Structure and Mechanism of the Diiron Benzoyl-Coenzyme A Epoxidase BoxB*§

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The coenzyme A (CoA)-dependent aerobic benzoate metabolic pathway uses an unprecedented chemical strategy to overcome the high aromatic resonance energy by forming the non-aromatic 2,3-epoxybenzoyl-CoA. The crucial dearomatizing reaction is catalyzed by three enzymes, BoxABC, where BoxA is an NADPH-dependent reductase, BoxB is a benzoyl-CoA 2,3-epoxidase, and BoxC is an epoxide ring hydrolase. We characterized the key enzyme BoxB from Azotobacter enansii by structural and Mössbauer spectroscopic methods as a new member of class I diiron enzymes. Several family members were structurally studied with respect to the diiron center architecture, but no structure of an intact diiron enzyme with its natural substrate has been reported. X-ray structures between 1.9 and 2.5 Å resolution were determined for BoxB in the diferric state and with bound substrate benzoyl-CoA in the reduced state. The substrate-bound reduced state is distinguished from the diferric state by increased iron-ligand distances and the absence of directly bridging groups between them. The position of benzoyl-CoA inside a 20 Å long channel and the position of the phenyl ring relative to the diiron center are accurately defined. The C2 and C3 atoms of the phenyl ring are closer to one of the irons. Therefore, one oxygen of activated O2 must be ligated predominantly to this proximate iron to be in a geometrically suitable position to attack the phenyl ring. Consistent with the observed iron/phenyl geometry, BoxB stereoselectively should form the 2S,3R-epoxide. We postulate a reaction cycle that allows a charge delocalization because of the phenyl ring and the electron-withdrawing CoA thioester.

After benzoyl-CoA is converted to benzoyl-CoA, the three enzymes BoxABC (benzoyl-CoA oxidizing) catalyze the crucial dearomatization reaction by epoxidation and following ring hydrolysis (Fig. 1). The key enzyme is BoxB. This 55-kDa monomeric protein catalyzes the transformation of benzoyl-CoA and oxygen with two electrons and two protons to the non-aromatic 2,3-epoxybenzoyl-CoA and water (4). BoxB is found with a sequence identity higher than 53% mainly in the family (8) as they contain two characteristic E-H motifs. Very low amino acid sequence identities are found to other proteins; the closest relative to BoxB in the database is the PaaA of phenylacetyl-CoA epoxidase. This 30-kDa PaaA subunit belongs to a related multicomponent epoxidase system (6), which was found in 16% of bacteria and converts phenylacetyl-CoA to 1,2-epoxyphenylacetyl-CoA. The crystal structure of the inactive heterotetrameric PaaAC complex has been recently reported in complex with its substrate (PDB code 3PW1) but in absence of the diiron center (7). BoxB is considered as a member of the class I diiron protein family (8) as they contain two characteristic EXXX motifs required for diiron ligation: E-X$_2$-Y$_2$-EERGH and D-X$_{28}$-EAAH. Amino acids in bold letters indicate the putative

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The atomic coordinates and structure factors (codes 3PER, 3PF7, 3Q1G, and 3PMS) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org).

§ The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1 and 2 and Figs. S1–S4.

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Benzoyl-CoA Epoxidase BoxB

FIGURE 1. Coenzyme A-dependent aerobic benzoate pathway. This pathway was mainly studied in the β-proteobacterium A. evansii. The enzymatic apparatus for benzoate oxidation is coded by 17 clustered box genes that may form an operon (5).

EXPERIMENTAL PROCEDURES

Materials—$^{57}$Fe metal powder (95.38% isotopic enrichment) was obtained from Campro Scientific (Berlin, Germany). Benzoyl-CoA was prepared according to published procedures (3), purified by reverse-phase HPLC in 40 mM ammonium acetate, pH 6.8 (4), and freeze-dried.

