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Reversible Biological Birch Reduction at an Extremely Low Redox Potential

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Abstract: The Birch reduction of aromatic rings to cyclohexadiene compounds is widely used in chemical synthesis and requires solvated electrons, the most potent reductants known in organic chemistry. Benzoyl-coenzyme A (CoA) reductases (BCR) are key enzymes in the anaerobic bacterial degradation of aromatic compounds and catalyze an analogous reaction under physiological conditions. Class I BCRs are FeS enzymes and couple the reductive dearomatization of benzoyl-CoA to cyclohexa-1,5-diene-1-carboxyl-CoA (dienoyl-CoA) to a stoichiometric ATP hydrolysis. Here, we report on a tungsten-containing class II BCR from *Geobacter metallireducens* that catalyzed the fully reversible, ATP-independent dearomatization of benzoyl-CoA to dienoyl-CoA. BCR additionally catalyzed the disproportionation of dienoyl-CoA to benzoyl-CoA/monoenoyl-CoA and the four- and six-electron reduction of benzoyl-CoA in the presence of a reduced low-potential bridged 2,2′-bipyridyl redox dye. Reversible redox titration experiments in the presence of this redox dye revealed a midpoint potential of $E^{0\prime} = -622$ mV for the benzoyl-CoA/dienoyl-CoA couple, which is far below the values of other known reversible substrate/product redox couples in enzymology. This work demonstrates the efficiency of reversible metalloenzyme catalysis, which in chemical synthesis can only be achieved under essentially irreversible conditions.

Introduction

The Birch reduction is a powerful and common synthetic tool in organic chemistry which is used industrially for dihydro additions to aromatic rings.[1,2] It proceeds via alternate single electron/proton transfer steps to the aromatic ring yielding cyclohexadiene compounds, which can be further reduced (Figure 1a). The rate-determining step is the first electron transfer yielding a nonaromatic radical anion. The redox potential of the one-electron reduction of nonactivated aromatic compounds is below −3 V.[3]

Considering the totally nonphysiological conditions of the Birch reduction in organic synthesis, it is surprising that enzymes exist in nature, which catalyze a similar reaction. For anaerobic bacteria that degrade aromatic growth substrates, the use of the dioxygen molecule for an oxidative attack on the aromatic ring is not an option. Instead, dearomatizing benzoyl-coenzyme A (benzoyl-CoA **1**) reductases (BCRs), key enzymes of the

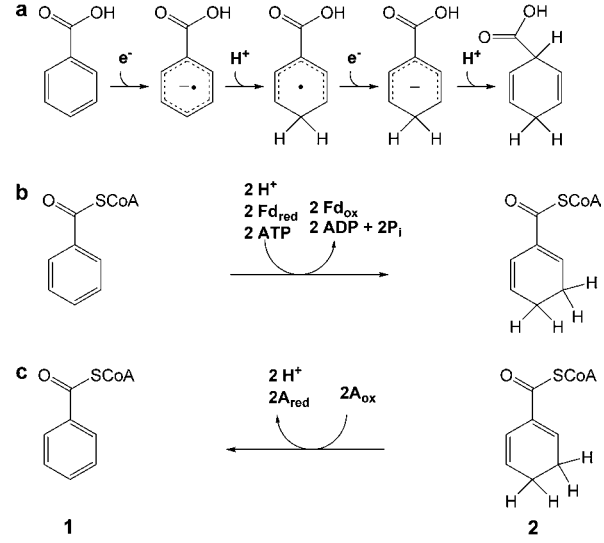

Figure 1. Reductive benzene ring dearomatization by the Birch reduction in organic synthesis and by benzoyl-CoA reductases (BCRs). (a) Reaction sequence of the Birch reduction of benzoic acid. Solvated electrons serve as electron donors, and the formation of the kinetically favored cyclohexa-2,5-diene isomer is shown. (b) Reaction catalyzed by class I BCRs (ATP-dependent). A reduced ferredoxin serves as an electron donor. (c) Reaction catalyzed by class II BCRs (ATP independent). The forward reaction of class II BCRs has not been determined, yet. A_{ox} = variable artificial one-electron acceptors.

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anaerobic aromatic metabolism, reduce the aromatic ring of the substrate with two electrons and specifically form cyclohexa-1,5-diene-1-carboxyl-CoA (dienoyl-CoA **2**, Figure 1b).[4,5] In most cases, no further reduction to the corresponding cyclic monoenoyl-CoA or cyclohexanecarboxyl-CoA is observed indicating a tight coordination of substrate binding, transfer of two electrons, and product release. The one-electron reduction potential of the benzoyl-CoA analogue *S*-ethyl thiobenzoic acid in an aprotic solvent to the corresponding radical anion is −1.9 V.[6] This value is more positive than that of the nonactivated benzoic acid due to the extended stabilization possibilities of the radical anion intermediate at the carbonyl functionality of the thioester.[7,8] The redox potential of the reaction benzoyl-CoA + $2e^-$ + $2H^+$ → dienoyl-CoA in an aqueous solution is unknown.

