Inactive pseudoenzyme subunits in heterotetrameric BbsCD, a novel short-chain alcohol dehydrogenase involved in anaerobic toluene degradation

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Introduction

Anaerobic toluene degradation proceeds by fumarate addition to produce (R)-benzylsuccinate as first intermediate, which is further degraded via β-oxidation by five enzymes encoded in the conserved bbs operon. This study characterizes two enzymes of this pathway, (E)-benzylidenedipropionyl-CoA hydratase (BbsH), and (S,R)-2-(α-hydroxybenzyl)succinyl-CoA dehydrogenase (BbsCD) from Thauera aromatica. BbsH, a member of the enoyl-CoA hydratase family, converts (E)-benzylidenedipropionyl-CoA to 2-(α-hydroxybenzyl)succinyl-CoA and was subsequently used in a coupled enzyme assay with BbsCD, which belongs to the short-chain dehydrogenases/reductase (SDR) family. The BbsCD crystal structure shows a C2-symmetric heterotetramer consisting of BbsC2 and BbsD2 dimers. BbsD subunits are catalytically active and capable of binding NAD+ and substrate, whereas BbsC subunits represent built-in pseudoenzyme moieties lacking all motifs of the SDR family required for substrate binding or catalysis. Molecular modeling studies predict that the active site of BbsD is specific for conversion of the (S,R)-diastereomer of 2-(α-hydroxybenzyl)succinyl-CoA to (S)-2-benzoysuccinyl-CoA by hydride transfer to the re-face of nicotinamide adenine dinucleotide (NAD)⁺. Furthermore, BbsC subunits are not engaged in substrate binding and merely serve as scaffold for the BbsD dimer. BbsCD represents a novel clade of related enzymes within the SDR family, which adopt a heterotetrameric architecture and catalyze the β-oxidation of aromatic succinate adducts.
use the same principal pathway for anaerobic toluene catabolism.

The initial step of anaerobic toluene catabolism is the highly unusual reaction of benzylsuccinate synthase (BSS; EC 4.1.99.11), which adds the double bond of a fumarate cosubstrate to the methyl group of toluene in a radical-based reaction, producing specifically the (R)-enantiomer of benzylsuccinate (Fig. 1) [4,6,9,10,11,12,13]. BSS is a glycylic radical enzyme consisting of three subunits and carries two [Fe₄S₄]-clusters per αβγ-unit [9,14]. The glycylic radical is essential for catalysis and is proposed to initiate the abstraction of a hydrogen atom from toluene via a conserved cysteine in close vicinity at the active site [15–20]. BSS is activated to the radical-containing state by the separate activating enzyme BssD (EC 1.97.1.4), which belongs to the family of S-adenosylmethionine-dependent radical enzymes [15] and is encoded in the conserved bss operon together with the genes for the three subunits of BSS and further genes of unknown function (Fig. 1) [9,21].

The first intermediate (R)-benzylsuccinate is further degraded via a specific β-oxidation pathway (Fig. 1) [11,22] involving five enzymes, which are encoded in a second conserved operon (bbs for β-oxidation of benzylsuccinate) in all anaerobic toluene degraders with known genome sequences. This part of the pathway starts with a succinyl-CoA-dependent CoA-transferase (BbsEF; EC 2.8.3.15) [11,23] activating (R)-benzylsuccinate to 2-(R)-benzylsuccinyl-CoA, followed by 2-(R)-benzylsuccinyl-CoA dehydrogenase (BbsG; EC 1.3.8.3), which generates the first oxidized intermediate of the pathway, 2-(E)-benzylidenesuccinyl-CoA [24]. Together with benzylsuccinate, (E)-benzylidenesuccinate was initially detected as dead-end metabolite in the culture supernatants of anaerobic toluene-degrading bacteria [2,26]. Later on, free (R)-benzylsuccinate was proven to be an actual intermediate of toluene degradation, whereas (E)-benzylidenesuccinate apparently originates from hydrolysis of the CoA thioester intermediate [4,9].

β-Oxidation of (E)-benzylidenesuccinate is proposed to continue with an enzyme of the enoyl-CoA hydratase (ECH) family and a short-chain alcohol dehydrogenase, which are encoded in all known bbs operons [12,22]. The bbsH gene codes for an ECH proposed to catalyze the addition of water to the double bond of 2-(E)-benzylidenesuccinyl-CoA to generate the alcohol intermediate 2-(α-hydroxybenzyl)succinyl-CoA (EC 4.2.1.-). Moreover, the bbs operons always contain two genes coding for apparent subunits of short-chain alcohol dehydrogenases (bbsCD), which are proposed to be involved in the subsequent oxidation of the alcohol intermediate to benzylsuccinyl-CoA, the keto-intermediate of the pathway (EC 1.1.1.-) [22]. It is unknown whether both gene products or only one constitute the subunits of this enzyme. The last step of benzylsuccinate β-oxidation is catalyzed by a 2-benzoylsuccinyl-CoA thiolase (EC 2.3.1.-) encoded by the bbsAB genes [22], which cleaves benzoylsuccinyl-CoA to succinyl-CoA, the CoA-donor for benzylsuccinate activation, and benzoyl-CoA, the central intermediate of anaerobic degradation of most aromatic compounds (Fig. 1).

In addition to toluene metabolism, several other anaerobic degradation pathways have been reported to be initiated by fumarate addition to a methyl group at an aromatic ring in various bacteria, namely, for m-xylene [10,20,27], p-cresol [28], 2-methylnaphthalene [29] or p-cymene [30]. In all these cases, bss- and bbs-like operons are present in the respective genome.

Fig. 1. Pathway of anaerobic toluene catabolism and organization of the corresponding operons in Thauera aromatica. (A) Organization of the bss and bbs operons. (B) Reactions of the toluene degradation pathway. The gene products involved are indicated in the same colors as the coding genes above. Note that involvement of BssD (pink) is essential as activating enzyme for BssABC. The intermediates studied in this report are (E)-benzylidenesuccinyl-CoA (1), (S,R)-2-(α-hydroxybenzoyl)succinyl-CoA (2), and (S)-2-benzoylsuccinyl-CoA (3). Their stereochemistry has been inferred from previous reports [24,25] and this study. Note that the (R)-conformation of the benzylic C-atom in 2 is equivalent to aliphatic (S)-3-hydroxyacyl-CoAs, which is commonly found in β-oxidation pathways.
sequences, which code for a BSS-like enzyme and its activating enzyme and for the enzymes of a conserved β-oxidation pathway, respectively, that are involved in degrading the respective compounds [13,31]. In this communication, we report on the biochemical and structural features of the third and fourth enzyme of the β-oxidation pathway for benzylsuccinate, 2-(E)-benzylidenesuccinyl-CoA hydratase (BbsH) and (S,R)-2-(α-hydroxybenzyl)succinyl-CoA dehydrogenase (BbsCD) from \( T.\ aromatica \).

**Results**

**Synthesis of (E)-benzylidenesuccinate and CoA-thioesters**

Since (E)-benzylidenesuccinate is not commercially available, it was synthesized chemically from benzaldehyde and dimethylsuccinate as described in methods. The synthetic procedure resulted in 10.6 g of pure (E)-benzylidenesuccinic acid with a yield of 78%, which was characterized via its UV/Vis spectrum and behaved identically to the material synthesized previously via a different protocol [24]. The compound was subsequently converted to the internal anhydride, which served as starting compound for the synthesis of thioester derivatives with either CoA or its shortened analogue N-acetylcysteamine (NAC) as described previously [11]. The identity of the produced thioesters was verified via UV-Vis spectroscopy and HPLC analysis. Two product peaks with identical UV-Vis spectra eluting at 2.5 and 3.5 min were detected after conversion with CoA, but only the later eluting compound (43% yield relative to CoA) was turned over by (E)-benzylidenesuccinyl-CoA hydratase and therefore represents the intermediate of the pathway, (E)-2-benzylidenesuccinyl-CoA. The earlier-eluting compound (32% yield relative to CoA) represents the wrong regioisomer, (E)-3-benzylidenesuccinyl-CoA. The thioesters were collected separately, lyophilized and stored. While most tests reported in this study were performed with purified (E)-2-benzylidenesuccinyl-CoA, we cannot exclude the presence of traces of the wrong regioisomer or of free CoA in the substrate solution, especially since both thioesters were rather sensitive against hydrolysis and showed increasing decay after storage for more than 3 months. Because of the rapid hydrolysis in neutral or alkaline solutions, enzyme assays with the (E)-benzylidenesuccinyl-CoA regioisomers were only possible under mildly acidic conditions (pH < 6.9). Conversion of (E)-benzylidenesuccinane anhydride with NAC yielded a single product peak after HPLC analysis which apparently represents the mixture of both possible regioisomers. As observed for the CoA thioesters, (E)-benzylidenesuccinyl-NAC was rapidly hydrolyzed at pH values > 6.9. Experimental extinction coefficients (\( \epsilon_{290} \)) of (E)-2-benzylidenesuccinyl-CoA prepared from two batches of independently synthesized benzylidenesuccinic acid were determined as 5600 and 5800 M\(^{-1}\)·cm\(^{-1}\), respectively, whereas \( \epsilon_{290} \) of (E)-2-benzylidenesuccinyl-NAC was determined as 7230 M\(^{-1}\)·cm\(^{-1}\). Enzyme activities were calculated based on the \( \epsilon_{290} \) values of the preparation used in the respective assay.

