Substrate induced radical formation in 4-hydroxybutyryl-CoA dehydratase

from Clostridium aminobutyricum

Jin Zhang\textsuperscript{1,2,\ast}, Peter Friedrich\textsuperscript{1,2,\ast}, Antonio J. Pierik\textsuperscript{3,4}, Berta M. Martins\textsuperscript{5}, Wolfgang Buckel\textsuperscript{1,2}

\textsuperscript{1}Laboratorium für Mikrobiologie, Fachbereich Biologie and Synmikro, Philipps-Universität, 35032 Marburg, Germany; \textsuperscript{2}Max-Plank-Institut für terrestrische Mikrobiologie, 35043 Marburg; \textsuperscript{3}Institut für Zytobiologie, Philipps-Universität, 35032 Marburg, Germany; \textsuperscript{4}Present address: Fachbereich Chemie, Universität Kaiserslautern, 67663 Kaiserslautern, Germany; \textsuperscript{5}Institut für Biologie, Strukturbiochemie/Biochemie, Humboldt-Universität zu Berlin, 10099 Berlin, Germany.

Dedicated to Dr. Peter Willadsen (Brisbane, Australia)

* JZ and PF contributed equally to this work and are considered both as first authors.

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Correspondence to: W. Buckel, Laboratorium für Mikrobiologie, Fachbereich Biologie, Karl-von-Frisch-Straße 8, 35032 Marburg. Tel +49 6421 28 22088, Fax +49 6421 28 28979, Email: buckel@staff.uni-marburg.de

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Abbreviations: 4HBD, 4-hydroxybutyryl-CoA dehydratase; MCAD, medium-chain acyl-CoA dehydrogenase; DTT, dithiothreitol.
4-Hydroxybutyryl-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 FAD in each subunit. Upon addition of crotonyl-CoA or the analogues butyryl-CoA, acetyl-CoA and CoASH, UV-visible and 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, H292E, C99A, C103A, 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, Y296W) or <1% (Y296F, T190V) dehydratase activities. E455Q, but neither Y296F nor E257Q, displayed the same spectral changes as the wild type enzyme after addition of crotonyl-CoA but at a much lower rate. The results suggest that upon substrate addition, Y296 is deprotonated by E455 and reduces FAD to FAD$^*$ aided by protonation from E257 via T190. In contrast to FADH$^*$, the tyrosyl radical could not be detected by EPR spectroscopy. FADH$^*$ appears to initiate the radical dehydration via an allylic ketyl radical that has been proposed 19 years ago. The mode of radical generation in 4HBD is without precedence in anaerobic radical chemistry. It largely differs from that in enzymes which use for this purpose coenzyme B$_{12}$, S-adenosylmethionine, ATP driven electron transfer or flavin-based electron bifurcation.

**Introduction**  
4-Hydroxybutyryl-CoA dehydratase (4HBD) catalyzes the reversible dehydration of 4-
hydroxybutyryl-CoA to crotonyl-CoA and the irreversible \( \Delta \)-isomerisation of vinylacetyl-CoA to crotonyl-CoA (Fig. 1). The enzyme was discovered in the fermentation of 4-aminobutyrate (\( \gamma \)-aminobutyrate, GABA) to ammonia, acetate and butyrate (Fig. 2) by \textit{Clostridium aminobutyricum} (1, 2) attributed to the cluster XI of the clostridia (3, 4). In this pathway 4-aminobutyrate is transaminated with 2-oxoglutarate to yield glutamate and succinic semialdehyde (4-oxobutanoate), which is reduced to 4-hydroxybutyrate. Activation to the CoA thioester and dehydration affords crotonyl-CoA, which disproportionates to butyrate and acetate. Energy is conserved via substrate level phosphorylation via acetylphosphate and via electron bifurcation at the electron transferring flavoprotein (Etf) and butyryl-CoA dehydrogenase (5, 6). The thereby obtained reduced ferredoxin recycles NADH mediated by NAD-ferredoxin oxidoreductase also called Rnf, which generates an electrochemical \( \text{Na}^+ \) gradient for ATP synthesis (7, 8). With one additional dehydrogenase, this pathway is used by \textit{Clostridium kluyveri} to reduce succinyl-CoA to butyrate (Fig. 2) (9). Autotrophic \( \text{CO}_2 \)-fixing Crenarchaeota synthesize succinyl-CoA by reductive carboxylations of acetyl-CoA. The pathway of Fig. 2 recycles the carrier molecule acetyl-CoA and forms a second one for biosynthesis (10, 11). The key enzyme of all these conversions is 4HBD, which links lipid and carbohydrate metabolisms. In this work we study the enzyme in more detail and propose a plausible mechanism of action.