There are two different classes of BCRs, which both form the identical dienoyl-CoA product (Figure 1b): class I BCRs couple electron transfer from a reduced ferredoxin to the aromatic ring to a stoichiometric ATP hydrolysis (2ATP/$2e^-$).[5,9,10] For this reason, the reactions catalyzed by class I BCRs are considered essentially irreversible. Class I BCRs have an $\alpha\beta\gamma\delta$-subunit composition and contain three [4Fe-4S] clusters. The $\alpha\delta$-subunits bind one ATP each and are responsible for ATP-driven electron transfer to the active site containing $\beta\gamma$-subunits. ATP hydrolysis-dependent electron transfer to a chemically inert substrate is a common feature of benzoyl-CoA reductases and nitrogenases.[11] ATP-dependent low potential electron transfers have also been described for the activating enzymes of 2-hydroxyacyl-CoA dehydratases[12,13] or B12-dependent dehalogenases.[14] BCR from the facultatively anaerobic *Thauera aromatica* is the best studied enzyme of class I BCRs. For this enzyme, initial evidence has been obtained that the stereospecific *trans*-dihydro addition of hydrogen atoms to benzoyl-CoA proceeds via radical intermediates according to the classical Birch reduction.[15,16]

The class II BCRs were recently discovered in the Fe(III)-respiring, obligately anaerobic *Geobacter metallireducens*.[17] In this organism, BCR is proposed to constitute a complex of eight benzoate inducible gene products (BamBCDEFGHI). The BamBC components have been isolated and characterized using an assay that followed the reverse reaction, the electron acceptor dependent aromatization of the product dienoyl-CoA.[17,18] The BamB is proposed to contain an active site tungstopterin and a [4Fe-4S] cluster; BamC is an electron transferring subunit containing three further FeS clusters.[17] Class II BCRs are proposed not to require ATP hydrolysis, and it was hypothesized that the BamDEFGHI components are involved in an uncharacterized electron activation process. Using Ti(III)-citrate or dithionite as electron donors, BamBC did not catalyze the reductive dearomatization of benzoyl-CoA.[17]

In this work, we demonstrate that BamBC was capable of catalyzing both the aromatization and dearomatization reactions in the absence/presence of external electron acceptors/donors, respectively, without coupling to an activation reaction. The redox potential of the benzoyl-CoA/dienoyl-CoA conversion was determined to be the most negative described for a fully reversible substrate/product couple in enzymatic catalysis. The results obtained provide initial insights into the energetics of the key reaction in anaerobic aromatic catabolism.

Materials and Methods

Previously Described Methodologies. Previous papers provide details for the cultivation of *G. metallireducens*[19] and the purification of BamBC under strictly anaerobic conditions.[17] The synthesis and purification of benzoyl-CoA **1**, dienoyl-CoA **2**, the three cyclomonoenoyl-CoA isomers, and [*ring*-^{13}C]-benzoyl-CoA/dienoyl-CoA was as described earlier.[20,21] Synthesis and ^{1}H NMR/^{13}C NMR purity control of 2,12-dimethyl-7,8-dihydro-6*H*-dipyrido[1,2-a:2′,1′-c][1,4]diazepinediium dibromide **4** was as described.[22]

BamBC Reduction and Disproportionation Assay. BamBC (1−5 μM) was reduced in 500 μL of reaction buffer containing 150 mM Mops/KOH, 15 mM $MgCl_2$, 150 mM NaCl, 5 mg/mL BSA, pH 6.8, with 0.15 mM dienoyl-CoA in the absence of an external electron acceptor. The decrease in absorption at 409 nm was followed by UV/vis spectroscopy. Samples (25 μL) were taken at different time points and analyzed by HPLC coupled to diode array detection as described.[16] The amounts of benzoyl-CoA and monoenoyl-CoA were determined by their extinction coefficients (ε[benzoyl-CoA]$_{260}$ = 21 300 M^{-1} cm^{-1} [17], ε[dienoyl-CoA]$_{260}$ = 20 900 M^{-1} cm^{-1},[17] ε[cyclohex-1-ene-1-carboxyl-CoA]$_{260}$ = 20 700 M^{-1} cm^{-1}, this work).

Isotope Exchange Assay. In the isotope exchange experiments, 0.25 mM dienoyl-CoA and 0.5 mM [*ring*-^{13}C]-benzoyl-CoA were added to 0.45 μM BamC in 500 μL of reaction buffer. Samples (25 μL) were taken at different time points and mixed with 100 μL of methanol. The supernatants were centrifuged twice at 16 000*g*. The samples were desalted by application to a preparative C18 reverse-phase HPLC column (Knauer) using an HPLC separation module (Waters 2695) as described.[16] Final elution was carried out with a mixture of 30% acetonitrile and 70% water. The samples were freeze-dried overnight.

Benzoyl-CoA Reduction Assay with Ti(III)-Reduced (4). The assay contained 5 mM Ti(III)-citrate, 5 mM **4**, and 0.4 mM benzoyl-CoA in 400 μL of reaction buffer. The reaction was started by addition of 1−3 μM BamBC (30 °C). Samples of 50 μL were taken at different time points and stopped by addition of 100 μL of methanol. After centrifugation, the samples were applied onto an HPLC (Waters 2695) in a gradient from 12% to 20% acetonitrile in 50 mM potassium phosphate, pH 6.8, within 13 min.