Cultivation of Bacteria—Azoarcus evansii KB740 (DSMZ6869) harboring chromosomally a C-terminal streptavidin affinity-tagged BoxB was grown aerobically with benzoate as the sole source of cell carbon and energy (3). The yield was 200 g of cells (wet mass) mol$^{-1}$ benzoate. Cells for Mössbauer measurements were grown with 200 mg of$^{57}$Fe to an optical density at 578 nm of 2, which corresponded to 0.5 g of cells (dry mass) liter$^{-1}$.

Protein Purification—Cell extracts from 100 g of cells were prepared according to Rather et al. (4). BoxB was purified and assayed as reported previously (11). Streptavidin affinity-tagged BoxB was purified in an anaerobic glove box under N$_2$/H$_2$ atmosphere without reducing agents (BoxBox) by affinity chromatography and assayed according to Rather et al. (4). BoxB and BoxC copurify. To quantitatively remove BoxC, BoxBox was further purified under anaerobic conditions by gel permeation chromatography (Amersham Biosciences, HiLoad 26/60, 320 ml, 2 ml min$^{-1}$) and stored at −70 °C in 10 mM Tris/ HCl, 100 mM KCl, pH 8.0, and 10% (v/v) glycerol (0.7 ml, 22 mg ml$^{-1}$) for crystallization. Protein concentration and purity were determined by standard methods. Protein activity was determined according to Rather et al. (4).

Determination of Metal Content—4.4 mg of BoxBox in 1.2 ml of 10 mM Tris/HCl, 150 mM KCl, pH 8.0, without glycerol was freeze-dried and analyzed via inductively coupled plasma-optical emission spectrometry (ICP-OES) at the Chemical Analysis Laboratory, University of Georgia, Athens, GA. As control, 1.2 ml of 10 mM Tris/HCl, 150 mM KCl, pH 8.0, was treated the same way and analyzed.

Mössbauer Measurements—$^{57}$Fe-labeled protein was purified only by affinity chromatography. Sample preparation was performed at 4 °C under anaerobic conditions. A sample of 600 μl of BoxBox$_{ox}$ (23.5 mg ml$^{-1}$, 0.427 μmol) in 10 mM Tris/HCl, pH 8.0, 2.5 mM desthiobiotin, and 10% (v/v) glycerol) was transferred into a Mössbauer cup, immediately frozen, and stored in liquid nitrogen. Mössbauer data were recorded with a conventional spectrometer with alternating constant acceleration of the source. The minimum line width was 0.24 mm s$^{-1}$ (full width at half-height). The sample temperature was maintained constant with an Oxford Instruments Variox cryostat. The γ-source ($^{57}$Co/Rh, 1.8 Gbq) was kept at room temperature, and isomer shifts are quoted relative to the iron metal at 300 K.

Crystallization—Prior to crystallization, the protein was concentrated to about 30 mg ml$^{-1}$ in a Vivaspin concentrator and washed with 10 mM Tris, pH 8.0, 0.1 M KCl or 10 mM PIPES, pH 7.0, 0.1 M KCl. Crystallization attempts were performed with the hanging drop method at 18 °C using a ratio of precipitant to protein solution of 1 μl:1 μl. BoxBox$_{ox}$-phosphate, BoxBox$_{ox}$-malonate, and BoxBox$_{ox}$-peg crystals were grown aerobically. For co-crystallization experiments, benzoyl-CoA was added to the protein.
solution prior to crystallization to a final concentration of 5 mm.
BoxB:BCA\textsubscript{red} crystals (BoxB with the substrate benzoyl-CoA under reducing conditions) were grown in an anaerobic glove box under \(\text{N}_2/\text{H}_2\) atmosphere. For reduction, BoxA, NADPH, and benzoyl-CoA were supplemented to the BoxB solution to a final concentration of 0.1 mg ml\(^{-1}\), 1 mm, and 5 mm, respectively. The residual oxygen in the protein solutions of BoxA and BoxB was removed by incubation in the glove box for 3 weeks. NADPH and benzoyl-CoA solutions were freshly prepared with anaerobic PIPES buffer. The crystallization and cryoprotection conditions are given in supplemental Table S1.