**Characterization of (E)-2-benzylidenesuccinyl-CoA hydratase (BbsH)**

All known anaerobic toluene-degrading bacteria contain a gene for a BbsH orthologue in their bbs operons, an ECH implicated in β-oxidation of benzylsuccinate [32,33]. The corresponding gene product is predicted to catalyze the hydration of (E)-2-benzylidenesuccinyl-CoA to the corresponding alcohol intermediate, 2-(α-hydroxybenzyl)succinyl-CoA [22]. The \( bbsH \) gene from \( T.\ aromatica \) was recombinantly expressed in \( E.\ coli \) BL21(DE3), and the produced protein (UniProt Q9KJE7) was purified via anion exchange chromatography on DEAE-Sepharose and subsequent chromatography on hydroxyapatite. Starting from 5 g of cells (wet mass), 7 mg of purified enzyme were obtained, which showed a specific activity of 27 ± 4 μmol·min\(^{-1}\)·(mg enzyme\(^{-1}\), corresponding to a 19-fold enrichment compared to cell extract (for details, see Table 1). In the course of purifying the

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**Table 1. Purification of (E)-2-benzylidenesuccinyl-CoA hydratase (BbsH).** Enrichment and yield were calculated based on the apparent enzyme activities in cell extracts of \( E.\ coli \) with overproduced BbsH. Due to thioesterase activities, the actual specific activities are supposed to be higher.

| Fraction             | Protein [mg] | Activity[a] | Specific activity[b] | Enrichment [fold] | Yield [%] |
|----------------------|-------------|-------------|---------------------|-------------------|----------|
| Cell extract         | 562         | 753         | 1.4                 | 1                 | 100      |
| DEAE pool            | 42          | 338         | 8                   | 6                 | 45       |
| Hydroxyapatite pool | 7           | 189         | 27                  | 19                | 25       |

[a]μmol·min\(^{-1}\); [b]μmol·min\(^{-1}\)·(mg protein\(^{-1}\)).
enzyme, it turned out that the photometrically recorded apparent activities in cell extracts contained significant unspecific background caused by *E. coli* thioesterases. Only after the first chromatographic separation, the fractions containing *(E)-2-benzylidenedisuccinyl-CoA* hydratase (called BbsH in the following) activity were free of thioesterase activity as tested by HPLC analysis.

The photometric assay to determine BbsH activity was based on the characteristic absorption properties of *(E)-2-benzylidenedisuccinyl-CoA* at 290 nm, which reflect the presence of the double bond in conjugation to the thioester carbonyl group [24]. The double bond is lost during the hydratase reaction, and therefore, the enzyme activity can be monitored by the loss of absorption at 290 nm. Because of the rapid hydrolysis of the thioesters at higher pH values, which leads to an increasing nonenzymatic background of absorption decrease at 290 nm, assays had to be performed in a pH range of 5.8–7.0 and were always accompanied with parallel controls without enzyme. Enzyme activity was based on the rate difference between the assays containing enzyme and the controls. An optimum pH of 6.2 was determined for the reaction, which allowed maximal turnover rates without significant thioester hydrolysis. Product formation from *(E)-2-benzylidenedisuccinyl-CoA* (1) was additionally verified by HPLC analysis, which showed the conversion of the substrate to an earlier-eluting product (2), which had lost absorption at 290 nm (Fig. 2A,B). Compound 2 showed a main peak at m/z 971.9 in HPLC-MS analysis, which matches the expected mass of 972.7 for the anion of 2-(α-hydroxybenzyl)succinyl-CoA.

Enzyme kinetics of BbsH was measured with the natural CoA thioesters as well as with the shorter NAC thioesters of *(E)-2-benzylidenedisuccinate*. The CoA and NAC thioesters were both accepted as substrates and yielded essentially the same results. The obtained data were plotted and analyzed by nonlinear curve fitting, using the equations for Michaelis–Menten and Hill kinetics with or without substrate inhibition. The best fit was obtained with a Hill kinetic model with integrated substrate inhibition according to Eqn 1 [34] (Table 2 and Fig. 2D).

![Fig. 2. Properties of *(E)-2-benzylidenedisuccinyl-CoA* hydratase (BbsH).](image)

(A) HPLC analysis of substrate and product. The upper chromatogram shows the substrate (1) directly after the start of the reaction (t = 0 min), the lower chromatogram the product formed after 15 min (2). (B) UV/Vis spectra of substrate and product (solid line: substrate *(E)-2-benzylidenedisuccinyl-CoA*, 1; dotted line: product 2-(α-hydroxybenzoyl)succinyl-CoA, 2). The arrow points to the wavelength used for enzyme assays (290 nm), which is indicative for the hydration of *(E)-2-benzylidenedisuccinyl-CoA*. (C) Mass spectrum of the product peak representing 2-(α-hydroxybenzoyl)succinyl-CoA. (D) Kinetic properties of BbsH with *(E)-benzylidenedisuccinyl-CoA* (regiosomer mixture). Error bars represent standard deviations, n = 3. Almost identical kinetic behavior was obtained with *(E)-benzylidenedisuccinyl-N-acetylcysteamine* (data not shown). (E) Homology model of the BbsH trimer with bound substrate, based on the closest known structural orthologue, methylthioacryloyl-CoA hydratase. (F) Detail view of the predicted active site of BbsH with its conserved catalytic residues, E110 and E130, and *(S,R)-2-(α-hydroxybenzyl)succinyl-CoA*. Structures are visualized using PYMOL 2.1.
Table 2. Kinetic parameters of (E)-2-benzylidene succinyl-CoA hydratase with different substrates.

|            | (E)-2-benzylidene succinyl-CoA | (E)-2-benzylidene succinyl-CoA |
|------------|---------------------------------|---------------------------------|
|            | N                               | N                               |
| Hill coeff. N | 4.0                             | 4.0                             |
| K_{0.5} [µM]  | 105 ± 4                         | 101 ± 3                         |
| V_{max} [µmol·min^{-1}·mg^{-1}] | 31.5 ± 0.5                     | 31 ± 2                          |
| K_{m} [µM]   | 230 ± 4                         | 226 ± 9                         |
| Inhibition coefficient M | 0.8 ± 0.1                      | 0.76                             |

The obtained kinetic parameters showed identical apparent values for both substrates, exhibiting a K_{0.5} value of 105 ± 4 µM, a V_{max} of 31.5 ± 0.5 µmol·min^{-1}·mg^{-1}; and a Hill coefficient (N) of 4, accompanied by identical substrate inhibition parameters, represented by a K_{m} constant of 230 ± 4 µM with an exponent (M) of 0.80 ± 0.1. These values indicate that substrate conversion by BbsH is positively cooperative, with a very high Hill coefficient, whereas substrate inhibition shows slightly negative cooperativity. At this point, we cannot exclude that the presence of the wrong regioisomer or free CoA/NAC in the substrate mixtures may have affected the substrate inhibition or cooperativity parameters.

The native molecular mass of BbsH was determined via size exclusion chromatography and Ferguson plot analysis after native PAGE, yielding apparent native masses of 110 ± 7 kDa and 123 ± 16 kDa, respectively. With a subunit mass of 28 kDa, these data fit best to a BbsH tetramer, but we interpret these data to indicate a trimeric composition of BbsH, because all known members of the well-characterized crotonase/ECH family occur as trimers and hexamers [35–37]. Accordingly, a structural model of a BbsH trimer was derived from the trimeric substructure of the methylthioacyrl-CoA hydratase (DmdD) hexamer with bound substrates, which is involved in dimethylsulfoniopropionate degradation (4IZD; 31% sequence identity) [36] (Fig. 2E). The BbsH model predicts a position of the substrate (E)-2-benzylidene succinyl-CoA in its active site that results in close contacts to the two conserved active site residues E110 and E130 (Fig. 2F).

Characterization of 2-(α-hydroxybenzyl)succinyl-CoA dehydrogenase (BbsCD)

In anaerobic toluene-degrading bacteria, all bhs operons coding for β-oxidation enzymes of benzylocetic acid contain two genes for subunits of a short-chain alcohol dehydrogenase, bbsC and bbsD (UniProt numbers Q9KJF2, Q9KJF1). Accordingly, one or both of the corresponding gene products were postulated to constitute the enzyme oxidizing 2-(α-hydroxybenzyl)succinyl-CoA to 2-benzoysuccinyl-CoA [22,32]. The bssCD genes from T. aromatica were cloned behind an IPTG-inducible trc promoter in pTrc99a and recombinantly expressed in E. coli DH5α, yielding large amounts of BbsC and BbsD in soluble form. The BbsCD complex was then purified via anion exchange chromatography on DEAE-Sepharose and subsequent chromatography on hydroxyapatite, leading to an essentially homogeneous preparation containing both BbsC and BbsD subunits in equal amounts (Table 3). We also constructed an analogous expression vector containing only bbsD with an N-terminal Strep-tag sequence by deletion of the bbsC gene from the expression vector via inverse PCR and religation, but did not obtain any produced protein with this plasmid.

The native molecular mass of BbsCD was determined via gel permeation chromatography and Ferguson plot analysis of native polyacrylamide gels, yielding apparent native masses of 61 ± 5 kDa and 58 ± 3 kDa, respectively. With subunit sizes of 26 kDa (BbsC) and 28 kDa (BbsD), this would best correspond to a dimeric or trimeric structure. However, after determining the actual X-ray structure of the enzyme, the composition of BbsCD was revealed as a stable α₂β₂ heterotetramer (see below), suggesting that the enzyme showed aberrant migration behavior in the tests used for native size determination.

Neither the substrate nor the product of the proposed reaction is commercially available or can be synthesized via a simple procedure. Therefore, we used a coupled enzyme assay for BbsCD, using the (E)-2-benzylidene succinyl-CoA hydratase BbsH as auxiliary enzyme to supply the substrate of BbsCD by conversion of (E)-2-benzylidene succinyl-CoA. As expected from previous experiments in cell extracts of *Thauera aromatica* [11], BbsCD accepted nicotinamide adenine dinucleotide (NAD)^* in, but not NADP^* as electron acceptor. Because of the strong sensitivity of the substrate against hydrolysis, the assay had to be performed at slightly acidic pH, and a pH optimum of 6.2 was determined for the coupled assay, the same as for the reaction of BbsH alone. The assays were started with acidic stock solutions of (E)-2-benzylidene succinyl-CoA and then monitored for absorbance increase at 365 nm due to NAD^* reduction. Remarkably, almost identical BbsCD activities were recorded in the presence or absence of the coupling enzyme BbsH as long as *E. coli* cell extracts containing BbsCD were used. The reaction
became only dependent on the addition of BbsH when purified fractions of BbsCD were used, suggesting that an *E. coli* enzyme unspecifically catalyzed the hydration of (E)-2-benzylidene succinyl-CoA. Moreover, the determination of activities in crude extracts was hampered by CoA-thioesterases, leading to less reliable apparent activity values as those measured with purified enzyme batches. Starting from 8.9 g of cells (wet mass), 14 mg of purified BbsCD was obtained, which showed a specific activity of 0.86 ± 0.01 mol·min⁻¹·(mg enzyme)⁻¹. Due to the competing thioesterases in the cell extract, the calculated enrichment factor of 3.1-fold is certainly underestimated (Table 3). The final BbsCD fraction was free of contamination by thioesterases or ECHs and homogeneous as judged by SDS/PAGE analysis.

