phenyl ring of benzoyl-CoA substrate gave 12 intense $^{13}$C NMR signals (Fig. 4) that were attributed to two products (compounds 2 and 3) by two-dimensional NMR experiments. More specifically, two well separated spin systems were gleaned from a HMQC-TOCSY experiment, establishing the connectivities between $^{13}$C and $^1$H atoms as well as between $^1$H atoms of the $^{13}$C-labeled ring moieties (Fig. 5 and Table I).

Each of the 12 $^{13}$C signals described above was split into a doublet or a pseudo-triplet by $^{13}$C-$^1$H coupling constants of 30–70 Hz. Apparently, each of the observed $^{13}$C atoms was directly bonded to two adjacent $^{13}$C atoms. This result suggests that both products retained the connectivity of the ring carbon atoms of the benzoyl substrate. Some of the signals showed additional fine splittings (coupling constants of 1–8 Hz, Table I) as a result of $^{13}$C-$^1$H couplings via two or three bonds.

Four of the six intense $^{13}$C NMR signals of compound 2 had chemical shifts values (121.9, 131.6, 137.4, and 140.6 ppm; cf. Fig. 4 and Table I) consistent with $sp^2$-hybridization of the corresponding carbon atoms. Two signals were detected at 27.7 and 22.8 ppm reflecting two $sp^2$-hybridized carbon atoms. This $^{13}$C NMR signature in conjunction with the detected $^{13}$C-$^{13}$C coupling pattern was consistent with a cyclic diene structure. Two-dimensional INADEQUATE, HMQC, and HMQC-TOCSY experiments (Table I and Fig. 5) afforded the entire network of carbon-carbon, carbon-proton, and proton-proton connectivities (28 detected homo- and heterocorrelations between $^1$H and $^{13}$C of the carbocyclic). Specifically, HMQC spectroscopy revealed correlations of the $^{13}$C NMR signals at 121.9, 131.6, and 140.6 ppm to the $^1$H NMR signals at 6.22, 6.02, and 6.97 ppm, respectively. The $^{13}$C signal at 137.4 ppm did not correlate to any $^1$H NMR signal in the HMOC experiment. This finding suggested a cyclohexadiene-1-carbonyl derivative. Carbon-carbon coupling constants (68.8 and 53.0 Hz) detected for the NMR signal at 137.4 ppm (C-1) as well as INADEQUATE spectroscopy showed that C-1 was adjacent to two sp-$^2$-hybridized carbon atoms with $^{13}$C NMR signals at 140.6 ppm (C-2) and 121.9 ppm (C-6). The signal at 121.9 ppm (C-6) gave an additional carbon-carbon correlation to the signal at 131.6 ppm (C-5), indicating a cyclohexa-1,5-diene-1-carbonyl system. The presence of a CoA residue was revealed by the $^1$H NMR data (not shown), thus establishing compound 2 as cyclohexa-1,5-diene-1-carbonyl-CoA.

The second product in the mixture (compound 3) was identified as 6-hydroxycyclohex-1-ene-1-carbonyl-CoA by the same
Products of Enzymatic Aromatic Ring Reduction

As reported earlier, dithionite can also act as an artificial electron donor for benzoyl-CoA reductase in T. aromatica (10). Cyclohexa-2,5-diene-1-carbonyl-CoA and cyclohexa-1,4-diene-1-carbonyl-CoA, which had been reported as products of benzoyl-CoA reduction catalyzed by benzoyl-CoA reductase in R. palustris (11), were not found in our reaction mixtures.

After prolonged incubation of [ring-\(^{13}\)C]benzoyl-CoA with benzoyl-CoA reductase, two minor products, [ring-\(^{13}\)C]cyclohexa-1-ene-1-carbonyl-CoA (Fig. 6, compound 6) and [ring-\(^{13}\)C]2-hydroxycyclohexane-1-carbonyl-CoA (7), were identified by NMR (data not shown). The NMR data of these compounds have been described elsewhere (10). We suggest tentatively that these compounds resulted from reduction of 2 by benzoyl-CoA reductase and subsequent hydration catalyzed by cyclohexa-1,5-diene-1-carbonyl-CoA hydratase (Fig. 6). The formation of these compounds was not observed when benzoyl-CoA was still present in the assay mixture as shown in Fig. 3 (A and B).

