Aromatizing Cyclohexa-1,5-diene-1-carbonyl-Coenzyme A Oxidase

CHARACTERIZATION AND ITS ROLE IN ANAEROBIC AROMATIC METABOLISM*

Bärbel Thiele†, Oliver Rieder‡, Nico Jehmlich*, Martin von Bergen*, Michael Müller†, and Matthias Boll†
From the †Institute of Biochemistry, University of Leipzig, Leipzig D-04103, Germany, ‡Institute of Pharmaceutical and Medical Chemistry, University of Freiburg, Freiburg D79104, Germany, and the Department of Proteomics, §Helmholtz Centre for Environmental Research-UFZ, Leipzig D-04318, Germany

Benzoyl-CoA reductases (BCRs) are key enzymes of anaerobic aromatic metabolism in facultatively anaerobic bacteria. The highly oxygen-sensitive enzymes catalyze the ATP-dependent reductive dearomatization of the substrate, yielding cyclohexa-1,5-diene-1-carbonyl-CoA (1,5-dienoyl-CoA). In extracts from anaerobically grown denitrifying *Thauera aromatica*, we detected a benzoate-induced, benzoyl-CoA-forming, 1,5-dienoyl-CoA:acceptor oxidoreductase activity. This activity co-purified with BCR but could be partially separated from it by hydroxyapatite chromatography. After activity staining on native gels, a monomeric protein with a subunit molecular weight of $M_\text{s}$, 76,000 was identified. Mass spectrometric analysis of tryptic digests identified peptides from NADH oxidase/2,4-dienoyl-CoA reductases/"old yellow" enzymes. The UV-visible spectrum of the enriched enzyme suggested the presence of flavin and Fe/S-cofactors, and it was bleached upon the addition of dioxygen as electron acceptor ($K_m$ = 10 $\mu$M) and therefore is referred to as 1,5-dienoyl-CoA oxidase (DCO). The likely product formed from dioxygen reduction was H$_2$O. DCO was highly specific for 1,5-dienoyl-CoA ($K_m$ = 27 $\mu$M). The initial rate of DCO followed a Nernst curve with half-maximal activity at +10 mV. We propose that DCO provides protection for the extremely oxygen-sensitive BCR enzyme when the bacterium degrades aromatic compounds at the edge of steep oxygen gradients. The redox-dependent switch in DCO guarantees that DCO is only active during oxidative stress and circumvents futile deearomatization/rearomatization reactions catalyzed by BCR and DCO.

The central intermediate generated by most anaerobic bacteria capable of using aromatic compounds as growth substrates is benzoyl-CoA, which then serves as the substrate for deearomatizing benzoyl-CoA reductases (BCRs)2 (1–5). With the exception of the enzyme from *Rhodopseudomonas palustris*, all BCRs appear to catalyze the two-electron reduction of benzoyl-CoA, yielding cyclohexa-1,5-diene-1-carbonyl-CoA (1,5-dienoyl-CoA; Fig. 1). The catalytic mechanism of BCR is considered to proceed in analogy to the Birch reduction in chemical synthesis and involves electron transfer steps at extremely low redox potentials (6). Two completely different classes of deearomatizing benzoyl-CoA reductases have been described. In BCR from facultative anaerobes, the electron transfer from reduced ferredoxin to benzoyl-CoA is coupled to a stoichiometric ATP-hydrolysis (1 ATP/electron transferred) (7, 8). The biochemistry of ATP-dependent BCR enzymes has so far only been studied with the enzyme from the denitrifying bacterium *Thauera aromatica*. It has an αβγε subunit composition with two functionally different modules: (i) the electron activation module is composed of the αδ β subunits (49 and 29 kDa, respectively) and harbors two ATP-binding sites and the so-called electron entry [4Fe-4S]1+/2+ cluster; (ii) the benzoyl-CoA reduction module is formed by the βγ subunits (48 and 44 kDa, respectively), which coordinate two further [4Fe-4S]1+/2+ clusters and bind a single benzoyl-CoA molecule (9). ATP binding and hydrolysis induce molecular switches that are proposed to facilitate the thermodynamically difficult electron transfers to the aromatic ring (10). Homologues of ATP-dependent benzoyl-CoA reductases appear to be present in all facultative anaerobes that use aromatic growth substrates (1, 3, 11). The reaction of BCR resembles that of nitorgenases in that both are extremely oxygen-sensitive enzymes involved in ATP-dependent electron transfer reactions at very negative redox potentials (12).

Much less is known about BCR enzymes from obligately anaerobic bacteria that degrade aromatic compounds. No genes coding for ATP-dependent BCRs are present in the genomes of strictly anaerobic *Geobacter metallireducens* (Fe[III]-respiring) (13) and *Syntrophus aciditrophicus* (fermenting) (14). Recent results strongly suggest that obligate anaerobes use a completely different, probably ATP-independent, class of BCR complex consisting of iron-sulfur-, selenium-, and molybdenum-containing protein components (13, 15).

In this work, a benzoate-induced benzoyl-CoA-forming 1,5-dienoyl-CoA:acceptor oxidoreductase activity was identified in extracts of several facultatively and obligately anaerobic bacteria grown on benzoate, which has not been described previously. The corresponding enzyme was identified in *T. aromatica* as a highly specific 1,5-dienoyl-CoA oxidase (DCO) and
characterized with regard to its role in anaerobic aromatic metabolism. We propose that DCO functions as an oxygen-scavenging enzyme, which is specifically involved in protection of the extremely oxygen-sensitive BCR.