The products formed in the coupled assay with BbsH and BbsCD were analyzed by HPLC analysis (Fig. 3A), indicating an initial conversion of (E)-2-benzylidene succinyl-CoA (1) to 2-(α-hydroxybenzyl) succinyl-CoA (2) catalyzed by BbsH, and the production of another later eluting CoA-thioester (3), which was expected to correspond to 2-benzoylsuccinyl-CoA (Fig. 3A). Our attempts to verify the identity of this new product by HPLC-MS analysis were unsuccessful, but we showed the presence of a keto group in (3) by adding phenylhydrazine (5) to the assay to convert it to a stable hydrazone (4). This resulted in a large shift of the elution position, implying the identity of (3) as benzyloxsuccinyl-CoA (Fig. 3A).

Enzyme kinetic measurements with BbsCD were performed using the coupled assay with tenfold excess of BbsH (by activity), and using (E)-2-benzylidene succinyl-CoA as starting substrate. We assumed that under these conditions conversion of (E)-2-benzylidene succinyl-CoA to 2-(α-hydroxybenzyl)succinyl-CoA is so much faster than the subsequent reaction catalyzed by BbsCD that it does not interfere in the BbsCD kinetics. With this constraint, we observed an apparent saturation kinetics of BbsCD activity with increasing substrate concentration, which was very well described by curve fitting with the Michaelis–Menten equation. The kinetic parameters were determined as apparent 

$$K_m = 96 \pm 8 \mu M$$ and an apparent $$V_{\text{max}} = 0.96 \pm 0.1 \mu \text{mol} \cdot \text{min}^{-1} \cdot (\text{mg protein})^{-1}$$ (Fig. 3B).

We also screened for potential artificial substrates of BbsCD by assaying the activity of purified enzyme for catalyzing the NADH-dependent reduction of aromatic ketones like acetophenone and chlorinated derivatives, which mimic the natural product benzoysuccinyl-CoA. BbsCD showed no reducing activity with acetophenone, but two chlorine-containing derivatives, 2,2-dichloroacetophene and 2,4'-dichloroacetophenone, were reduced with specific activities of 3.5 ± 1 \mu mol·min⁻¹·(mg protein)⁻¹ and 7.0 ± 2 \mu mol·min⁻¹·(mg protein)⁻¹, respectively. The pH optimum for reduction of these artificial substrates was at pH 6.2, like the one for the forward reaction. However, the kinetic behavior of these reactions only fitted to highly cooperative kinetic models, revealing apparent Hill coefficients of 4 (Fig. 3C).

### Structural investigation of BbsCD as a heterotetramer with \(\alpha_2\beta_2\) topology

The complex structure of BbsCD from *T. aromatica* with bound NAD⁺ was solved at a resolution of 2.25 Å. The final refinement provided a model with \(R_{\text{work}}\) and \(R_{\text{free}}\) factors of 0.184 and 0.210, respectively (see Table 4). Electron density is defined for the BbsC chains from G7 to I250 and for the BbsD chains from G2 to G248. Edman sequencing of the two proteins from a proteomic analysis of tolune-induced proteins has revealed before that BbsD lacks the N-terminal methionine, while BbsC had a blocked N terminus [22]. BbsCD is a heterotetramer comprising two BbsC and two BbsD polypeptides. Given that BbsC and BbsD belong both to the SDR protein family, they are structurally very similar with an r.m.s.d. of 0.96 Å for 212 Cα positions. Despite their common protein fold, the BbsCD tetramer contains only two NAD⁺ molecules, which are bound to the BbsD subunits with well-defined omit electron densities. Unfortunately, we were not able to generate BbsCD crystals, in which the substrate, 2-(α-hydroxybenzyl)succinyl-CoA, or substrate analogues were present. As reported before for

### Table 3. Purification of 2-(α-hydroxybenzyl)succinyl-CoA dehydrogenase (BbsCD). Enrichment and yield were calculated based on the apparent enzyme activities in cell extracts of *E. coli* after heterologous production.

| Fraction         | Protein [mg] | Activity a | Specific activity b | Enrichment [fold] | Yield [%] |
|------------------|--------------|------------|---------------------|-------------------|-----------|
| Cell extract     | 763          | 250        | 0.28                | 1                 | 100       |
| DEAE pool        | 150          | 48         | 0.32                | 1.2               | 22        |
| Hydroxyapatite pool | 14          | 12         | 0.86                | 3.1               | 5.5       |

\(a[\mu\text{mol} \cdot \text{min}^{-1}]\), \(b[\mu\text{mol} \cdot \text{min}^{-1} \cdot (\text{mg protein})^{-1}]\).
phenylhydrazine. Compounds and retention times: 15 min; bottom: same products after derivatization with benzylidenesuccinyl-CoA (3.8 min), acyl carrier protein (ACP) reductase [38], BbsCD is the heterotetrameric human mitochondrial 3-ketoacyl-acyl carrier protein (ACP) reductase [38], BbsCD is arranged in a symmetry-breaking composition of homodimeric α2 and β2 components. Moreover, the subunits BbsC and BbsD are twisted toward each other by 90° in horizontal and 180° in vertical direction (Fig. 4A). All other members of the SDR family with solved structures represent homotetramers [34,39,40,41,42].

The BbsCD subunits comprise a Rossmann fold with its central seven-stranded parallel β-sheet element and six flanking α-helices (Fig. 4B), which is typical for NAD+ binding proteins like the members of the SDR family [40,43,44]. Adjacent to the Rossmann fold, four 310-helices are present which follow directly after strands 4-7 of the β-sheet in the sequence. Despite their structural similarity, the BbsC and BbsD subunits differ for their additional α-helices at the periphery of the Rossmann fold: BbsC contains only one (α7), whereas BbsD contains two helices (α6 and α7; Fig. 4B).

Symmetric interactions within the BbsC dimer are mainly stabilized by hydrogen bonds between the α5-helices of the respective monomers (S160, G164); in contrast, the BbsD dimer is stabilized by polar interactions of the α4-helices (E103, D107, K115). In both

### Table 4. Statistics for data collection and refinement of BbsCD.

|                  | BbsCD-NAD+ (7PCS) | BbsCD:Gd(OAc)3 |
|------------------|-------------------|----------------|
| Wavelength [Å]   | 0.894             | 1.711          |
| Space group      | P 31              | P 31           |
| Cell dimensions  | 68.8, 68.6, 192.0; 69.8, 69.8, 192.6 |  |
| Resolution [Å]   | 43.54–2.25        | 44.01–2.65     |
|                  | (2.37–2.25)       | (2.79–2.65)    |
| Measured & unique reflections | 155211, 46066 | 158730, 30477 |
|                  | (22492, 6818)     | (23792, 4508)  |
| Rmerge           | 0.049 (0.39)      | 0.060 (0.53)   |
| Wilson B factor [Å²] | 37.6             | 49.1           |
| Mosaicity [%]    | 0.156             | 0.122          |
| Completeness [%] | 96.1 (96.4)       | 99.9 (99.9)    |
| Solvent content [%] | 52.3             |                |
| Multiplicity     | 3.4 (3.3)         | 5.2 (5.3)      |

Refinement

|                  | 29.7–2.25 |  |
| Rwork, Rfree [%] | 18.4, 21.0 |  |
| Reflections (work, test) | 45095, 961 |  |
| Residues         | 994       |  |
| B factor all [Å²] | 52.7     |  |
| Protein, NAD+ [Å²] | 53.3, 36.5 |  |
| Water [Å]        | 43.7      |  |
| Water molecules  | 297       |  |
| r.m.s.d. bonds [Å] | 0.006    |  |
| r.m.s.d. angles [°] | 0.772   |  |
subunits the first and the second of the 310-helices contribute to structural stabilization by forming interactions with the α5-helix. The asymmetric interactions between the BbsC and BbsD homodimers involve mainly the loop region between α8 and β7 of BbsC1 and the β7 strand with its preceding loop of BbsD1 (and vice versa between BbsC2 and BbsD2).

The BbsCD tetramer harbors two catalytically incompetent BbsC pseudoenzyme subunits

Although the BbsC and BbsD subunits exhibit almost identical Rossmann folds, the BbsC subunits are found by our analyses to act only as built-in pseudoenzyme subunits, as they are unable to bind NAD⁺ or substrate on their own. While the catalytically active BbsD subunits contain the characteristic Ser-Tyr-Lys triad necessary for catalysis in enzymes of the SDR family [40,44,45,46], BbsC is not only missing this conserved sequence motif (see below), but also the GXXXXGXG dinucleotide binding motif for NAD⁺ binding (BbsD: G12-G18; [43]) and the conserved aspartate (BbsD: D36) that determines NAD⁺ specificity by interacting with the 2’- and 3’-hydroxyl group of the adenosine ribose moiety [47] (Fig. 5A). Instead, three bulky residues (D17, E92 and M185) block the active site of BbsC. Calculation of the electrostatic surface potentials of the subunits shows a normal NAD⁺ binding pocket in BbsD, but a highly negatively charged counterpart in BbsC which is incompatible for interacting with the negatively charged diphosphate moiety of NAD⁺ (Fig. 5B).