As reported earlier, dithionite can also act as an artificial electron donor for benzoyl-CoA reductase with rates comparable with other artificial electron donors (5). Surprisingly, HPLC analysis of the products formed from incubation of [ring-\(^{14}\)C]benzoyl-CoA with dithionite and a protein mixture containing benzoyl-CoA reductase and 1,5-dienoyl-CoA hydratase revealed a product pattern (Fig. 3B) that was different from that obtained with titanium(III) citrate as electron donor. On the other hand, two novel peaks reflecting compounds 8 and 9 were detected (Fig. 3B). Moreover, when dithionite (5 mM) was added to a benzoyl-CoA reductase assay that had been started with titanium(III) citrate as electron donor, the HPLC pattern shown in Fig. 3A changed to that shown in Fig. 3C.

Compound 8 and compound 9 formed by enzymatic conversion of [ring-\(^{13}\)C]benzoyl-CoA were analyzed by NMR spectroscopy (Table I). The detected \(^{13}\)C NMR chemical shifts (120–140 ppm) and coupling signatures (double doublets or triplets) were consistent with cyclic hexamonoene motifs for both compounds. Notably, each of the \(^{13}\)C atoms correlated to directly bonded \(^1\)H atoms in the HM-NMR experiment, thus indicating that the C-1 positions of both compounds carried a hydrogen atom and were not involved in a double bond. INADEQUATE and HMQC-TOCSY experiments afforded the entire network of carbon-carbon and carbon-proton bonds establishing compound 9 as [ring-\(^{13}\)C]cyclohexa-2,5-diene-1-carbonyl-CoA and compound 8 as [ring-\(^{13}\)C]6-hydroxycyclohexa-2,5-diene-1-carbonyl-CoA (Fig. 7).

No cyclic diene was detected with dithionite as electron donor.

To clarify the effects of electron donors on the product pattern, we analyzed the effect of sodium dithionite on a benzoyl-CoA reductase free preparation of 1,5-dienoyl-CoA hydratase by HPLC. Under equilibrium conditions, similar amounts of cyclohexa-1,5-diene-1-carbonyl-CoA (peak 2) and 6-hydroxycyclohexa-1-ene-1-carbonyl-CoA (peak 3) were present in assays of the hydratase (Fig. 8A). After addition of sodium dithionite, both compounds were converted into 6-hydroxycyclohexa-1,5-diene-1-carbonyl-CoA (Fig. 8, B and C, peaks 8). Fig. 8B shows that an additional intermediate apolar product (reflecting peak 10) is formed after 1 min of incubation. After 10 min of incubation this peak disappeared in the chromatogram (Fig. 8C). It is conceivable that peak 10 represents cyclohexa-2,5-diene-1-carbonyl-CoA (Fig. 7, compound 10), which can be converted rapidly into compound 8 by the addition of water. When sodium dithionite was replaced by 1 mM sodium sulfate, the formation of compound 8 could not be detected, whereas a mixture containing 1 mM sodium sulfate and 1 mM titanium(III) citrate had the same effect as sodium dithionite (not shown). In the absence of 1,5-dienoyl-CoA hydratase, no spontaneous reaction of dithionite with 2 and 3 could be observed.

For the energetics of benzoyl-CoA reduction, the transfer of the first electron to the aromatic ring of benzoyl-CoA yielding a radical anion is considered as the crucial step in the reaction catalyzed by benzoyl-CoA reductase (9). A highly negative redox potential is expected for this reaction. The natural substrate benzoyl-CoA is not suitable for the direct determination of this potential because irreversible follow-up reactions occur in protic solvents. Therefore, the substrate analog S-ethyl-
The use of multiply \(^{13}\)C labeled substrate facilitated approximately viable; minor waves appeared in the reverse scan at a potential of versus an Ag/AgCl reference electrode. The reaction was reversed and analyzed by cyclic voltammetry.

Thiobenzoate, which is soluble in aprotic solvents, was synthesized and analyzed by NMR spectroscopy and enabled NMR signal assignments by two-dimensional INADEQUATE, HMQC, and HMQC-TOCSY spectroscopy and enabled NMR analysis with product mixtures.

Specifically, the NMR data established cyclohexa-1,5-diene-1-carboxylate (compound 2) as the product of benzoyl-CoA reduction with titanium(III) or with reduced [8Fe-8S]-ferredoxin from \(T.\) aromatica as electron donors. This follow-up product probably results from protonation of the radical anion yielding a free radical. Residual traces of water in the solvent could be the proton donor for such species.

**DISCUSSION**

The structures of six CoA thioesters obtained after incubation of \([\text{ring-}^{13}\text{C}]\)benzoyl-CoA with purified benzoyl-CoA reductase (BcrCBAD protein) and 1,5-dienoyl CoA hydratase (Dch protein) from \(T.\) aromatica were established by NMR analysis. The use of multiply \(^{13}\)C labeled substrate facilitated signal assignments by two-dimensional INADEQUATE, HMQC, and HMQC-TOCSY spectroscopy and enabled NMR analysis with product mixtures.