**EXPERIMENTAL PROCEDURES**

**Experiments under Anoxic Conditions**—Experiments under anoxic conditions were performed in a glove box (Coy Laboratories, Ann Arbor, MI) under an atmosphere containing 5% H\(_2\) and 95% N\(_2\) (by volume).

**Growth of Bacterial Cells and Preparation of Cell Extracts**—All bacteria used in this study, listed in the following, were anaerobically cultured in a mineral salt medium with benzoate or acetate as sole carbon source, as described previously (15–19): *T. aromatica* (DSM 6984), *Azoarcus evansii* KB 740 (DSM 6869), *G. metallireducens* (DSM 7210), *Desulfococcus multivorans* (DSM 2059), *S. aciditrophicus* (culture collection of M. McInerney, University of Oklahoma), and *R. palustris* (ATTC 17001).

For the preparation of crude extracts, frozen cells were suspended in 20 mM triethanolamine hydrochloride-KOH buffer, pH 7.3 (1 g of cells in 2 ml of buffer), 10 mM MgCl\(_2\), 10% (w/v) glycerol, and 0.1 mg of DNase I. Cell lysates were obtained by passage through a French pressure cell at 137 megapascals. After centrifugation at 100,000 g (1 h at 4 °C), the supernatant was used for further studies.

**Protein Purification and Sample Preparation**—Purification of DCO included three chromatographic steps using DEAE-Sepharose (Fast Flow; GE Healthcare), ceramic hydroxyapatite (Bio-Rad), and Mono Q-Sepharose (GE Healthcare). Buffer A is defined as 20 mM triethanolamine-KOH (pH 7.8), 4 mM MgCl\(_2\), and 10% (w/v) glycerol. Approximately 15 ml of crude extract was applied to a DEAE-Sepharose column (20-ml column volume; 3-ml min\(^{-1}\) flow rate), which had been equilibrated with buffer A. The column was washed with two column volumes of buffer A, followed by four column volumes of buffer A, including 50 mM KCl. DCO was eluted using a linear gradient from 50 to 150 mM KCl in buffer A over 8 column volumes. The enzyme eluted at \(\approx 120\) mM KCl. In the next step, the pooled DEAE fractions with DCO enzyme activity were applied to a ceramic hydroxyapatite column (10-ml column volume; 3-ml min\(^{-1}\) flow rate), which had been equilibrated with buffer A. The column was developed with a linear gradient from 0 to 30 mM potassium phosphate over five column volumes achieved by mixing buffer A and 50 mM potassium phosphate (pH 7.8), including 10% (w/v) glycerol. Minor amounts of DCO activity were found in the 1 M KCl step (pool DCOH1) and at low phosphate concentrations (2–5 mM potassium phosphate; pool DCOH2). The majority of the DCO coeluted with BCR between 12 and 15 mM potassium phosphate (pool DCO\(_{12}\)). Notably, the DCO\(_{12}\) pool was identical with BCR preparations after hydroxyapatite chromatography. The hydroxyapatite pools were concentrated in microconcentrators (30 kDa exclusion limit; Vivaspin 6; Sartorius) by centrifugation. The buffer of the samples was exchanged to buffer A by passage over a Sephadex G-25 column (PD10; GE Healthcare). Approximately 25–30 mg aliquots of protein from the hydroxyapatite pools were separately applied to a Mono Q-Sepharose column (1-ml column volume; 1-ml min\(^{-1}\) flow rate), which had been equilibrated with buffer A. The column was washed with one column volume of buffer A and three column volumes of buffer A, including 100 mM KCl. Then a linear gradient from 100 to 160 mM KCl in buffer A over 14 bed volumes was applied. DCO activity of hydroxyapatite pools (DCO\(_{11}\) and DCO\(_{12}\)) free from BCR eluted with 120 mM KCl (DCO\(_{21}\) and DCO\(_{33}\)), respectively. DCO\(_{11}\) and BCR eluted in two overlapping peaks at 120 and 140 mM KCl (DCO\(_{33}\)). Small molecular weight compounds were removed by passage over a Sephadex G-25 column (PD10; GE Healthcare), which was run in 20 mM triethanolamine hydrochloride-KOH buffer, pH 7.3.

**Synthesis of Dihydrobenzoic Acid Isomers**—2,5-Dihydro-1-carboxybenzene was synthesized via a cyclization reaction of propionic acid and 1,3-butanediene in a pressure bottle at 130 °C and 18 bars (20). 1,4-Dihydro-1-carboxybenzene was synthesized via Birch reduction of benzoic acid using sodium dissolved in liquid ammonia at –78 °C (21).

2,3-Dihydro-1-carboxybenzene was synthesized in a two-step process. In the first step a cyclization reaction of acrylic acid and acetoxy-1,3-butadiene was conducted in refluxing toluene to give 2-acetoxycyclohex-3-ene-carboxylic acid (22). The intermediate was stirred in 10% sulfuric acid at 40 °C to yield the product.

**Synthesis of CoA-Thiol Esters**—Benzoyl-CoA was synthesized from benzoic acid anhydride and CoA (23). 1,5-Dienoyl-CoA and 6-hydroxycyclohex-1-ene-1-carboxyl-CoA were enzymatically synthesized from benzoyl-CoA using enriched benzoyl-CoA reductase from *T. aromatica* (24); the product was purified by preparative HPLC as described earlier (24).

