Substrate-Induced Radical Formation in 4-Hydroxybutyryl Coenzyme A Dehydratase from Clostridium aminobutyricum

Jin Zhang,a,b Peter Friedrich,a,b Antonio J. Pierik,c,* Berta M. Martins,d Wolfgang Buckela,b

Laboratorium für Mikrobiologie, Fachbereich Biologie und Synmikro, Philipps-Universität, Marburg, Germany; Max-Plank-Institut für terrestrische Mikrobiologie, Marburg; Institut für Zytobiologie, Philipps-Universität, Marburg, Germany; Institut für Biologie, Strukturbiologie/Biochemie, Humboldt-Universität zu Berlin, Berlin, Germany

4-Hydroxybutyryl-coenzyme A (CoA) dehydratase (4HBD) from Clostridium aminobutyricum catalyzes the reversible dehydration of 4-hydroxybutyryl-CoA to crotonyl-CoA and the irreversible isomerization of vinylacetyl-CoA to crotonyl-CoA. 4HBD is an oxygen-sensitive homotetrameric enzyme with one [4Fe-4S]2+ cluster and one flavin adenine dinucleotide (FAD) in each subunit. Upon the addition of crotonyl-CoA or the analogues butyryl-CoA, acetyl-CoA, and CoA, UV-visible light and electron paramagnetic resonance (EPR) spectroscopy revealed an internal one-electron transfer to FAD and the [4Fe-4S]2+ cluster prior to hydration. We describe an active recombinant 4HBD and variants produced in Escherichia coli. The variants of the cluster ligands (H292C [histidine at position 292 is replaced by cysteine], H292E, C99A, C103A, and C299A) had no measurable dehydratase activity and were composed of monomers, dimers, and tetramers. Variants of other potential catalytic residues were composed only of tetramers and exhibited either no measurable (E257Q, E455Q, and Y296W) hydratase activity or <1% (Y296F and T190V) dehydratase activity. The E455Q variant but not the Y296F or E257Q variant displayed the same spectral changes as the wild-type enzyme after the addition of crotonyl-CoA but at a much lower rate. The results suggest that upon the addition of substrate, Y296F is deprotonated by E455 and reduces FAD to FADH2, aided by protonation from E257 via T190. In contrast to FADH2, the tyrosyl radical could not be detected by EPR spectroscopy. FADH2 appears to initiate the radical dehydration via an allylic ketyl radical that was proposed 19 years ago. The mode of radical generation in 4HBD is without precedent in anaerobic radical chemistry. It differs largely from that in enzymes, which use coenzyme B12.

Received 21 September 2014 Accepted 20 November 2014

Accepted manuscript posted online 1 December 2014


Editor A. M. Spormann

Address correspondence to Wolfgang Buckel, buckel@staff.uni-marburg.de.

* Present address: Antonio J. Pierik, Fachbereich Chemie, Universität Kaiserslautern, Kaiserslautern, Germany.

J.Z. and P.F. contributed equally to this work and are both considered first authors.

Dedicated to Peter Willadsen (Brisbane, Australia).

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.03099-14.

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doi:10.1128/AEM.03099-14
and one histidine (H292) via Nɛ2 (Fig. 3) (12). FAD, located about 10 Å apart from the cluster, occupies the same position as in the structurally related medium-chain acyl-CoA dehydrogenase (MCAD) from pig liver (20). MCAD shares only 16% sequence identity with 4HBD and contains no cluster. The structure of 4HBD further showed two channels filled with water molecules that reach from the protein surface to the [4Fe-4S]2+ cluster. Into one channel, called the “substrate channel,” leading through the gap between the cluster and FAD and surrounded by H292, E455, and Y296, 4-hydroxybutyryl-CoA was modeled in such a way that the 4-hydroxyl group approaches Fe1 of the cluster, whereas the thioester carbonyl is hydrogen bonded to the backbone NH from A460 and from 2'-OH of FAD (12), as found in MCAD. It has been proposed that the hydroxyl group displaces H292 and coordinates to Fe1, whereby the distances shown in Fig. 3 do not change significantly. This location of the substrate brings the 2Re hydrogen close to H292 and the 3Si hydrogen close to N5 of FAD, enabling the anti-elimination of both hydrogens, as observed in MCAD catalysis (13, 15, 16). The second channel, called the “water channel,” is located perpendicular to the substrate channel and aligned by E455 and E257 (12). Probably the product water leaves through this channel.

The proposed mechanism of the dehydration involves the removal of the 2Re proton by H292, followed by one-electron oxidation of the enolate to the enoxy radical with FAD (12, 21). The formation of the enoxy radical lowers the pK of the C3 hydrogens to 37°C (25). Escherichia coli DH5α and Top10 strains were grown aerobically on standard I medium (Merck, Darmstadt, Germany). The E. coli BL21-CodonPlus(DE3) strain, containing an extra constructed plasmid—pOxFtac-SL2 [BL21-CodonPlus(DE3)-GroEL strain (27)—was cultivated with carbenicillin (50 μg/ml) and chloramphenicol (50 μg/ml). The plasmids used for cloning, pASK-IBA3(+) and pASK-IBA7, have an hydrotetraycine-inducible promoter, a carbicillin resistance gene, and a C-terminal or N-terminal Strep-tag for protein purification.

Cloning, mutagenesis, and expression. The gene of 4HBD (abfD) was amplified (Phusion DNA polymerase and EasyStart PCR mix; Molecular Bioproducts, Inc., San Diego, CA, USA) and ligated into the PCR2.1-TOPO plasmid (Invitrogen, USA) for sequencing. The oligonucleotides used for amplification were from MWG AG Biotech, Ebersberg, Germany (forward, 5'-ATGGTAGGTCTCAAATGTTAATGACAGCAAGGACATATTG-3', and reverse, 5'-ATGTTAGTCTCAGGCGTGTATTATTCCAGGCATGGCGCTTACG-3'). Afterwars, the gene was inserted (Bsal digested) into the expression vectors pASK-IBA3(+) and pASK-IBA7, which were used to transform E. coli strains DH5α and BL21. The abfD gene was sequenced by MWG AG Biotech (Ebersberg, Germany). DNA and protein sequence comparisons were performed using the ClustalW (28) and WebLogo (29, 30) programs.

For each site-directed mutagenesis, two complementary primers were designed for PCR (Table 1). The reactions were performed in an Easystart

FIG 1 Reactions catalyzed by 4HBD. The removal of the 2Re and the 3Si protons (red) from 4-hydroxybutyryl-CoA has been established (16). We assume that the isomerization of vinylacetoyl-CoA obeys the same stereocenter.