X-ray Structure Analysis—Data were collected at the Swiss Light Source (SLS) beamline PXII using an MAR CCD225 detector and processed with XDS (13) and the CCP4 program suite (14). The data quality was examined with PHENIX (15). The initial structure was determined with a BoxBox-phosphate crystal using the single wavelength anomalous dispersion method based on the two intrinsic iron ions (see Table 1). The positions of the iron atoms were detected with SHELXD (16), and the phases were subsequently calculated with SHARP (17). After solvent flattening (18) of the electron density, an initial model was automatically built with BUCCANEER (19), which could be manually completed with COOT (20). The refinement was carried out using REFMAC5 (21) and PHENIX (15). The structures of the other crystal forms were solved with the molecular replacement method using PHASER (22), and the refinement was carried out with PHENIX and REFMAC5.

The optimal segmentation for TLS refinement of the different structures with REFMAC5 and PHENIX was obtained using the TLSMD server (23). The BoxB structures were nearly identical, and the root mean square deviations varied between 0.3 and 0.4 Å but revealed differences in the active site region. The homology model of the [4Fe-4S]-containing domain of BoxA and the homology model of the BoxC dimer were obtained using Protein Data Bank (PDB) entries 2FDN and 2W3P, respectively, and the SWISS-MODEL server (24). The structures were analyzed using MolProbity (25). The different crystal contact regions within the different crystal forms and the small contact area confirmed the presence of BoxB as a monomer. Fig. 2 and supplemental Figs. S1, B and C, S2A, S3, and S4 were prepared with PyMOL (Schrödinger, LLC). Supplemental Fig. S2B was prepared with CHIMERA (26). Crystal parameters, data collection, and refinement statistics are given in Table 1.

RESULTS AND DISCUSSION

Spectral Characterization of the Oxidized Diiron Center of BoxB—Recombinant BoxB of A. euviss was produced as a C-terminal streptavidin affinity-tagged variant and purified under anaerobic conditions without reducing agents (BoxB\textsubscript{ox}) (4). The metal content of BoxB\textsubscript{ox} was determined by inductively coupled plasma-optical emission spectrometric measurements, revealing two irons per BoxB monomer (2.1 ± 0.1). Because the oxidized protein is colorless (9), a Rieske-type [2Fe-2S] cluster or heme irons could be excluded. Zero-field Mössbauer studies of \(^{57}\)Fe-labeled BoxB\textsubscript{ox} showed a Lorentzian quadrupole doublet with isomer shift \(\delta = 0.45\) mm s\(^{-1}\), electric quadrupole splitting \(\Delta E_Q = 0.69\) mm s\(^{-1}\), and line width \(I_{\text{FWHM}} = 0.65\) mm s\(^{-1}\). These values are typical of high spin Fe\textsuperscript{III} in an octahedral coordination shell with hard ligands such as nitrogen or oxygen (from carboxylates or water). Similar values were found for other non-heme diiron enzymes in the oxidized state (27). Interestingly, the low electric quadrupole splitting, being in the normal range for ferric compounds, is consistent with the presence of one or more \(\mu\)-hydroxo groups coordinated to the diiron center, but it particularly rules out a bridging \(\mu\)-oxo group. In contrast, complexes with the corresponding Fe\textsuperscript{III}–O–Fe\textsuperscript{III} core show much larger quadrupole splitting in the range 0.9–2.4 mm s\(^{-1}\) (except diferric porphyrins and other systems with macroyclic and highly covalent ligand systems). This is due to the pronounced charge anisotropy caused by the uniquely short iron-oxo bond (28, 29).