MD analyses of BbsCD benzoylsuccinyl-CoA-NADH define stereochemistry of β-oxidation of benzylsuccinate

Given the absence of stereochemical information about the configurations of the BbsCD substrate and product, 2-(α-hydroxybenzyl)succinyl-CoA or benzoylsuccinyl-CoA, we performed molecular dynamics (MD) analysis of the BbsCD-NADH complex with either (S)- or (R)-configured benzoylsuccinyl-CoA to determine its stereoisomer preference. To define initial structures of these ternary BbsCD complexes, we used our structure of the BbsCD-NAD⁺ complex and the homologous
suggesting that the (\(S\))-benzoylsuccinyl-CoA complex shows that the pro-(S) hydrogen of the C4-carbon of NADH points toward the si-side of the carbonyl group of (S)-benzoylsuccinyl-CoA (H\(_S\) – carbon distance: 2.92 ± 0.17 Å), leading to the formation of (S,\(R\))-configured 2-(\(\alpha\)-hydroxybenzyl)succinyl-CoA after reductive hydride transfer. From this analysis, we may also infer that this diastereoisomer is the cognate product generated by the previous enzyme of the pathway, BbsH (Figs 1 and 2F) [44,45].

Substrate recognition by BbsCD

The conserved catalytic mechanism within the enzymes of the SDR family involves a catalytic triad generally consisting of S141, Y153 and K157 [35,36,43,47]. These residues are all preserved in BbsD, but not in BbsC (Fig. 6C). The tyrosine plays a major role in the oxidation/reduction reactions, whereas the lysine helps to position the NAD\(^+\), and the serine forms hydrogen bonds to the substrate/intermediate/product [44,46,48,49]. Accordingly, in the BbsCD-NAD\(^+\) structure K157 forms an intimate hydrogen bond to the 3'-hydroxyl of the nicotinamide nucleotide (3.0 Å), and likewise the hydroxyl group of Y153 bonds to the 2'-hydroxyl group (2.6 Å). Furthermore, our MD simulations show the formation of the expected hydrogen bond between the hydroxyl group of S141 and the carbonyl oxygen of (S)-benzoylsuccinyl-CoA (2.9 Å). Other predicted interactions between (S)-benzoylsuccinyl-CoA and BbsD include a salt bridge between the product’s carboxymethyl moiety and R142 (Fig. 6D, C-C distance between guanidino and carboxyl groups 4.5 Å). The phenyl group of (S)-benzoylsuccinyl-CoA is positioned in a hydrophobic pocket of BbsD made up by the side chains of M190, W191 and L194. This pocket is created during substrate binding by a blockwise movement of the helical segment S189-E193 toward (S)-benzoylsuccinyl-CoA (Fig. 6B, D). Interestingly, we did not observe any strong interactions in our MD simulations between the BbsC subunits and the CoA tail of (S)-benzoylsuccinyl-CoA, which might have been expected based on the structures of other 3-hydroxyacyl-CoA dehydrogenases [40,50,51], but we cannot exclude such contacts in the absence of a real structure of the ternary complex.

Phylogenetic analysis of BbsCD

Close orthologues of the BbsCD subunits occur in many known anaerobic hydrocarbon-degrading bacteria, and
they are always encoded by a pair of adjacent genes within larger operons. Moreover, all of these proteins are localized in \textit{bbs}-like apparent operons, which always contain at least one additional gene coding for a BbsH ortholog, and in most cases all other genes for orthologues of BbsA-H (Fig. 1). Some of these operons are involved in anaerobic toluene degradation, others have been implicated in other anaerobic degradation pathways initiated by fumarate addition, for example, for \textit{p}-cresol, \textit{p}-cymene or 2-methylnaphthalene [13,30, 52,53,54,55]. BbsC and BbsD form related, but distinct branches within the SDR enzyme family (Fig. 7, red

Fig. 6. Stereochemical preference and substrate recognition of BbsCD. (A) MD analysis of (S)- or (R)-benzoylsuccinyl-CoA binding at the active site of BbsCD. Substrate binding dynamics was calculated over 3.8 or 5.2 µs, respectively, and the distance of the carbonyl group to the NADH cofactor is shown as measure for binding stability. (B) Overlay of nine representative conformations of BbsCD with bound NADH and (S)-benzoylsuccinyl-CoA from the MD analysis. The determined X-ray structure without bound substrate is shown in black. Note the large conformational shift of the helical domain S189.E193 of BbsD upon substrate binding (lower right). (C) Detail view of nine different conformations of NADH and (S)-benzoylsuccinyl-CoA in the active site from the MD analysis. Note the indicated glycerol molecule of the determined original structure (black C atoms), which interferes with the bound substrate molecules and had to be removed prior to the MD analysis. All observed conformations allow appropriate positioning of the residues of the catalytic triad (K157, Y152, S141) toward NADH and (S)-benzoylsuccinyl-CoA. (D) Positioning of the benzoylsuccinyl-moiety in the active site for two different conformations from the MD analysis. The interactions of the pro(S)-hydrogen of NADH toward the si-side of the β-carbonyl atom of the substrate, H-bridging between S141 and the carbonyl-O atom, and the salt bridge between R142 and the carboxymethyl group are shown in the upper panel, the embedding of the phenyl group of the substrate in the hydrophobic binding pocket between L194, M190, and W191 is shown in the lower panel. Structures are visualized using PYMOL 2.1.
branches) and are not affiliated to any of the previously recognized 460 SDR subfamilies [56]. Most of the BbsC and BbsD sequences even show a similar branching pattern between the respective source organisms, suggesting a long co-evolution of the two adjacent genes (Fig. 7).

**Discussion**

We show in this study that the bbsH and bbsCD genes code for specific enzymes involved in the β-oxidation of benzylsuccinate, namely, (E)-benzylidenesuccinyl-CoA hydratase and (S,R)-2-(α-hydroxybenzyl)succinyl-CoA dehydrogenase. To assay BbsCD activity, we generated 2-(α-hydroxybenzyl)succinyl-CoA in situ from (E)-2-benzyldienesuccinyl-CoA, using BbsH as auxiliary enzyme. The identity of 2-(α-hydroxybenzyl)succinyl-CoA was confirmed by HPLC-MS analysis, whereas its oxidized derivative benzoylsuccinyl-CoA was verified by chemical conversion to a hydrazone with phenylhydrazine.

BbsH is a typical enzyme of the ECH family, which accepts only one of the (E)-benzylidenesuccinyl-CoA regioisomers and exhibits cooperative enzyme kinetics with substrate inhibition. As known for many enzymes converting CoA thioesters [57], BbsH accepts CoA or NAC thioesters of (E)-benzylidenesuccinate. It is surprising that both substrates are turned over with equal kinetic parameters, but this is consistent with the modeled product binding mode in which the adenosine, phosphate, and pantoic acid residues of CoA remain outside of the binding pocket (Fig. 2E). Like other ECH enzymes, BbsH is predicted to adopt a trimeric quaternary structure with a substrate binding pocket in each subunit (Fig. 2E), whereas a tetrameric composition, as initially suggested by the experimental data, or larger hexameric assemblies as found for DmdD [37], its homology modeling template, can be ruled out. The model of the BbsH:product complex also fits with the predicted reaction mechanism of BbsH (Fig. 1). The two conserved active site residues, E110 and E130, are positioned in H-bonding distance with C2 of the succinyl moiety and the hydroxyl group of the benzylc...
carbon, when (S,R)-2-(α-hydroxybenzyl)succinyl-CoA is modeled into the structure (Fig. 2F). However, despite the apparently good fit, ECH enzymes are often not completely stereospecific and may also exhibit epimerization reactions as side activities [58,59]. Accordingly, predictions of the stereochemical properties of BbsH have to be taken with caution.

BbsCD is an unusual member of the short-chain dehydrogenase/reductase (SDR) family of alcohol dehydrogenases, exhibiting a heterotetrameric αβ2 composition with two active and two inactive subunits. These characteristics are corroborated by the observed necessity of having both genes coexpressed to produce stable recombinant protein BbsCD, because using an otherwise identical expression plasmid containing the bbsD gene without bbsC resulted in complete loss of any protein production. However, only the BbsD subunits contain the conserved amino acids necessary for catalysis as well as for NAD+ binding, whereas all of these residues are missing in BbsC, and some aberrant residues even block access of substrates or cofactors, consistent with the lack of bound NAD+ in BbsC in the X-ray structure (Fig. 4A).

In terms of catalysis, BbsCD showed the same relatively low pH optimum as BbsH (pH 6.2), but a normal Michaelis–Menten kinetics, when using (E)-2-benzylideneacetoacetyl-CoA in the coupled assay with BbsH. The much higher specific activity of BbsH compared with that of BbsCD and the lack of cooperative behavior and substrate inhibition in the coupled assay suggest that BbsCD is not limited by the rate of 2-(α-hydroxybenzyl)succinyl-CoA production via BbsH. This implies that the actual activity of BbsCD was recorded without interference of the auxiliary enzyme BbsH. The activities of BbsCD in reducing chloro-substituted acetophenones indicate its general ability to catalyze the reverse reaction, which could not be assayed with its physiological substrate benzyloxsuccinyl-CoA, due to its unavailability. Given that the kinetic data for these substrates cannot be compared to those of the forward reaction, the observed high cooperativity of the reactions may either suggest inhibitory effects of the chlorinated analogues, or potential conformational interactions between the BbsCD subunits.