Because even highly purified BCR preparations (purity >95%) contained traces of a highly active cyclohexa-1,5-dienoyl-CoA hydrolase, the synthesis of 1,5-dienoyl-CoA always resulted in a 1:1 ratio of 1,5-dienoyl-CoA and its hydrated product, 6-hydroxycyclohex-1-ene-1-carboxyl-CoA (6-OH-cyclohexenoyl-CoA). For this reason, both 1,5-dienoyl-CoA and 6-OH-cyclohexenoyl-CoA were used as substrates in the DCO assay, in which both were converted to their hydrated/dehydrated product, respectively. If 1,5-dienoyl-CoA hydrolase activity in highly enriched DCO preparations was negligible, heterologously expressed hydratase from *G. metallireducens* (24) was added to the assay mixture with an activity that was at least 20-fold higher than that of DCO.
Cyclohex-1-ene-1-carbonyl-CoA (1-monoenoyl-CoA) and 3-monoenoyl-CoA were synthesized from the free acids and CoA via succinimimid ester synthesis as described (25). 2-Monoenoyl-CoA was enzymatically synthesized by a BCR/1,5-dienoyl-CoA hydratase-containing enzyme fraction using dithionite as electron donor as described earlier (26). 1,3-, 1,4-, and 2,5-dienoyl-CoA were synthesized from the free acids and CoA via modified succinimimid ester synthesis. Due to the spontaneous oxidative aromatization in the presence of oxygen, synthesis of 2,5-dienoyl-CoA was performed under anaerobic conditions in the glove box. Succinimidyl ester synthesis was stopped after 2 h by filtration. The succinimidyl esters were not lyophilized but were directly used in CoA-ester synthesis. Ether extraction after CoA-ester synthesis was abandoned. Analytical and preparative HPLC of 2,5-dienoyl-CoA was performed with anaerobically prepared solvents.

**Assay for Benzoyl-CoA Reductase**—The MgATP- and benzoyl-CoA-dependent oxidation of methyl viologen catalyzed by BCR was spectrophotometrically monitored at 730 nm ($\varepsilon_{730} = 2.4 \text{ mm}^{-1} \text{ cm}^{-1}$) as described (7). For activity determination in crude extracts, a discontinuous HPLC assay was used as described earlier (26).

**Assay for 1,5-Dienoyl-CoA:Acceptor Oxidoreductase**—The activity was determined by either a continuous or a discontinuous assay at 30 °C. The continuous spectrophotometric assay is based on the absorption of oxidized 2,6-dichlorophenol indophenol (DCPIP); $\varepsilon_{660} = 14.3 \text{ mm}^{-1} \text{ cm}^{-1}$, determined by UV-visible spectroscopy. The typical assay mixture (400 µl) contained 100 mM MOPS-KOH, pH 7.3, 0.15 mM DCPIP, 0.2 mM 1,5-dienoyl-CoA, and 10–20 µg of DCOHA3. This assay was used routinely to measure 1,5-dienoyl-CoA oxidation activity of enriched DCO.

To test alternative electron acceptors, DCPIP was replaced by 0.2 mM oxidized phenazine methosulfate ($\varepsilon_{935} = 18.5 \text{ mm}^{-1} \text{ cm}^{-1}$), methylene blue ($\varepsilon_{615} = 6.31 \text{ mm}^{-1} \text{ cm}^{-1}$), and diethylsafranin ($\varepsilon_{480} = 11.0 \text{ mm}^{-1} \text{ cm}^{-1}$) or by 0.5 mM oxidized benzyl viologen ($\varepsilon_{630} = 7.6 \text{ mm}^{-1} \text{ cm}^{-1}$) and methyl viologen ($\varepsilon_{605} = 11.9 \text{ mm}^{-1} \text{ cm}^{-1}$). All extinction coefficients were determined via UV-visible spectroscopy.

In the discontinuous assay (125 µl in the assay buffer described above) 25–µl aliquots were taken at different time points. After the addition of 5 µl of 10% (w/v) formic acid, the samples were centrifuged and the supernatant was analyzed by C18 reversed phase HPLC as described earlier (26). The HPLC assay was used for product analysis and for testing activities in crude extracts. For conversion of 2,5-dienoyl-CoA, complete sample preparation and HPLC analysis were performed under anaerobic conditions using anaerobically prepared solvents. To test oxygen as electron acceptor, the assay was performed under aerobic conditions under continuous stirring.

**NADH:Acceptor Oxidoreductase Activities**—For testing NADH:DCPIP oxidoreductase activity, the spectrophotometric enzyme assay using DCPIP as electron acceptor described above was used; 1,5-dienoyl-CoA was replaced by 0.2 to 1 mM of NADH. NADH:O2 oxidoreductase activity was tested in a spectrophotometric assay based on the absorption of NADH ($\varepsilon_{340} = 6.2 \text{ mm}^{-1} \text{ cm}^{-1}$) under aerobic conditions. The typical assay mixture contained 100 mM MOPS-KOH, pH 7.3, air-saturated, 0.2 mM NADH, and 50–100 µg of DCOHA3.

**Studies of Kinetics and Stoichiometry**—The $K_m$ value of DCO for 1,5-dienoyl-CoA was determined using the spectrophotometric assay with DCPIP as electron acceptor. The following concentrations of 1,5-dienoyl-CoA were used: 0, 8, 15, 35, 70, 150, 350, and 680 µM. The initial rates were fitted to Michaelis-Menten curves. The $K_m$ value for benzoyl-CoA was determined in the same assay at concentrations of 25, 50, 100, and 200 µM for 1,5-dienoyl-CoA and 0, 25, 50, 100, 175, 200, 230, 330, and 730 µM for benzoyl-CoA. Data fitting was generally performed with the Prism software package (GraphPad, San Diego, CA).

