Coenzyme A-dependent Aerobic Metabolism of Benzoate via Epoxide Formation

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In the aerobic metabolism of aromatic substrates, oxygenases use molecular oxygen to hydroxylate and finally cleave the aromatic ring. In the case of the common intermediate benzoate, the ring cleavage substrates are either catechol (in bacteria) or 3,4-dihydroxybenzoate (protocatechuic, mainly in fungi). We have shown before that many bacteria, e.g., Azoarcus evansii, the organism studied here, use a completely different mechanism. This elaborate pathway requires formation of benzoate-CoA, followed by an oxygenase reaction and a nonoxygenolytic ring cleavage. Benzoyl-CoA transformation is catalyzed by the iron-containing benzoyl-CoA oxygenase (BoxB) in conjunction with an FAD and iron-sulfur centers containing reductase (BoxA), which donates electrons from NADPH. Here we show that benzoyl-CoA oxygenase actually does not form the 2,3-dihydrodiol even when NADPH is used as the electron donor (4). Second, ring cleavage is hydrolytic rather than oxygenolytic (7). Third, none of the classical enzymes is involved in any step, except \( \beta \)-ketothiolase, which cleaves \( \beta \)-ketoadipyl-CoA into succinyl-CoA and acetyl-CoA (8). Fifteen genes coding for the benzoyl-CoA oxidation (box) pathway are clustered on the A. evansii chromosome (8). These box genes code for the following functions: a putative ATP-dependent benzoate transport system, a benzoate-CoA ligase, a benzoyl-CoA oxygenase and reductase, a ring-opening enzyme, enzymes for \( \beta \)-oxidation of CoA-activated intermediates, a thioesterase, and a lactone hydrolase, as well as completely unknown enzymes belonging to new protein families (8).

Benzoate is converted to benzoate-CoA ligase forming benzoyl-CoA (8–10). Benzoyl-CoA conversion requires NADPH, \( \mathrm{O}_2 \) and two protein components, BoxA and BoxB (3). BoxA is a homodimeric iron-sulfur flavoprotein (46-kDa subunit), which acts as a reductase (3). In the absence of BoxB, BoxA catalyzes the benzoyl-CoA-stimulated artificial electron transfer from NADPH to \( \mathrm{O}_2 \) to produce \( \mathrm{H}_2\mathrm{O}_2 \) (3). Physiologically, BoxA uses NADPH to reduce BoxB, a monomeric 55-kDa iron protein that acts as benzoyl-CoA oxidase (11). The product of benzoyl-CoA oxidation was tentatively identified by NMR spectroscopy as its dihydrodiol derivative, 2,3-dihydro-2,3-dihydroxycyclohexyl-CoA (11). This suggested that BoxAB (native enzyme BoxA in conjunction with recombinant enzyme BoxB\text{BoxBBoxA}) acts as a benzoyl-CoA dioxygenase/reductase. The benzoyl-CoA oxidase system has very low similarity to known oxygenase systems (11).

Unexpectedly, benzoyl-CoA transformation by BoxAB was greatly stimulated when an enoyl-CoA hydratase/isomerase-like protein, BoxC, was added (11). BoxC, a homodimeric enzyme (61-kDa subunits), catalyzes the hydrolytic conversion of the BoxAB product, which inactivates BoxAB, to 3,4-dehydrodiphenyl-CoA semialdehyde and formic acid (7). It contains domains characteristic for enoyl-CoA hydratases/isomerases, besides a large central domain with no significant similarity to sequences in the database (7).

BoxD, a homodimer composed of 54-kDa subunits, is a NADP\(^+\)-specific aldehyde dehydrogenase that oxidizes the...
product of BoxC, 3,4-dehydrodiphenyl-CoA semialdehyde, to the corresponding acid, 3,4-dehydrodiphenyl-CoA (12). The further metabolism probably requires a kind of β-oxidation leading to β-ketoadipyl-CoA, the last intermediate at which the conventional β-ketoadipate pathway and the unorthodox new pathway merge (4, 7).

To elucidate the exciting mechanism of benzoyl-CoA oxidation catalyzed by BoxAB, we applied 18O labeling studies and analyzed the products by mass spectrometry. It turned out that the product contained only one additional oxygen and could be derivatized by an epoxide trapping agent. In summary, the results indicate that BoxAB is a benzoyl-CoA epoxidase forming 2,3-epoxybenzoyl-CoA rather than a benzoyl-CoA dioxygenase/reductase forming the 2,3-dihydrodiol of benzoyl-CoA. BoxC acts on this epoxide or its oxepin tautomer by adding two molecules of water and thus eliminating ring C2 as formic acid. This new principle is widely distributed and has a counterpart in a novel pathway of phenylacetyl-CoA oxidation that also involves coenzyme A thioesters, epoxide, and oxepin intermediates.2

**EXPERIMENTAL PROCEDURES**

*Materials—*Oxygen-18O (normalized, >97 atom %) and water-18O (normalized with respect to hydrogen, >97 atom %) were obtained from Campen Scientific (Berlin, Germany). Glucose 6-phosphate dehydrogenase from baker's yeast (268 units mg−1 protein, 0.91 mg ml−1) was obtained from Fluka Analytical (Buchs, Switzerland). Vector pASK-IBA43plus, anhydrotetracycline, and desthiobiotin were obtained from IBA GmbH (Göttingen, Germany). Restriction enzymes KpnI and BamHI and T4 DNA ligase were obtained from Fermentas (St. Leon-Rot, Germany). Vector pASK-IBA43plus, anhydrotetraacycline (Buchs, Switzerland). Vector pASK-IBA43plus, anhydrotetracycline is made at 37 °C in lysogeny broth medium with 50 μg of ampicillin ml−1 in a 10-liter fermentor (300 rpm). At an optical density (light path, 1 cm) at 578 nm (A578 nm) of 0.5, 200 μg of anhydrotetracycline liter−1 was added for induction. The cells were harvested in the exponential growth phase at an A578 nm of 1.6. The culture was cooled down to 4 °C, and the cells were harvested by centrifugation and stored at −70 °C. The yield was 20 g of cells (wet mass).