MATERIALS AND METHODS

Chemicals and biochemicals. Acetyl-CoA, butyryl-CoA, and crotonyl-CoA, prepared from their corresponding acid anhydrides and free coenzyme A, were purified using C18 Sep-PakTM columns (Waters, USA) (23). 4-Hydroxybutyryl-CoA and vinylacetoyl-CoA were prepared in situ via 4-hydroxybutyrate-CoA-transferase from the corresponding acids and acetyl-CoA (24, 25). γ-[2-3H]butyrolactone was obtained from unlabeled 0.5 M γ-butyrolactone (Sigma-Aldrich) by equilibration in 5 M NaOH at 95°C for 72 h. Acidification followed by extraction into dichloromethane yielded 85% of the product (93% label as determined by nuclear magnetic resonance [NMR]). γ-[3,5-2H2]butyrolactone was obtained from 0.5 M ethyl 4,4-dimethoxybutanoate (Sigma-Aldrich) by heating at 95°C in deuterium oxide at pH 1 to 2 for 40 h. The resulting aldehyde was reduced with NaN3 at pH 7 (1), after which any unwanted label at C-2 was removed by heating at 95°C in H2O at pH 10 for 16 h. The final product was isolated as described above (91% yield and 97% label as determined by NMR) (1). Prior to use, the lactones were hydrolyzed with 2 NaOH equivalents at ambient temperature for 10 min. [1-3,5-2H2]tyrosine was synthesized, using a modification of a previously described method (26), from unlabeled 1.1 M L-tyrosine in 10 ml deuterium oxide to which as much as 0.005 M Na2SO4 was added as required to dissolve the amino acid. The solution was heated to 150°C in a microwave reactor, and the proton/deuterium exchange was followed by 1H NMR. After 2 h, only the aromatic protons at C-3 and C-5 suffered 94% exchange and the tyrosine was quantitatively recovered.

Restriction enzymes for cloning were obtained from Fermentas GmbH (St. Leon-Rot, Germany). Phusion high-fidelity DNA polymerase was from Finnzymes (Espoo, Finland). The expression vector pASK-IBA and Streptactin Sepharose column were from IBA GmbH (Göttingen, Germany).

Bacterial strains and plasmids. Clostridium aminobutyricum (DSM 2634), listed as Clostridium sp., was obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DMSZ, Braunschweig, Germany). It grows anaerobically on a medium with 4-aminobutyrate as the main carbon source at 37°C (25). Escherichia coli DH5α and Top10 strains were grown aerobically on standard I medium (Merck, Darmstadt, Germany). The E. coli BL21-CodonPlus(DE3) strain, containing an extra constructed plasmid—pOxFtac-SL2 [BL21-CodonPlus(DE3)-GroEL strain (27)—was cultivated with carbenicillin (50 μg/ml) and chloramphenicol (50 μg/ml). The plasmids used for cloning, pASK-IBA3(+) and pASK-IBA7, have an hydrotetraycine-inducible promoter, a carbicillin resistance gene, and a C-terminal or N-terminal Strep-tag for protein purification.

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tube (Molecular Bioproducts, Inc., San Diego, CA, USA), using 20 ng template, pASK-IBA3 (+) carrying \textit{abfD}, 30 pmol primer, 10 μl 1% Triton X-100, and 0.5 U Phusion polymerase in a total reaction mixture volume of 50 μl. PCR was performed by initial denaturation at 95°C for 5 min followed by 15 cycles, each consisting of denaturation at 95°C for 30 s, annealing at 66°C for 30 s, and extension at 72°C for 5 min (1 min/kb). The PCR product (a complete plasmid) was desalted (Millipore dialysis paper for 30 min), and the template DNA was removed (DpnI for 1 h).

Before transformation into \textit{E. coli} DH5α cells, the plasmid used was dia
erized again. The \textit{abfD}-containing plasmids obtained from colonies were sequenced and transferred into the expression system, \textit{E. coli} strain BL21 CodonPlus(DE3)-GroEL.

\textit{E. coli} strain BL21-CodonPlus(DE3)-GroEL was transformed with the pASK-IBA3 (+) plasmid carrying \textit{abfD}. The transformed strain was grown aerobically at 20 to 25°C on standard LB medium supplemented with 2 mM Fe(III)citrate, 0.4 mM riboflavin, carbenicillin (50 μg/ml), and

FIG 2 Proposed pathway of 4-aminobutyrate fermentation in \textit{C. aminobutyricum}. Enzymes (indicated by boldface numbers): 1, 4-aminobutyrate aminotransferase (EC2.6.1.9); 2, glutamate dehydrogenase (EC1.4.1.2); 3, 4-hydroxybutyrate dehydrogenase (NAD⁺, \textit{C. aminobutyricum}, EC1.1.1.61) or NADP⁺ specific (\textit{M. sedula} and \textit{N. maritima}); 4, 4-hydroxybutyrate CoA-transferase (\textit{C. aminobutyricum}) (EC2.8.3.1) or 4-hydroxybutyryl-CoA ligase (\textit{M. sedula} and \textit{N. maritima}) (6.2.1.2); 5, 4HBD (EC4.2.1.120); 6, 3-hydroxybutyryl-CoA dehydratase (crotonase, EC4.2.1.17); 7, 3-hydroxybutyryl-CoA dehydrogenase (EC1.1.1.35); 8, thiolase (EC2.3.1.9); 9 + 10, phosphate acetyl-transferase (EC2.3.1.8) and acetate kinase (EC2.7.2.1); 11, electron-transferring flavoprotein/butyryl-CoA dehydrogenase complex (5, 6) (similar to EC1.3.8.1); 12, NAD ferredoxin oxidoreductase (Rnf) (EC1.18.1.13) (5); 13, succinyl-CoA reductase (NADPH; EC1.2.1.1). The blue and red hydrogens refer to the labeling experiment described later in the text.
enzymes comprised vinylacetyl-CoA Δ-isomerase activity, an alternative method to determine this activity was applied later in this work, using 100 mM potassium phosphate, pH 7.4, 1 mM vinylacetate, 0.2 mM NADPH, 30 mM KHCO₃, 0.5 mM acetyl-CoA, 1.2 U 4-hydroxybutyrate CoA-transferase (24), and 0.5 U crotonyl-CoA reductase/carboxylase (35, 36). The reaction was initiated by adding 4HBD, and the oxidation of NADPH was measured at 340 nm. All assays were repeated at least three times. In most cases, different enzyme preparations were used also.

Analysis of the quaternary structure. To determine the molecular mass, the recombinant 4HBD solution was loaded on a HiLoad 26/60 Superdex 200 column (GE Healthcare, Freiburg, Germany) equilibrated with 50 mM Tris-HCl, pH 7.4, and 150 mM NaCl. The column was eluted with the same buffer at a flow rate of 0.5 ml min⁻¹, and the proteins were detected by their absorption at 280 nm. Aldolase from rabbit muscle (158 kDa), catalase from human serum (232 kDa), ferritin from horse spleen (440 kDa), and bovine thyroglobulin (669 kDa) were used as molecular-mass standards.