HPLC-MS/MS Analysis of CoA Esters. All experiments were carried out on an Agilent 1100 series binary HPLC system (Agilent Technologies) coupled with a 4000 QTRAP linear ion trap mass

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spectrometer (AB Sciex) equipped with a TurboIon spray. The MS was operated in negative ion and selected reaction monitoring (SRM) mode. Separation of CoA esters was achieved on a Chromolith SpeedROD RP-18e 50 mm × 4.6 mm from Merck at 40 °C with eluent A (95% 20 mM aqueous ammonium acetate, 5% acetonitrile) and eluent B (50% 20 mM aqueous ammonium acetate, 50% acetonitrile) at a flow rate of 400 μL/min using gradient elution. The characteristic mass transitions for ^{12}C benzoyl-, dienoyl-, monoenoyl-, and cyclohexanoyl-CoA were monitored at 870.2/408.1 *m/z*, 872.2/408.1 *m/z*, 874.2/408.1 *m/z*, and 876.2/408.1 *m/z*, respectively; those for the [*ring*-^{13}C]-equivalents were six mass units higher.

Determination of the Extinction Coefficients and Redox Potential of (4). For the determination of the extinction coefficients of **4**, UV−vis spectra were recorded with a UV-1650PC Shimadzu spectrophotometer in gastight quartz cuvettes under anaerobic conditions. For reduction of **4**, a solution in reaction buffer without BSA was complemented with 0.15 mM dienoyl-CoA in the presence of BamBC (0.04 μM). The extinction coefficients were determined by measuring the change of absorbance upon addition of distinct amounts of a 0.64 μM 2,6-dichlorophenolindophenol stock solution.

The determination of the one-electron redox potential of **4** was performed by cyclic voltammetry on a 25 μL droplet containing 0.5 mM **4** in 50 mM Mops, pH 7.5, using an argon-purged electrochemical cell, with a nitric acid activated glassy carbon working electrode (Le Carbon Lorraine), a Ag/AgCl reference electrode (Radiometer), and a platinum counter electrode as previously described.[23] The electrodes were connected to a PSTAT10 potentiostat (Ecochemie) controlled by GPES software (version 4.7). The scan rate was 10 mV/s. All reported potentials have been recalculated to the normal hydrogen electrode (NHE) by the addition of +0.197 V.

Redox Titration of the Benzoyl-CoA/Dienoyl-CoA Couple. The equilibrium concentrations of dienoyl-CoA and benzoyl-CoA were determined in the presence of BamBC and **4** in reaction buffer. Initial concentrations for dienoyl-CoA varied from 0.05 to 3.0 mM and for oxidized **4** from 0.5 to 20 mM. At different time points, 25−50 μL samples were taken and mixed with 100 μL of methanol. The concentrations of benzoyl-CoA and dienoyl-CoA were determined by HPLC as described. The respective amount of reduced electron acceptor was determined spectrophotometrically using $\varepsilon_{512} = 3700\ M^{-1}\ cm^{-1}$ at concentrations below 4 mM and $\varepsilon_{555} = 2370\ M^{-1}\ cm^{-1}$ at higher concentrations. The equilibrium redox potential was determined according to the Nernst equation for two reversible redox pairs using $E^{0\prime} = -673.5$ mV for the one-electron reduction potential of **4**. The ratio benzoyl-CoA/dienoyl-CoA was plotted against the redox potential, and the curve obtained was fitted to the Nernst equation using the GraphPad Prism4 software package.

Inhibition of BamBC-Catalyzed Dienoyl-CoA Aromatization by Benzoyl-CoA and Monoenoyl-CoA. The determination of the K_i values were performed according to a Dixon plot analysis. The assay was carried out with 0.1, 0.2, and 0.4 mM dienoyl-CoA. In each case, benzoyl-CoA and monoenoyl-CoA were added in concentrations varying from 0 to 2 mM. The reciprocals of the initial reaction rates were plotted against the concentration of the inhibitor (Dixon plot). The K_i values were determined using the GraphPad Prism4 software package.

Results

With the recent discovery of BamBC as the first member of the ATP-independent class II BCRs, a dearomatizing enzyme system became available that opened a door to elucidate the thermodynamic basis of the Biological Birch reduction. The reaction catalyzed by BCRs was considered to operate at the negative redox potential limit in enzyme catalysis.

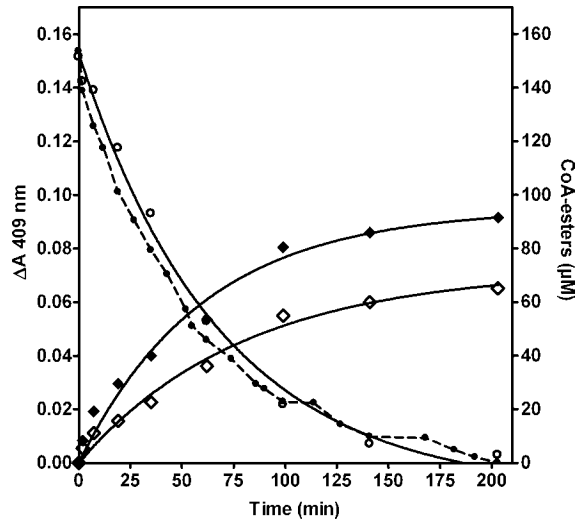

Figure 2. FeS cluster reduction and disproportionation reaction of BamBC. Dotted line: reduction of the iron sulfur clusters as followed by absorbance change at 409 nm (left *y*-axis). Straight lines: increase/decrease of CoA esters as determined by HPLC analysis (right *y*-axis): ○ dienoyl-CoA **2**; ◆, benzoyl-CoA **1**; ◇, monoenoyl-CoA **3**; ●, FeS cluster reduction. The data were fitted to exponential curves using the GraphPad software package.