4HBD from \textit{C. aminobutyricum} is a homotetrameric enzyme (4 \( \times \) 56 kDa) containing one \([4\text{Fe}-4\text{S}]^{2+}\) cluster and one FAD in each subunit (2, 12). The dehydration of 4-hydroxybutyryl-CoA to crotonyl-CoA involves the \textit{anti}-elimination of the activated 2\textit{Re}-hydrogen (\( pK_a \) 7-8) (13) and the non-activated 3\textit{Si}-hydrogen (\( pK_a \) ca. 40) (Fig. 1) as well as the replacement of the 4-hydroxyl...
group by hydrogen with retention of configuration, probably via an allylic ketyl radical intermediate (14-16). Upon incubation of the enzyme under air, the dehydratase activity was lost within one hour, whereas the isomerase activity decreased by 60 – 90% and remained stable at these levels for at least 24 hours (17-19). Most likely FAD and the [4Fe-4S]^{2+} cluster are not required for the isomerization.

The crystal structure of 4HBD suggested that the [4Fe-4S]^{2+} cluster is coordinated by three cysteines (C99, C103, C299) and one histidine (H292) via Né2 (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 'substrate channel', leading through the gap between the cluster and FAD, 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 significantly change. This location of the substrate brings the 2Re-hydrogen close to H292 and the 3Si-hydrogen close to NS of FAD enabling the anti-elimination of both hydrogens as observed in MCAD catalysis (13, 15, 16). The second channel, called '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 from 40 to 14 (22). The now facilitated removal of the 3Si-proton yields the allylic ketyl radical that readily eliminates the hydroxyl group bound to Fe1 of the cluster. The one-electron oxidation of the enolate, however, is in contrast to MCAD, where FAD, in a structurally identical position, removes a hydride (or two electrons and a proton), which in the case of 4HBD would lead to the dead-end product 4-hydroxycrotonyl-CoA. Therefore the question arises how 4HBD manages to take away only one electron from the enolate. To resolve this major drawback of the proposed radical mechanism, we showed by EPR and UV-vis spectroscopy that the substrate crotonyl-CoA as well as butyryl-CoA, acetyl-CoA and CoASH induced an internal electron transfer leading to a partial one-electron reduction of the [4Fe-4S]^{2+} cluster and the FAD. To elucidate the electron source and the role of the conserved residues around the active site, we produced an active recombinant 4HBD with a C-terminal Streptag in *Escherichia coli* and prepared a series of variants, which we analyzed by their enzymatic activities and as well as by EPR and UV-vis spectroscopy. The results lead to a modification of our previous mechanistic scheme (14, 21) involving a putative tyrosyl radical.

### 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 by C18 Sep-PakTM columns (Waters, USA) (23). 4-Hydroxybutyryl-CoA and vinylacetyl-CoA were prepared in situ via 4-hydroxybutyrate CoA-transferase from the corresponding acids and acetyl-CoA (24, 25). \(\gamma\)-[2-\(^2\text{H}_2\)]Butyrolactone was obtained from unlabeled 0.5 M \(\gamma\)-butyrolactone (Sigma-Aldrich) by
equilibration in 5 M NaO\textsubscript{2}H at 95 °C for 72 h. Acidification followed by extraction into dichloromethane yielded 85% of the product (93 % label by NMR). γ-[3-\textsuperscript{2}H\textsubscript{2}]Butyrolactone was obtained from 0.5 M ethyl 4,4-dimethoxybutanoate (Sigma-Aldrich) by heating in deuterium oxide at 95 °C and pH 1-2 for 40 h. The resulting aldehyde was reduced with NaBH\textsubscript{4} at pH 7 (1), after which any unwanted label at C2 got removed by heating at 95 °C in H\textsubscript{2}O at pH 10 for 16 h. The final product was isolated as above (91% yield; 97% label by NMR) (1). Prior to use, the lactones were hydrolyzed with 2 equivalents NaOH at ambient temperature for 10 min. L-[3,5-\textsuperscript{2}H\textsubscript{2}]Tyrosine was synthesized by a modification of the described method (26) from unlabeled 1.1 M L-tyrosine in 10 ml deuterium oxide to which as much \textsuperscript{2}H\textsubscript{2}SO\textsubscript{4} 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 \textsuperscript{1}H-NMR. After 2 h, only the aromatic protons at C3 and C5 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 Finnzyms (Espoo, Finland). Expression vectors pASK-IBA and Strep-Tactin 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 main carbon source at 37°C (25). *Escherichia coli* DH5α and TOPO 10 strains were grown aerobically on Standard-I medium (Merck, Darmstadt, Germany). The *E. coli* BL21-CodonPlus(DE3) strain containing an extra constructed plasmid – pOFXtac-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 anhydrotetracycline inducible promoter, a carbenicillin 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; “Easystart PCR Mix” from Molecular Bioproducts Inc., San Diego, USA) and ligated into PCR®-2.1-TOPO plasmid (Invitrogen, USA) for sequencing. The oligonucleotides used for amplification were from MWG AG Biotech, Ebersberg, Germany; forward: 5’-ATGGTAGGTCCTCAAAATGTAAATGACAGCAGAACAGTACATTG-3’. reverse: 5’-ATGGTAGGTCCTCAGCGGTTTAATTCCAGCGATTGCCTAGC-3’.