Reconstitution of the [4Fe-4S] cluster. To reconstitute the cluster in 4HBD, three separate solutions were prepared in 100 mM Tris-HCl, pH 7.4: (i) 200 mM diithiothreitol, (ii) 100 mM FeCl₃, and (iii) 30 mM Na₂S (37, 38). All steps were performed at 20°C in the anaerobic chamber. The enzyme solution was incubated with diithiothreitol (final concentration, 5 mM, for 30 min). Then, FeCl₃ and Na₂S (5 and 10 mol/mol 4HBD, molecular mass = 216 kDa) were added and the mixture was incubated for 90 min. To remove the precipitated iron sulfide, the mixture was centrifuged (10,000 × g for 5 min). The solution was concentrated by using a Centricon centrifugal filter (100 kDa).

Non-heme iron was quantified with the iron chelator 3-(2-pyridyl)-5,6-bis(5-sulfo-2-furyl)-1,2,4-triazine (disodium salt trihydrate) (ε₅₇₇ nm = 32 mM⁻¹ · cm⁻¹) as described previously (39, 40). To measure the FAD content in 4HBD, the protein solution was denatured (80°C for 10 min) and centrifuged (10,000 × g for 5 min) to remove the precipitated protein. The yellow supernatant was characterized by its absorption spectrum at 375 nm and 445 nm (ε₄₄₅ nm = 11.3 mM⁻¹ · cm⁻¹) (41).

UV-vis and EPR spectroscopy. To completely convert the FAD of 4HBD (2.8 U/mg) to the quinone form, the enzyme was oxidized with 5 mM potassium hexacyanoferrate(III) (5 min at 17°C) in 50 mM potassium phosphate, pH 7.4, followed by removal of the oxidizing agent (3X volume reduction to 5% by using a 30-kDa-cutoff Centricon ultrafiltration centrifugal filter and refilling with fresh buffer). Each sample was diluted for UV-vis measurements (total volume of 500 μl, 1.6 mg protein/

TABLE 1 Primers used for site-directed mutagenesis

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer nucleotide sequence (5′→3′)</th>
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<tbody>
<tr>
<td>H292C</td>
<td>CAGCAAGGTTCGATACGTCTCGACAGACATAGC GGG</td>
</tr>
<tr>
<td>H292E</td>
<td>CAGCAAGGTTCGATACGTCTCGACAGACATAGC GGG</td>
</tr>
<tr>
<td>C99A</td>
<td>CAGCACGAGAGCCGATCAGCATCATCGAGATG TTAG</td>
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<td>C103A</td>
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</tr>
<tr>
<td>A460G</td>
<td>GAGACGCGGTCCACAGAGGCTGATGAGG TGAG</td>
</tr>
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</table>

FIG 3 Modeled localization of 4-hydroxybutyryl-CoA in the active center of 4HBD. Carbons are in silver, oxygens in red, nitrogens in blue, sulfurs in yellow, and irons in light brown. The dashed lines represent distances at hydrogen bonding interactions (values in Å). The distances between Y296(OH) and Fe1 (4.7 Å), and between Y296(OH) and FAD(N-5) (6.6 Å) are not hydrogen bonding interactions (12). The CoA moiety is truncated; only the cysteamine part and the carbonyl group of β-alanine are depicted.
ml, 4.5 U, and 7.1 μM). The control sample was measured from 300 to 800 nm before adding 1 mM CoA substrate.

EPR measurements were performed on a Bruker X-band EMX-6/1 EPR spectrometer with an ER-4102 standard universal TE102 rectangular cavity. The temperature was maintained at 77 K in a liquid nitrogen finger Dewar or was controlled by an Oxford Instruments ESR-900 helium flow cryostat and ITC4 temperature controller. Each sample contained preoxidized 4HBD (10 mg/ml and 3.6 U/mg), which was mixed with 5 mM substrate and frozen in liquid nitrogen for EPR analysis within less than 1 min.

**Analysis of a potential proton migration.** Three passages were required to adapt *C.aminobutyricum* to a medium in which 50 mM 4-aminobutyrate was replaced by 50 mM 4-hydroxybutyrate and 10 mM ammonium sulfate. Finally, the bacteria were shifted to a medium with 50 mM either 4-hydroxy[2-2H]butyrate or 4-hydroxy[3-2H] butyrate as the substrate. After growth for 24 h at 37°C, the supernatants of the medium were acidified (H2SO4, pH 1). The volatile organic acids were isolated by steam distillation (42), neutralized with NaOH, evaporated to dryness, dissolved in D2O, and analyzed by 1H and 2H NMR. For the in vitro analysis, the reaction mixture (10-ml volume) consisted of 50 mM potassium phosphate, pH 7.4, 140 mM 4-hydroxy[2-2H]butyrate, 1.3 mM acetyl-CoA, 10 mM CoA, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.1 mM NADH, and 175 mM sodium formate. The following proteins were added: 4-hydroxybutyrate CoA-transferase (1.0 U/ml), 4HBD (0.13 U/ml), electron-transferring flavoprotein/butyryl-CoA dehydrogenase complex from *Clostridium tetanomorphum* (4.5 U/ml, ferricenium assay) (6, 43, 44), ferredoxin from *C. tetanomorphum* (1 mg/ml) (6), formate dehydrogenase from *Candida boidinii* (3.13 U/ml; Sigma-Aldrich), and hydrogenase from *Clostridium pasteurianum* (4.5 U/ml) (45). The reaction was carried out under anoxic conditions for 18 h at room temperature. The reaction was stopped by the addition of HCl to pH 1, and the volatile acids were isolated by steam distillation (42). The distillate was basified by NaOH and dried under reduced pressure. For NMR analysis, the alkaline residue was first dissolved in D2O for 1H NMR and thereafter evaporated again and redissolved in H2O for 2H NMR.