Disproportionation Reaction. A previous study revealed that only dienoyl-CoA was capable of reducing the four FeS clusters and the tungstopterin cofactor of BamBC. This finding was explained by an extremely low redox potential of both the redox centers of BamBC and the benzoyl-CoA/dienoyl-CoA couple.[17]

To investigate further the electron transfer from dienoyl-CoA to oxidized BamBC, the stoichiometry and kinetics of dienoyl-CoA oxidation/BamBC cofactor reduction were studied in the absence of an external electron acceptor by HPLC (CoA esters) and UV/vis spectroscopy (FeS cluster reduction). The redox centers of purified BamBC were reduced by dienoyl-CoA in a slow reaction with an initial rate of 11 nmol/mg/min FeS clusters reduced as monitored by the time-dependent decrease of absorption at 409 nm (Figure 2). Surprisingly, an approximately 10-fold excess of dienoyl-CoA was required to reduce the FeS clusters of BamBC completely. HPLC analysis of the CoA esters revealed that during BamBC reduction dienoyl-CoA consumption was accompanied by the formation of benzoyl-CoA and cyclohex-1-ene-1-carboxyl-CoA (monoenoyl-CoA **3**, see also Figure 6, Discussion section).

Obviously, the dienoyl-CoA-reduced tungsten cofactor of BamBC transferred electrons to both the FeS clusters and, after benzoyl-CoA/dienoyl-CoA exchange, to a second dienoyl-CoA, thereby reducing it to monoenoyl-CoA. This disproportionation reaction followed the same time course as the FeS cluster reduction but at a 5-fold higher rate; the initial rate was 0.1% of the rate of dienoyl-CoA oxidation with methyl viologen as an electron acceptor (57.2 μmol/min/mg). In a typical experiment, the stoichiometry of the disproportionation reaction was 0.58 mol of benzoyl-CoA and 0.42 mol of monoenoyl-CoA formed per mole of dienoyl-CoA oxidized; it remained constant in the course of the reaction. The imbalance between benzoyl-CoA and monoenoyl-CoA formation can be rationalized by the electrons used for FeS cluster reduction. This finding explains why an excess of dienoyl-CoA was required to reduce BamBC completely. The total recovery of electrons derived from the disproportionation reaction/BamBC reduction was maximally 91%.

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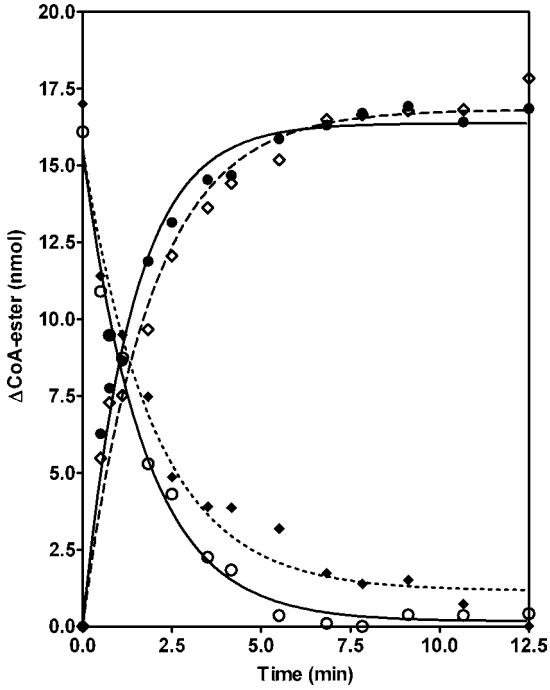

Figure 3. Time course of $^{12}C/^{13}C$ isotope exchange reaction of BamBC. The reaction was started with a 2:1 ratio of [*ring*-^{13}C]-benzoyl-CoA (0.5 mM) and ^{12}C-dienoyl-CoA (0.25 mM) in the presence of BamBC. Samples taken were analyzed by HPLC-MS/MS; for SRM chromatograms, see Supporting Information Figure S1. The *y*-axis shows the difference in the concentrations of the individual CoA esters. Reduction of BamBC by dienoyl-CoA (○) yielded benzoyl-CoA (◇) (straight lines). Reduced BamBC converted [*ring*-^{13}C]-benzoyl-CoA (◆) to [*ring*-^{13}C]-dienoyl-CoA (●) (dotted lines), demonstrating the reversibility of the reaction. The data were fitted to exponential curves using the GraphPad software package.

The monoenoyl-CoA formed during the disproportionation reaction was not further reduced to cyclohexanecarboxyl-CoA **5** by reduced BamBC. It was not oxidized to dienoyl-CoA or benzoyl-CoA using oxidized BamBC and high-potential artificial electron acceptors such as 2,6-dichlorophenol indophenol. These findings suggest that the redox potential of the dienoyl-CoA/monoenoyl-CoA couple was too positive for electron transfer to the active site of BamBC. Both monoenoyl-CoA and benzoyl-CoA served as competitive inhibitors of dienoyl-CoA aromatization with apparent K_i values of 0.2 ± 0.02 mM and 0.34 ± 0.02 mM, respectively. These findings indicate that benzoyl-CoA and monoenoyl-CoA have a considerable affinity for oxidized BamBC.