*Preparation of Cell Extracts—*All of the steps were performed at 4 °C under anaerobic conditions. Frozen cells were suspended in an equal volume of 20% (v/v) glycerol containing 0.05 mg of DNase ml−1. The suspension was passed through a French pressure cell at 137 MPa and centrifuged (1 h, 100,000 × g).

*Enzyme Purification—*All of the steps were performed at 4 °C under anaerobic conditions. BoxA was purified and assayed according to Mohamed et al. (3). BoxBStrep was purified by affinity chromatography. Cell extract (20–150 ml, 70 mg of protein ml−1, 100,000 × g supernatant) was applied to a column of Strep-Tactin Superflow (25 ml; IBA GmbH, Göttingen, Germany), which was equilibrated with 200 ml of 10 mM Tris/Tris pH 8.0 (buffer A), at a flow rate of 3 ml min−1. The column was washed with 60 ml of buffer A, 180 ml of buffer A containing 250 mM KCl, and 60 ml of buffer A. Protein was eluted with 60 ml of buffer A containing 2.5 mM desthiobiotin. Eluted protein (30 ml, 20 mg) was concentrated to 1–5 ml (Amicon; 30 kDa). The enzymes were stored at −70 °C with 10% (v/v) glycerol. BoxHisCStrep was purified similar to BoxBStrep; the column was washed with 60 ml of buffer A and 180 ml of buffer A containing 100 mM KCl BoxDStrep was purified as described (12).

*Protein Analysis—*Protein concentration was determined by the Bradford method (15) and the BC assay kit as described in the instruction manual (Uptima, Interchem, Montlucon Cedex, France) using bovine serum albumin as standard. SDS-polyacrylamide (11.5%) gel electrophoresis used the Laemmli method (15) and Coomassie Blue staining (16).

*Stoichiometry of BoxAB Reaction—*NADPH oxidation at 30 °C was measured spectrophotometrically at 377 nm (ε[NADPH]377 nm extrapolated 1,620 m−1 cm−1). Either limiting concentrations of benzoyl-CoA or oxygen were applied. Air-saturated (30 °C) assay mixtures (500 μl) with 100 mM Tris/HCl buffer, pH 8.0, contained either 0.4, 0.1, or 0.05 mM benzoyl-CoA. They were mixed with 0.04 mg of BoxA ml−1, 1.02 mg of 2 R. Teufel, V. Mascaraque, W. Ismail, M. Voss, J. Perera, W. Eisenreich, W. Haehnel, and G. Fuchs, unpublished results.

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BoxB\textsubscript{Strep} ml\textsuperscript{-1}, and 0.96 mg of Box\textsubscript{HisC\textsubscript{Strep}} ml\textsuperscript{-1}. After the addition of 0.6 mM NADPH, the assay mixture was covered immediately with 200 \mu l of paraffin oil to avoid further oxygen uptake. NADPH oxidation observed after the BoxABC (native enzyme BoxA in conjunction with recombinant enzyme BoxB\textsubscript{Strep} and recombinant enzyme Box\textsubscript{HisC\textsubscript{Strep}}) reaction caused by BoxA was subtracted. The slow, endogenous, benzoyl-CoA-independent NADPH oxidation with O\textsubscript{2}, which is catalyzed by BoxA, was monitored as a constant slow absorption decrease at 377 nm, when benzoyl-CoA was consumed. This blank reaction was extrapolated back to the zero time point. This extrapolated A\textsubscript{377} value was subtracted from the initial A\textsubscript{377} value.

**Enzyme Assays for BoxAB, BoxABC, and BoxABCD**—Standard assay mixtures (0.04 – 1 ml) containing 0.6 mM NADPH and 0.2 mM benzoyl-CoA in 10 mM Tris/HCl buffer, pH 8.0, were mixed at 4 °C with 0.08 mg of BoxDMal ml\textsuperscript{-1}. Routinely, a NADPH regenerating system was included consisting of 3.3 mM MgCl\textsubscript{2}, 3.3 mM glucose 6-phosphate, and 2 units of glucose 6-phosphate dehydrogenase ml\textsuperscript{-1}. The reaction was started by the addition of 0.64 mg of BoxB\textsubscript{Strep} ml\textsuperscript{-1}. In some experiments, 0.16 mg of Box\textsubscript{HisC\textsubscript{Strep}} ml\textsuperscript{-1} was added, and in some cases additionally 0.08 mg of BoxDMal ml\textsuperscript{-1}. Labeling assays were performed in a closed tube (7.5 ml) with 50% \textsuperscript{18}O\textsubscript{2}, 50% \textsuperscript{16}O\textsubscript{2} (v/v) gas phase or with 50% H\textsubscript{2}\textsuperscript{18}O, 50% H\textsubscript{2}\textsuperscript{16}O (v/v). The assay mixtures were stirred for 24 °C for 30 min. The enzymatic reaction was stopped by adding a 5-fold volume of ethanol (–20 °C). After incubation for 20 min at –20 °C, the denatured protein was removed by centrifugation. The supernatant was evaporated under reduced pressure at 30 °C. The residue was resolved in 100 \mu l of H\textsubscript{2}O, and the products were purified by reverse phase HPLC.\textsuperscript{3}

**BoxAB Reaction in the Presence of N,N-Diethyldithiocarbamate (DTC)**—Standard assay mixtures (500 \mu l) included 10 mM DTC. Aliquots (40 \mu l) were taken at different points (t = 0, 2, 4, 6, 8, 10, 15, 20, 40, and 60 min), and the samples were analyzed.