The structural model of the active site of BbsD with bound NADH and (S)-2-benzoxyloxsuccinyl-CoA is consistent with the general reaction mechanism of alcohol dehydrogenases of the SDR family, which usually catalyze either alcohol oxidation or ketone reduction. The catalytic triad of BbsD differs from that used by other (S)-3-hydroxyacyl-CoA dehydrogenases (Had) (Fig. 7) involved in fatty acid oxidation or other β-oxidation pathways. They employ a conserved triad of Ser, His, and Asn (e.g. S137, H158, N190 in the human enzyme [51]), where the His residue acts as general base [50,51,60]. However, most enzymes of the SDR family contain the same Ser/Tyr/Lys triad as observed in BbsD, for example, the acetoacetyl-CoA reductases (PhaB) producing (R)-3-hydroxybutyryl-CoA for polyhydroxybutyrate (PHB) biosynthesis or secondary alcohol dehydrogenases (Ped, Hped), which appear among the most related SDR clades to BbsD and BbsC (Fig. 7). A scheme of the hydrogen-bonding networks of the residues of the catalytic triad of BbsD with NAD+ or NADH and the respective CoA thioesters is shown in Fig. 8. These interactions lead to a local decrease of the pKa value of Y153, allowing it to be deprotonated to a tyrosinate, which acts as base for proton abstraction from (S,R)-2-(α-hydroxybenzyl)succinyl-CoA in a concerted reaction with hydride abstraction by NAD+ (Fig. 8). The stereochemistry of substrate and product as determined by MD modeling indicates that the hydrogen of the (R)-configured benzylic C-atom (Cα) of (S,R)-2-(α-hydroxybenzyl)succinyl-CoA is transferred to the re side of the nicotinamide ring of NAD+ in the oxidative direction. Conversely, the reaction in reductive direction involves transfer of the pro-(S) hydrogen of NADH to the si side of the carbonyl group of (S)-benzoxyloxsuccinyl-CoA (Fig. 8). The substrate is intrinsically bound in a well-defined conformation, involving specific binding sites for the carbonyl-CoA arm, the carboxymethyl substituent, and the phenyl ring (Fig. 6D). Interestingly, the (R)-configuration of the benzylic carbon of (S,R)-2-(α-hydroxybenzyl)succinyl-CoA corresponds...
to an (S)-configuration of aliphatic 3-hydroxyacyl-CoA substrates (because of different substituent priorities for naming the compounds). (S)-configured 3-hydroxyacyl-CoA intermediates are often found in \(\beta\)-oxidation/condensation pathways with notable exceptions, for example, for the synthesis of PHB [61].

As revealed by the phylogenetic analysis of SDR enzymes, the stereochemical preference of the enzymes affiliated to different branches does not appear to be highly conserved during evolution. For example, various clades of 1-phenylethanol dehydrogenases shown in Fig. 7 are either specific for the (S)- (Ped/Ped2 or Hped2) or (R)-enantiomers (Hped1 or (R)-Adh) [34,42]. In spite of the large evolutionary distance between BbsCD and standard \(\beta\)-oxidation enzymes, they apparently exhibit common mechanistic properties, such as similar conformation changes of the substrate-binding domain, which tightens around the bound CoA thioesters in an induced-fit mechanism [40].

The unique C2 symmetric arrangement of the BbsCD structure in which dimers of two inactive BbsC and two active BbsD subunits are combined to a heterotetrameric quaternary structure, confirmed an early notion based on sequence data that BbsC subunits do not contribute to catalysis, but merely serve for the structural and/or regulatory integrity of the catalytic BbsD subunits [22]. In the concept of pseudoenzymes, nonfunctional enzyme paralogues regulate catalytic outputs either by protein–protein interactions or by competition for ligands, integrating signaling events, or inducing switching between active and inactive conformations of enzymes or the formation or dissolution of subcellular complexes [62–64]. While pseudoenzymes mostly act as separate entities from the enzymes affected, BbsCD is the first example from the SDR family, where the built-in BbsC pseudoenzyme subunits serve as scaffolding and potentially regulatory modules. In this way, they resemble noncatalytic pseudoenzyme-like subunits of some eukaryotic enzymes, for example, 20S proteasomes, plant pyridoxal-5’-phosphate synthases, or trypanosomal protein:arginine-methyltransferases [62,65,66,67]. The inactive BbsC subunits seem to be important for the functioning of BbsCD, as evident from the observed genetic coupling in operons of every known version of these two genes. The phylogenetic analysis suggests that BbsC and BbsD have originated from a homotetrameric ancestral protein and divergently evolved after functional differentiation, leading to the observed situation of a fully functional BbsD subunit complexed with a BbsC subunit lacking any of the residues involved in catalysis. It is interesting to note that the total absence of conserved residues is only valid for the BbsC subunits from facultative anaerobic denitrifying Betaproteobacteria (labeled yellow in Fig. 7). The highly similar BbsC orthologues from strictly anaerobic Fe(III)- or sulfate-reducing Deltaproteobacteria or Firmicutes (labeled green in Fig. 7) still contain the conserved residues of the catalytic triad and D17, which interacts with the ribose of the adenosyl moiety of NAD\(^+\), but have already lost the diphosphate-binding motif. It appears therefore that the BbsC subunits of the latter enzymes may still be in a more preliminary evolutionary state of losing catalytic activity, while those of the enzymes in Betaproteobacteria are fully switched to pseudoenzyme state.

**Materials and methods**

**Synthesis of (E)-benzylidenesuccinyl-thioesters**

(E)-benzylidenesuccinate was prepared by following a previously described method [68]. Briefly, a solution of 35 mmol benzaldehyde and 44 mmol dimethylsuccinate in 5 mL tert-butanol were added slowly over 4 h under reflux to a mixture of 39 mmol K-tert-butanolate in 25 mL tert-butanol. After additional 3 h of incubation, tert-butanol was evaporated, the precipitate was dissolved in 15 mL methanol. 25 mL of 15% NaOH in methanol was added and the solution was heated under reflux for 12 h, concentrated by rotating evaporation and resolved in H\(_2\)O (38 mL). After threefold extraction with 25 mL ethyl acetate, the aqueous solution was acidified with HCl to pH 3.0 and again extracted three times with 25 mL ethyl acetate. The organic phases were pooled, dried with Na\(_2\)SO\(_4\), and concentrated by rotating evaporation. (E)-Benzyldienesuccinate was precipitated from the solution with ethyl acetate and hexane and dried at 60 °C. CoA or NAC thioesters of (E)-benzyldienesuccinate were prepared via the internal anhydride as described previously [11,69]. The stability of the thioesters at different pH values or in the presence of extracts containing thioesterase activity was checked in control assays by UV-spectroscopy (loss of absorption at 235 nm) and by HPLC analysis. Hydrolysis of the (E)-benzyldienesuccinyl-thioesters was also visible as loss of UV-absorption at 290 nm in the BbsH enzyme assays.

**Cloning of bssH, bbsCD, and bbsD**

A 1.1 kb EcoRI fragment of the chromosomal DNA of *T. aromatica* encoding the *bssH* gene was cloned into

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Pseudoenzyme subunits in the SDR enzyme BbsCd

S. von Horsten et al.

BbsH was purified chromatographically in two steps. First, cell extract (0.5 g total protein) was applied to a DEAE column (DEAE FastFlow, 15 mL) equilibrated with buffer 1 (B1) (10 mM Tris/HCl pH 8, 2 mM MgCl2, 10% glycerol). After washing with 5 column volumes (CV) of B1, the protein was eluted with a linear gradient of 0–100 mM KCl in B1 over 15 CV. BbsH eluted with 30–40 mM KCl.

Purification of BbsCD

BbsCD was purified chromatographically in two steps. Cell extract (0.75 g total protein) was applied to a DEAE column (30 mL DEAE FastFlow, GE Healthcare, Boston, MA, USA) equilibrated with B1. After washing with 5 CV of B1, the protein was eluted with a linear gradient of 0–150 mM KCl in B1 over 20 CV. BbsCD eluted with 50–100 mM KCl. Active fractions were pooled, diluted with B2 to a salt concentration below 30 mM KCl, and applied to a hydroxyapatite column (20 mL MacroPrep Ceramic Hydroxyapatite Type II, Bio-Rad) equilibrated with B2. After washing the column with 3 CV B2, BbsCD was eluted with a linear gradient of 0–100 mM KCl in B2 over 15 CV. BbsCD eluted at concentrations of 30–40 mM salt.

Protein analytic methods

Native molecular masses of proteins were estimated by gel filtration in relation to the retention volumes of standard proteins (gel filtration calibration kit HMW, GE Healthcare), and by Ferguson plot analysis [72] of the proteins separated by native PAGE (using polyacrylamide concentrations of 6%–10%). Bovine serum albumin (BSA) oligomers and ovalbumin were used as mass standards. Protein concentrations were determined by Coomassie dye binding with BSA as a standard [73]. Separation of proteins by discontinuous SDS/PAGE (13% polyacrylamide) was performed as described previously [74].

Enzyme assays

Catalytic activity of BbsH was determined spectrophotometrically at 30 °C by following the decrease in absorption at 290 nm with (E)-benzylidenesuccinyl-CoA or (E)-benzylidenesuccinyl-NAC as substrates. The decrease in absorption is caused by the loss of the double bound of the substrate as a result of its hydration with experimentally determined extinction coefficients of 5800 M⁻¹·cm⁻¹ and 7230 M⁻¹·cm⁻¹ for the CoA and NAC thioester, respectively. The assay mixture (1 mL) contained 50 mM MES/NaOH pH 6.2 and 0.1–0.3 mg BbsH. The reaction was started with 0.2 mM substrate.

The substrate of BbsCD, 2-(α-hydroxybenzyl)succinyl-CoA, is not commercially available or easily synthesized. Therefore, oxidation of 2-(α-hydroxybenzyl)succinyl-CoA was followed by the reduction of NAD⁺ at 365 nm (ε = 3400 M⁻¹·cm⁻¹) in a coupled spectrophotometric assay (1 mL) containing 50 mM MES/NaOH pH 6.2, 0.2 mM NAD⁺, 0.5–1 mg BbsH, and 0.1–0.3 mg BbsCD. The reaction was started with 0.2 mM (E)-benzylidenesuccinyl-CoA, which was quickly converted to 2-(α-hydroxybenzyl)succinyl-CoA by surplus amounts of BbsH. If indicated, the ketone product of BbsCD (benzoylsuccinyl-CoA) was derivatized to the respective phenylhydrazone by adding 500–750 µM phenylhydrazine to the assay mixture. The reverse reaction with acetophenone (respectively, chlorinated derivatives thereof) was followed by the oxidation of NADH at 365 nm with the assay conditions described above, but NAD⁺ was

Heterologous expression of bssH and bbsD

The recombinant cells containing the expression plasmids for either bssH, bbsCD, or bbsD were grown at 20 °C in LB medium containing 100 µg·ml⁻¹ ampicillin and expression was induced by centrifugation and cell extracts were prepared as described elsewhere [71]. BbsH and BbsCD were produced in large amounts, as evident from the appearance of additional protein bands after SDS/PAGE analysis after induction with IPTG, whereas expression of the bbsD gene alone did not yield any detectable product, suggesting that the BbsD subunit may not be stable in the absence of BbsC.