Aromatization/Dearomatization Reaction without External Acceptors/Donors. To test whether dienoyl-CoA reduced BamBC was competent to catalyze benzoyl-CoA dearomatization, the enzyme was incubated with a mixture of ^{12}C-dienoyl-CoA (0.25 mM) and [*ring*-^{13}C]-benzoyl-CoA (0.5 mM) in the absence of an external electron acceptor. The higher concentration of benzoyl-CoA was used to minimize the disproportionation side reaction. HPLC analysis in conjunction with mass spectrometric analysis of samples taken at different time points revealed the BamBC- and time-dependent decrease of ^{12}C-dienoyl-CoA and [*ring*-^{13}C]-benzoyl-CoA and the concomitant increase of both ^{12}C-benzoyl-CoA and [*ring*-^{13}C]-dienoyl-CoA (Figure 3, Supporting Information Figure S1).

Formation of ^{12}C-benzoyl-CoA from ^{12}C-dienoyl-CoA was via the aromatization reaction described above, whereas the formation of [*ring*-^{13}C]-dienoyl-CoA can only be explained by the reductive dearomatization of [*ring*-^{13}C]-benzoyl-CoA by dienoyl-CoA-reduced BamBC. After completion of the reaction, the initial 2:1 ratio of $^{13}C/^{12}C$ added was equally distributed between benzoyl-CoA and dienoyl-CoA. The initial rate of the isotope exchange was 19.4 μmol/min/mg, which corresponds to 34% of the dienoyl-CoA aromatization rate with methyl viologen as the electron acceptor (57.2 μmol/min/mg). After prolonged incubation, the formation of [*ring*-^{13}C]-monoenoyl-CoA was observed, which can be explained by the slow, BamBC-catalyzed disproportionation of the [*ring*-^{13}C]-dienoyl-CoA formed.

Midpoint Potential of the Benzoyl-CoA/Dienoyl-CoA Redox Couple. The results obtained indicate that in the absence of an external electron acceptor the conversion of dienoyl-CoA to benzoyl-CoA by BamBC was fully reversible. To determine the two-electron reduction potential of benzoyl-CoA in aqueous solution, redox titration experiments were carried out by the determination of the equilibrium concentrations of benzoyl-CoA/dienoyl-CoA and the oxidized/reduced forms of an appropriate redox dye in the presence of BamBC. Methyl viologen ($E^{0\prime} = -456$ mV, 0.5 mM)[24] was completely reduced by 0.2 mM dienoyl-CoA/BamBC without reaching equilibrium. When methyl viologen was replaced by 1,1′,2,2′-tetramethyl viologen (0.2 mM, $E^{0\prime} = -536$ mV as determined by cyclic voltammetry, kind gift of R.K. Thauer, Marburg), still 90−95% of the redox dye was reduced by 0.2 mM dienoyl-CoA in the presence of a surplus of benzoyl-CoA (1 mM) indicating that the equilibrium was just reached. For this reason, a number of bridged 2,2′-bipyridyl salts with redox potentials much more negative than those of nonbridged 4,4′-bipyridyl salts such as methyl viologens were synthesized as potential redox dyes for the redox titration experiment. The 4,4′-dimethyl derivative of the propylene-bridged 2,2′-biypridyl, 2,12-dimethyl-7,8-dihydro-6*H*-dipyrido-[1,2-*a*:2′,1′-*c*][1,4]diazepinediium dibromide **4**, was chosen as a promising candidate for the redox titration experiments as it was readily reduced by dienoyl-CoA/BamBC to the colored free radical (Figure 4a,b). The one-electron reduction potential of **4** in the buffer used was $E^{0\prime} = -673.5$ mV as determined by cyclic voltammetry (Supporting Information Figure S2). One-electron reduced **4** decayed to an unknown species in a slow, linear reaction as evidenced by a time-dependent decrease of the UV/vis spectrum. The rate of this linear background reaction reached maximally 10% of the initial rate of the dienoyl-CoA-dependent reduction of **4**, which allowed a reliable correction for this decay.

The redox titration typically started with the fully oxidized form of **4**, and the time-, dienoyl-CoA-, and protein-dependent reduction of **4** was followed until the equilibrium was reached. At saturating dienoyl-CoA concentrations, the rate of the reduction of **4** by dienoyl-CoA was nearly identical to the one obtained with methyl viologen as acceptor (57.6 μmol/min/mg). The equilibrium concentrations of dienoyl-CoA and benzoyl-CoA were determined by HPLC analysis and those of the oxidized/reduced form of **4** by UV/vis spectroscopy. By varying the initial concentrations of dienoyl-CoA, benzoyl-CoA, and **4**, the equilibrium redox potential was poised to values between −660 and −540 mV. Reduction of **4** by dienoyl-CoA was barely achieved at potentials below −650 mV. Addition of benzoyl-CoA to an equilibrated sample shifted the equilibrium toward the oxidized state of **4**. This finding indicated the presence of a true redox equilibrium which could be reached from the oxidative and reductive direction. The data obtained were fitted