**Purification of Products by Reverse Phase HPLC**—Aliquots were applied to a column of Lichrospher 100 RP 18E, 5.0 \mu m, 125 x 4 mm (Wicom, Heppenheim, Germany), equilibrated with 40 \% ammonium acetate (NH\textsubscript{4}Ac), pH 6.8, containing 5% (v/v) acetonitrile (ACN) at a flow rate of 1 ml min\textsuperscript{-1}. An ACN gradient in the same buffer was used: 2 min to 5%, 1 min to 10%, 11 min to 30%, 1 min from NH\textsubscript{4}Ac at 30% to water at 30%, 3 min to 50%, 3 min back to 5%, 3 min from water plus 5% to NH\textsubscript{4}Ac plus 5%, and 6-min equilibration 5% in NH\textsubscript{4}Ac. Elution was monitored with an UV diode array detector routinely at 260 nm. The amount of the CoA thioesters was estimated based on the assumption that they exhibited identical absorption coefficients at 260 nm as benzoyl-CoA (ε\textsubscript{260} nm = 21,100 M\textsuperscript{-1} cm\textsuperscript{-1}). The retention times were as follows: 1.0 min for polar products; 3.5 min for product of BoxA, BoxB\textsubscript{Strep}, Box\textsubscript{HisC\textsubscript{Strep}} and BoxD\textsubscript{Mal}; 6.4 min for product of BoxA, BoxB\textsubscript{Strep} and Box\textsubscript{HisC\textsubscript{Strep}}; 7.0 min for product of BoxA and BoxB\textsubscript{Strep}; 9.5 min for benzoyl-CoA; and 12.0 min for derivative product of BoxA and BoxB\textsubscript{Strep} with DTC. The fractions of 0.5 ml were collected and frozen at –20 °C.

**Analysis by Mass Spectrometry (MS)**—Samples of HPLC fractions were transferred by a syringe pump or via nano-reverse phase HPLC into the nano-electrospray ionization source of a Finnigan LTQ-FT mass spectrometer (Thermo Electron Corporation, Waltham, MA) for online mass detection assembled from a linear ion trap and an ion cyclotron (7 Tesla magnet) with Fourier transform ion cyclotron resonance mass spectrometry.

**Analysis by Direct Coupling of HPLC, MS, and MS/MS**—Benzoyl-CoA and the product of the enzymatic reaction were injected with a FAMOS autosampler (Dionex) and desalted by transfer with an Agilent HPLC 1100 to a reverse phase trap column (Zorbax Eclipse XDB-C18, 5 \mu m; 0.1 x 15 mm). The sample was eluted at 200 nl min\textsuperscript{-1} with an ACN gradient from a quaternary HPLC pump (Ultimate, Dionex) and separated on a fused silica emitter of 0.075 x 105 mm (Proxen) packed with Pro C18, 3 \mu m (YMC). Elution started with 100% A (H\textsubscript{2}O, 3% ACN, 0.1% formic acid) and 0% B (H\textsubscript{2}O, 80% ACN, 0.1% formic acid) for 11 min, followed by successive linear gradients in 4 min to 5% B, 20 min to 30% B, and 21 min to 70% B and terminated by 2 min at 70% B. The fused silica emitter connected to –2 kV was mounted in the nano-electrospray interface of the LTQ-FT. The mass spectrometer was operated in the data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey MS spectra (from m/z 250 to 1800) were acquired in the FT-ICR with a resolution of 25,000. The most intense ion was isolated for high resolution (50,000) measurement in the FT-ICR with a 10-Da mass range. These ions were then fragmented in the linear ion trap using collision-induced dissociation and recorded at low resolution (MS/MS scan). The latter ions were dynamically excluded for the following 30 s. The total cycle time was ~0.3 s.

**Analysis of Oxygen Exchange by Mass Spectrometry**—3,4-Dehydrodiapyl-CoA semialdehyde was enzymatically synthesized without \textsuperscript{18}O and purified by reverse phase HPLC. 100 \mu l of unlabeled semialdehyde was mixed with an equal amount of H\textsubscript{2}\textsuperscript{18}O (resulting in 50% H\textsubscript{2}\textsuperscript{18}O, 50% H\textsubscript{2}\textsuperscript{16}O) and analyzed by MS over a period of 14 min after different incubation times (t = 1 min, 15 min, 45 min, and 17 h). As control, 100 \mu l of unlabeled semialdehyde was mixed with 100 \mu l of H\textsubscript{2}\textsuperscript{16}O and analyzed.