Purification of BbsH

BbsH was purified chromatographically in two steps. First, cell extract (0.5 g total protein) was applied to a DEAE column (DEAE FastFlow, 15 mL) equilibrated with buffer 1 (B1) (10 mM Tris/HCl pH 8, 2 mM MgCl₂, 10% glycerol). After washing with 5 column volumes (CV) of B1, the protein was eluted with a linear gradient of 0–200 mM KCl in B1 over 10 CV. BbsH eluted with 75–125 mM KCl. Active fractions were pooled, diluted with buffer 2 (B2) (10 mM Tris/HCl pH 7.5, 10% glycerol) to a salt concentration below 30 mM KCl, and applied to a hydroxyapatite column (20 mL MacroPrep Ceramic Hydroxyapatite Type II, Bio-Rad, Hercules, CA, USA) equilibrated with B2. The column was washed with 3 CV of B2 and the protein was eluted with a linear gradient of 0–100 mM KCl in B2 over 15 CV. BbsH eluted with 30–40 mM KCl.

Purification of BbsCD

BbsCD was purified chromatographically in two steps. Cell extract (0.75 g total protein) was applied to a DEAE column (30 mL DEAE FastFlow, GE Healthcare, Boston,
substituted by 0.4 mm NADH. The reaction was started by adding 0.5 mm of the respective ketones. The pH optima of BbsH and BbsCD were determined using 50 mm MES/NaOH (pH 5.8-6.5) or 50 mm MOPS/NaOH (pH 6.5-7.5), respectively, as assay buffers.

**HPLC analysis**

Free aromatic acids and thioesters were analyzed via HPLC using a RP-C18 column (Chromolith® Performance RP18 endcapped, Merck, Darmstadt, Germany) and detected by their absorption at 220 or 260 nm in a diode array detector, respectively. Enzyme assay mixtures were acidified by addition of 10% (v/v) NaHSO₄ and centrifuged prior to HPLC analysis to remove the precipitated protein. Aromatic acids were separated using isocratic conditions (20% acetonitrile in 32 mM formic acid pH 3). Thioesters were separated by applying a linear gradient of 3%–20% acetonitrile in 50 mm MES/NaOH pH 6.2 over 30 min. Observed retention times were as follows: (R)-benzylsuccinate 6.5 min; (E)-benzylidenesuccinate 8.5 min; (R)-benzoylsuccinyl-CoA 6.5 min; benzoylsuccinyl-CoA phenylhydrazone 22 min; 2-(α-hydroxybenzyl)succinyl-CoA 3 min; 3-(E)-benzylidenesuccinyl-CoA 2.5 min; 2-(E)-benzylidenesuccinyl-CoA 3.8 min; (E)-benzylidenesuccinyl-N-acetyltyrosine 4.5 min. HPLC-MS analysis (liquid chromatography coupled to mass spectrometry) of 2-(α-hydroxybenzyl)succinyl-CoA was performed on a Surveyor HPLC system coupled to a LCQ Advantage ESI-MS system (Thermo-Fisher, Bremen, Germany). Samples were separated via a Reprosil C18 column, 4 x 125 mm, 5 µm particle size (Wicom, Heppenheim, Germany) at a flow rate of 1 mL·min⁻¹ in 0.5% triammonium bicarbonate pH 8.5, which was eluted by means of a gradient from 0% to 10% Acetonitrile over 10 min. Mass spectrometric analysis was carried out with an ESI system in negative mode. The capillary temperature was set to 330 °C, the voltage to 10 V.

**Crystallization of BbsCD**

BbsCD was concentrated to 31 mg·mL⁻¹, and crystals were grown using the sitting-drop vapor-diffusion, incubated at 18 °C. Crystallization attempts were performed with a Honeybee 963 robot system (Zinsser Analytic) and commercially available screens (Qiagen, Hilden, Germany) in a 96-well format. The crystal for the native dataset and for the Gd⁺-soaked datasets were solved by molecular replacement using the CCP4 suite [75] and CCP4 [76] package. Initial single-wavelength anomalous diffraction (SAD) phasing was performed with the PHENIX suite [77] using the heavy atom derivative method.

**Data collection and structure determination**

Crystals for the native and the Gd³⁺-soaked datasets were incubated in a cryoprotection buffer (reservoir buffer supplemented with 30% glycerol) prior to freezing in liquid nitrogen. For the crystals from cocrystallization, a cryoprotection buffer containing 0.2 M (NH₄)₂SO₄, 0.1 M sodium cacodylate, pH 6.5, 30% (v/v) PEG 8000, and 30% (v/v) glycerol was used. The native and the Gadolinium-soaked dataset were collected at beamline 14.1 and the dataset for the cocrystallization at beamline 14.3 of BESSY-II (Berlin Elektronenspeicherring-Gesellschaft für Synchrotronstrahlung, Berlin, Germany). All datasets were processed using the XDS [75] and CCP4 [76] package. The electrostatic surface potential was generated by APBS (DeLano Scientific LLC, South San Francisco, CA, USA).

**Molecular dynamics analysis of substrate binding by BbsCD**

Molecular dynamics simulations of BbsCD using the available crystal structure (PDB: 7PCS) were done with the Amber18 suite [82] using the ff14sb force field for the protein and the TIP3P water model. Parameters for NADH and CoA cofactors were taken from the R.E.DD.B repository (project code F-90) [83]; benzoylsuccinate parameters were derived by antechamber using the Amber GAFF force field. The electrostatic surface potential was generated by APBS (parameters: ion strength (+1/−1): 0.15, (+2/−2): 0; ion radius (+1): 2.0, (−1): 1.8, (+2/−2): 2.0; grid size: x, y, z = −1; 10 grid points/Å³). PDB files were generated using the PDB2PQR server [81].

**Performance RP18®**

In the context of BbsCD crystal structure, we applied the Amber18 suite for the molecular dynamics analysis of substrate binding. The crystal structure from the PDB code 7PCS was used as the input for the simulations. The simulations were performed with the Amber18 suite using the ff14sb force field for the protein and the TIP3P water model. Parameters for NADH and CoA cofactors were taken from the R.E.DD.B repository. The electrostatic surface potential was generated using APBS with the following parameters: ion strength (+1/−1): 0.15, (+2/−2): 0; ion radius (+1): 2.0, (−1): 1.8, (+2/−2): 2.0; grid size: x, y, z = −1; 10 grid points/Å³. PDB files were generated using the PDB2PQR server [81].

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cutoff, and a Monte Carlo barostat. Subsequent trajectories with lengths of 100–600 ns were processed and evaluated in jupyter notebooks using pytraj 2.0.5 (https://github.com/Amber-MD/pytraj [84]); AmberTools20; and NGLview 2.7.7 [85]). The total length of these production trajectories for the BbsCD-NADH-S-benzylolsuccinyl-CoA complex amounts to 5.19 μs, for the BbsCD-NADH-R-benzylolsuccinyl-CoA complex to 3.80 μs.

Structural model of BbsH

The structure of BbsH was predicted by homology modeling, using the MODELLER program 10.0 [86] using the structure of methylthioacryloyl-CoA hydratase as template, which exhibits 30% identity to BbsH [37] (PDB 4IZD). Figures were generated using PYMOL 2.1 (DeLano Scientific LLC).

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Conflict of interest

The authors declare no conflict of interest.

Peer Review

The peer review history for this article is available at https://publons.com/publon/10.1111/febs.16216.

Data accessibility

The structural data were deposited to the PDB database and are available under the accession code 7PCS.

Author contributions

SvH refined the crystal structure of BbsCD; MLL performed most biochemical experiments with BbsH and BbsCD; YG crystallized BbsCD and solved its structure; KS and IS provided expression vectors and enzymes for structural and biochemical studies; LOE evaluated and interpreted the structural data, performed MD calculations, and wrote the manuscript; JH designed the project, interpreted the structural data, and wrote the manuscript. Data deposition footnote: The atomic coordinates and structure factors have been deposited with the protein data bank (ID code 7PCS).

References

1 Lovley DR & Lonergan DJ (1990) Anaerobic oxidation of toluene, phenol, and p-cresol by the dissimilatory iron-reducing organism, GS-15. Appl Environ Microbiol 56, 1858–1864.
2 Evans PJ, Ling W, Goldschmidt B, Ritter ER & Young LY (1992) Metabolites formed during anaerobic transformation of toluene and o-xylene and their proposed relationship to the initial steps of toluene mineralization. Appl Environ Microbiol 58, 496–501.
3 Rabus R & Widdel F (1996) Utilization of alkylbenzenes during anaerobic growth of pure cultures of denitrifying bacteria on crude oil. Appl Environ Microbiol 62, 1238–1241.
4 Biegert T, Fuchs G & Heider J (1996) Evidence that anaerobic oxidation of toluene in the denitrifying bacterium Thauera aromatica is initiated by formation of benzylouccinate from toluene and fumarate. Eur J Biochem 238, 661–668.
5 Beller HR & Spormann AM (1997) Anaerobic activation of toluene and o-xylene by addition to fumarate in denitrifying strain T. J Bacteriol 179, 670–676.
6 Beller R & Heider J (1998) Initial reactions of anaerobic metabolism of alkylbenzenes in denitrifying and sulfate-reducing bacteria. Arch Microbiol 170, 377–384.
7 Zengler K, Heider J, Rosselló-Mora R & Widdel F (1999) Phototrophic utilization of toluene under anoxic conditions by a new strain of Blastochloris sulfoviridis. Arch Microbiol 172, 204–212.
8 Beller HR & Edwards EA (2000) Anaerobic toluene activation by benzylouccinate synthase in a highly enriched methanogenic culture. Appl Environ Microbiol 66, 5503–5505.
9 Leuthner B, Leutwein C, Schulz H, North P, Haehnel W, Schultz E, Schäger H & Heider J (1998) Biochemical and genetic characterization of benzylsuccinate synthase from Thauera aromatica: a new glycol radical enzyme catalysing the first step in anaerobic toluene metabolism. Mol Microbiol 28, 615–628.
10 Krieger CJ, Beller HR, Reinhard M & Spormann AM (1999) Initial reactions in anaerobic oxidation of m-xylene by the denitrifying bacterium Azoarcus sp. strain T. J Bacteriol 181, 6403–6410.
11 Leutwein C & Heider J (1999) Anaerobic toluene-catabolic pathway in denitrifying *Thauera aromatica*: activation and β-oxidation of the first intermediate, (R)-(−)-benzylsuccinate. Microbiology **145**, 3265–3271.