Structural Basis—BoxB was crystallized aerobically in the diferric oxidation state BoxBox\textsubscript{ox}. Three different crystal structures were obtained and termed BoxB\textsubscript{ox-phosphate}, BoxB\textsubscript{ox-malonate} and BoxB\textsubscript{ox-peg} based on the precipitants phosphate, malonate, and partly polyethylene glycol (PEG) contributive in iron ligation (supplemental Table S1). In addition, BoxB was co-crystallized with the substrate benzoyl-CoA under anaerobic and reducing conditions by supplementing the enzyme solution with NADPH and catalytic amounts of BoxA inside an anaerobic glove box. EPR spectroscopic measurements also using the physiological NADPH/BoxA system for reduction resulted in a semireduced Fe\textsuperscript{II}Fe\textsuperscript{III} center. In vitro the fully reduced Fe\textsuperscript{II}Fe\textsuperscript{II} center was only achieved with the stronger reducing agent dithionite in the presence of methyl viologen. Because of the deviating conditions concerning solution composition, time, and radiation used for x-ray and EPR studies, we cannot specify whether the diiron center in the crystal is present in the semireduced Fe\textsuperscript{II}Fe\textsuperscript{III} or the completely reduced Fe\textsuperscript{II}Fe\textsuperscript{II} state. We named the corresponding structure BoxB:BCA\textsubscript{red}. Interestingly, BoxB in the diferric oxidation state (BoxBox\textsubscript{ox-peg}) did not co-crystallize with benzoyl-CoA under non-reducing conditions despite similar crystallization conditions as used for growing the BoxB:BCA\textsubscript{red} crystals (supplemental Table S1). This suggests that benzoyl-CoA can only be bound to the reduced state, as reported for the substrate methane and the oxygenase component of methane monooxygenase (30). The structure of BoxB was determined in the BoxB\textsubscript{ox-phosphate} state using the single wavelength anomalous dispersion method based on the anomalous signal of the two intrinsic iron atoms. The BoxB structures of the other crystal forms were obtained by the molecular replacement method. The BoxBox\textsubscript{phosphate}, BoxB\textsubscript{ox-malonate}, BoxBox\textsubscript{ox-peg} and BoxB:BCA\textsubscript{red} structures were refined at 2.1, 1.9, 2.5, and 2.3 Å, respectively. The detailed composition of the individual states and the corresponding crystallographic parameters are summarized in Table 1. The extracted structural information is based on average values of the different molecules in the asymmetric unit.

Overall Structure—BoxB is present in solution (9) and in the crystal as a monomer (Fig. 2A). In comparison, other family members such as stearyl-ACP A9 desaturase, ribonucleotide reductase R2, and methane monooxygenase are homodimers, heterotetramers, and heterohexamers, respectively (8, 31, 32).

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4 L. J. Rather, E. Bill, and G. Fuchs, unpublished data.
BoxB is architecturally composed of eight largely parallel α-helices complemented by several short helices, irregular elements, and three small sheets (Fig. 2A and supplemental Fig. S1A). The core of the enzyme is formed by the canonical four-helix bundle (helices B, C, E, and F), into which the catalytic diiron center is embedded. Only a small surface region (17 × 25 Å) of the four-helix bundle in front of helix F is directly accessible to the bulk solvent. We postulate this region as docking site for the iron-sulfur flavoprotein BoxA to transfer electrons from NADPH to the diiron center of BoxB (see supplemental Fig. S2). The residual surface of the four-helix bundle is encircled by helices A, D, G, and H, an N-terminal β-hairpin, and an irregular stretch preceding helix B.