**RESULTS**

**Conversion of Benzoyl-CoA with NADPH and Oxygen and Characterization of the Product**—Benzoyl-CoA was transformed by the recombinant enzyme BoxB\textsubscript{Strep} in conjunction with the native enzyme BoxA (here referred to as BoxAB) with O\textsubscript{2} and NADPH as electron donor. The stoichiometry of the oxygen-dependent reaction was 1 NADPH oxidized and 1 O\textsubscript{2} consumed per 1 benzoyl-CoA transformed (Table 1). This ratio could be interpreted as the result of a dioxygenase/reductase reaction leading to the nonaromatic cis-2,3-dihydrodiol of benzoyl-CoA. The product was purified by HPLC and analyzed by electrospray ionization mass spectrometry (Fig. 1). However,
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TABLE 1
Stoichiometry of benzoyl-CoA, NADPH, and O₂ of the reaction catalyzed by BoxAB

| BCoA | Limiting Factor | Consumption of NADPH | Conclusion |
|------|-----------------|----------------------|------------|
| [mM] |                  | [mM]                |            |
| 0.4  | O₂              | 0.235               | \( n_{\text{NADPH}} = n_{\text{Oxygen}} \) |
| 0.1  | BCoA            | 0.102               | \( n_{\text{NADPH}} = n_{\text{BCoA}} \) |
| 0.05 | BCoA            | 0.052               | \( n_{\text{BCoA}} = n_{\text{Oxygen}} \) |

FIGURE 1. Analysis of the product formed from benzoyl-CoA by BoxAB with NADPH and O₂. A, reverse phase HPLC with an acetonitrile gradient in 40 mM NH₄Ac, pH 6.8. B, analysis of the product eluting at 7 min (15.5% acetonitrile) by electrospray ionization mass spectrometry at a resolution of 100,000. \( m/z = 888.144 \) agrees with the monoisotopic mass of benzoyl-CoA plus one oxygen atom (expected \( MH^+ = 888.1436 \)). The peak at 910.127 represents the same product but associated with Na⁺ instead of H⁺.

no mass of benzoyl-CoA plus two oxygen atoms and two hydrogen atoms (expected \( MH^+ = 906 \)) was found. The observed value of \( m/z = 888.144 \) agrees well with the monoisotopic mass of benzoyl-CoA plus one oxygen atom (expected \( MH^+ = 888.1436 \)). The peak at 910.127 represents the same product but associated with Na⁺ instead of H⁺. Because not even traces of a product carrying two additional oxygen atoms could be observed, the product as isolated is not the expected benzoyl-CoA dihydrodiol. It is rather a hydroxylated benzoyl-CoA or an epoxide of benzoyl-CoA.

Still, the possibility exists that the actual product of the reaction was a labile dihydrodiol, which during isolation becomes stabilized by rearomatization through water elimination, thus forming 2- or 3-hydroxybenzoyl-CoA as a dead-end product. We therefore used direct coupling of HPLC, MS, and MS/MS in a fast experiment to minimize possible side reactions and to detect possible dihydroxylated intermediates. Direct injection is gentle compared to freeze-drying of the HPLC sample and dissolving it again. The fragments of CoA served as an indicator that the detected parent mass even of trace unknown products is indeed a derivative of CoA. Fragmentation of CoA in a mass spectrometer has been reported, but only the mass of 428 was attributed to adenosine 3',5'-bisphosphate (17). We observed this mass and additional masses representing fragments of acyl-CoA thioesters. The fragments contain either the aromatic ring or the CoA adenylic moiety (Fig. 2). The method proved valid when tested with benzoyl-CoA (Fig. 2A). We observed in addition a fragment with a mass of 365.2, which is likely caused by an additional loss of phosphoric acid (97.97) (463.13 - 97.97 = 365.15). A related elimination of phosphate is known for phosphoserine leading to dehydroalanine (18). The product of BoxAB showed a minor 479.1 fragment and a major fragment of 381.2, which is likely formed from the minor fragment by an additional loss of phosphoric acid as in the case of benzoate (Fig. 2B).

These results indicate that the product detected under these gentle conditions was also a benzoyl-CoA derivative carrying only one additional oxygen atom. A monohydroxylated, aromatic derivative like 2-hydroxybenzoyl-CoA or 3-hydroxybenzoyl-CoA is an unlikely candidate for the following nonoxynolytic cleavage of the ring, and the benzoyl oxidation gene cluster does not code for a ring-cleaving dioxygenase. In case of an epoxide, one may expect a hydratase yielding the trans-2,3-dihydrodiol of benzoyl-CoA, but a corresponding hydratase gene is not found in the gene cluster.