12 Heider J (2007) Adding handles to unhandy substrates: anaerobic hydrocarbon activation mechanisms. *Curr Opin Chem Biol* **11**, 188–194.

13 Heider J, Szaleniec M, Martins BM, Seyhan D, Buckel W & Golding BT (2016) Structure and function of benzylsuccinate synthase and related fumarate-adding glycol radical enzymes. *J Mol Microbiol Biotechnol* **26**, 29–44.

14 Hilberg M, Pierik AJ, Bill E, Friedrich T, Lippert ML & Heider J (2012) Identification of FeS clusters in the glycol-radical enzyme benzylsuccinate synthase via EPR and Mössbauer spectroscopy. *J Biol Inorg Chem* **17**, 49–56.

15 Selmer T, Pierik AJ & Heider J (2005) New glycol radical enzymes catalysing key metabolic steps in anaerobic bacteria. *Biol Chem* **386**, 981–988.

16 Funk MA, Marsh ENG & Drennan CL (2015) Substrate-bound structures of benzylsuccinate synthase reveal how toluene is activated in anaerobic hydrocarbon degradation. *J Biol Chem* **290**, 22398–22408.

17 Funk MA, Judd ET, Marsh ENG, Elliott SJ & Drennan CL (2014) Structures of benzylsuccinate synthase elucidate roles of accessory subunits in glycol radical enzyme activation and activity. *Proc Natl Acad Sci U.S.A* **111**, 10161–10166.

18 Seyhan D, Friedrich P, Szaleniec M, Hilberg M, Buckel W, Golding BT & Heider J (2016) Elucidating the stereochemistry of enzymatic benzylsuccinate synthesis with chirally labeled toluene. *Angew Chemie Int Ed Engl* **55**, 11664–11667.

19 Szaleniec M & Heider J (2016) Modeling of the reaction mechanism of enzymatic radical C-C coupling by benzylsuccinate synthase. *Int J Mol Sci* **17**, 514.

20 Salii I, Szaleniec M, Zein AA, Seyhan D, Sekuşa A, Schühle K, Kaplieva-Dudek I, Linne U, Meckenstock RU & Heider J (2021) Determinants for substrate recognition in the glycol radical enzyme benzylsuccinate synthase revealed by targeted mutagenesis. *ACS Catal* **11**:3361–3370. https://doi.org/10.1021/acscatal.0c04954

21 Hermuth K, Leuthner B & Heider J (2002) Operon structure and expression of the genes for benzylsuccinate synthase in *Thauera aromatica* strain K172. *Arch Microbiol* **177**, 132–138.

22 Leuthner B & Heider J (2000) Anaerobic toluene catabolism of *Thauera aromatica*: the *bbs* operon codes for enzymes of β oxidation of the intermediate benzylsuccinate. *J Bacteriol* **182**, 272–277.

23 Leutwein C & Heider J (2001) Succinyl-CoA:(R)-benzylsuccinyl-CoA-transferase: an enzyme of the anaerobic toluene catabolic pathway in denitrifying bacteria. *J Bacteriol* **183**, 4288–4295.

24 Leutwein C & Heider J (2002) (R)-Benzylocacetyl-CoA dehydrogenase of *Thauera aromatica*, an enzyme of the anaerobic toluene catabolic pathway. *Arch Microbiol* **178**, 517–524.

25 Migaud ME, Chee-Sanford JC, Tiedje JM & Frost JW (1996) Benzylfumaric, benzylmaleic, and Z- and E-phenylitaconic acids: Synthesis, characterization, and correlation with a metabolite generated by Azotobacter toluticus tol-I during anaerobic toluene degradation. *Appl Environ Microbiol* **62**, 974–978.

26 Zeyer J, Kuhn EP & Schwarzenbach RP (1986) Rapid microbial mineralization of toluene and 1,3-dimethylbenzene in the absence of molecular oxygen. *Appl Environ Microbiol* **52**, 944–947.

27 Verfürth K, Pierik AJ, Leutwein C, Zorn S & Heider J (2004) Substrate specificities and electron paramagnetic resonance properties of benzylsuccinate synthases in anaerobic toluene and m-xylene metabolism. *Arch Microbiol* **181**, 155–162.

28 Müller JA, Galushko AS, Kappler A & Schink B (2001) Initiation of anaerobic degradation of p-cresol by formation of 4-hydroxybenzylsuccinate in *Desulfovibrio cetonicum*. *J Bacteriol* **183**, 752–757.

29 Annweiler E, Materna A, Safinowski M, Kappler A, Richnow HH, Michaelis W & Meckenstock RU (2000) Anaerobic degradation of 2-methylpropanol by a sulfate-reducing enrichment culture. *Appl Environ Microbiol* **66**, 5329–5333.

30 Strikstra A, Trautwein K, Jarling R, Wöhlbrand L, Dörries M, Reinhardt R, Drozdowska M, Golding BT, Wilkes H & Rabus R (2014) Anaerobic activation of p-cymene in denitrifying betaproteobacteria: methyl group hydroxylation versus addition to fumarate. *Appl Environ Microbiol* **80**, 7592–7603.

31 Boll M, Estelmann S & Heider J (2020) Anaerobic degradation of hydrocarbons: mechanisms of hydrocarbon activation in the absence of oxygen. In Anaerobic Utilization of Hydrocarbons, Oils, and Lipids (Boll M, ed), pp. 3–29. Springer, New York. https://doi.org/10.1007/978-3-642-30141-4_71

32 Heider J & Schühle K (2013) Anaerobic biodegradation of hydrocarbons including methane. In The Prokaryotes: Prokaryotic Physiology and Biochemistry (Rosenberg E, DeLong EF, Thompson F, Lory S & Stackebrandt E, eds), pp. 601–630. Springer, New York. https://doi.org/10.1007/978-3-642-30411-4_80

33 Boll M, Estelmann S & Heider J (2020) Catabolic pathways and enzymes involved in the anaerobic degradation of monocyclic aromatic compounds. In Anaerobic Utilization of Hydrocarbons, Oils, and Lipids (Boll M, ed), pp. 85–133. Springer, New York. https://doi.org/10.1007/978-3-319-50391-2_6

34 Hoffken HW, Duong M, Friedrich T, Breuer M, Hauer B, Reinhardt R, Rabus R & Heider J (2006) Crystal structure and enzyme kinetics of the (S)-specific
Pseudoenzyme subunits in the SDR enzyme BbsCD

S. von Horsten et al.

1. Phenylethanol dehydrogenase of the denitrifying bacterium strain EbN1. *Biochemistry* **45**, 82–93.

2. Holden HM, Benning MM, Haller T & Gerlt JA (2001) The crotonase superfamily: divergently related enzymes that catalyze different reactions involving acyl coenzyme a thioesters. *Acc Chem Res* **34**, 145–157.

3. Hamed RB, Batchelar ET, Clifton JI & Schofield CJ (2008) Mechanisms and structures of crotonase superfamily enzymes — how nature controls enolate and oxygenation reactivity. *Cell Mol Life Sci* **65**, 2507–2527. https://doi.org/10.1007/s00018-008-8082-6

4. Tan D, Crabb WM, Whitman WB & Tong L (2013) Crystal structure of DmdD, a crotonase superfamily enzyme that catalyzes the hydration and hydrolysis of methylthioacryloyl-CoA. *PLoS ONE* **8**, e63870.

5. Venkatesan R, Sah-Teli SK, Awoniyi LO, Jiang G, Prus P, Kastaniotis AJ, Hiltnen JK, Wierenga RK & Chen Z (2014) Insights into mitochondrial fatty acid synthesis from the structure of heterotetrameric 3-ketoacyl-ACP reductase/3R-hydroxyacyl-CoA dehydrogenase. *Nat Commun* **5**, 4805.

6. Jörnvall H, Landreh M & Östberg LJ (2015) Alcohol dehydrogenase, SDR and MDR structural stages, present update and altered era. *Chem Biol Interact* **234**, 75–79.

7. Tanaka N, Nonaka T, Nakamura K & Hara A (2005) SDR structure, mechanism of action, and substrate recognition. *Curr Org Chem* **9**, 89–111. https://doi.org/10.2174/138527201091375751

8. Ladenstein R, Winberg JO & Benach J (2008) Medium-and short-chain dehydrogenase/reductase gene and protein families: Structure-function relationships in short-chain alcohol dehydrogenases. *Cell Mol Life Sci* **65**, 3918–3935. https://doi.org/10.1007/s00018-008-8590-4

9. Büsing I, Höffken HW, Breuer M, Wölflbrand L, Hauer B & Rabus R (2015) Molecular genetic and crystal structural analysis of 1-(4-Hydroxyphenyl)-ethanol dehydrogenase from “aromatoleum aromaticum” EbN1. *J Mol Microbiol Biotechnol* **25**, 327–339.

10. Bellamacina CR (1996) The nicotinamide dinucleotide binding motif: a comparison of nucleotide binding proteins. *FASEB J* **10**, 1257–1269.

11. Oppermann UCT, Filling C & Jörnvall H (2001) Forms and functions of human SDR enzymes. *Chem Biol Interact* **130-132**, 699–705.