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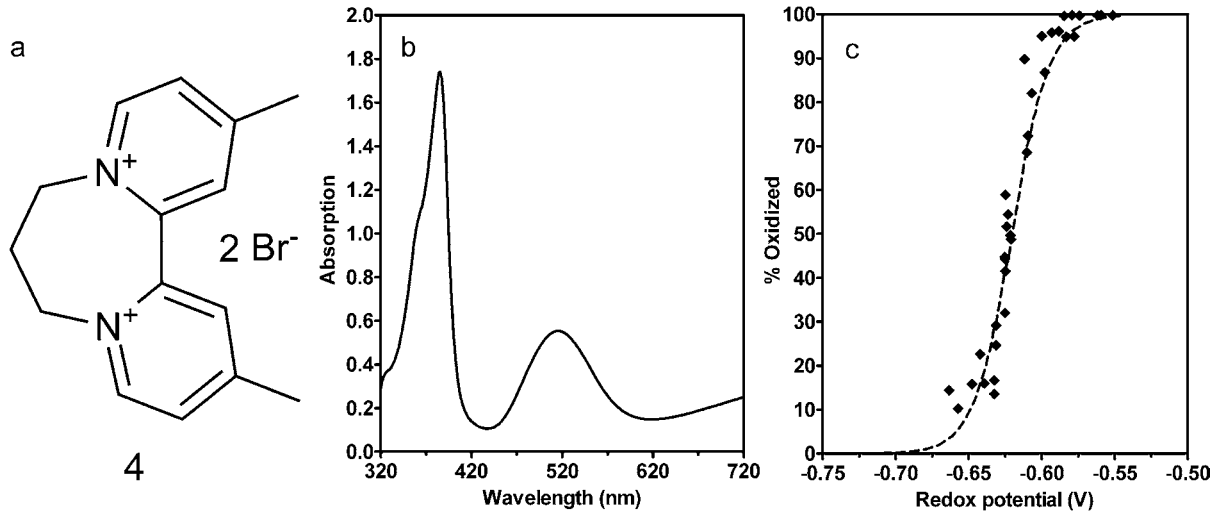

Figure 4. Redox titration of benzoyl-CoA/dienoyl-CoA redox couple in the presence of **4** as redox dye. (a) Structure of **4** used for the redox titration. (b) UV/vis spectrum of BamBC reduced **4** to the free radical with maxima at 385 and 512 nm. (c) Redox titration of the benzoyl-CoA/dienoyl-CoA couple in the presence of **4**. Benzoyl-CoA and dienoyl-CoA concentrations were determined by HPLC, and the concentrations of the oxidized and reduced forms of **4** were determined by UV/vis spectroscopy; the percentage of benzoyl-CoA concentration at the equilibrium is shown (oxidized form of the redox couple). $E^{0\prime}$ of the one-electron reduction potential of **4** was 673.5 mV (Supporting Information Figure S2). The values obtained were fitted to a Nernst equation with $E^{0\prime} = -622$ mV and $n = 2$ with $r^2 = 0.95$.

to a Nernst equation with $E^{0\prime}$ (benzoyl-CoA/dienoyl-CoA) $= -622$ mV, $n = 2$, and $r^2 = 0.95$ (Figure 4c).

Dearomatization Reaction with an External Electron Donor. The full reversibility of the BamBC reaction during the redox titration experiment suggested that dienoyl-CoA-reduced **4** was competent to serve as an electron donor for benzoyl-CoA reduction by BamBC. To test this assumption further, we partially reduced 5 mM **4** with 5 mM Ti(III)-citrate ($E^{0\prime} \sim -720$ mV at pH 7)[25] and used it as the ultimate electron donor for benzoyl-CoA reduction. Notably, Ti(III)-citrate in the absence of **4** did not serve as an electron donor for BamBC-catalyzed benzoyl-CoA dearomatization which is in agreement with earlier observations.[17] HPLC analysis of samples taken at different time points revealed the time-, protein-, and electron donor dependent decrease of benzoyl-CoA and the formation of reduced cyclic products (Figure 5). The expected dienoyl-CoA product was only transiently formed in traces at the beginning of the reaction. In contrast, the four-electron reduced 1- and 2-isomers of monoenoyl-CoA continuously increased and reached a maximal concentration. While the latter remained constant, prolonged incubation resulted in a decrease of the 1-monoenoyl-CoA isomer formed with a concomitant increase of an apolar product, which was identified by mass spectrometry as the six-electron-reduced cyclohexanecarboxyl-CoA (Figures 5 and 6). When benzoyl-CoA was replaced by dienoyl-CoA, a similar product pattern was observed including the transient formation of the 1-monoenoyl-CoA isomer and its conversion to cyclohexanecarboxyl-CoA. This finding confirms that dienoyl-CoA was an intermediate during benzoyl-CoA reduction to monoenoyl-CoA/cyclohexanecarboxyl-CoA.

The initial rate of two-electron transfer to the substrates benzoyl-CoA and dienoyl-CoA was estimated to be 25% of the aromatization/dearomatization with **4** as electron acceptor/donor (14.7 μmol/min/mg). The rate of benzoyl-CoA reduction was highest at pH 7 and could not be increased at higher pH values. Thus, the expected lowering of the redox potential of Ti(III)-citrate at pH values above 7[25] had no stimulatory effect on the benzoyl-CoA reduction rate.

Discussion

The reactions catalyzed by the class II BamBC from *G. metallireducens* and the corresponding rates determined in this work are summarized in Figure 6. The reversible benzoyl-CoA dearomatization/dienoyl-CoA aromatization in the presence of $\mathbf{4}_{ox/red}$ as electron acceptor/donor (redox titration experiment) (Figure 6a), the reversible aromatization/dearomatization reactions in the absence of an electron acceptor ($^{12/13}$C exchange between dienoyl-CoA and benzoyl-CoA) (Figure 6b), the dearomatization of benzoyl-CoA with Ti(III)-citrate reduced **4** as electron donor (Figure 6c), and the disproportionation of dienoyl-CoA to benzoyl-CoA and monoenoyl-CoA in the absence of an external electron acceptor (Figure 6d) are shown. In this work, the forward reaction for a tungsten-containing class II BCR has been demonstrated for the first time.