³⁰O Labeling of the Product after Transformation of Benzoyl-CoA by BoxAB in the Presence of ¹⁸O₂—The free product of benzoyl-CoA oxygenase BoxAB cannot be unreasonably labile; nevertheless, a transient formation of a labile dihydrodiol might be possible. If a dihydrodiol transiently emerges in the course of the reaction, this should be a cis-dihydrodiol in case of a dioxygenase/reductase and a trans-dihydrodiol in the case of epoxide formation followed by water addition. To test the different options, benzoyl-CoA was transformed in \( H₂-¹⁸O \) in the presence of 50% \( ^{18}O₂ \), 50% \( ^{16}O₂ \); isolated by reverse phase HPLC; and analyzed by mass spectrometry. In the first case, half of the diol should be unlabeled, and the other half should carry two \( ^{18}O \); water elimination should yield 50% unlabeled and 50% \( ^{18}O \)-labeled benzoyl-CoA derivative carrying one oxygen atom. In the second case, 50% should be unlabeled and 50% carrying one \( ^{16}O \) atom; random water elimination would yield 75% unlabeled and 25% benzoyl-CoA carrying one \( ^{18}O \). Two molecule species with equal concentrations were observed (masses of 888.147 and 890.151); one corresponding to a benzoyl-CoA derivative carrying one additional \( ^{18}O \) (888.1436) and the other corresponding to benzoyl-CoA carrying one additional \( ^{18}O \) (890.1480) (Fig. 3). Again, no molecule species carrying two oxygen atoms were detected.
18O Labeling of Products after Transformation of Benzoyl-CoA by BoxAB in the Presence of H218O or 18O2 and Subsequent Transformation by Enoyl-CoA Hydratase BoxC and Aldehyde Dehydrogenase BoxD—The products of the transformation of benzoyl-CoA with the three enzymes BoxABC are considered established: 3,4-dehydroadipyl-CoA semialdehyde and formic acid derived from C2 of the aromatic ring (7). Formic acid cannot be detected because of its small size (m/z < 50 cannot be detected), nor can a derivative of higher mass be obtained without the loss of one oxygen atom of formate. BoxD catalyzes the oxidation of the CoA-linked C6 semialdehyde to the dicarboxylic acid under incorporation of water (12). Transformations were conducted with 16O2 in 50% H218O, 50% H216O. When BoxAB or BoxABC were added, solely the mass peaks of unlabeled products were observed (Table 2). The mass peaks corresponded to benzoyl-CoA carrying one additional oxygen atom and 3,4-dehydroadipyl-CoA semialdehyde, respectively. This finding seems to exclude the possibility that oxygen from water is incorporated into the C6 product formed by BoxABC. Correspondingly, the assay with BoxABCD (native enzyme BoxA in combination with recombinant enzyme BoxBStrep, recombinant enzyme BoxHisCStrep and recombinant enzyme BoxDMal) yielded one product exhibiting two mass peaks with equal intensity corresponding to unlabeled and singly 18O-labeled 3,4-dehydroadipyl-CoA semialdehyde, respectively. This finding seems to exclude the possibility that oxygen from water is incorporated into the C6 product formed by BoxABC. Correspondingly, the assay with BoxABCD (native enzyme BoxA in combination with recombinant enzyme BoxBStrep, recombinant enzyme BoxHisCStrep and recombinant enzyme BoxDMal) yielded one product exhibiting two mass peaks with equal intensity corresponding to unlabeled and singly 18O-labeled 3,4-dehydroadipyl-CoA semialdehyde, respectively. This finding seems to exclude the possibility that oxygen from water is incorporated into the C6 product formed by BoxABC. Correspondingly, the assay with BoxABCD (native enzyme BoxA in combination with recombinant enzyme BoxBStrep, recombinant enzyme BoxHisCStrep and recombinant enzyme BoxDMal) yielded one product exhibiting two mass peaks with equal intensity corresponding to unlabeled and singly 18O-labeled 3,4-dehydroadipyl-CoA semialdehyde, respectively.

FIGURE 2. **Mass spectrometry after collision-induced dissociation fragmentation of benzoyl-CoA (A) and of the product obtained from benzoyl-CoA by BoxAB (B).** Detected ions were isolated and fragmented in the linear ion trap (MS/MS). The parent mass in A was 872.15, and that in B was 888.14. Three fragments are the same in both spectra showing masses of 410.1, 428.1, and 508.1 and are attributed to the adenylate containing part of CoA as indicated by the formulas. The fragments of 365.2 and 463.1 in A correspond to those of 381.2 and 479.1 in B with the exact difference of a single oxygen atom and represent fragments of the part with the thioester of benzoic acid. For details, see text.

FIGURE 3. **Isotopic resolved mass spectrometric analysis of the product of benzoyl-CoA conversion by BoxAB in the presence of 50% 18O2.** The product was isolated by reverse phase HPLC and analyzed by mass spectrometry (conditions as in Fig. 1). The peak at 889.15 originates from benzoyl-CoA with one 13C. The mass difference of the benzoyl-CoA derivative with two 13C of low abundance could not be resolved from the peak with one 13C. The two peaks at 891.159 and 892.161 are benzoyl-CoA with one or two 13C, respectively, plus one 18O.
corresponds to unlabeled 3,4-dehydrodipip-CoA, and mass peak 896 corresponds to single $^{18}$O-labeled 3,4-dehydrodipip-CoA. Because the $^{18}$O-labeled dipip C6 carboxyl group is derived from C3 of the 3,4-dehydrodipip-CoA semialdehyde (see Fig. 6), C3 of the semialdehyde must have been linked to $^{18}$O. However, the observed missing labeling of the semialdehyde seemingly is contradictory to this conclusion because 50% of this product should also contain $^{18}$O. This inconsistency can be explained if the free carbonyl oxygen of the aldehyde (product of BoxABC) rapidly exchanges $^{18}$O with $^{16}$O from water via the aldehyde hydrate, when the sample was prepared and the product was isolated in unlabeled water. This reaction exchange is well known (19). 3,4-Dehydrodipip-CoA semialdehyde hydrate was observed before in NMR studies of the reaction mechanism of BoxC (7).

**Table 2**

$^{18}$O Incorporation from $^{18}$O$^{16}$O or $^{18}$O$^{2}$ into products derived from benzoyl-CoA

Benzoyl-CoA was converted in the presence of either 50% $^{18}$O$^{16}$O or 50% $^{18}$O$^{2}$ by the combined action of BoxAB, BoxABC, or BoxABCD. The products were purified by reverse phase HPLC (cf. Fig. 1) and analyzed by mass spectrometry (cf. Fig. 3).

|               | BoxAB          | BoxABC         | BoxABCD        |
|---------------|----------------|----------------|----------------|
|               | 888.14         | 890.15         | 878.16         |
| %             | %              | %              | %              |
| 50% $^{18}$O | 50% $^{16}$O   | 100            | 0%             |
| 50% $^{2}$   | 50% $^{2}$     | 100            | 0%             |
|               | 54             | 46             | 55             |
|               | 45             | 46             | 54             |

$^{18}$O Incorporation from $^{18}$O$^{16}$O or $^{18}$O$^{2}$ into products derived from benzoyl-CoA.