Specifically, the NMR data established cyclohexa-1,5-diene-1-carboxylate (compound 2) as the product of benzoyl-CoA reduction with titanium(III) or with reduced [8Fe-8S]-ferredoxin from \(T.\) aromatica as electron donors. This is in agreement with the results of Koch et al. (8), who identified this compound as an early product in experiments with crude cell extracts of \(T.\) aromatica.

The formation of this cyclic hexadiene implicates a 1,2-addition mechanism. Interestingly, Gibson and Gibson (11) detected cyclohexa-1,4-diene-1-carboxylate (compound 4) and cyclohexa-1,4-diene-1-carboxylate (compound 5) as products of benzoate reduction with titanium(III) or with reduced [8Fe-8S]-ferredoxin from \(T.\) aromatica and \(R.\) palustris show high similarity (78% sequence identity) (22, 23). Interestingly, Gibson and Gibson (11) detected cyclohexa-2,5-diene-1-carboxylate (compound 4) and cyclohexa-1,4-diene-1-carboxylate (compound 5) as products of benzoate re-

### TABLE I

| Position | Chemical shift \(^{13}\)C | Coupling constant \(\pm J_{\text{CC}}\) | Correlation experiments |
|----------|-------------------------|-------------------------------|------------------------|

| Position | Chemical shift \(^{13}\)C | Coupling constant \(\pm J_{\text{CC}}\) | Correlation experiments |
|----------|-------------------------|-------------------------------|------------------------|

\(\text{ppm Hz} \)

| Benzoyl-CoA (1) | 138.3(t) | 57.8(2/6), 9.1 | 2/6 |
| 2/6 | 129.2(t) | nd | 2/6, 3/5, 4 | 1, 3/5 |
| 3/5 | 131.2(t) | nd | 3/5, 2/6, 4 | 2/6, 4 |
| 4 | 136.5(t) | 53.5(3/5), 9.5 | 4, 3/5, 2/6 | 3/5 |

### 6-Hydroxy-cyclohex-1-ene-1-carboxyl-CoA (3)

| 1 | 140.7(dd) | 68.9(2), 47.1(6), 4.2 | 2.6 |
| 2 | 147.4(dd) | 68.9(1), 38.2(3), 3.2 | 1.3 |
| 3 | 28.1(t) | 38.2(4), 2.21 | 2.4 |
| 4 | 18.0(t) | 33.8(5), 3 | 3.5 |
| 5 | 32.2(dd) | 19.1(5), 1.67 | 4.6 |
| 6 | 64.0(dd) | 46.8(1), 37.0(5) | 5.1 |

### Cyclohex-1,5-diene-1-carboxyl-CoA (2)

| 1 | 137.4(d) | 68.8(2), 53.0(6), 6.6 | 2.6 |
| 2 | 140.8(d) | 68.4(1), 38.7(3), 7.6, 3.1 | 1.3 |
| 3 | 24.7(dd) | 38.7(2), 34.8(4), 6.0, 2.8 | 2.5 |
| 4 | 22.8(t) | 37.6(3), 5 | 3.5 |
| 5 | 131.6(t) | 68.8(6), 7.0 | 4.6 |
| 6 | 121.9(dd) | 67.8(5), 53.5(1), 6.2, 1.4 | 5.1 |

### Cyclohex-2-ene-1-carboxyl-CoA (9)

| 1 | 51.8(dd) | 40.2, 32.6 | 2.6 |
| 2 | 125.1(dd) | 69.4, 40.6 | 1.3 |
| 3 | 134.3(dd) | 67.3, 37.0 | 2.4 |
| 4 | 26.3(t) | 35.0 | 3.5 |
| 5 | 22.0(t) | 33.2 | 4.6 |
| 6 | 128.7(dd) | 29.4 | 5.1 |

### 6-Hydroxy-cyclohex-2-ene-1-carboxyl-CoA (8)

| 1 | 54.0(t) | 35.9 | 2.6 |
| 2 | 124.2(dd) | 69.5, 38.0 | 1.3 |
| 3 | 132.9(dd) | 70.0, 36.0 | 2.4 |
| 4 | 25.3(t) | 32.9 | 3.5 |
| 5 | 24.4(t) | 33.2 | 4.6 |
| 6 | 59.2(t) | 34.9 | 5.1 |

\(\text{a} \) Referenced to external 3-trimethylsilylpropane 1-sulfonate.