Comparison with Structures of Other Diiron Proteins—A superposition between BoxB and other enzymes of the class I diiron protein family indicated a high structural relationship of the four-helix bundles despite very low sequence similarity. According to the Dali server (33), the root mean square deviations between BoxB (106:163, 201:264) and methane monooxygenase (1FZ1, 100:157, 198:262), toluene/o-xylene diiron monooxygenase (1T0Q, 90:147, 186:250), stearyl-ACP Δ9 desaturase (1AFR, 91:156, 186:249), ribonucleotide reductase (1SYY, 75:133, 182:248), p-aminobenzoate N-oxidase (3CHH, 90:149,188:248), and PaaA (3PVT, 28:85, 116:175) were 2.4, 2.4, 3.1, 2.4, 3.4, and 1.7 Å, respectively. The structure-based sequence identity was 12, 17, 16, 12, 12, and 19%, respectively. Helices D, G, and H flanking the four-helix bundle are, in principle, found in all family members, although their length and orientation differ (Fig. 2A and supplemental Fig. S1A).

The Oxidized Diiron Center—In the dифференци BoxBox-ox-malate/BoxBox-ox-phosphate and BoxBox-ox-peg structures, both iron sites are highly occupied, as reflected by the low temperature factor of the irons (Table 1), which are comparable with those of the surrounding amino acids. The diiron center is anchored to the conserved residues of the four-helix bundle, as illustrated in Fig. 2B and supplemental Fig. S3 (with electron density) for first sphere interactions and in supplemental Fig. S1B for second sphere interactions.

In the oxidized substrate-free BoxB structures, the two iron sites of the diiron center have a distance of ~3.5 Å and are octahedrally ligated. Fe1 is coordinated to Glu-120-O and His-153-Nε2, and Fe2 is coordinated to Asp-211-Oε2 and His-243-Nε2 in a nearly symmetric manner. The third ligand of Fe1 in the BoxBox-malate state, and presumably in all oxidized states, is a solvent molecule, which was assigned as H2O. H2O appears to be more suitable than a hydroxo group for forming hydrogen bonds to the adjacent carboxylate oxygens of Glu-120 and malonate. The equivalent position at Fe2 is occupied by Glu-240-COO−, His-243-Nε2, and Gln-254-Nε2, by the distance of 2.0 Å to the irons, indicated by the microenvironment (Glu-150, Glu-240, and His-243) and the absent interaction between its carboxylate group and the iron-sulfur protein.

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In the BoxBox-ox-malate and BoxBox-ox-phosphate structures, the two malonate carboxylate/phosphate oxygens of the precipitants bridge the two iron ions in a bidentate manner and complete