Afterwards the gene was inserted (BsaI 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 ClustalW (28) and Weblogo (29, 30) programs.

For each site-directed mutagenesis two complementary primers were designed for PCR (Table 1). The reaction was performed in an “Easystart” tube (Molecular Bioproducts Inc., San Diego, USA) and in a total volume of 50 µl, 20 ng template, pASK-IBA3(+) carrying abfD, 30 pmol primer, 10 µl 1% Triton X-100 and 0.5 U Phusion polymerase were added. PCR was performed by initial denaturation at 95 °C for 5 min followed by 15 cycles, each consists of denaturation at 95 °C for 30 seconds, annealing at 66 °C for 30 seconds and extension at 72 °C for 5 min (1 min/kb). The PCR product (a complete plasmid) was desalted (Millipore dialysis paper, 30 min) and the template DNA was removed (DpnI, 1 hour). Before transformation into E. coli DH5α cells, the plasmid used...
was dialyzed again. The *abfD* containing plasmids obtained from colonies were sequenced and transferred into the expression system, *E. coli* BL21 CodonPlus (DE3)-GroEL strain. 

*E. coli* strain BL21-CodonPlus (DE3)-GroEL was transformed with the pASK-IBA3(+) plasmid carrying *abfD*. The transformed strain was grown aerobically at 20-25 °C on Standard-I medium supplemented with 2 mM Fe(III)citrate, 0.4 mM riboflavin, carbenicillin (50 µg/ml) and chloramphenicol (50 µg/ml). At OD_578⁰ = 0.5 – 0.6, the culture was induced with 0.22 µM anhydrotetracycline (100 µg/l) and incubated overnight. Cells pellets were harvested by centrifugation at 6,000 × g and washed with anaerobic water in a Coy anaerobic chamber under 95% N₂ and 5% H₂ (Ann Arbor MI, USA). All subsequent procedures were performed in this chamber. The harvested cells were suspended (50 mM potassium phosphate pH 7.4, 2 mM dithiothreitol, 200 mM NaCl) for ultrasonication on ice and the debris was removed by ultracentrifugation (100,000 × g, 1 hour, 4 °C). The filtered cell-free extract was applied directly to a Strep-Tactin column, equilibrated with buffer (100 mM Tris-HCl, pH 8.0, 150 mM NaCl and 2 mM dithiothreitol). After washing with 50 ml buffer the protein was eluted with the same buffer containing 2.5 mM desthiobiotin. The purified 4HBD was concentrated with a 100 kDa Centricon and stored at −80 °C.

**Enzyme assays.** The specific activity of 4HBD (1, 31, 32) was measured anaerobically in a coupled assay based on the determination of the formed crotonyl-CoA by β-oxidation to acetyl-CoA. The cuvette (d = 1 cm) contained 100 mM potassium phosphate, pH 7.4, 2 mM EDTA, 2 mM dithiothreitol, 2 mM NAD⁺, 1 mM sodium 4-hydroxybutyrate or vinylacetate, 0.1 mM CoASH, 0.1 mM acetyl-phosphate, 1 mM acetyl-CoA, 1.0 U 4-hydroxybutyrate CoA-transferase (24) and a
mixture of auxiliary enzymes from *Acidaminococcus fermentans* (0.3 mg/ml) (33). After 3 min incubation at room temperature, the reaction was started by adding 4HBD. The formation of NADH was measured at 340 nm using an absorption coefficient of 6.3 mM$^{-1}$·cm$^{-1}$ (34). Up to $\Delta A =$ 0.8 min$^{-1}$ the assay responded linearly to the amount of 4HBD added. As described in ref. 31, the mixture of auxiliary enzymes was prepared by chromatography of a cell-free extract from *A. fermentans* on a DEAE Sephacel column. The fractions exhibiting glutaconate CoA-transferase activity were collected, dialyzed and concentrated. This crude mixture also contained crotonase, 3-hydroxybutyryl-CoA dehydrogenase, thiolase and phosphate acetyltransferase which are required for the assay of 4HBD (see Fig. 2). Because some preparations of the auxiliary enzymes comprised vinylacetyl-CoA $\Delta$-isomerase activity, an alternative method to determine this activity was applied later in this work: 100 mM potassium phosphate, pH 7.4, 1 mM vinylacetate, 0.2 mM NADPH, 30 mM KHCO$_3$, 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 also with different enzyme preparations were used.