12. Persson B, Kallberg Y, Bray JE, Bruford E, Dellaporta SL, Favia AD, Duarte RG, Jörnvall H, Kavanagh KL, Kedishvili N et al. (2009) The SDR (short-chain dehydrogenase/reductase and related enzymes) nomenclature initiative. *Chem Biol Interact* **178**, 94–98.

13. Ghosh D, Wawrzak Z, Weeks CM, Duax WL & Erman M (1994) The refined three-dimensional structure of 3α,20β-hydroxysteroid dehydrogenase and possible roles of the residues conserved in short-chain dehydrogenases. *Structure* **2**, 629–640. https://doi.org/10.1016/S0969-2126(00)00064-2

14. Javidpour P, Pereira JH, Goh EB, McAndrew RP, Ma SM, Friedland GD, Kcsajing JD, Chhabra SR, Adams PD & Beller HR (2014) Biochemical and structural studies of NADH-dependent FabG used to increase the bacterial production of fatty acids under anaerobic conditions. *Appl Environ Microbiol* **80**, 497–505.

15. Hermansen LF, Bergman T, Jörnvall H, Hushby G, Ranlov I & Sletten K (1995) Purification and characterization of amyloid-related transthyretin associated with familial amyloidotic cardiomyopathy. *Eur J Biochem* **227**, 772–779.

16. Tanaka N, Nonaka T, Nakanishi M, Deyashiki Y, Hara A & Mitsui Y (1996) Crystal structure of the ternary complex of mouse lung carbonyl reductase at 1.8 Å resolution: the structural origin of coenzyme specificity in the short-chain dehydrogenase/reductase family. *Structure* **4**, 33–45.

17. Kim J, Chang JH & Kim KJ (2014) Crystal structure and biochemical properties of the (S)-3-hydroxybutyryl-CoA dehydrogenase PaaH1 from Ralstonia eutropha. *Biochem Biophys Res Commun* **448**, 163–168. https://doi.org/10.1016/j.bbrc.2014.04.101

18. Barycki JJ, O’Brien LK, Bratt JM, Zhang R, Sanishvili R, Strauss AW & Banaszak LJ (1999) Biochemical characterization and crystal structure determination of human heart short chain L-3-hydroxyacyl-CoA dehydrogenase provide insights into catalytic mechanism. *Biochemistry* **38**, 5786–5798.

19. Rabus R, Boll M, Heider J, Meckenstock RU, Buckel W, Einsle O, Ermrler U, Golding BT, Gunsalus RP, Kronke PMH et al. (2016) Anaerobic microbial degradation of hydrocarbons: from enzymatic reactions to the environment. *J Mol Microbiol Biotechnol* **26**, 5–28.

20. Bergmann FD, Selesi D & Meckenstock RU (2011) Identification of new enzymes potentially involved in anaerobic naphthalene degradation by the sulfate-reducing enrichment culture N47. *Arch Microbiol* **193**, 241–250.

21. Selesi D, Jehmlich N, Von Bergen M, Schmidt F, Ratteei T, Tischler P, Luenders T & Meckenstock RU (2010) Combined genomic and proteomic approaches identify gene clusters involved in anaerobic 2-methyllnaphthalene degradation in the sulfate-reducing enrichment culture N47. *J Bacteriol* **192**, 295–306.

22. Vogt MS, Schühle K, Köbler S, Peschke P, Chowdhury NP, Kleinsorge D, Buckel W, Essen LO & Heider J (2019) Structural and functional characterization of an electron transfer flavoprotein involved in toluene degradation in strictly anaerobic bacteria. *J Bacteriol* **201**, e00326–e419.
56 Persson B & Kallberg Y (2013) Classification and nomenclature of the superfamily of short-chain dehydrogenases/reductases (SDRs). *Chem Biol Interact* **202**, 111–115.

57 Franke J & Hertweck C (2016) Biomimetic thioesters as probes for enzymatic assembly lines: synthesis, applications, and challenges. *Cell Chem Biol* **23**, 1179–1192.

58 Feng Y, Hofstein HA, Zwahlen J & Tonge PJ (2002) Effect of mutagenesis on the stereochmistry of enoyl-CoA hydratase. *Biochemistry* **41**, 12883–12890.

59 Bell AF, Feng Y, Hofstein HA, Parikh S, Wu J, Rudolph MJ, Kisker C, Whitby A & Tonge PJ (2002) Stereoselectivity of enoyl-CoA hydratase results from preferential activation of one of two bound substrate conformers. *Chem Biol* **9**, 1247–1255.

60 Barycki JJ, O’Brien LK, Strauss AW & Banaszak LJ (2000) Sequestration of the active site by interdomain shifting: Crystallographic and spectroscopic evidence for distinct conformations of L-3-hydroxyacyl-CoA dehydrogenase. *J Biol Chem* **275**, 27186–27196.

61 Kim KJ & Kim J (2015) Crystal structure and biochemical properties of ReH16-A1887, the 3-ketoacyl-CoA thiolase from Ralstonia eutropha H16. *Biochem Biophys Res Commun* **459**, 547–552. https://doi.org/10.1016/j.bbrc.2015.02.148

62 Ribeiro AJM, Das S, Dawson N, Zaru R, Orchard S, Thornton JM, Orego C, Zeqiraj E, Murphy JM & Eyers PA (2019) Emerging concepts in pseudoenzyme classification, evolution, and signaling. *Sci Signal* **12**, eaat9797.

63 Eyers PA & Murphy JM (2016) The evolving world of pseudoenzymes: Proteins, prejudice and zombies. *BMC Biol* **14**, 98.

64 Adrain C (2020) Pseudoenzymes: dead enzymes with a lively role in biology. *FEBS J* **287**, 4102–4105.

65 Dell’Aglio E, Boycheva S & Fitzpatrick TB (2017) The pseudoenzyme PDX1.2 sustains vitamin B6 biosynthesis as a function of heat stress. *Plant Physiol* **174**, 2098–2112.

66 Kafková L, Debler EW, Fisk JC, Jain K, Clarke SG & Read LK (2017) The major protein arginine methyltransferase in *Trypanosoma brucei* functions as an enzyme-prozyme complex. *J Biol Chem* **292**, 2089–2100.

67 Striebel F, Kress W & Weber-Ban E (2009) Controlled destruction: AAA+ ATPases in protein degradation from bacteria to eukaryotes. *Curr Opin Struct Biol* **19**, 209–217.

68 Caro Y, Masaguer CF & Raviña E (2003) Preparation of (R)(−) and (S)(+)-3-hydroxymethyl-1-tetralone tosylates, key intermediates in the synthesis of new CNS drugs, via resolution of precursors. *Tetrahedron Asymmetry* **14**, 381–387.

69 Schachter D & Taggart JV (1953) Benzoyl coenzyme A and hippurate synthesis. *J Biol Chem* **203**, 925–934. https://doi.org/10.1016/S0021-9258(19)52362-6

70 Amann E, Ochs B & Abel KJ (1988) Tightly regulated tac promoter vectors useful for the expression of unfused and fused proteins in Escherichia coli. *Gene* **69**, 301–315.

71 Heider J, Boll M, Breeke S, Breinig S, Ebenau-Jehle C, Feil U, Gad’on N, Laempe D, Leuthner B, Mohamed MES et al. (1998) Differential induction of enzymes involved in anaerobic metabolism of aromatic compounds in the denitrifying bacterium *Thauera aromatica*. *Arch Microbiol* **170**, 120–131.

72 Ferguson KA (1964) Starch-gel electrophoresis—application to the classification of pituitary proteins and polypeptides. *Metabolism* **13**, 985–1002. https://doi.org/10.1016/S0026-0495(64)80018-4

73 Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.

74 Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

75 Kabsch W (2010) XDS. *Acta Crystallogr D* **66**, 125–132.

76 Collaborative Computational Project, Number 4 (1994) The CCP4 suite: programs for protein crystallography. *Acta Crystallogr D* **50**, 760–763.

77 Adams PD, Afonine PV, Bunkoczi G, Chen VB, Davis IW, Echols N, Headd JJ, Hung LW, Kapral GJ, Grosse-Kunstleve RW et al. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D* **66**, 213–221.

78 Emsley P & Cowtan K (2004) Coot: model-building tools for molecular graphics. *Acta Crystallogr D* **60**, 2126–2132.

79 Murshudov GN, Vagin AA & Dodson EJ (1997) Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D* **53**, 240–255.

80 Baker NA, Sept D, Joseph S, Holst MJ & McCammon JA (2001) Electrostatics of nanosystems: application to microtubules and the ribosome. *Proc Natl Acad Sci USA* **98**, 10037–10041.

81 Dolinsky TJ, Nielsen JE, McCammon JA & Baker NA (2004) PDB2PQR: an automated pipeline for the setup of Poisson-Boltzmann electrostatics calculations. *Nucleic Acids Res* **32**, W665–W667.

82 Lee TS, Cerutti DS, Mermelstein D, Lin C, Legrand S, Giese TJ, Roitberg A, Case DA, Walker RC & York DM (2018) GPU-accelerated molecular dynamics and free energy methods in Amber18: performance
enhancements and new features. *J Chem Inf Model* **58**, 2043–2050.
83 Dupradeau FY, Cézard C, Lelong R, Stanislawiak É, Pécher J, Delepine JC & Cieplak P (2008) R.E.DD.B.: a database for RESP and ESP atomic charges, and force field libraries. *Nucleic Acids Res* **36**, gkm887.
84 Roe DR & Cheatham TE (2013) PTRAJ and CPPTRAJ: software for processing and analysis of molecular dynamics trajectory data. *J Chem Theory Comput* **9**, 3084–3095.
85 Nguyen H, Case DA & Rose AS (2018) NGLview-interactive molecular graphics for Jupyter notebooks. *Bioinformatics* **34**, 1241–1242.
86 Webb B & Sali A (2016) Comparative protein structure modeling using MODELLER. *Curr Protoc Bioinforma* **54**, 5–6.