To determine the stoichiometry between DCPIP reduction and 1,5-dienoyl-CoA oxidation, samples were removed at different time points from the cuvette during a spectrophotometric assay and analyzed by HPLC. The ratio of DCPIP reduced/benzoyl-CoA formed was determined.

The $K_m$ value for oxygen was determined using the Fibox-3-trace oxygen sensor (PreSens GmbH, Regensburg, Germany) and the corresponding optodes as described (27). A half-microcuvette, containing an optode, was filled to the top with 100 mM MOPS-KOH, pH 7.3, under anaerobic conditions and sealed with a rubber stopper to prevent formation of a headspace. Then 0.5 mM 1,5-dienoyl-CoA was added using a gas-tight Hamilton syringe. The following oxygen concentrations were adjusted by adding different amounts of air: 0.8, 3.6, 6.3, 14.6, 16.4, 73, 110, and 152 µM. The reaction was started by the addition of DCO. The time-dependent reduction of oxygen was measured several times, and the initial activities were determined via linear regression. Only fits with regression coefficients of >0.95 were used. The oxygen concentration-dependent initial rate was fitted to Michaelis-Menten curves. The stoichiometry of 1,5-dienoyl-CoA oxidized/dioxygen reduced was determined by analyzing samples for production of benzoyl-CoA using HPLC; the samples were taken at time points with defined amounts of oxygen consumed.

**Redox Titration of DCO Activity**—For determination of the dependence of the initial rate of DCO on the redox potential, DCPIP ($E^\circ = 220 \text{ mV}$) was replaced by either of the following electron acceptors: 0.5 mM benzyl viologen, 0.5 mM methyl viologen, 0.1–0.3 mM phenazine methosulfate, 0.1–0.5 mM diethylsafranin, and 0.1–0.5 mM methylene blue (for midpoint potentials, see Table 5). The redox potential was varied by adding different amounts of reduced electron acceptor to a constant amount of 0.1 mM oxidized electron acceptor, thereby altering the ratio acceptor$_{ox}$/acceptor$_{red}$. Sodium dithionite was used for reduction. The amounts of oxidized and reduced acceptor were estimated from absorption, and the redox potential was calculated by means of the Nernst equation. The initial activities were plotted against the redox potentials and fitted (without activities from benzyl and methyl viologen) with a nonlinear regression method to the Nernst equation.

**Activity Staining of Native Gels**—Native gel electrophoresis and activity staining were performed under anaerobic conditions in the anaerobic chamber using a 4% (w/v) acrylamide stacking gel and an 8% (w/v) separating gel. Electrophoresis was performed at 4 °C with 15 mA. After electrophoresis, the gel was incubated with 50 ml of 0.06 mM DCPIP and 1.2 mM...
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3-(4′,5′-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide in water for 10 min at room temperature. A paper filter soaked with 0.5 mM 1,5-dienoyl-CoA in 100 mM MOPS-KOH, pH 7.3, was incubated on the gel for 10 min at room temperature. Electrons were transferred from 1,5-dienoyl-CoA via DCPIP to 3-(4′,5′-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide, which formed a stable dark-colored complex upon reduction.

**Determination of Native Mass**—The native molecular weight of DCO was determined by analytical gel filtration via Superdex 200 (10/300 GL column; GE Healthcare; 25-ml column volume; 0.5-ml min⁻¹ flow rate) using 20 mM triethanolamine-HCl buffer, pH 7.8, 4 mM MgCl₂, 100 mM KCl. The column was calibrated with catalase (Mr 245,000), BCR (Mr 171,000), lactate dehydrogenase (Mr 140,000), bovine serum albumin (Mr 67,000), carboanhydrase (Mr 29,000), and lysozyme (Mr 14,000).

**Mass Spectrometry Analysis**—Protein bands of interest were cut from denaturing gels and digested overnight using trypsin (Sigma) as described (28). The cleaved peptides were eluted, concentrated by vacuum centrifugation, and thereafter separated by reverse phase nano-liquid chromatography (LC1100 series; Agilent Technologies, Paolo Alto, CA; column: Zorbax 300SB-C18, 3.5 μm, 150 × 0.075 mm; eluate: 0.1% formic acid, 0–60% acetonitrile). The peptides were identified by on-line tandem mass spectrometry (LC/MSD TRAP XCT mass spectrometer; Agilent Technologies). Thereafter, a data base search was conducted using the tandem mass spectrometry ion search by a Mascot in-house version (MASCOT 2.2.1.; Matrix Science, London, UK) against all bacterial entries of NCBInr (National Center for Biotechnology Information, Bethesda, MD) with the following accuracies: a peptide tolerance of ±1.2 Da and 0.8 Da for the tandem mass spectrometry fragments was applied.

**UV-visible Spectroscopy**—The spectra were recorded using 140 μl of oxidized DCOQ2 (15 μM) in a gas-tight, sealed quartz microcuvette. Reduction was carried out by the addition of 25 μl of 0.2 mM 1,5-dienoyl-CoA. The further addition of 1,5-dienoyl-CoA did not change the observed spectrum.

**RESULTS**

**1,5-Dienoyl-CoA:Acceptor Oxidoreductase Activities in Anaerobic Bacteria**—BCR from *T. aromatica* accepts electrons from donors with midpoint potentials below −400 mV, such as reduced ferredoxin, reduced methyl viologen, sodium dithionite, or Ti(III)-citrate. Since all of these reactions are strictly coupled to ATP hydrolysis, they are considered to be thermodynamically irreversible (26, 29).