**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 dithiothreitol, (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 dithiothreitol (final concentration 5 mM, 30 min). Now FeCl₃ and Na₂S (5 and 10 mol/mol 4HBD, m = 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, 5 min). The solution was concentrated by Centricon (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) (ε₅₃⁷ nm = 32 mM⁻¹·cm⁻¹) as described (39, 40). To measure the FAD content in 4HBD, the protein solution was denatured (80 °C, 10 min) and centrifuged (10,000 × g, 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-visible 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, 17 °C) in 50 mM potassium phosphate pH 7.4 followed by removal of the oxidizing agent (3 × volume reduction to 5% by a 30 kDa cut-off Centricon ultrafiltrator and refill with fresh buffer). Each sample was diluted for UV-vis measurements (total volume 500 µl, 1.6 mg protein/ml, 4.5 U, 7.1 µM). The control sample was measured from 300 – 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 pre-oxidized 4HBD (10 mg/ml, 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 substrates. After growth for 24 hours at 37 °C, the supernatants of the media 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 (volume 10 ml) consisted of 50 mM potassium phosphate pH 7.4, 140 mM 4-hydroxy[2-^2H]butyrate, 1.3 mM acetyl-CoA, 10 mM CoASH, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.1 mM NADH, and 175 mM sodium formate. 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* (Sigma-Aldrich, 3.13 U/ml), 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 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 re-dissolved in H₂O for ²H-NMR.
Results and Discussion

Kinetic isotope effects with regiospecifically $^2$H-labelled 4-hydroxybutyrates and 4HBD isolated from *C. aminobutyricum*. 4-Hydroxy[2-$^2$H$_2$]butyrate was obtained by heating unlabelled $\gamma$-butyrolactone in 5 M NaO$_2$H. Heating 4,4-dimethoxybutyrate, a protected succinic semialdehyde, in deuterium oxide at pH 1-2 afforded 4-oxo[3-$^2$H$_2$]butyrate, which was reduced with unlabelled sodium borohydride to 4-hydroxy[3-$^2$H]butyrate. Because 4-hydroxybutyryl-CoA is unstable due to lactonisation (25), it was generated in situ from 4-hydroxybutyrate, acetyl-CoA and 4-hydroxybutyrate CoA-transferase. To measure the kinetic constants of 4-hydroxybutyryl-CoA, the concentration of acetyl-CoA was varied from 10 – 400 µM and that of 4-hydroxybutyrate (1 mM) was kept constant. Actually the apparent $K_m$ and $V_{max}$ values of acetyl-CoA were determined. The unlabelled substrate yielded $K_m = 22 \pm 1 \mu M$, $V_{max} = 7.6 \pm 0.3 s^{-1}$, $V_{max} K_m^{-1} = 0.34 s^{-1} \mu M^{-1}$; 4-hydroxy[2-$^2$H]butyrate gave $K_m = 27 \pm 1 \mu M$, $V_{max} = 4.7 \pm 0.3 s^{-1}$, $V_{max} K_m^{-1} = 0.17 s^{-1} \mu M^{-1}$; 4-hydroxy[3-$^2$H]butyrate gave $K_m = 20 \pm 1 \mu M$, $V_{max} = 7.6 \pm 0.3 s^{-1}$, $V_{max} K_m^{-1} = 0.27 s^{-1} \mu M^{-1}$. From these data the kinetic isotope effects (KIE) were calculated as $^2$H($k_{cat}/K_m$) = 2.0 ± 0.2 for 4-hydroxy[2-$^2$H]butyrate and 1.3 ± 0.2 for 4-hydroxy[3-$^2$H]butyrate. By comparison with MCAD, [2-$^2$H$_2$]butyrly-CoA exhibited a KIE of 2 and with [3-$^2$H$_2$]butyrly-CoA a KIE of 3 was obtained (13, 46). Hence the abstraction of the $2Re$-proton by a basic residue, which in 4HBD is most likely H292 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 barrier-less radical mechanism (see below).
Spectroscopic analysis of 4HBD isolated from *C. aminobutyricum*. 4-HBD has the remarkable property to increase 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-visible 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 flavin quinone (380 and 438 nm) overlaid with those of the [4Fe-4S]⁺⁺⁺⁺⁺⁺ cluster at 420 nm. Upon addition of the substrate crotonyl-CoA, an immediate reduction of the quinone was observed (Fig. 4, absorption decrease at 438 nm) accompanied with a long-wavelength band that appeared in the region from 500 to 800 nm (Fig. 4, absorption increases at 590 and 739 nm). The absorption increase at 590 nm could be due to the neutral FAD semiquinone (λ<sub>max</sub> = 590 nm) (48) and that at 739 nm to a charge transfer band. 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 slower reaction 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 CoASH, acetyl-CoA or butyryl-CoA (Fig. 4). Thus, bleaching the FAD quinone at 438 nm with butyryl-CoA or CoASH 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 CoASH only exhibited a slow increase at these wavelengths. Notably, using ferricenium hexafluorophosphate as electron acceptor (49)
4HBD did not catalyze the oxidation of butyryl-CoA.