### Table 1: Data collection and refinement statistics

| Data collection statistics | BoxBox-ox-phosphate peak | BoxBox-ox-phosphate native | BoxBox-ox-malate | BoxBox-ox-peg | BoxB:BCAred |
|---------------------------|-------------------------|---------------------------|-----------------|--------------|-------------|
| Space group               | C222,                   | C222,                     | C2              | C2           | C2          |
| Wavelength (Å)            | 1.738                   | 0.9999                    | 1.000           | 1.000        | 1.2137      |
| Resolution (Å)            | 50.0-2.7 (2.8-2.7)       | 50.0-2.1 (2.2-2.1)        | 50.0-1.9 (2.0-1.9) | 50.0-2.5 (2.6-2.5) | 50.0-2.3 (2.5-2.3) |
| No. of unique reflections | 88,005 (8511)           | 96,760 (12,375)           | 124,905 (15,532) | 76,352 (8215) | 98,025 (21,689) |
| Redundancy                | 4.0 (3.5)               | 3.5 (3.2)                 | 5.2 (4.3)       | 3.8 (3.7)    | 3.6 (3.8)   |
| Unit cell                 |                         |                           |                 |              |             |
| a, b, c (Å)               | 113.5, 214.6, 139.0     | 112.3, 213.9, 139.0      | 112.8, 213.5, 137.2 | 109.4        | 108.3       |
| β [°]                     |                         | 4                          | 2               |              | 4           |
| Number of molecules in the asymmetric unit | 2 | 2 | 2 | 4 | 4 |
| Completeness (%)          | 97.7 (91.8)             | 99.2 (98.3)                | 96.2 (84.7)     | 99.0 (96.3)  | 99.2 (99.9) |
| Rmerge [%]                | 11.0 (68.3)             | 7.5 (67.7)                 | 9.4 (71.2)      | 9.4 (71.2)   | 6.4 (40.7)  |
| I/σ(I)                    | 10.8 (2.7)              | 11.8 (2.3)                 | 13.3 (2.7)      | 8.6 (1.8)    | 12.1 (3.4)  |
| Refinement statistics     |                         |                           |                 |              |             |
| Ligands per monomer       | 2 Fe2+, 2 PO4−, 4 glycerol | 2 Fe2+, 1 malonate, 0.5 Cl− | 2 Fe2+, 1 Cl−, 1 SO4− | Fe2+, Fe2+H+, 1 Cl−, 1 benzoyl-CoA, 2 SO4−, 0.5 glycerol, 1 propanediol, 0.25 tetraethylene glycol | 22.4 (33.0)/17.4 (25.0) |
| Rfree/Rmerge (%)          | 20.3 (35.9)/17.2 (29.3) | 21.7 (48.6)/18.2 (46.1)   | 23.5 (42.0)/20.6 (40.0) | 20.0 (22.3)/17.4 (25.0) | 91.1/100/0  |
| Ramachandran favored/allowed/disallowed [%] | 90.9/100/0 | 91.9/100/0 | 91.5/100/0 | 91.1/100/0 |
| Average B-factor protein/solvent/iron/substrate [Å²] | 43.2/46.6/34.7/— | 28.7/41.0/22.2/— | 52.7/44.2/45.2/— | 52.6/47.6/71.1/68.4 |
| r.m.s.d. [Å]              | 0.011                   | 0.012                      | 0.015           | 0.020        |
| Bond length [Å]           | 1.096                   | 1.205                      | 1.317           | 1.724        |

* r.m.s.d., root mean square deviation.
the distorted octahedral coordination shell of both irons (Fig. 2B and supplemental Fig. S3). We assume that O₂ binds between the two bridging oxygens (Fig. 2D). Correspondingly, in the BoxB<sub>ox-peg</sub> structure, the potential O₂ ligation site was partly occupied. The density was tentatively interpreted as a PEG fragment and/or a hydroxo group.

The Reduced Diiron Center with Bound Benzoyl-CoA—In the BoxB<sub>red</sub> structure, the irons are also ligated to Glu-120-O<sub>e1</sub>, His-153-N<sub>ε1</sub>, Asp-211-O<sub>η2</sub>, and His-243-N<sub>ε1</sub>, but the iron-ligand distances are significantly longer when compared with those found in the oxidized BoxB structures (supplemental Table S2). The carboxylate of Glu-240 appears to be ligated to Fe₂ in a bidentate manner. The solvent molecule connected to Fe₁ is not visible, but its presence in the BoxB:BCA<sub>red</sub> state cannot be completely excluded at 2.3 Å resolution. Despite the similar distance of 3.5 Å between the two irons of the oxidized and reduced states, the irons do not superimpose exactly; in particular, Fe₂ is displaced by ~0.7 Å (Fig. 2B). In the BoxB:BCA<sub>red</sub> state, the irons have high temperature factors (71 Å<sup>2</sup>) relative to the surrounding protein (40 Å<sup>2</sup>). This could be due to increased iron-ligand distances, a possible heterogeneity effect, and the surprising absence of ligands bridging the irons.

In the oxidized states, the non-polar C<sub>B</sub> and C<sub>y</sub> atoms of Glu-150 contact the Val-146 peptide oxygen and a solvent molecule (supplemental Fig. S1B). Induced by reduction and substrate binding, the side chain of Glu-150 is rotated away from the diiron center. Thus, the Glu-150 carboxylate group in the BoxB:BCA<sub>red</sub> state predominantly occupies the same polar binding site as the solvent molecule in the oxidized states and is hydrogen-bonded to Ser-123, Gln-127, and Asn-147. Because weak residual electron density (20–40% occupancy) is present at the same position as in the oxidized states, Glu-150 seems to occupy a split conformation in the BoxB:BCA<sub>red</sub> structure (supplemental Fig. S3C).