**RESULTS AND DISCUSSION**

Kinetic isotope effects with regiospecifically 2H-labeled 4-hydroxybutyrylates and 4HBD isolated from *C.aminobutyricum*. 4-Hydroxy[2-2H]butyrate and 4HBD isolated from *C.aminobutyricum*. 4-Hydroxy[2-2H]butyrate was obtained by heating unlabeled γ-butyrolactone in 5 mM NaO2H. Heating 4,4-dimethoxybutyrate, a protected succinic semialdehyde, in deuterium oxide at pH 1 to 2 afforded 4-oxo[3-2H]butyrate, which was reduced with unlabeled sodium borohydride to 4-hydroxy[3-2H]butyrate. Because 4-hydroxybutyryl-CoA is unstable due to lactonisation (25), it was generated in situ from 4-hydroxybutyrate, acetyl-CoA, and 4-hydroxybutyryl-CoA transferase. To measure the kinetic constants of 4-hydroxybutyryl-CoA, the concentration of acetyl-CoA was varied from 10 to 400 μM and that of 4-hydroxybutyrate (1 mM) was kept constant. Thus, the apparent Km values of acetyl-CoA were determined. The unlabeled substrate yield K m = 22 ± 1 μM, Vmax = 7.6 ± 0.3 s−1, Vmax/Km = 0.34 s−1 μM−1; 4-hydroxy[2-2H]butyrate gave K m = 27 ± 1 μM, Vmax = 4.7 ± 0.3 s−1, Vmax/Km = 0.17 s−1 μM−1; and 4-hydroxy[3-2H]butyrate gave K m = 20 ± 1 μM, Vmax = 7.6 ± 0.3 s−1, Vmax/Km = 0.27 s−1 μM−1. From these data, the kinetic isotope effects (KIE) were calculated as 2H(Kcat/Km) = 2.0 ± 0.2 (mean ± standard deviation) for 4-hydroxy[2-2H]butyrate and 1.3 ± 0.2 for 4-hydroxy[3-2H]butyrate. By comparison with MCAD, 2-2H2]butyryl-CoA exhibited a KIE of 2, and with [3-2H]butyryl-CoA, a KIE of 3 was obtained (13, 46). Hence, the abstraction of the 2H proton by a basic residue, which is most likely H92 in 4HBD and a conserved glutamate in MCAD, yielded almost the same KIE. In contrast, the higher KIE of MCAD observed with the 3Re hydrogen (equivalent to 3Si in 4-hydroxybutyryl-CoA) is proposed to be due to a hydride transfer to FAD, whereas in 4HBD, the 3Si proton is most likely abstracted via a barrierless radical mechanism (see below).

**Spectroscopic analysis of 4HBD isolated from *C. aminobutyricum*.** 4HBD has the remarkable property of increasing its specific activity from 100% to 140% after a brief (≤1 min) exposure to air. Prolonged exposure to air, however, completely abolishes the activity with a half-life of about 20 min. This initial activation is due to the oxidation of the neutral flavin semiquinone and the [4Fe−4S]+ cluster, as revealed by UV-vis and EPR spectroscopy (2, 17, 47). A more defined oxidation of 4HBD without the subsequent inactivation was done with an excess of hexacyanoferrate(III), followed by gel filtration. Therefore, all spectroscopic experiments were performed with the hexacyanoferrate(III)-treated enzyme, which was EPR silent and showed the visible absorbencies of a flavinquinone (380 and 438 nm) overlaid with those of the [4Fe−4S]+ cluster at 420 nm. Upon the addition of the substrate crotonyl-CoA, an immediate reduction of the quinone was observed (Fig. 4; note the decrease in absorption at 438 nm), accompanied by a long-wavelength band that appeared in the region from 500 to 800 nm (Fig. 4; note the increases in absorption at 590 and 739 nm). The increase in absorption at 590 nm could be due to the neutral FAD semiquinone (λmax = 590 nm) (48), and that at 739 nm to a charge transfer band. The results of preliminary stopped-flow experiments indicated that the spectral changes at 438 nm occurred with a rate constant of >400 s−1, followed by a 100-times-lower reaction rate of ca. 4 s−1, equal to the turnover of the dehydration (19). Similar but smaller and much slower spectral changes were detected by replacing crotonyl-CoA with CoA, acetyl-CoA, or butyryl-CoA (Fig. 4). Thus, bleaching the FAD quinone at 438 nm with butyryl-CoA or CoA required about an hour, whereas acetyl-CoA induced no significant changes above the background level. Interestingly, butyryl-CoA and acetyl-CoA caused an immediate increase of the long-wave absorption bands, measured at 590 and 739 nm, whereas CoA only exhibited a slow increase at these wavelengths. Notably, when ferricenium hexafluorophosphate was used as the electron acceptor (49), 4HBD did not catalyze the oxidation of butyryl-CoA.

The EPR data (Fig. 5) corroborated the results of the UV-vis spectroscopic experiments. All components analyzed, crotonyl-CoA as well as CoA, acetyl-CoA, or butyryl-CoA, induced similar EPR spectra, showing not only the FAD semiquinone (g-factor [i.e., position of an EPR signal in the spectrum; gΔ = 2.004 to 2.007]) but also features which are attributed to a reduced [4Fe−4S]+ cluster (gΔ = 2.031 to 2.032, g∥ = 1.97, and g⊥ = 1.91). This raises the question of the origin of the electron that reduces FAD and the [4Fe−4S]+ cluster. The slight shoulder in the spectrum induced by butyryl-CoA (Fig. 5, arrows) could stem from an aliphatic ketyl radical that cannot react further due to the lack of the hydroxyl group at C-4. The smooth spectra induced by acetyl-CoA and CoA are consistent with the inability of these compounds to form such a radical.

**Sequence analysis of 4HBD.** The amino acid sequence of 4HBD from *C.aminobutyricum* was reported earlier (32) but was in mismatch with the electron density map (12). The new sequence established in this work revealed nine nucleotide changes that led to the mutation of R43 to A (R43A), G167 to D, and D357...
to G (GenBank accession number CAB60035.2) (see Table S1 in the supplemental material). With these changes, the sequence is now in accord with the structural data (12). Highly conserved regions were found by comparing the amino acid sequences annotated as 4HBD to dehydratase/isomerases participating in 4-aminobutyrate metabolism, 4-hydroxyphenylacetate 3-hydroxylases, or phenol hydroxylases. The closest relationships revealed were sequences from the more than 100 *Clostridium difficile* strains (50) [renamed *Peptoclostridium difficile* (51)], with 83% identity, followed among others by sequences from *Porphyromonas gingivalis* (76%) (52), *Clostridium kluyveri* (74%) (9,53), and *Fusobacterium nucleatum* (72%) (54). Relatives of 4HBD are also found in the archaea, including *Archaeoglobus fulgidus* (56%) (55), *Metallosphaera sedula* (43%) (11), and *Nitrosopumilus maritimus* (58%) (56). All sequences contain the [4Fe-4S] cluster coordination motif, CX3CXnHX6C (12) and the conserved amino acids T190, Y296, E455, and E257 (C. *aminobutyricum* numbering) near the active center, as well as the more peripheral K300, A460, and R90 (see Table S1). Several homologous sequences, annotated as dehydratase/isomerase or 4-hydroxyphenylacetate 3-hydroxylase, lack the [4Fe-4S] cluster cysteines. On the other hand, the putative 4HBD of *Syntrophus aciditrophicus* (46% sequence identity) contains the cluster’s cysteines and histidine but lacks Y296, E455, and E257. The results with the variants shown below strongly indicate that this enzyme should have neither a dehydratase nor an isomerase activity.