\(\text{b} \) Signal multiplicities in parentheses (t = pseudotriplet; dd = double doublet).

\(\text{c} \) Determined from one-dimensional \(^{13}\)C NMR spectra; coupled atoms to the respective index atoms are in parentheses.

\(\text{d} \) w, weak signal intensity.
duction in cell suspension experiments with R. palustris. These compounds were identified by gas chromatography/mass spectrometry techniques after derivatization of the free acids to the corresponding methyl esters. The discrepancy to our results might have different reasons. Either benzoyl-CoA reduction proceeds via different mechanisms in R. palustris as discussed elsewhere (3, 22), or the diene isomers detected by GC/MS were artificially formed during derivatization of the products.

In the so-called Birch reduction of benzoic acid with elemental sodium or lithium as reductants, 2,5-cyclohexadiene-1-carboxylate (compound 4) is formed under kinetic control (24). The electron withdrawing character of the acid group of benzoate stabilizes the anion radical at carbon 1 of the ring; the second electron adds in para position because of electrostatic repulsion. However, in the presence of a strong base, the thermodynamically more stable conjugated 1,5-diene can be obtained (25, 26). This base-catalyzed rearrangement seems to occur in the catalytic cycle of T. aromatica.

The formation of the hex-1-ene compound (compound 6) at a slow rate (5) explained by a two-electron reduction of the 1,5-diene (compound 2) catalyzed by benzoyl-CoA reductase. Although the hex-1-ene (compound 6) and the 2-hydroxyhexane compound (compound 7) may play an important role in the aromatic metabolism of R. palustris (3, 22), their formation in T. aromatica appears as an in vitro effect without metabolic relevance.

The natural electron donor, reduced ferredoxin, and the artificial reductant titanium(III) citrate yielded the same products. Surprisingly, cyclohex-2-ene-1-carbonyl-CoA (compound 9) and 6-hydroxycyclohex-2-ene-1-carbonyl-CoA (compound 8) were identified as products of benzoyl-CoA reductase and cyclohexa-1,5-diene-1-carbonyl-CoA hydratase when dithionite was used as an artificial electron donor. Our results strongly suggest that the artificial formation of the 6-hydroxy-2-ene (compound 8) was a product of dienoyl-CoA hydratase activity via a set of artificial isomerization and hydration reactions. In this case, cyclohexa-2,5-diene-1-carbonyl-CoA (compound 10) is suggested as an intermediate isomerization product (Fig. 7).

Under anaerobic conditions in the presence of benzoyl-CoA reductase, MgATP, and an excess of dithionite, this hypothetical diene is further reduced to cyclohexa-2,5-diene-1-carbonyl-CoA (compound 9). The following observations support our suggestions: (i) When sodium dithionite was added to a benzoyl-CoA reductase/1,5-dienoyl-CoA hydratase assay that had been started with Ti(III) citrate as electron donor and benzoyl-CoA as substrate, the product pattern shifted immediately from the cyclohexa-1,5-diene/6-hydroxymonene mixture (reflecting peaks 2 and 3 in the HPLC chromatogram; cf. Fig. 8A) to a cyclohexa-2-ene/6-hydroxy-2-ene mixture (reflecting peaks 9 and 8 in the HPLC chromatogram; cf. Fig. 8C). (ii) Under aerobic conditions, when the hydratase was fully active and benzoyl-CoA reductase was irreversibly inactivated, addition of dithionite induced the formation of 6-hydroxycyclohex-2-ene-1-

![Fig. 7. Product formation by 1,5-dienoyl-CoA hydratase (Dch protein) and benzoyl-CoA reductase (BerCBAD protein) from T. aromatica in the presence of dithionite.](image)

![Fig. 8. HPLC chromatograms of products formed from 6-hydroxycyclohex-1-ene-1-carbonyl-CoA by 1,5-dienoyl-CoA hydratase in the absence and presence of sodium dithionite. A, after 10 min aerobic incubation of 6-hydroxycyclohex-1-ene-1-carbonyl CoA (compound 3) with dienoyl-CoA hydratase. Peak 2 representing cyclohexa-1,5-diene-1-carbonyl-CoA (compound 2) cochromatographed with a reference sample. B, after addition of 1 mM sodium dithionite followed by 1 min of incubation at 37 °C under aerobic conditions. C, the same assay as in B, but after 10 min of aerobic incubation at 37 °C.](image)

![Fig. 9. Hypothetical mechanism of benzoyl-CoA reduction by benzoyl-CoA reductase in T. aromatica.](image)
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Acknowledgments—We thank A. Werner and F. Wendling for expert help with the preparation of the manuscript.

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