The EPR data (Fig. 5) corroborated the UV-visible spectroscopic experiments. All analyzed components, crotonyl-CoA as well as CoASH, acetyl-CoA or butyryl-CoA, induced similar EPR spectra showing not only the FAD semiquinone ($g_2 = 2.004-2.007$) but also features which are attributed to a reduced $[4\text{Fe}-4\text{S}]^{2+}$ cluster ($g_1 = 2.031-2.032$; $g_3 = 1.97$; $g_4 = 1.91$). This raises the question about the origin of the electron that reduces FAD and the $[4\text{Fe}-4\text{S}]^{2+}$ cluster. The slight shoulder in the spectrum induced by butyryl-CoA (indicated by arrows in Fig. 5) could stem from an allylic ketyl radical that cannot react further due to the lack of the hydroxyl group at C4. The smooth spectra induced by acetyl-CoA and CoASH 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, which led to the revision of R43 to A, G167 to D, and D357 to G (GenBank: [CAB60035.2](http://aem.asm.org/)), see Supplement Table 1. These changes are now in accord with the structural data (12). Highly conserved regions were found by comparisons of the amino acid sequences annotated as 4HBD, dehydratase/isomerases participating in 4-aminobutyrate metabolism, 4-hydroxyphenylacetate 3-hydroxylases or phenol hydroxylases. The closest relationships revealed the sequences from the over 100 *Clostridium difficile* strains (50) with 83% identity, renamed as *Peptoclostridium difficile* (51), followed among others by *Porphyromonas gingivalis* (76%) (52), *Clostridium kluyveri* (74%) (9, 53) and *Fusobacterium nucleatum* (72%) (54). Relatives of 4HBD are also found in the archaea *Archaeoglobus fulgidus* (56%), *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 Supplement Table 1. 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 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 coding for 4-hydroxybutyryl-CoA dehydratase (32) in *E. coli* BL21 yielded large amounts of insoluble protein. After many trials we identified the following crucial parameters for a successful abfD expression: (i) growth temperature 20-25 °C; (ii) concentration of the inducer anhydrotetracycline, 100 µg/l (0.23 µM); (iii) coexpression of the chaperon genes groEL; (iv) maintenance of the exponential growth phase during the production phase (OD₅₇₈ = 0.5 – 0.8); (v) 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, which was C-terminally fused to a Streptag for affinity purification on a Strep-Tactin 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 that is only weakly coordinated by H296 as indicated by the unusual long N-Fe bond of 2.4 Å (12, 14). The obtained specific activity was similar to those obtained with the enzyme preparations purified from *C. aminobutyricum*: 7.3 U mg⁻¹ (2), 2–9 U mg⁻¹ (17) and 2–16.7 U mg⁻¹ (19). Based on our method (57) Könneke et al. (56) recently produced 4HBD from *C. aminobutyricum* and the almost 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 the 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 FeCl₃ and Na₂S, 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, or H292E, yielded proteins without measurable activity and 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 consisted of tetramers and dimers (Table 2). Hence, the [4Fe-4S] cluster may also contribute to stabilize the quaternary structure. The maintenance of the homotetrameric structure in all other mutants underpins this suggestion. Especially H292C contradicted our expectation of an increased stability of the cluster.
In comparison to the $\text{N}_2\text{E}^2$ of histidine, the thiolate of the cysteine residue probably is too far away to coordinate Fe1. In summary these data underpin that the $[4\text{Fe}-4\text{S}]$ cluster is essential for activity. Therefore it is surprising why the low activity of some of the variants could not be improved by cluster reconstitution.

The variant Q101E 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 fixing 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 variant A460G 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 higher flexibility of glycine disturbs the protein structure. M149S revealed 7\% of the dehydratase activity. M149 is part of the 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 in the next chapter. E455 is at 3.6 Å from the modeled C4 position of substrate (Fig. 3). The retention of configuration during the formation of the methyl group of crotonyl-CoA (16) suggests E455 as 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 distance from the hydroxyl group of T190 which could form a hydrogen bond at 3.3 Å distance to N5 of the isoalloxazine ring (Fig. 3). A replacement of T190 by valine or E257 by glutamine resulted in an over 99% drop-off of 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 of T190 in 4HBD (58). A threonine-aspartate dyad corresponding to T190/E257 in 4HBD has been detected only in the related 4-hydroxyphenylacetate 3-hydroxylase (59). The experiments of Fig. 7 underpin these considerations. As already shown in Fig. 4, 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 slower 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 FADH•. The EPR experiments in Fig. 8 support this interpretation.

The question arises on the origin of the electron required for the reduction of FAD. Although the electron could come from the substrate and butyryl-CoA, acetyl-CoA or CoASH are less likely donors. Furthermore, the data of Figures 4 and 7 as well as 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 is converted to a radical as observed in flavin monoamine oxidases (60). This would fit well to the observed inactivity of Y296F in the electron transfer (Figs. 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 – 10.5 Å apart from N5 of FAD. We made the 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 the distance of 2.6 Å, which in the variant E455Q could be carried out by the more distantly located H292 (3.7 Å) causing the slower rates of Fig. 7. The resulting phenolate could be much easier oxidized by FAD than the protonated 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 $g = 2.004$-$2.007$ was detected at 27 K (Fig. 9) and at 77 K. The spectra are compared with that of 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 have been 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 lower temperature and broadened beyond detection at higher temperature (Figs. 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 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 base to remove the 2Re-proton of vinylacetyl-CoA, whereas E455 may protonate the C4 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 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.