**Gene expression and purification.** Initial attempts to express the gene *abfD* that codes for 4-hydroxybutyryl-CoA dehydratase in *E. coli* BL21 yielded large amounts of insoluble protein. After many trials, we identified the following crucial parameters for successful expression of *abfD*: (i) growth temperature of 20 to 25°C, (ii) a 100 g/liter (0.23 M) concentration of the inducer anhydrotetracycline, (iii) coexpression of the chaperon genes *groEL*, (iv) maintenance of the exponential growth phase during the production phase (OD578 of 0.5 to 0.8), and (v) the presence of 2 mM Fe(III)citrate and 0.4 mM riboflavin in the medium (57). Without added Fe, the specific activity of the purified dehydratase was 6-fold lower, and without riboflavin, the activity was zero. The heterologous expression of pASK-IBA(3) carrying *abfD* resulted in the production of recombinant protein that was C-terminally fused to a Strep-tag for affinity purification on a StrepTactin column (Fig. 6). Pure 4HBD was obtained with a specific dehydratase activity of 2.2 ± 0.3 U mg⁻¹. It contained 11.8 ± 0.1 mol Fe and 4.4 ± 0.2 mol FAD per mol homotetramer (Table 2). After reconstitution with FeCl₃ and Na₂S, the specific activity increased to 4.5 ± 0.2 U mg⁻¹, and the iron content also increased, to 14.8 mol Fe (theoretically, 16 Fe and 4 FAD are required for the homotetrameric dehydratase). Probably the increase in specific activity is due to the insertion of the fourth iron (Fe₁) into the cluster, which is only weakly coordinated by H296, as indicated by the unusual long N-Fe bond of 2.4 Å (12, 14). The specific activity obtained was similar to those obtained with the enzyme preparations purified from *C. aminobutyricum* (7.3 U mg⁻¹ [2], 2 to 9 U mg⁻¹ [17],

**FIG 4** Visible absorption changes of 4HBD (1.6 mg/ml, 4.5 U, and 7.1 μM) isolated from *C. aminobutyricum* after the addition of 5 mM substrate or analogue: red, crotonyl-CoA; black, butyryl-CoA; magenta, acetyl-CoA; blue, CoA; brown, no substrate.

**FIG 5** EPR spectra of 4HBD (41.7 μM) at 10 K, frozen in liquid nitrogen 1 min after incubation with substrates (5 mM) crotonyl-CoA (red) and CoA (blue). The spectrum with butyryl-CoA (black) revealed an additional signal shoulder between 338 and 343 mT (signal around g₃ = 1.966), highlighted with arrows, which could stem from a substrate-derived radical. Total spin concentrations are 2.9% ± 0.2% radical per active site. Microwave power, 2 mW; microwave frequency, 9.459 GHz; modulation amplitude, 1.25 mT; modulation frequency, 100 kHz.
and 2 to 16.7 U mg\(^{-1}\) \(^{19}\)). Based on our method \(^{57}\), Könneke et al. \(^{56}\) recently produced 4HBD from \(C.\) aminobutyricum and the nearly oxygen-insensitive 4HBD from \(N.\) maritimus in \(E.\) coli, both with specific activities of 20 U mg\(^{-1}\). Probably the higher activities were due to the use of synthetic genes adapted to the codon usage of \(E.\) coli.

**Variants of 4HBD and their dehydratase activities.** The successful expression of the abfD gene in \(E.\) coli allowed evaluation of the function of the conserved residues in the active center of 4HBD by site-directed mutagenesis. The variants (Table 2) were produced and purified under the same conditions as the recombinant wild-type enzyme. After reconstitution with FeCl\(_3\) and Na\(_2\)S, the activities of all mutants remained unchanged, although all samples contained more iron.

The mutations of the amino acids of 4HBD that may coordinate the [4Fe-4S] cluster, C99A, C103A, C299A, H292C, and H292E, yielded proteins without measurable activity and with less iron (50%) and FAD (75%) (Table 2). Moreover, the three cysteine mutations caused dissociation of the tetrameric protein into dimers and monomers, whereas H292C and H292E proteins consisted of tetramers and dimers (Table 2). Hence, the [4Fe-4S] cluster may also contribute to the stabilization of the quaternary structure. The maintenance of the homotetrameric structure in all other mutants underpins this suggestion. In particular, the H292C protein contradicted our expectation of an increased stability of the cluster. In comparison to the Ne2 of histidine, the thiolate of the cysteine residue probably is too far away to coordinate Fe1. Altogether, these data underpin the idea that the [4Fe-4S] cluster is essential for activity. Therefore, it is surprising that the low activity of some of the variants could not be improved by cluster reconstitution.

The Q101E variant retained about 4% dehydratase activity. Q101 is located in the N-terminal domain and anchors the loop containing C99 and C103 to the middle domain via interactions at hydrogen bonding distance with N194. Moreover, Q101 also helps to fix C99 and C103 through interactions at hydrogen bonding distance between its side chain and the carbonyl group of C103 and between its carbonyl group and the amide group of C103. Thus, the mutation Q101E is most probably able to keep this network of interactions. The A460G variant was produced because NH of A460 could form a hydrogen bond to the thioester carbonyl of the substrate \(^{12}\). The activity of this variant is surprisingly low (2%), although NH of glycine could also form a hydrogen bond to the thioester carbonyl. Probably the greater flexibility of glycine disturbs the protein structure. The M149S variant revealed 7% of the dehydratase activity. M149 is one of the residues that coordinate Fe1 and between its carbonyl group and the amide group of C103. Thus, the mutation Q101E is most probably able to keep this network of interactions. The A460G variant was produced because NH of A460 could form a hydrogen bond to the thioester carbonyl of the substrate \(^{12}\). The activity of this variant is surprisingly low (2%), although NH of glycine could also form a hydrogen bond to the thioester carbonyl. Probably the greater flexibility of glycine disturbs the protein structure. The M149S variant revealed 7% of the dehydratase activity. M149 is one of the residues that coordinate Fe1 and