The full reversibility of benzoyl-CoA dearomatization in aqueous solution (Figure 6a,b) is particularly remarkable as the reversal of the analogous Birch reduction in organic synthesis would couple cyclohexadiene aromatization to the reduction of alkali cations to the corresponding metals. The redox potential of the benzoyl-CoA/dienoyl-CoA couple appears to be at the negative limit for a fully reversible substrate/product couple redox in biochemistry. Due to the lack of electron donors with potentials more negative than -500 mV, electron transfer reactions in living systems below this potential are usually driven by exergonic reactions such as photoactivation [e.g., photosystem I[26]] or ATP hydrolysis [e.g., nitrogenase].[11,13] They are therefore all considered as essentially irreversible under physiological conditions. However, very low potential reversible electron transfer has been reported between redox intermediates in the catalytic cycle of radical/SAM superfamily enzymes. In the case of lysine 2,3-aminomutase, reversible electron transfer for the reductive cleavage of *S*-adenosylmethionine occurs at a

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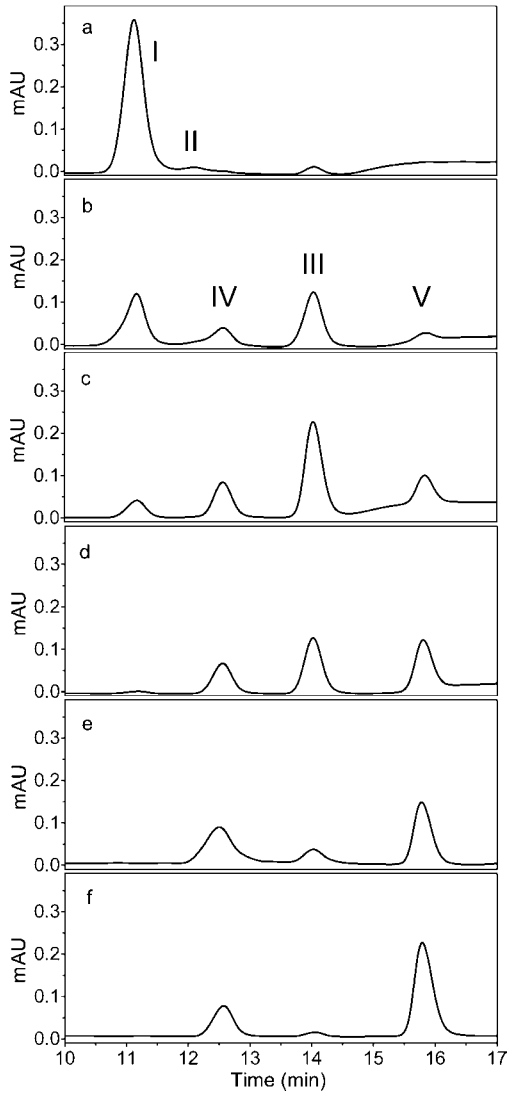

Figure 5. Benzoyl-CoA dearomatization by BamBC with Ti(III)-citrate-reduced **4** as electron donor. HPLC diagrams of CoA ester analysis are shown after (a) 0.5 min, (b) 4 min, (c) 10 min, (d) 21 min, (e) 40 min, and (f) 64 min of incubation. (I) = benzoyl-CoA, (II) = dienoyl-CoA, (III) = 1-monoenoyl-CoA, (IV) = 2-monoenoyl-CoA, (V) = cyclohexanecarboxyl-CoA. The 1- and 2-isomers of monoenoyl-CoA and cyclohexanecarboxyl-CoA were identified by coelution with standards and by mass spectrometric analysis.

[4Fe-4S] cluster at −600 mV,[27] a value comparable to that of the benzoyl-CoA/dienoyl-CoA redox couple. Although benzoyl-CoA dearomatization must be coupled to a currently unknown activation reaction in vivo, the results from both the isotope exchange activity of BamBC and the reversible redox titration in the presence of **4** clearly demonstrate the full reversibility of the reaction in vitro.

Depending on the type and concentration of the electron donor used, BamBC catalyzed either a specific reversible two-electron reduction yielding dienoyl-CoA or the irreversible four- or six-electron reduction to different products. The latter process can be rationalized by an uncoupling of electron transfer to the active site of BamBC and dienoyl-CoA release. Obviously over-reduction of the bound dienoyl-CoA is favored in reduced versus oxidized BamBC. The four- and six-electron reductions of