Fate of the 2Re- and 3Si-protons of 4-hydroxybutyryl-CoA. The conversion of 4-hydroxybutyryl-CoA to crotonyl-CoA involves the abstraction of the 2Re- and 3Si-protons (16). Whether one of these protons is used to replace the hydroxyl group was checked using 4-hydroxy[2- and 3-2H]butyrates as growth substrates for *C. aminobutyricum*. Though the natural substrate is 4-aminobutyrate, the bacterium could be adapted to ferment 4-hydroxybutyrate. After growth, the products and the residual substrate were isolated by steam distillation and their deuterium content was analyzed by 1H- and 2H-NMR. The 1H-NMR spectrum obtained with the products of the 4-hydroxy[2-2H]butyrate fermentation showed signals pertaining to butyrate (B2-B4), acetate and residual 4-hydroxybutyrate (HB2-HB4) (Fig. 10A). The integrals of the 2H-NMR signals revealed no deuterium at C3 and C4 of butyrate (signals B3 and B4), whereas at C2 of butyrate (B2) only
0.22 \textsuperscript{2}H rather than the expected 1.00 \textsuperscript{2}H were present. The \textsuperscript{2}H-NMR spectrum (red traces in Fig. 10A) showed little deuterium incorporation into C4 of butyrate. This could support a possible proton migration from C2 to C4 during catalysis of 4HBD concomitant with a dominating proton exchange with the solvent. More likely, however, the observed deuterium incorporation at C4 stems from the reversible oxidation of [2-\textsuperscript{2}H]crotonyl-CoA to [\textsuperscript{2}H]acetyl-CoA and unlabeled acetyl-CoA (Fig. 2), which also explains the loss of deuterium from C2 and the relatively high deuterium content of acetate. The reversibility of this oxidation is demonstrated by bacteria able to reduce 2 acetyl-CoA to butyrate, e.g. \textit{C. kluyveri}, which use the same enzymes as in the reverse pathway (5).

4-Hydroxy[3-\textsuperscript{2}H]butyrate as initial substrate was almost completely consumed. By \textsuperscript{1}H-NMR the ratio of the signals for butyrate (B4 2.92: B3 1.17: B2 2.0) indicates retention of 0.83 deuterium atoms at B3, which is close to the theoretical value of 1.00. The \textsuperscript{2}H-NMR spectrum shows no signals at B2 or B4 above background level indicating no migration event originating from C3 (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-\textsuperscript{2}H]butyrate into the methyl group of butyrate, we repeated the experiment in vitro. Therefore we generated in situ 4-hydroxy[2-\textsuperscript{2}H]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 formed crotonyl-CoA was irreversibly reduced by NADH to butyrate using the electron bifurcating electron transferring flavoprotein/butyryl-CoA dehydrogenase complex from \textit{Clostridium tetanomorphum} together with ferredoxin and hydrogenase (5). Formate and formate dehydrogenase acted as NADH regenerating system. In summary:
4-Hydroxybutyrate$^-$ + acetyl-CoA + 2 formate$^-$ + 2 H$^+$ → 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 C2 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 C3 of the enolate of 4-hydroxybutyryl-CoA. The spectroscopic data of 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$^*$ is formed, which can only accept one electron, and the abstraction of a hydride from C3 is prevented. Since butyryl-CoA induces similar spectral changes (Figs. 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 N5 of FAD (Fig. 3) allows such a transfer. Most likely the tyrosyl radical interacts with the neutral FAD semiquinone and the reduced [4Fe-4S]$^+$ cluster (4.7 Å apart), which change the typical spectrum (Fig. 9, lower trace) to those shown above or in Fig. 5 (crotonyl-CoA, red trace).

The question arises 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 also the inactive substrate analogue butyryl-CoA induces a fairly rapid absorbance change in the >500 nm region, whereas that induced by CoASH, 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 formed FADH⁻ anion 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 partial reduced [4Fe-4S]⁺ 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 formed H₂O could be facilitated.

The mechanistic scheme (Fig. 11) proposes several protonation/deprotonations. 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 (FAD•−) to FAD•+, 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. E257 has to emit its proton via the water channel into the medium. The eliminated hydroxyl group 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 2 H2O, could be released (64). Re-oxidation of FADH•− to FAD•+ by the dienox radical occurs without proton movement. E455 is located in a position to protonate the dienolate at C4 from the same side from which the hydroxyl group has left (16). After release of crotonyl-CoA, an electron from FADH•+ flows back to Y296•− to yield FAD and Y296−, whereby the proton is taken up by T190/E257. The reformed phenolate gets its proton from the water channel via E455 to regenerate Y296.