In HPLC assays, which monitored the formation and consumption of CoA esters, we observed that soluble crude extracts of *T. aromatica* cells grown anaerobically on benzoate catalyzed the protein-, time-, and electron acceptor-dependent formation of benzoyl-CoA from 1,5-dienoyl-CoA. For technical reasons, the reaction was routinely started with 6-OH-monoenoyl-CoA (0.2 mM) (Fig. 2). The time-dependent formation of benzoyl-CoA using DCPIP as electron acceptor from HPLC analysis of samples taken at 0 min (A), 0.5 min (B), 3 min (C), and 6 min (D) is shown. Detection of all compounds was carried out by UV detection at 260 nm.

**TABLE 1**

| Organism     | Growth substrate | Activity (nmol min⁻¹ mg⁻¹) |
|--------------|------------------|----------------------------|
| *T. aromatica* | Benzoate         | 250                        |
| *A. evansi*   | Acetate          | ≤0.1                       |
| *R. palustris*| Benzoate         | 4                          |
| *G. metallireducens* | Benzoate | 8                          |
| *D. multivorans* | Acetate         | 190                        |
| *S. aciditrophicus* | Benzoate    | 8                          |

As shown below, evidence will be provided that molecular oxygen serves as the most likely *in vivo* electron acceptor for 1,5-dienoyl-CoA oxidation. For this reason, the 1,5-dienoyl-CoA-aromatizing activity will be henceforth referred to as DCO activity, although the enzyme was routinely tested with DCPIP as artificial electron acceptor in an anaerobic assay. The presence of MgADP or MgATP (5 mM each) had no effect on the activity, and exposure of cell extracts to air for 1 h did not
Since aromatization of 1,5-dienoyl-CoA was not coupled to ATP synthesis, it could not be interpreted as a true reversal of the oxygen-sensitive BCR reaction. Using DCPIP as electron acceptor, DCO activities were also determined in extracts from other facultative and obligate anaerobes grown on an aromatic growth substrate, albeit at largely differing rates (Table 1). Notably, in extracts from the obligately anaerobic G. metallireducens, this activity was in the range of the activity observed in T. aromatica extracts and was clearly induced during growth on benzoate.

Separation of DCO and BCR Activities—The BCR preparations usually used for enzymatic assays (purity 80%, 0.3 μmol min⁻¹ mg⁻¹) also contained a DCO activity (1.5–3 μmol min⁻¹ mg⁻¹). To test whether the aromatizing activity was an artificial side reaction of BCR or whether it was catalyzed by an as yet uncharacterized benzoate-induced enzyme, attempts to establish a protein purification protocol for separating DCO from BCR activities were carried out. The aim of this initial isolation of the 1,5-dienoyl-CoA-rearomatizing enzyme was to identify unambiguously the 1,5-dienoyl-CoA-aromatizing enzyme and to study its putative role in anaerobic aromatic metabolism.

Both BCR and DCO activities coeluted during anaerobic DEAE-Sepharose ion exchange chromatography at 120 mM KCl (pH 7.8). After subsequent chromatography on hydroxyapatite, all of the BCR activity eluted between 12 and 15 mM (yield 60–70%), as established (7) and again contained the major part of DCO activity referred to as DCO₃ (Table 2). A minor, but significant, fraction of DCO eluted in the 1M KCl wash fraction (DCOH₁) and at low phosphate concentrations (2–5 mM; DCOH₂). Notably, the latter two fractions did not exhibit BCR activity, which suggested at least a partial separation of DCO and BCR activities. All three DCO fractions containing activity were individually further purified by a third chromatographic step using Mono Q-Sepharose. The DCOH₃ fraction applied to this column coeluted with all of the BCR between 120 and 160 mM KCl (referred to as DCOQ₃). In contrast, the DCOH₁ and DCOH₂ fractions devoid of BCR activity and both eluting at 120 mM KCl were enriched with higher yields and specific activities compared with DCOH₃ (referred to as DCOQ₁ and DCOQ₂; Table 2 and Fig. 3A). In summary, with the protocol established, it was possible to separate DCO and BCR activity, with DCOQ₂ exhibiting the highest DCO-specific activity (Table 2).

Isolation and Molecular Properties of DCO—Since SDS-PAGE analysis of DCOQ₂ still contained a number of protein bands at different intensities (Fig. 3A), a further purification step was performed with the DCOQ₁–₃ fractions. After native gel electrophoresis, an activity stain using 1,5-dienoyl-CoA as a substrate revealed two major bands with molecular masses of 35 kDa and 25 kDa, corresponding to the DCOQ₁ and DCOQ₂ fractions, respectively.

### Table 2

| Purification step     | Protein (mg) | Activity (μmol min⁻¹) | Specific activity (μmol min⁻¹ mg⁻¹) | Yield (%) | Purification |
|-----------------------|--------------|-----------------------|-------------------------------------|-----------|--------------|
| Crude extract         | 525          | 63                    | 120                                 | 70        | 1            |
| DEAE-Sepharose         | 113          | 90                    | 793                                 | 100       | 6.6          |
| Hydroxyapatite (total)| 54           | 68                    | 1264                                | 74        | 10.5         |
| Hydroxyapatite: DCO₁  | 11           | 11                    | 1020                                | 12        | 8.5          |
| Hydroxyapatite: DCO₂  | 6            | 7                     | 1204                                | 8         | 10.0         |
| Hydroxyapatite: DCO₃  | 32           | 50                    | 1569                                | 56        | 13.1         |
| Mono Q: DCOQ₁         | 3            | 7                     | 2250                                | 8         | 18.8         |
| Mono Q: DCOQ₂         | 2            | 6                     | 2420                                | 7         | 20.2         |
| Mono Q: DCOQ₃         | 30           | 31                    | 1050                                | 35        | 8.8          |