**FIGURE 4.** Mechanism (A) and kinetic behavior (B) of oxygen exchange between the carbonyl oxygen of 3,4-dehydrodipip-CoA semialdehyde and oxygen from water (50% $^{18}$O$^{16}$O, 50% $^{18}$O$^{2}$). Benzoyl-CoA was converted by BoxABC, and the 3,4-dehydrodipip-CoA semialdehyde was isolated by reverse phase HPLC (conditions as in Fig. 1). Samples were collected after different incubation times and immediately injected within 14 min into the electrospray ionization source of a Finnigan LTQ-FT mass spectrometer.

Evidence for an Epoxide as the Actual Product of BoxAB—The possibility that a dihydrodiol intermediate rearramizes under elimination of water can be excluded, because the product of BoxAB is cleaved by BoxC nonoxygenolytically, which would not be possible in the case of an aromatic ring. The remaining option of how benzoyl-CoA may be activated by BoxAB using 1 O$_2$ plus 1 NADPH is the formation of an epoxide most likely between C2 and C3 of the ring. The second oxygen atom is released as H$_2$O. We set out to obtain a derivative with DTC, which reacts with epoxides even under gentle enzyme assay conditions under opening of the epoxide ring (20, 21). DTC did not disturb the enzymatic reaction. The 2,3-epoxide of the benzene ring of benzoyl-CoA, 2,3-epoxybenzoyl-CoA, is expected to yield two possible DTC adducts depending on the steric hindrance of the products: a main product (little steric hindrance) and a side product (more steric hindrance) (Fig. 5A).

When benzoyl-CoA was transformed at 24 °C with BoxAB, the reaction did not go to completion because of inactivation of the enzymes by its product (Fig. 5B) (11). This alone indicates that the BoxAB product is chemically reactive. When DTC was added in excess, the reaction went to completion, and nearly stoichiometric amounts of a new product appeared (Fig. 5B), which migrated in reversed phase HPLC at 12–14 min. The derivative apparently did not inactivate BoxAB anymore. The new product peak was collected and analyzed by UV-visible spectroscopy (Fig. 5C) and MS (Fig. 5D).

The UV-visible spectrum of the BoxAB product showed an absorption maximum at 260 nm ($\epsilon_{260}$ nm = 14,000 M$^{-1}$ cm$^{-1}$ at pH 6.8) and another characteristic maximum at 310 nm ($\epsilon_{260}$ nm = 7,000 M$^{-1}$ cm$^{-1}$ at pH 6.8) compared with benzoyl-CoA ($\epsilon_{260}$ nm = 21,100 M$^{-1}$ cm$^{-1}$ at pH 6.8) (11) (Fig. 5C). The DTC derivative had nearly lost the absorption at 310 nm, which would be consistent with an opening of the epoxide (Fig. 5C). The MS spectrum showed a main mass peak at 1037.177 that agrees well with the theoretical mass of the DTC adduct of 2,3-epoxybenzoyl-CoA (expected mass 1037.1769) (Fig. 5D). The same derivative was obtained when the product of BoxAB was isolated and then treated with DTC. This indicates that the...
epoxide is not a transiently formed intermediate in the BoxAB catalyzed reaction, but it is a true product. Benzene epoxide is known to be in equilibrium with its tautomeric oxepin form (22). 2,3-Epoxbenzoyl-CoA might undergo an epoxide-oxepin valence tautomerism (Figs. 5E and 6). An oxepin is an unsaturated seven-membered heterocycle having six carbon atoms, one oxygen atom, and three double bonds. This oxepin is not aromatic because it does not obey the Hückel rule. We conclude that benzoyl-CoA is oxidized by BoxAB to an epoxide, which is nonaromatic, thus allowing a nonoxygenolytic ring cleavage catalyzed by the following enzyme BoxC. BoxAB therefore is not a dioxygenase/reductase but an epoxidase.

DISCUSSION

Product of BoxAB—In previous work the product of the oxygen- and NADPH-dependent transformation of benzoyl-CoA by BoxAB was tentatively assigned to cis-2,3-dihydro-2,3-dihydroxybenzoyl-CoA, and the enzyme system BoxAB was named benzoyl-CoA, NADPH:oxygen oxidoreductase (2,3-hydroxylating) (11). Although the NMR data were consistent with a cis-diol configuration, they did not provide unequivocal evidence (11). In the conventional metabolism of aromatic compounds, a cis-dihydrodiol undergoes oxidation and rearomatization to a dihydroxy aromatic central intermediate is then cleaved by ring-cleaving dioxygenases, yet a putative diol dehydrogenase gene could not be found in the benzoate oxidation gene cluster. In addition, the BoxB amino acid sequence shows no similarity to known oxygenase subunits of ring hydroxylating or ring-cleaving dioxygenases. This study revealed that the BoxAB enzyme system forms an epoxide and should be renamed benzoyl-CoA, NADPH:oxygen oxidoreductase.
(2,3-epoxide-forming). The suggested trivial name is benzoyl-CoA 2,3-epoxidase (EC 1.14.13).