**TABLE 2** Characterization of wild-type and mutants of 4HBD

<table>
<thead>
<tr>
<th>4HBD</th>
<th>Sp act of dehydratase (U/mg)</th>
<th>Amt of iron/tetramer (mol/mol)</th>
<th>Amt of FAD/tetramer (mol/mol)</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>2.2 ± 0.3</td>
<td>11.8 ± 0.1</td>
<td>4.4 ± 0.2</td>
<td>Tetramer</td>
</tr>
<tr>
<td>Wild type(^{a})</td>
<td>4.5 ± 0.2</td>
<td>14.8</td>
<td>ND(^{b})</td>
<td>ND</td>
</tr>
<tr>
<td>H292C</td>
<td>&lt;0.005</td>
<td>8.1 ± 0.1</td>
<td>3.1 ± 0.1</td>
<td>Tetramer, dimer</td>
</tr>
<tr>
<td>H292E</td>
<td>&lt;0.005</td>
<td>7.8 ± 0.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C99A</td>
<td>&lt;0.005</td>
<td>7.7</td>
<td>2.9</td>
<td>Tetramer</td>
</tr>
<tr>
<td>C103A</td>
<td>&lt;0.005</td>
<td>8.0</td>
<td>3.2</td>
<td>Tetra-, di-, and monomer</td>
</tr>
<tr>
<td>C299A</td>
<td>&lt;0.005</td>
<td>7.7 ± 0.2</td>
<td>3.0</td>
<td>Tetra-, di-, and monomer</td>
</tr>
<tr>
<td>Y296F</td>
<td>0.030 ± 0.003</td>
<td>11.0 ± 0.2</td>
<td>4.4 ± 0.2</td>
<td>Tetramer</td>
</tr>
<tr>
<td>Y296F(^{a})</td>
<td>0.030 ± 0.003</td>
<td>13.8</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Y296W</td>
<td>&lt;0.005</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>E455Q</td>
<td>&lt;0.005</td>
<td>10.2 ± 0.2</td>
<td>3.8 ± 0.1</td>
<td>Tetramer</td>
</tr>
<tr>
<td>E455Q(^{a})</td>
<td>&lt;0.005</td>
<td>12.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>E257Q</td>
<td>&lt;0.005</td>
<td>11.0 ± 0.1</td>
<td>3.5</td>
<td>Tetramer</td>
</tr>
<tr>
<td>Q101E</td>
<td>0.20 ± 0.02</td>
<td>13.1</td>
<td>3.2</td>
<td>Tetramer</td>
</tr>
<tr>
<td>M149S</td>
<td>0.30 ± 0.02</td>
<td>11.4</td>
<td>3.0</td>
<td>Tetramer</td>
</tr>
<tr>
<td>T190V</td>
<td>0.020 ± 0.001</td>
<td>10.6</td>
<td>2.9</td>
<td>Tetramer</td>
</tr>
<tr>
<td>K300Q</td>
<td>0.20 ± 0.02</td>
<td>13.6</td>
<td>3.4</td>
<td>Tetramer</td>
</tr>
<tr>
<td>A460G</td>
<td>0.09 ± 0.01</td>
<td>10.3</td>
<td>3.5</td>
<td>Tetramer</td>
</tr>
</tbody>
</table>

\(^{a}\) Superscript \(R\) indicates a protein reconstituted with FeCl\(_3\) and Na\(_2\)S.

\(^{b}\) ND, not determined.
delineating residues of the substrate channel. Upon changing to serine, the channel might become wider and more polar, affecting the entrance and positioning of the substrate.

Besides H292, the most important catalytic residues appear to be E455, T190, E257, and Y296. The inactive but homotetrameric E455Q and E257Q variants show that these residues are absolutely necessary for dehydratase activity, whereas the isomerase activity was retained, especially in the latter variant, as shown below. E455 is 3.6 Å from the modeled C-4 position of the substrate (Fig. 3). The retention of the configuration during the formation of the methyl group of crotonyl-CoA (16) suggests that E455 is a catalytic acid, since it is positioned at the correct angle of the planar dienolate intermediate proposed in the mechanism. E257 is located on the Si side of the FAD isoalloxazine ring at 3.0 Å from the hydroxyl group of T190, which could form a hydrogen bond at 3.3 Å to N-5 of the isoalloxazine ring (Fig. 3). The replacement of T190 by valine or E257 by glutamine resulted in a drop-off of over 99% in the specific activity (Table 2). We consider that T190 and E257, located at the water channel, are involved in the protonation of the initially formed FAD semiquinone anion and in the translocation of the 3Si proton from 4-hydroxybutyryl-CoA. Remarkably, the acyl-CoA dehydrogenases contain a conserved threonine at the same position as T190 in 4HBD (58). A threonine-aspartate dyad corresponding to T190/E257 in 4HBD has been detected only in the related 4-hydroxphenylacetate 3-hydroxylase (59).

The experiments whose results are shown in Fig. 7 underpin these considerations. As already shown by the results in Fig. 4, the addition of crotonyl-CoA to 4HBD induces an immediate drop in the absorbance at 438 nm and a transient increase at 590 nm. The E445Q variant exhibited the same behavior but at a much lower rate, indicating a direct role of E445 in acid base catalysis but only an indirect role in electron transfer. In contrast, E257Q, Y296F, and Y296W hardly responded to the addition of substrate, which we interpret as the involvement of the E257/Y296 dyad in the reduction of FAD to its blue semiquinone FADH2. The results of EPR experiments shown in Fig. 8 support this interpretation.

The question of the origin of the electron required for the reduction of FAD arises. Although the electron could come from the substrate and butyryl-CoA, acetyl-CoA and CoA are less likely donors. Furthermore, the data shown in Fig. 4 and 7, as well as the results of the preliminary stopped-flow experiments, indicate that the electron transfer occurs before the actual dehydration. Therefore, we speculate that the conserved tyrosine 296 could be the electron source and that it is converted to a radical, as observed in flavin monoamine oxidases (60). This would fit well with the observed inactivity of Y296F in the electron transfer (Fig. 7 and 8), whereby the low residual dehydratase activity of Y296F (0.7%) (Table 2) could be due to a more distant electron source, perhaps M149, which is 8.5 to 10.5 Å apart from N-5 of FAD. We made a Y296W variant because the indole moiety could be oxidized to a stable radical. The unexpected complete loss of dehydratase activity of this variant suggested that the relatively large indole moiety did not fit into the active site. Probably E455 deprotonates Y296 at a distance of 2.6 Å, which in the E455Q variant could be carried out by the more distantly located H292 (3.7 Å), causing the lower rates seen in the results shown in Fig. 7. The resulting phenolate could be much more easily oxidized by FAD than the protonated...
The E257Q and Y296F compared with those of the wild type (Table 3). The E257Q and Y296F with Saccharomyces cerevisiae cells of the yeast ribonucleotide reductase, which can be detected in whole detected at 27 K (Fig. 9) or at 77 K. The spectra inFig. 9 are of such a radical should be measurable. Most likely, the interaction of the tyrosyl radical in 4HBD isolated from E. coli with an authentic tyrosyl radical of the aerobic ribonucleotide reductase, which can be detected in whole cells of the yeast Saccharomyces cerevisiae W303 (61). Since all three spectra were taken under identical conditions: temperature, 27 K; microwave power, 2 mW; microwave frequency, 9.465 GHz; modulation amplitude, 0.45 mT; and modulation frequency, 100 kHz.

form. However, in the dehydratase purified from E. coli cells grown on (S)-[phenyl-3,5-2H₂]tyrosine, no significant change of the line width of the isotropic signal at g = 2.004 to 2.007 was detected at 27 K (Fig. 9) or at 77 K. The spectra in Fig. 9 are with that of an authentic tyrosyl radical of the aerobic ribonucleotide reductase, which can be detected in whole cells of the yeast Saccharomyces cerevisiae. All three spectra were taken under identical conditions: the presence of such a radical should be measurable. Most likely, the interaction of the tyrosyl radical in 4HBD with the nearby [4Fe-4S] cluster and the flavin semiquinone affords a very different spectrum, which could possibly be concealed by other signals at a lower temperature and broadened beyond detection at a higher temperature (Fig. 8 and 9).