FIGURE 3. Purification of DCO from T. aromatica. A, SDS-PAGE of fractions containing DCO-activity. MW, molecular weight standard; Mono Q 1–3, pools obtained after chromatography on DEAE-Sepharose and hydroxyapatite were individually further purified on Mono Q (see "Experimental Procedures"). Fraction 3 contained all subunits of BCR, whereas fractions 1 and 2 were BCR-free. The highest DCO specific activity was in fraction 2. B, native PAGE with DCO activity staining. 1–3, Mono Q1–3 pools from SDS-PAGE in A. C, SDS-PAGE after native gel electrophoresis of Mono Q pools. The DCO activity bands obtained in B were cut out of the gel and applied to SDS-PAGE. 1–3, electrophoresis of Mono Q pools 1–3 after native PAGE.
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By a careful analysis of the binding affinity of DCO toward the electron acceptor dioxygen using optical oxygen sensors (optodes), a $K_w$ value of $10 \pm 2 \mu M$ was obtained. The stoichiometry between benzoyl-CoA formed from 1,5-dienoyl-CoA and DCPIP/O$_2$ reduced, catalyzed by DCO was obtained. The stoichiometry is presented in Table 6. The stoichiometry was obtained by measuring the initial rates at various 1,5-dienoyl-CoA concentrations. A fit of the data obtained with a Michaelis-Menten curve gave $K_m = 27 \pm 3 \mu M$ and $V_{max} = 6.0 \pm 0.3 \mu mol \text{mg}^{-1} \text{min}^{-1}$ ($r^2 = 0.98$). Especially at low 1,5-dienoyl-CoA concentrations, the reaction was not linear. To investigate this observation further, the enzyme was preincubated with varying benzoyl-CoA concentrations, and the reaction was started with different amounts of 1,5-dienoyl-CoA. A fit of the data obtained to non-linear regression curves revealed a competitive inhibition of DCO by the product benzoyl-CoA with a $K_i = 50 \mu M$ (not shown).

To determine the stoichiometry between 1,5-dienoyl-CoA oxidized and DCPIP reduced, samples were taken at different time points during a spectrophotometric assay in which the amount of reduced DCPIP was continuously determined. The samples were quantitatively analyzed for 1,5-dienoyl-CoA by HPLC. The data obtained gave rise to the expected stoichiometry of one DCPIP reduced per 1,5-dienoyl-CoA oxidized (Table 3). Notably, oxidized DCPIP is a two-electron acceptor.

UV-visible Spectroscopy of DCO and Possible Redox Cofactors—The high similarities of peptide fragments obtained from the 76-kDa polypeptide with flavo/FeS proteins suggested the presence of similar redox cofactors in DCO. Indeed, DCO preparations of highest purity (DCO$_{Q2}$) had a yellow color, indicating the presence of redox cofactors as expected. A UV-visible spectroscopic analysis was carried out (Fig. 4).

The enzyme as isolated was considered to be fully oxidized. It exhibited a spectrum with distinct features between 350 and 600 nm and absorption maxima at 415 and 440 nm. Both are characteristic for the presence of Fe/S clusters (usual absorption maxima between 390 and 450 nm) and/or flavins (usual absorption maxima at 370 and 450 nm). The extinction coefficients are presented in Table 6. After the anaerobic addition of 20 $\mu M$ 1,5-dienoyl-CoA, the spectrum clearly bleached in the region between 400 and 520 nm, suggesting electron transfer from 1,5-dienoyl-CoA to the redox cofactors of DCO. The corresponding difference spectrum is shown in Fig. 4B. It exhibits a major peak at 466 nm with $\Delta e_{475\text{ox-red}} = 5200 \text{ M}^{-1} \text{cm}^{-1}$.

**TABLE 3**

| Electron acceptor | Benzoyl-CoA formed | Benzoyl-CoA formed/electron acceptor reduced |
|-------------------|--------------------|---------------------------------------------|
| DCPIP$_{ox}$ | 0 | 0 |
| DCPIP$_{ox}$ | 6 | 6 |
| DCPIP$_{ox}$ | 14 | 13 |
| DCPIP$_{ox}$ | 22 | 22 |
| DCPIP$_{ox}$ | 31 | 33 |
| O$_2$ | 17 | 48 |
| O$_2$ | 38 | 72 |
| O$_2$ | 58 | 83 |

*Substrate and DCPIP/3-(4’,5’-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide as electron acceptor/staining reagent was carried out. As shown in Fig. 3B, a major band at ~75,000 developed upon the addition of 1,5-dienoyl-CoA. This band was excised from the gel and subjected to denaturing SDS-PAGE. As shown in Fig. 3C (lane 2), a protein band around $M_r$ 76,000 ± 1,000 was highly enriched by this procedure; in addition, an $M_r$ 110,000 protein band was present at less than 10% intensity. The presence of a major protein band after activity staining on native gels at $r$ 10% intensity. The presence of a major protein band after activity staining on native gels at $M_r$ 76,000 and the presence of a major band of the same size after SDS gel electrophoresis suggested that DCO is composed of a single $M_r$ 76,000 subunit. To unambiguously verify this assumption, the native mass was also determined by gel filtration using the DCO$_{Q2}$ fraction. The DCO activity eluted at approximately $M_r = 95,000 \pm 5000$, which is in accordance with a monomeric composition of DCO.