In summary the proposed mechanism of 4HBD is without precedence in anaerobic radical chemistry. The radical generating system Y296 – (FAD + cluster) with concomitant protonation of FAD•+ by T190 – E257 – H2O channel is quite different from other anaerobic radical generators such as coenzyme B12, S-adenosylmethionine (radical SAM enzymes), the ATP-dependent activator of 2-hydroxyacyl-CoA dehydratases and flavin-based electron bifurcation (6, 14, 65). The formation of 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 remove two
electrons from the enolate (Fig. 1), which would result in the dead-end product 4-hydroxycrotonyl-CoA. Finally, the authors are fully aware that further experiments are required to confirm this mechanism. One goal would be a crystal structure of 4HBD together with 4-hydroxybutyryl-CoA or crotonyl-CoA, which, despite many efforts, could not be achieved yet (12).

In contrast, crystallization of the radical enzyme 2-hydroxyisocaproyl-CoA dehydratase together with its substrate worked very well (66). This could be due to crystallization of the inactive enzyme without radical formation by the ATP-dependent electron transfer (67). Hence, further crystallographic studies with 4HBD and substrates should be performed with the inactive Y296F or E257Q variants. Another important goal would be the simulation of the EPR-spectrum (Fig. 5).

The successful production of recombinant 4HBD paves the way for biotechnology, especially due to the discovery of the almost oxygen-insensitive 4HBD from *N. maritimus* (56). Notably the radical species in this enzyme must be somehow shielded from oxygen access. That this is possible demonstrates class I ribonucleotide reductase in which a tyrosine radical is produced by oxygen (68). In biotechnology the synthesis of 4-hydroxybutyryl-CoA from acetate (Fig. 2) could lead to succinate, 1,4-butanediol (69) or glutamate. 4-Hydroxybutyryl-CoA also serves as precursor for polymerization to polyhydroxyalkanoates (70). Although the equilibrium constant \( K' = \frac{[\text{crotonyl-CoA}]}{[\text{4-hydroxybutyryl-CoA}]} = 4.2 \) (2) favors the synthesis of 4-hydroxybutyryl-CoA from crotonyl-CoA, to our knowledge no natural pathway is known yet which uses 4HBD in this direction.

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acknowledge Professor Bernard T. Golding (University of Newcastle upon Tyne) for his advice to synthesize γ-[3-2H₂]butyrolactone. This work was supported by the Max-Planck-Institut für terrestrische Mikrobiologie, the Deutsche Forschungsgemeinschaft (DFG), and Synmikro of the Philipps-Universität Marburg.

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55. Klenk HP, Clayton RA, Tomb JF, White O, Nelson KE, Ketchum KA, Dodson RJ, Gwinn M, Hickey EK, Peterson JD, Richardson DL, Kerlavage AR, Graham DE, Kyrpides NC, ...


### Table 1. Primers used for site-directed mutagenesis.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Nucleotide sequence (5’→ 3’)</th>
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<tr>
<td>H292C</td>
<td>GAAAGATTTGCTGGATCTGCAAGACAGTCATACGCCG</td>
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<tr>
<td>H292E</td>
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<td>Y296W</td>
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<td>E257Q</td>
<td>CACGTCGCGCGACACGAGCTTTAGTCGATTCG</td>
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<td>E455Q</td>
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<td>A460G</td>
<td>GAACCTGAAATCCATGAGTTGGCCTCCCTACGGCTTAGG</td>
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Table 2. Characterization of wild type and mutants of 4HBD

<table>
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<tr>
<th>Mutant</th>
<th>Specific activity of dehydratase (U/mg)</th>
<th>Iron/tetramer (mol/mol)</th>
<th>FAD/tetramer (mol/mol)</th>
<th>Structure</th>
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<tr>
<td>Wild type</td>
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<td>4.4 ± 0.2</td>
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<td>3.1 ± 0.1</td>
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<tr>
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<td>n.d.</td>
<td>n.d.</td>
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<td>3.5</td>
<td>tetramer</td>
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<td>0.09 ± 0.01</td>
<td>10.3</td>
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<td>tetramer</td>
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n.d., not determined.

*reconstituted protein with FeCl₃ and Na₂S.
Table 3. Comparison of vinylacetyl-CoA isomerase specific activities before and after exposure to air.