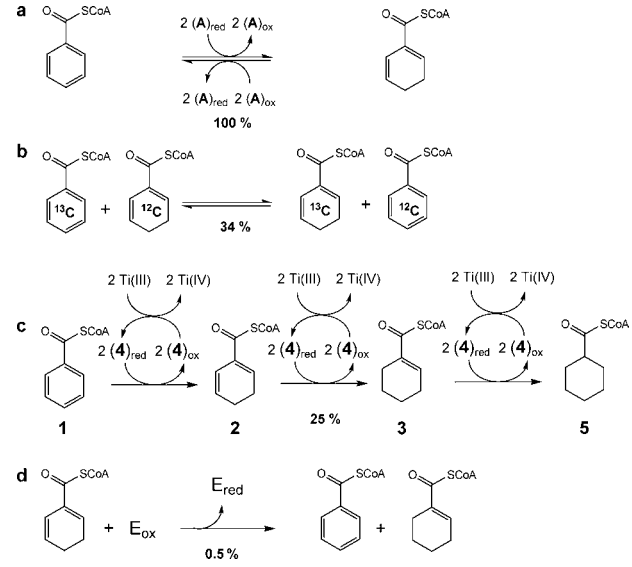

Figure 6. Reactions catalyzed by BamBC. (a) Aromatization/dearomatization of dienoyl-CoA/benzoyl-CoA in the presence of an external electron acceptor/donor (A). With A = methyl viologen, the reaction was essentially irreversible in the direction of benzoyl-CoA formation, whereas with A = **4**, it was fully reversible. The specific activities were similar with both viologens (∼57 μmol/min/mg, referred to as 100%). (b) Reversible aromatization/dearomatization reaction in the absence of an external electron as evidenced by the $^{12}C/^{13}C$ isotope exchange reaction. (c) Dearomatization of benzoyl-CoA in the presence of Ti(III)-reduced **4** (A) to four- and six-electron reduced products. (d) Disproportionation of dienoyl-CoA in the absence of an external electron acceptor with oxidized BamBC. During this reaction, a portion of electrons are transferred to oxidized BamBC; therefore, the ratio of benzoyl-CoA/monoenoyl-CoA formed depended on the enzyme concentration used. The numbers presented below the individual reactions refer to relative specific activities.

benzoyl-CoA have to be considered as artificial reactions in the presence of excess artificial reductant as the subsequent enzymatic steps of the benzoyl-CoA degradation pathway rely on the release of the dienoyl-CoA product.[28,29]

BamB belongs to the family of aldehyde:ferredoxin oxidoreductases (AOR), which usually oxidize aldehydes to the corresponding carboxylic acids with a ferredoxin as the electron acceptor.[30] As the $E^{0\prime}$ values of typical carboxylic acid/aldehyde couples are between −530 and −560 mV, such reactions were not considered to be reversible under physiological conditions.[31] However, previous work provided evidence that this reaction can be at least partially reversed in vitro using low potential artificial electron donors and high concentrations of the carboxylic acid.[32,33] With BamBC, a member of the AOR family catalyzes a fully reversible redox reaction at the even more negative redox potential of $E^{0\prime} = -622$ mV. Tungsten cofactor containing enzymes of the AOR family appear to be the biocatalysts of choice for electron transfer reactions at the negative redox potential limit in enzymatic catalysis.

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The determination of the redox potential of the benzoyl-CoA/dienoyl-CoA couple provides novel insights into the energetics of a key reaction in the anaerobic aromatic metabolism. If H_2 ($E^{0\prime} = -414$ mV for the $2H^+/H_2$ redox couple), or a reduced ferredoxin at a similar redox potential, served as the electron donor for benzoyl-CoA reduction ($E^{0\prime} = -622$ mV), the resulting ΔG^0 for the two-electron reduction would yield a value of around +40 kJ mol^{-1}. Thus, hydrolysis of a single MgATP to MgADP + P_i ($\Delta G^0 \sim -50$ kJ mol^{-1} under cellular concentrations)[34] would be thermodynamically sufficient to drive benzoyl-CoA dearomatization. However, class I BCRs couple electron transfer from reduced ferredoxin ($E^{0\prime} = -435$ mV)[35] to benzoyl-CoA to the hydrolysis of even two MgATP, which can be rationalized based on the mechanism (one ATP hydrolyzed for each electron transferred). Accordingly, benzoyl-CoA dearomatization in ATP-dependent BCRs is essentially irreversible. Class I BCRs are distributed in facultative anaerobes such as denitrifyers or bacteria with an anoxic photosynthesis and so they can afford an ATP hydrolyzing BCR.

Genes coding for ATP-independent class II BCRs have so far only been found in genomes of obligately anaerobic bacteria where energy conservation is at a premium.[17,18,36] In these enzymes, two possible ATP-independent processes for an electron transfer to the aromatic ring were recently discussed and are driven either by a membrane potential or by electron bifurcation.[17] In the case of the former scenario, a coupling to the transport of two Na^+/H^+ across the membrane to the cytoplasm would be sufficient to drive benzoyl-CoA reduction with reduced ferredoxin as the electron donor (assuming $\Delta G^0 = -20$ kJ/mol for the H^+/Na^+ transport). In the case of an electron bifurcation process, an endergonic electron transfer is tightly coupled to an exergonic one.[37,38] Accordingly, reduction of benzoyl-CoA by reduced ferredoxin has to be coupled to that of a second electron acceptor with $E^{0\prime} \geq -220$ mV. If a low-potential ferredoxin with $E^{0\prime} \sim -500$ mV, e.g., generated by 2-oxoglutarate:ferredoxin oxidoreductase, served as an electron donor, even NAD^+ could serve as an appropriate second electron acceptor for a bifurcation driven benzoyl-CoA reduction process. In this context, it is interesting to note that the class II BCR complex from *G. metallireducens* is predicted to contain three modules (BamGHI) with similarities to the soluble NAD^+/NADH-binding modules of NADH:quinone oxidoreductases.[17,18]

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Supporting Information Available: The HPLC/MS analysis of the $^{12/13}C$ exchange reaction (Figure S1) and the cyclic voltammetric determination of the midpoint potential of **4** (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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