Comparison of Diiron Ligation—The diiron ligation structure of BoxB is well conserved in all class I diiron proteins, implying a related oxygen activation mechanism (34). BoxB<sub>ox-malonate</sub> and methane monooxygenase<sub>ox</sub> (35) (and tolu-
ene/o-xylene diiron monooxygenase$_{\text{co}}$ (36)) only reveal minor differences in the ligand pattern. The carboxylate chelated to Fe$_2$ originates from an aspartate in BoxB (Asp-211) and PaaAC (7) and from a glutamate in the other family members. This change might be correlated with the different $\pi$-helix character of helix E (one $\pi$-helix turn in BoxB and PaaA and two in methane monooxygenase (32)). The variable character of Glu-240 for BoxB was also found in other family members. For example, one carboxylate oxygen bridges the two iron ions in the reduced state of methane monooxygenase, in contrast to two carboxylate oxygens in stearyl-ACP $\Delta^9$ desaturase (32, 37). The different side chain arrangement of Glu-150 in the oxidized and reduced substrate-bound state is unique in class I diiron center proteins. The Fe–Fe distances are rather variable among the family members; the value for BoxB is in this range.

The Substrate Binding Site—The approximate position of the benzoyl-CoA binding site can be assigned on the basis of a protein surface analysis as a 20 Å long channel extends from the protein surface to the diiron center in the protein interior (Fig. 2C). The overall architecture of the channel is formed by helices B and E, by the C-terminal end of helix G, by the segment following helix D, and by the small helix 303:313. It most resembles that of PaaA (7) and next that of stearyl-ACP $\Delta^9$ desaturase (37). Because of the longer stearyl chain of the latter, the substrate channel protrudes deeper into the protein than in the case of BoxB. The channel is shortened by the side chains of Ser-123 and Phe-206 in BoxB, and its exit is displaced by a positional change of helix G and the following segment when compared with stearyl-ACP $\Delta^9$ desaturase. Interestingly, the channel entrance between helices B and E is smaller in BoxB, PaaA, and stearyl-ACP $\Delta^9$ desaturase than in methane monooxygenase or toluene/o-xylene diiron monooxygenase, although BoxB, PaaA, and stearyl-ACP $\Delta^9$ desaturase use larger substrates. In methane monooxygenase and toluene/o-xylene monooxygenase, longer and more hydrophobic side chains define the channel entrance (34). In agreement with the polar character of CoA, the channels of BoxB and PaaA are the most hydrophilic ones.

The benzoyl-CoA binding mode was accurately established based on the 2.3 Å BoxB:BCA$_{\text{red}}$ structure (Fig. 2C). The benzoyl-CoA site is highly occupied and clearly defined in the electron density except the phosphorylated adenosine moiety of CoA, which is partially disordered. This moiety positioned at the solvent-accessible entrance of the channel might be influenced in the current crystal form by contacts to a neighboring molecule (supplemental Fig. S1C). Nevertheless, benzoyl-CoA adopts the characteristic J-shaped conformation with a bent adenosine and a small kink after the thioester group. The catalytic relevant phenyl ring is accommodated by a rather hydrophobic pocket, which is built up of Thr-119, Ser-123, Phe-193, Phe-206, and Asp-211 (supplemental Fig. S1C). Substrate binding in BoxB and PaaA (7) is related, although the contacting residues are only moderately conserved. In BoxB, the phenyl ring is buried less deeply inside the channel than in PaaA, perhaps due to the presence of the diiron center.