Mass Spectrometric Analysis and Identification of Similar Enzymes—The $M_r$ 76,000 protein was digested by trypsin, and the peptides obtained were subsequently analyzed by mass spectrometry. Using the MASCOT platform, the best matches were obtained with peptides from a deduced gene product in the genome of *Aromatoleum aromaticum*, which was annotated as NADH oxidase/NoxB-2. The protein identification was based on six peptides (sequence coverage, 10%; protein summary score, 280), from which five featured individual ion scores of >40, indicating high identity or extensive homology (probability >95%). It has to be taken into account that the genome of *T. aromatica* is not yet sequenced, which resulted in the observed low recovery. Further hits were obtained with other flavin-dependent oxidoreductases, including NADH oxidases, 2,4-dienoyl-CoA reductase, and old yellow flavoprotein-like proteins from different organisms.

In addition, the protein band at 110 kDa, which occasionally occurred at varying intensities, was identified as an aconitase-like protein based on a cross-species homology (sequence homology to *Azoarcus* sp. BH72; peptides matched, 14; sequence coverage, 17%; protein summary score, 744).

**FIGURE 4. UV-visible spectra of DCO.** Spectra were recorded in anaerobic buffer in sealed cuvettes. A, full range spectrum. Solid line, DCOQ2 (15 $\mu M$) as isolated in the oxidized state; dotted line, after the addition of 20 $\mu M$ 1,5-dienoyl-CoA. B, difference spectrum of as isolated enzyme minus as isolated enzyme in the presence of 1,5-dienoyl-CoA.

**TABLE 3** Stoichiometry between benzoyl-CoA formed from 1,5-dienoyl-CoA and DCPIP/O$_2$ reduced, catalyzed by DCO

| Electron acceptor | Benzoyl-CoA formed | Benzoyl-CoA formed/electron acceptor reduced |
|-------------------|--------------------|---------------------------------------------|
| DCPIP$_{ox}$ | 0 | 0 |
| DCPIP$_{ox}$ | 6 | 6 |
| DCPIP$_{ox}$ | 14 | 13 |
| DCPIP$_{ox}$ | 22 | 22 |
| DCPIP$_{ox}$ | 31 | 33 |
| O$_2$ | 17 | 48 |
| O$_2$ | 38 | 72 |
| O$_2$ | 58 | 83 |
Redox titration of the initial rate was carried out. For this purpose, different redox potentials were poised by altering the ratio of acceptor$_{ox}$/acceptor$_{red}$. Symbols indicate the electron acceptors used: benzyl viologen (▲), diethylsafranin (●), methylene blue (■), phenazine methosulfate (■), and DCPIP (▲).

**FIGURE 5. Redox titration of DCO activity.** The initial activities plotted against the redox potential follow a Nernst equation with a midpoint potential of +10 mV. Activities were determined spectrophotometrically using 0.1 mM oxidized electron acceptor. The redox potential was varied by altering the ratio of acceptor$_{ox}$/acceptor$_{red}$. Symbols indicate the electron acceptors used: benzyl viologen (▲), diethylsafranin (●), methylene blue (■), phenazine methosulfate (■), and DCPIP (▲).

**DISCUSSION**

**Properties of DCO and Comparison with Other Enzymes**—The properties of DCO from *T. aromatica* are summarized in Table 6. Although the gene coding for DCO from *T. aromatica* has not been identified, mass spectrometric analysis of tryptic digests revealed significant similarities between DCO and 2,4-dienoyl-CoA reductases, NADH oxidases, and old yellow enzymes. Although the latter often exist as tetramers of a 35–40-kDa subunit with a single flavin cofactor (30), bacterial NADH oxidases and 2,4-dienoyl-CoA reductases usually consist of a single 75-kDa subunit and may contain one or more
flavin cofactors and a [4Fe-4S] cluster (31–33). The UV-visible spectrum of DCO indeed provided initial evidence that the enzyme is a flavo/FeS protein, and it was shown that 1,5-dienoyl-CoA reduced these cofactors.

DCO, old yellow enzymes, and 2,4-dienoyl-CoA reductases use \( \alpha,\beta \)-unsaturated carbonyl compounds as electron donors (30, 32–37). Whereas the latter two reduce their substrates most often with NADPH, only DCO catalyzes an irreversible oxidation of its substrate. In contrast to most other homologous enzymes, DCO has an unique substrate specificity for 1,5-dienoyl-CoA as electron donor for dioxygen reduction, whereas old yellow enzymes in particular have been shown to use various artifical \( \alpha,\beta \)-unsaturated carbonyl compounds. Remarkably, an old yellow enzyme was shown to aromatize cyclic enones (36). The NADH oxidase partial activity of DCO was very poor (<0.3% of 1,5-dienoyl-CoA-aromatizing activity), which precludes that it plays a role in vivo. However, the NADH:DCPIP oxidoreductase activity of DCO suggests that NADH indeed can bind to DCO. In summary, compared with other NADH oxidases/old yellow enzymes, DCO has a high substrate specificity and is induced by growth on benzoate, which suggests a specific role for it in anaerobic aromatic metabolism.

Role of DCO in Anaerobic Aromatic Metabolism—The exergonic aromatization reaction of DCO was not coupled to ATP synthesis. The re aromatization of a product formed by ATP-dependent BCR initially suggested a futile ATP hydrolysis cycle without any benefit for the cell. However, the low \( K_m \) values for the substrates and its strong induction during growth on aromatic compounds indicate that DCO activity has an important function in vivo. Below, we discuss possible specific roles of DCO in anaerobic aromatic metabolism.