Vinylacetyl-CoA Δ-isomerase activity of 4HBD and variants. The isomerization of vinylacetyl-CoA to crotonyl-CoA was determined by an enzymatic assay with the NADPH-dependent crotonyl-CoA carboxylase/reductase as an auxiliary enzyme (36). To detect the function of the conserved amino acids during the isomerization procedure, the activities of all variants before and after oxidation by air (24 h at 20°C) were determined and compared with those of the wild type (Table 3). The E257Q and Y296F variants retained high isomerase activities, which, in contrast to that of the wild type, were hardly affected by incubation with air. The slight decrease of the isomerase activity of the Y296F variant could be due to its residual, <1% dehydratase activity. This confirms our expectation that these residues are involved in the electron transfer and, therefore, are not necessary in the isomerization. Hence, a functional electron transfer from Y296 to FAD appears to be required for the inactivation by air, because the tyrosyl radical and the FAD semiquinone should readily react with oxygen. The H292E and E455Q variants revealed low isomerase activities, supporting our view that H292 and E455 catalyze the isomerization. H292 could act as a base to remove the 2Re proton of vinylacetyl-CoA, whereas E455 may protonate the C-4 methylene group to form the methyl group. The behavior of the variants of the three cysteines coordinating the [4Fe-4S] cluster cannot be readily explained. As shown by the data in Table 2, they not only affect the iron content and, thus, the composition of the cluster but also disturb the quaternary structure of the dehydratase. The unexpected high isomerase activity of the C299A variant that was not affected by air could be due to a stable [2Fe-2S] cluster.

**Table 3** Comparison of vinylacetyl-CoA isomerase specific activities catalyzed by 4HBD before and after its exposure to air

<table>
<thead>
<tr>
<th>4HBD</th>
<th>Vinylacetyl-CoA Δ-isomerase activity (U/mg) with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Freshly purified 4HBD</td>
</tr>
<tr>
<td>Wild type</td>
<td>18.5</td>
</tr>
<tr>
<td>H292C</td>
<td>2.1</td>
</tr>
<tr>
<td>H292E</td>
<td>0.2</td>
</tr>
<tr>
<td>C99A</td>
<td>0.25</td>
</tr>
<tr>
<td>C103A</td>
<td>3.1</td>
</tr>
<tr>
<td>C299A</td>
<td>14.0</td>
</tr>
<tr>
<td>Y296F</td>
<td>9.6</td>
</tr>
<tr>
<td>E455Q</td>
<td>1.3</td>
</tr>
<tr>
<td>E257Q</td>
<td>17.0</td>
</tr>
</tbody>
</table>

4-Hydroxy[3-2H₂]butyrate as the initial substrate was almost completely consumed. By ¹H NMR, the ratio of the signals of the protons (16). Whether the observed deuterium incorporation at C-4 stems from the reversible oxidation of [2-2H₂]crotonyl-CoA to [1H]acetyl-CoA and unlabeled acetyl-CoA (Fig. 2), which also explains the loss of deuterium from C-2 and the relatively high deuterium content of acetate. The reversibility of this oxidation is demonstrated by bacteria able to reduce two molecules of acetyl-CoA to one molecule of butyrate, e.g., C. kluveri, which use the same enzymes as in the reverse pathway (3).
close to the theoretical value of 1.00. The $^2$H NMR spectrum shows no signals above the background level at B2 or B4, indicating no migration event originating from C-3 (Fig. 10B). As expected, acetate contains almost no deuterium due to the complete loss during oxidation of 3-hydroxybutyryl-CoA to acetoacetyl-CoA.

To verify or exclude an incorporation of deuterium from 4-hydroxy[2-2H$_2$]butyrate into the methyl group of butyrate, we repeated the experiment in vitro. Therefore, we generated in situ 4-hydroxy[2-2H$_2$]butyryl-CoA from the free acid and acetyl-CoA by CoA-transfer followed by dehydration, catalyzed by 4-hydroxybutyrate CoA-transferase and 4HBD, respectively. The crotonyl-CoA formed was irreversibly reduced to butyrate by NADH using the electron-bifurcating electron-transferring flavoprotein/butyryl-CoA dehydrogenase complex from Clostridium tetanomorphum together with ferredoxin and hydrogenase (5). Formate and formate dehydrogenase acted as an NADH-regenerating system. To summarize, 4-hydroxybutyrate$^{-}$ + acetyl-CoA $\rightarrow$ formate$^{-}$ + 2 H$^+$ $\rightarrow$ butyryl-CoA + acetate$^{-}$ + 2 CO$_2$ + H$_2$ + H$_2$O.

After completion of the reaction, the thioesters were hydrolyzed with NaOH and the fatty acids were isolated by steam distillation. Analysis by NMR revealed no deuterium incorporation into the methyl group of butyrate. Thus, it appears very likely that the in vivo incorporation of deuterium from C-2 into the methyl group was due to butyrate synthesis from [2H]acetyl-CoA (Fig. 2).
Proposal of an alternative mechanism involving a tyrosyl radical. As outlined in the introduction, the main argument against the previously postulated mechanism is the action of FAD to remove only one electron from C-3 of the enolate of 4-hydroxybutyryl-CoA. The spectroscopic data for the wild type and the variants suggest a substrate-induced electron transfer to FAD, as well as to the [4Fe-4S] cluster, before the actual dehydration takes place. Thus, FADH$_2$ which can only accept one electron, is formed and the abstraction of a hydride from C-3 is prevented. Since butyryl-CoA induces similar spectral changes (Fig. 4 and 5), 4HBD should not catalyze the oxidation of butyryl-CoA to crotonyl-CoA, even in the presence of an effective electron acceptor, as observed experimentally. Because the substrate-induced electron transfer is abolished in the Y296F variant, we propose the deprotonated Y296 as the one-electron donor rather than the substrate itself. The 6.6-Å distance between the phenolate oxygen of the tyrosine residue and N-5 of FAD (Fig. 3) allows such a transfer. Most likely the tyrosyl radical interacts with the neutral FAD semiquinone and the reduced [4Fe-4S]$^{2-}$ cluster (4.7 Å apart), which changes the typical spectrum (Fig. 9, bottom trace) to the top two traces in Fig. 5 or to the red trace (crotonyl-CoA) in Fig. 5. The question arises of why this electron transfer from Y296 to FAD and to the cluster is induced by the substrate. In its absence, the substrate channel is filled with polar water molecules (12), which may prevent an electron transfer. In the presence of substrate, however, the water molecules are displaced and the medium between Y296 and FAD becomes more hydrophobic, which may enable the electron transfer. Confirmation of this view comes from the fact that the inactive substrate analogue butyryl-CoA also induces a fairly rapid absorbance change in the region of >500 nm, whereas that induced by CoA, which cannot replace the water molecules, is very slow (Fig. 4). In the ferredoxin-NADP$^+$ reductases from chloroplasts, an electron transfer occurs between ferredoxin and FAD over a similar distance of 6 Å. The crystal structure and site-directed mutagenesis of the reductase revealed the importance of hydrophobic residues between the two redox centers (62, 63).