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<tr>
<th>Mutant</th>
<th>Vinylacetyl-CoA Δ-isomerase activity (U/mg)</th>
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<td></td>
<td>Freshly purified 4HBD</td>
</tr>
<tr>
<td>Wild type</td>
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</tr>
<tr>
<td>H292C</td>
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</tr>
<tr>
<td>H292E</td>
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</tr>
<tr>
<td>C99A</td>
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<tr>
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<tr>
<td>C299A</td>
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</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>
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FIG. 1. Reactions catalyzed by 4HBD. The removal of the 2\textit{Re}- and the 3\textit{Si}-protons (red) from 4-hydroxybutyryl-CoA has been established (16). We assume that the isomerization of vinylacetyl-CoA obeys the same stereochemistry.
FIG 2 Proposed pathway of 4-aminobutyrate fermentation in *C. aminobutyricum*. Enzymes: 1, 4-aminobutyrate aminotransferase (EC 2.6.1.9); 2, glutamate dehydrogenase (EC 1.4.1.2); 3, 4-hydroxybutyrate dehydrogenase (*NAD^+*, *C. aminobutyricum*, EC 1.1.1.61) or NADP^+ specific (*M. sedula* and *N. maritima*); 4, 4-hydroxybutyrate CoA-transferase (*C. aminobutyricum*) (EC 2.8.3.-) or 4-hydroxybutyryl-CoA ligase (*M. sedula* and *N. maritima*) (6.2.1.-); 5, 4HBD (EC 4.2.1.120); 6, 3-hydroxybutyryl-CoA dehydratase (crotonase, EC 4.2.1.17); 7, 3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.35); 8, thiolase (EC 2.3.1.9); 9 + 10, phosphate acetyl-transferase (EC 2.3.1.8) and acetate kinase (EC 2.7.2.1); 11, electron transferring flavoprotein/butyryl-CoA dehydrogenase complex (5, 6) (similar to EC 1.3.8.1); 12, NAD ferredoxin oxidoredutase (Rnf) (EC 1.18.1.3) (5); 13, Succinyl-CoA reductase (NADPH; EC 1.2.1.-). The blue and red hydrogens refer to the labelling experiment described later in the text.
FIG 3 Modeled localization of 4-hydroxybutyryl-CoA in the active center of 4HBD. Carbons 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(N5) (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.
**FIG 4** Visible absorption changes of 4HBD (1.6 mg/ml; 4.5 U; 7.1 µM) isolated from *C. aminobutyricum* after addition of 5 mM substrate or analogue: Crotonyl-CoA, red crosses; butyryl-CoA, black triangles; acetyl-CoA, magenta squares; CoASH, blue diamonds; without substrate, brown crossed vertical bars.
FIG 5 EPR-spectra of 4HBD (41.7 µM) at 10 K, frozen in liquid nitrogen one minute after incubation with different substrates (5 mM); crotonyl-CoA, red; CoASH, blue. The spectrum with butyryl-CoA, black, revealed an additional signal shoulder between 338-343 mT (signal around $g_3 = 1.966$) 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.
FIG 6 Production of recombinant 4HBD in *E. coli* and purification on a Strep-Tactin column. Lane m, protein markers; lane 1, supernatant of cell-free extract before induction; lane 2, after induction by 0.22 µM anhydrotetracycline; lane 3, pellet of cell-free extract after induction by 0.22 µM anhydrotetracycline; lane 4, 10 µg purified 4HBD.
FIG 7 Visible absorption changes of wild type (WT) 4HBD and variants after addition of 5 mM crotonyl-CoA. Each assay contained purified wild type or variant 4HB at a concentration of 0.5 mg protein ml$^{-1}$. 
FIG 8. EPR spectra of 4HBD wild type (46 µM), E257Q (55 µM), Y296F (19 µM) and E455Q (55 µM) variants at 77 K. One min before freezing the samples of pre-oxidized enzyme were mixed with 5 mM crotonyl-CoA. Microwave power, 1.27 mW; microwave frequency, 9.450 GHz; modulation amplitude 1.0 mT; modulation frequency, 100 kHz.
**FIG 9.** EPR spectra of 46 µM wild type 4HBD (top) and 25 µM wild type 4HBD isolated from *E. coli* cells grown with (S)-[phenyl 3,5-²H₁]tyrosine (middle, scaled up 3.3-fold as compared to the top spectrum). Both samples of pre-oxidized enzymes were mixed with 5 mM crotonyl-CoA 1 min before freezing. For comparison, the lower spectrum exhibits the tyrosine radical present in whole yeast cells (*S. cerevisiae*). 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; modulation frequency, 100 kHz.
FIG 10. NMR spectra of the steam volatile acids produced by *C. aminobutyricum* using 4-hydroxy[2-2H₂]butyrate (A) or 4-hydroxy[3-2H₂]butyrate (B) as carbon source. An overlay of ¹H NMR (black) and ²H-NMR spectra (red) is shown. The integrals are shown above the peaks as black curves (¹H-NMR) or beneath as red bars (²H-NMR).
**FIG 11.** Proposed mechanism of 4HBD. The amino acids proposed to be involved in each step are indicated in italics, blue acting as base, red acting as acid (Säure in German). The release of H₂O from Fe1 of the [4Fe-4S]²⁺ cluster could be facilitated by reduction to [4Fe-4S]⁺; see text for details.