Recycling of Dead End Monoenoyl-CoA or Dienoyl-CoA Isomers—An obvious scenario could be that the 1,5-dienoyl-CoA is not the in vivo substrate of DCO. DCO could be involved in recycling other dead-end dienoyl-CoA or monoenoyl-CoA isomers, which may be formed by BCR. For example, upon prolonged incubation, the 1,5-dienoyl-CoA formed by BCR is slowly further reduced by BCR to cyclohex-1-ene-1-carbonyl-CoA (7). Recycling could be performed either by oxidative aromatization or by isomerization of dead-end dienoyl-CoA iso mers to the 1,5-dienoyl-CoA intermediate. However, none of the tested monoenoyl-CoA or alternative dienoyl-CoA isomers was converted, either by oxidation or by isomerization.

**TABLE 6**

Properties of DCO from *T. aromatica*

| Property                   | Value                              |
|----------------------------|------------------------------------|
| Substrates \((K_m)\)       | 1,5-Dienoyl-CoA (27 \( \mu \)M), O\(_2\) (10 \( \mu \)M) |
| Products                   | 2ATP + 2\( \text{H}_2\)O             |
| Specific activity          | \(\approx 6\) \( \mu \)mol min\(^{-1}\) mg\(^{-1}\) |
| Subunit                    | 76,000 ± 1,000 (SDS-PAGE)          |
| Native mass                | 95,000 ± 5,000 (gel filtration)    |
| Catalytic number           | 7.7 s\(^{-1}\)                     |
| Competitive Inhibitor      | Benzoyl-CoA \((K_i = 50 \mu M)\)  |
| Potential of half-maximal activity | 10 mV                          |
| Cofactors                  | Flavins/FeS                        |
| Absorption coefficients at maxima | \( \varepsilon_{281} = 123,000 \text{ M}^{-1} \text{ cm}^{-1} \) |
| Effect of oxygen           | No significant inhibition          |

**Figure 6**. Redox- and oxygen-dependent activities of DCO and BCR. A, at negligible oxygen concentrations, the cellular redox potential is far below 0 mV; reduced ferredoxin is available as electron donor for the BCR reaction. The DCO activity is switched off, since no oxygen detoxification is required. A futile ATP hydrolysis cycle is avoided. B, at oxygen concentrations higher than 10 \( \mu \)M, the redox potential becomes positive; reduced ferredoxin becomes limiting for the BCR reaction. DCO is switched on, since protection of BCR against oxidative damage is required.

**β-Oxidation of Fatty Acids**—The amino acid sequence of DCO peptides suggested similarities to bacterial 2,4-dienoyl-CoA reductases, and both enzymes use similar CoA ester substrates. Thus, a similar in vivo function might be anticipated. Bacterial 2,4-dienoyl-CoA reductases play a role in β-oxidation of unsaturated fatty acids with cis-double-bonds at even-numbered carbon atoms (7, 32, 33, 37). However, *T. aromatica* neither grows on such compounds nor contains such fatty acids (16). Moreover, DCO did not reduce 1,5-dienoyl-CoA with NADH, NADPH, dithionite, or Ti(III)-citrate as electron donors to a monoenoyl-CoA compound. Thus, DCO only acts as a highly specific, irreversible oxidase.

**Adaptation to Oxidative Stress**—*T. aromatica* is a facultative anaerobe that can be isolated from aerobic and microaerophilic but also from fully anaerobic soils when nitrate is abundant as an electron acceptor. Facultatively anaerobic bacteria like *T. aromatica* thrive in narrow oxygen gradients and must therefore be able to adapt rapidly to changing oxygen concentrations. A particular problem for bacteria such as *T. aromatica* that degrade aromatic compounds is that the extremely oxygen-sensitive BCR has a lifetime in air of only a few seconds (7). Consequently, even a very short exposure to oxygen would immediately result in an irreversible inactivation of the enzyme. For this reason, the benzoate-induced DCO represents an attractive enzymatic tool to remove molecular oxygen rapidly. The low \( K_m \) values for dioxygen and 1,5-dienoyl-CoA support this assumption. Furthermore, DCO becomes activated by a redox-dependent switch at \( E^0 = +10 \text{ mV} \). This switch guarantees that no futile 1,5-dienoyl-CoA-aromatization occurs when the redox potential is low and BCR is active (Fig. 6). On the other hand, it enables a rapid activation of DCO during oxidative stress. The co-purification of BCR and DCO through several chromatographic steps suggests an association of both enzymes, which would facilitate the use of the product formed by BCR for oxygen detoxification.

The advantage for the cell of using 1,5-dienoyl-CoA as electron donor for dioxygen reduction can be explained by the fact that the product, benzoyl-CoA, is the common intermediate of
both the anaerobic and aerobic benzoate degradation pathways in *T. aromatica* (5). Thus, after prolonged oxygen exposure and concomitant induction of genes for aerobic aromatic degradation, the benzoyl-CoA formed by DCO can be directly used for the aerobic pathway.

There are a number of reports about the putative roles of NADH oxidases and old yellow enzymes in oxygen detoxification (38–41). In comparison with many of these assumed oxygen-scavenging enzymes, DCO appears to have a very high affinity for dioxygen. The requirement for keeping oxygen levels as low as possible when an oxygen-sensitive enzyme is synthesized has been well documented for the protection of nitrogense against oxygen damage under several conditions (42–45).

**DCO Activities in Anaerobic Bacteria**—Using DCPIP as electron acceptor, DCO activities were found in all tested anaerobic bacteria that degrade aromatics, and in the case of *T. aromatica* and *G. metallireducens* /H11002 for Environmental Sciences) for help with the oxygen consumption measurements using optodes. We thank Gary Sawers (Halle, Germany) for careful proofreading of the manuscript.

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