In view of the new findings, the former NMR data were re-evaluated (Table 3). The product that was studied before and in this work had the same UV-visible spectrum and therefore appears to be identical. The true nature of the product may well be 2,3-epoxybenzoyl-CoA (7-oxabicyclo (4.1.0) hepta-2,4-diene-2-carboxyl-CoA), as the comparison between data-based and rule-based chemical shifts indicates. Still, these NMR data do not prove the proposed structure. NMR spectroscopy in this instance does not allow discriminating unambiguously between a cis-dihydrodiol and an epoxide. This is because both compounds exhibit cis carbon-oxygen bonds at C2 and C3 of the ring. The tautomeric oxepin has no carbon-carbon bond between C2 and C3; thus it would have different chemical shifts and signal splitting (Table 3), which were not observed. Taking into account the DTC derivative and the spectral properties of the product, we consider the epoxide as the true product and not only as a transient intermediate. BoxB has a counterpart in phenylacetyl-CoA oxygenase, which also forms an epoxide of an aromatic CoA thioester that is converted to an oxepin form.2

Thus this common unprecedented epoxide formation represents a new paradigm of aerobic aromatic metabolism.

Occurrence of the New Benzoate Pathway and Relation of Benzoyl-CoA Oxygenase (BoxB) to Other Enzymes—A BLAST search (BLASTP 2.2.22+) (23, 24) with BoxBC from A. evansi (NCBI accession numbers Q9AI75 and Q84HH6) revealed that 4–5% of all fully sequenced eubacterial genomes (mostly α- and β-proteobacteria) harbor the two key genes of the CoA-dependent benzoate oxidation pathway, which is lacking in Archaea (supplemental Table S1). For comparison, 7% of the species harbor benzoate 1,2-dioxygenase benABC and cis-diol dehydrogenase benD genes characteristic for the classical benzoate pathway involving ring-cleaving dioxygenases. Some species contain even both options (1.3%) (supplemental Table S1), which might be required for high turnover of benzoate or if reduced oxygen tension is present, as has been suggested for Burkholderia xenovorans LB400 (6, 25). These percentages indicate that the new pathway is not a minor route. The amino acid sequence similarity and identity for BoxB are between 97 and 92% and between 72 and 57%, respectively.

The active center of BoxB is a dinuclear iron center4 resembling the active site of soluble methane monooxygenase (EC 1.14.13.25, Protein Data Bank entry 1MMO) (26), ribonucleoside-diphosphate reductase (EC 1.17.4.1, Protein Data Bank entry 1RIB) (27), multicomponent phenol hydroxylase (EC 1.14.13.7, Protein Data Bank entry 2NN) (28), toluene/o-xylene monooxygenase (Protein Data Bank entry 1T0Q) (29), Δ9 stearoyl-acyl carrier protein desaturase (EC 1.14.99.6, Protein

4 L. Rather, T. Weinert, U. Demmer, E. Bill, U. Ermler, and G. Fuchs, unpublished results.

| Position | Chemical Shifts |
|----------|-----------------|
|          | observed [ppm]  | Predicted for diol [ppm] | Predicted for epoxide [ppm] | Predicted for oxepin [ppm] |
|          |                 | Data based | Rule based | Data based | Rule based | Data based | Rule based |
| δ13 C    |                  |            |            |            |            |            |            |
| 1        | 136.9 (dd)      | nd         | 147        | nd         | 147        | nd         | 124        |
| 6        | 132.9 (dd)      | 130        | 143        | 131        | 143        | 124.0      | 128.9      |
| 5        | 129.1 (dd)      | 126        | 128        | 128        | 128        | 135.3      | 129.9      |
| 4        | 135.5 (dd)      | 125        | 130        | 128        | 130        | 110.3      | 106.7      |
| 3        | 64.4 (b)        | 72         | 73         | 56         | 52         | 120.5(d)   | 141.8(d)   |
| 2        | 64.4 (b)        | 73         | 74         | 54         | 58         | 153.7(d)   | 156.3(d)   |

TABLE 3
Comparison of observed 13C NMR data of the product of BoxAB versus simulation of 13C NMR data of 2,3-dihydro-2,3-dihydroxybenzoyl-CoA and 2,3-epoxybenzoyl-CoA and its oxepin tautomer

The 13C NMR spectroscopic features of the product of BoxAB originally were interpreted as indicating a dihydrodiol structure (11). The predicted chemical shifts for 2,3-dihydro-2,3-dihydroxybenzoyl-CoA and 2,3-epoxybenzoyl-CoA and its oxepin were recalculated based on known average data (data based) or on numerical rules (rule based) using SpecInfo. b, broad; d, doublet; dd, doublet of doublets, nd, not determined.
CoA-dependent Aerobic Metabolism of Benzoate via an Epoxide

Possible Mechanism of BoxAB and Catalysis of BoxC Revisited—BoxAB introduces one oxygen atom from molecular oxygen into benzylo-CoA to form 2,3-epoxybenzoyl-CoA. The other oxygen atom is eliminated as water, which fuels the reaction and renders it irreversible. Oxygen becomes activated at the di-iron center, and the enzyme structure and properties of the active site are topics of current studies. BoxC converts the di-iron center, and the enzyme structure and properties of the active site are topics of current studies. BoxC converts the di-iron center, and the enzyme structure and properties of the active site are topics of current studies. BoxC converts the di-iron center, and the enzyme structure and properties of the active site are topics of current studies.

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BoxC converts the product of BoxAB, 2,3-epoxybenzoyl-CoA, possibly via its oxepin form, to an aldehyde plus formic acid by integration of two water molecules (Fig. 6). BoxC was formerly termed benzylo-CoA-dihydrodiol lyase and should be renamed 2,3-epoxybenzoyl-CoA dihydrolyase. The quick removal of the reactive epoxide by BoxC is probably vital. In the absence of BoxC, BoxAB becomes inactivated in the course of the reaction; in vivo BoxC may even form a complex with BoxB (11).