In the new mechanism of the dehydration of 4-hydroxybutyryl-CoA (Fig. 11), we postulate that the FAD semiquinone rather than the FAD quinone oxidizes the enolate (or, in the reverse direction, the dienolate) to the enoxy radical (dienoxy radical). The FADH$^-$ anion formed, in combination with the T190/E257 dyad, probably acts as a more efficient base to remove the 3Si proton. Alternatively, the enolate could be directly oxidized to the allylic ketyl radical via a proton-coupled electron transfer (PCET). Because the FAD is now reduced to the hydroquinone, further oxidation of the allylic ketyl radical to 4-hydroxycrotonyl-CoA is not possible. The mechanism depicted in Fig. 11 does not account for the observed partially reduced [4Fe-4S]$^{2-}$ cluster. Possibly the electron withdrawn from Y296 is delocalized between the flavin (6.6 Å apart) and the [4Fe-4S] cluster (4.7 Å apart). Because the reduced cluster has a lower Lewis acidity, the release of the H$_2$O formed could be facilitated.

The mechanistic scheme (Fig. 11) proposes several protona-
tion/deprotonation events. The addition of 4-hydroxybutyryl-CoA to 4HBD expels three water molecules from the substrate channel and the hydroxyl group interacts with Fe1 of the cluster, whereby H292 is liberated to act as a base. The substrate-induced electron transfer from Y296 to FAD and to the [4Fe-4S] cluster could be assisted by deprotonation of Y296 with E455. After the electron transfer, the dyad E257/T190 could neutralize the FAD semiquinone anion (FADH−) to FADH, whereby the proton stems from the water channel. Then, H292 removes the 2Re proton to yield the enolate, which is oxidized and deprotonated to the allylic ketyl radical by the FADH−→T190−E257 triad connected by hydrogen bonds. E257 has to emit its proton via the water channel into the medium. The hydroxyl group eliminated at Fe1 is most likely protonated by H292 and discharged as H2O, also through the water channel. Notably, such a channel has also been detected in quinolinate synthase, through which the products HPO42− and 2H2O could be released (64). Reoxidation of FADH− to FADH· by the dienoyl radical occurs without proton movement. E455 is located in a position to protonate the dienolate at C-4 from the same side from which the hydroxyl group has left (16). After the release of crotonyl-CoA, an electron from FADH· flows back to Y292 to yield FAD and Y296·, whereby the proton is taken up by T190/E257. The re-formed phenolate gets its proton from the water channel via E455 to regenerate Y296.

To summarize, the proposed mechanism of 4HBD is without precedence in anaerobic radical chemistry. The radical-generating system consists of Y296, FAD, and [4Fe-4S], whereby the initially formed FAD− gets a proton from the water channel via E257 and T190. This system is quite different from those of other anaerobic enzymes, which use coenzyme B12, S-adenosylmethionine (radical SAM enzymes), ATP-driven electron transfer, or flavin-based electron bifurcation (6, 14, 65). The electron transfer, the dyad E257/T190 could neutralize the FADH− in 4HBD ensures a one-electron oxidation of the substrate, which is necessary to form the allylic ketyl radical that eliminates the hydroxyl group. Furthermore, FADH· cannot transfer electrons to the substrate, which is necessary to form the allylic ketyl radical by the FADH·−T190−E257 triad connected by electron transfer, or flavin-based electron bifurcation (6, 14, 65). The adenylsulfur and FAD-containing 4-hydroxybutyryl-CoA dehydratase/ vinylacetyl-CoA delta 3-delta 2-isomerase from Clostridium anabinobutyricum. Eur J Biochem 215:421−429. http://dx.doi.org/10.1111/j.1432-1033.1993.tb18049.x.

This work was supported by the Max-Planck-Institut für terrestrische Mikrobiologie, the Deutsche Forschungsgemeinschaft (DFG), and Syn-mikro of the Philipps-Universität Marburg.

ACKNOWLEDGMENTS

We are indebted to Írfin Çinkaya (Teva Pharmaceuticals, Turkey) for helpful advice and for data from his Ph.D. thesis (19). We thank Dallir J. Netz (Philips-Universitats-Marburg) for providing E. coli strain BL21 CodonPlus (DE3)-GroEL. We acknowledge Bernard T. Golding (University of Newcastle upon Tyne) for his advice on synthesizing γ-[3-2H]butyrolactone.

REFERENCES

Electron-nuclear double resonance spectroscopy investigation of 4-hy-
droxybutyryl-CoA dehydrogenase from Clostridium symbiosum: toward a 4-
hydroxybutyryl-CoA dehydrogenase catalyzing the reaction: 4-
hydroxybutyryl-CoA + H2O → 4-hydroxybutyrate + CoA. Biochim Biophys 
6233.2001.

Hewitson KS, Ollagnier-de Choudens S, Sanakis Y, Shaw NM, Baldwin 
JE, Münch RM, Thauer RK. 2003. Complete genome sequence of the oral 
pathogenic bacterium Clostridium tetanomorphum. J Bacteriol 185:1278–
10.1037/pnas.85.18.6677.

Pohl B, Raichle T, Ghisla S. 1986. Studies on the reaction mechanism of 
general acyl-CoA dehydrogenase. Determination of selective isotope ef-

Synthesis of 13C-labeled gamma-hydroxybutyrates for EPR studies with 
JIUB/01417-07.

Koivunen S. 2008. Aufreinigung und Charakterisierung des Butyryl-CoA 
Dehydrogenase/ETF Komplexes aus Clostridium tetanomorphum. Di-
ploma Thesis, Philipps-Universität Marburg, Marburg, Germany.

Massey V, Palmer G. 1966. On the existence of spectrally distinct classes of 
flavoprotein semiquinones. A new method for the quantitative produc-

Lehman TC, Hale DE, Bhala A, Thorpe C. 1990. An acyl-coenzyme A 
dehydrogenase assay utilizing the ferricenium ion. Anal Biochem 186: 

Kurka H, Ehrenreich A, Ludwig W, Monot M, Rupnik M, Barbutf F, 
Indra A, Dupuy B, Liebl W. 2014. Sequence similarity of Clostridium 
difficile strains studied by analysis of conserved genes and genome content is re-
10.1371/journal.pone.0086535.

Yutin N, Galperin MY. 2013. A genomic update on clostridial phylogeny: 
Gram-negative spore formers and other misplaced clostridia. Environ 

Nelson KE, Fleischmann RD, DeBoy RT, Paulsen IT, Fouts DE, Eisen 
JA, Daugherty SC, Dodson RJ, Fauron CM, Johnson J, Nusbaum C, 
Margolies R, Skrzypek M, Huson DH, Garrett M, Clayton LA, 
Theron T, Los Alamos National Laboratory. 2002. Complete genome sequence of 

Seedorf H, Fricke WF, Veith B, Brüggemann H, Liesegang H, Stritt-
matter A, Mietheke M, Buckel W, Hinderberger J, Li F, Hagemeier C, 
Thauer RK, Gottschalk G. 2008. The genome of Clostridium kluyveri, a