Although a few diiron center protein-substrate (analog) complexes were structurally characterized (7, 36, 38), the BoxB:BCA$_{\text{red}}$ structure reveals the first one where the substrate is bound to its binding site in a catalytically relevant orientation relative to the diiron center. The shortest distance between the phenyl ring of benzoyl-CoA and the diiron center is 3.5 Å between Fe1 and C2, whereas the corresponding distance to Fe2 is 4.9 Å (Fig. 2D). The residue Gln-116, which is also involved in malonate binding, is hydrogen-bonded to the thioester oxygen and cysteamine nitrogen of benzoyl-CoA and thereby determines the position of the phenyl ring. The benzoyl thioester group snugly fits into the narrowest segment of the channel with the smallest diameter of $\sim$6.5 Å between Thr-119 and Gly-214 (Fig. 2C and supplemental Fig. S1C). Therefore, the chemical reaction is completely shielded from bulk solvent.

**Proposed Association of BoxB with BoxA and BoxC**—The catalytic machinery that epoxidizes benzoyl-CoA and hydrolyzes the epoxide consists of the three proteins BoxA, BoxB, and BoxC, which somehow must be associated in vivo. Our postulated molecular architecture is reminiscent of other multicomponent monooxygenases regarding basic principles of electron supply, catalysis, and stimulation, with the exception that BoxC also catalyzes the subsequent ring cleavage of the epoxide (Fig. 1). For creating a complete picture of the BoxABC system, we tentatively modeled the transient BoxAB and BoxBC associations. In the BoxAB model, the distance between the distal [4Fe-4S] center of BoxA and the diiron center of BoxB is 13.5 Å and thus allows electron transfer at physiological rates (supplemental Fig. S2). In the BoxBC model, the substrate binding site of one BoxC monomer is close to that of BoxB. The association between BoxB and the ring-cleaving dihydrolation BoxC (39) may prevent an undesirable release of the reactive epoxide and may guarantee its quick removal by dihydrolase (supplemental Fig. S4).

**Proposed Catalytic Cycle**—The combination of structural and spectroscopic data provides new insights into the catalytic mechanism of BoxB outlined in Fig. 3. The resting state (Box$_{\text{ox}}$) is defined as ferric diiron complex bridged by two $\mu$-hydroxo groups (Fe$_1$$^{III}$–(OH)$_2$–Fe$_2$$^{III}$) as found also for other multicomponent monooxygenases. In most BoxB$_{\text{ox}}$ structures, one bridging hydroxyl group is replaced by a ligand from the crystallization solution. According to EPR data, the reaction cycle is initiated by reducing BoxB with BoxA/NADPH to a mixed-valent Fe$_1$$^{III}$Fe$_2$$^{II}$ state, which is in agreement with theoretical studies on methane monooxygenase (40). In parallel, one or both bridging hydroxido groups are released as H$_2$O. Upon reduction, the interactions between the diiron center and the surrounding polypeptide are decreased, and the (positional) mobility of the iron ions is increased, suggesting a fluctuating Fe–Fe distance during the catalytic cycle. Benzoyl-CoA binding (BoxB:BCA$_{\text{red}}$) and the weaker iron-ligand interactions in the reduced state induce a rotation of Glu-150 away from the hydrophobic phenyl group, which moves a polar position (Fig. 2, B and C). We suggest that benzoyl-CoA binding and the displacement of Glu-150 allows O$_2$ to move to a position that is favorable for activation (Fig. 2D). O$_2$ activation induced by transferring the second electron would be directly coupled to the reaction, which is useful as the production of reactive oxygen species and the waste of reducing power are thus prevented.

Although the exact geometry of the active site prior to the oxygen attack remains elusive, the spatial relationship between
The epoxide ring is subsequently closed to the 2,3-epoxybenzoyl-CoA adduct (T). The relative orientation of benzoyl-CoA and the diiron center clearly determine the side of the phenyl ring to which the generated oxygen must point (Fig. 2D). According to these geometric considerations, BoxB should stereoselectively produce the 2S,3R-epoxide. Finally, the enzyme releases the product and returns to the resting state (BoxB<sub>red</sub>).

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