The crystal structure of BoxC without substrate was solved, and benzyl-CoA 2,3-dihydrodiol was modeled into the active center (44). This model needs revision in view of the true epoxide substrate. Water addition may be catalyzed consecutively by the same amino acids. Mainly two glutamate residues function as essential concerted acid/base catalysts (7, 44). It is obvious from the \( ^{18} \text{O} \) labeling experiment that BoxC opens the \( ^{18} \text{O} \)-2,3-epoxide by adding \( \text{OH}^- \) regiospecifically at ring C2 of the epoxide or its oxepin tautomer, leading to a common seven-membered ring (Fig. 6). This results in \( ^{18} \text{O} \) being linked to ring C3, which gives rise to the C6 carbonyl group of 3,4-dehydroadi palp-CoA semialdehyde formed by BoxC, in which \( ^{18} \text{O} \) was retained. The electron-withdrawing effect of the CoA activated carboxyl group facilitates ring opening of the epoxide by the addition of \( \text{OH}^- \) to form a dialdehyde. The enolate anion intermediate is stabilized by an oxanion hole characteristic for enoyl-CoA hydratases/isomerases (7, 44). Protonation at ring C1 prepares the intermediate for the next addition of \( \text{OH}^- \) at ring C2, which leads to elimination of the C2 atom as formic acid. Finally, a second protonation at ring C1 leads to the product 3,4-dehydroadi palp-CoA semialdehyde (7). The production of formic acid explains the odd finding that genes for enzymes of benzoate and formate metabolism are coinduced with withdrawing effect, which stabilizes negative charge and thus activates the aromatic ring. Furthermore, thioester formation allows an efficient trapping of aromatic acids within the cell, and CoA intermediates, especially the epoxide, might be less toxic. Because the energy-rich thioester bond is retained in the products, the energy initially spent is not lost. Benzylo-CoA 2,3-epoxide BoxAB catalyzes the introduction of one oxygen atom to form 2,3-epoxybenzoyl-CoA. The 2,3-epoxybenzoyl-CoA dihydrolyase BoxC integrates two water molecules to form the open chain intermediate 3,4-dehydroadi palp-CoA semialdehyde; formic acid is split off. 3,4-Dehydroadi palp-CoA semialdehyde dehydrogenase BoxD oxidizes the semialdehyde to its corresponding acid, 3,4-dehydroadi palp-CoA (4, 12). Modified \( \beta \)-oxidation leads to \( \beta \)-ketoadi palp-CoA, which is finally cleaved into acetyl-CoA and succinyl-CoA by \( \beta \)-ketoadi palp-thio lase (8).

The overall stoichiometry of aerobic benzoate degradation via CoA ligation follows the equation: Benzoate + ATP + 2 CoA + O$_2$ + 3 H$_2$O + NAD$^+$ \rightarrow Acetyl-CoA + Succinyl-CoA + Formic acid + AMP + PP$^+$ + NADH + H$^+$. Aerobic benzoate degradation via the \( \beta \)-ketoadipate pathway follows the equation: Benzoate + CoA + 2 O$_2$ + H$_2$O \rightarrow Acetyl-CoA + Succinate + CO$_2$. Hence, the new pathway uses less oxygen and produces reduced products, NADPH and formic acid.

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Data Bank entry 1AFR (30), p-aminobenzoate N-oxygenase (Protein Data Bank entry 3CHH) (31), and alkene monooxygenase (EC 1.14.13.69) (32). Soluble methane monooxygenase (33, 34) and alkene monooxygenase (32, 35) are also able to form epoxides. The dinuclear iron center in general is thought to be able to perform epoxidation (36, 37). It should be stressed that other epoxide-forming enzymes exist that are not related to BoxB. Some examples are cytochrome P450 (EC 1.14.14.1) (38), vitamin-K reductase (EC 1.1.4.1 and 1.1.4.2) (39, 40), squa-lene monooxygenase (EC 1.14.99.7) (41, 42), and zeaxanthine epoxidase (EC 1.14.13.90) (43).

Summarizing the New Pathway—Benzoate appears to be transported into the bacterium by an ABC transporter system and immediately becomes activated by benzoate-CoA ligase forming benzylo-CoA (Fig. 6) (8–10). In the following reactions all of the intermediates are coenzyme A thioesters. This may be advantageous because the CoA activated group has an electron-withdrawing effect, which stabilizes negative charge and thus activates the aromatic ring. Furthermore, thioester formation allows an efficient trapping of aromatic acids within the cell, and CoA intermediates, especially the epoxide, might be less toxic. Because the energy-rich thioester bond is retained in the products, the energy initially spent is not lost. Benzylo-CoA 2,3-epoxide BoxAB catalyzes the introduction of one oxygen atom to form 2,3-epoxybenzoyl-CoA. The 2,3-epoxybenzoyl-CoA dihydrolyase BoxC integrates two water molecules to form the open chain intermediate 3,4-dehydroadi palp-CoA semialdehyde; formic acid is split off. 3,4-Dehydroadi palp-CoA semialdehyde dehydrogenase BoxD oxidizes the semialdehyde to its corresponding acid, 3,4-dehydroadi palp-CoA (4, 12). Modified \( \beta \)-oxidation leads to \( \beta \)-ketoadi palp-CoA, which is finally cleaved into acetyl-CoA and succinyl-CoA by \( \beta \)-ketoadi palp-thio lase (8).

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