Enzyme Mechanisms

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The Complete Stereochemistry of the Enzymatic Dehydration of 4-Hydroxybutyryl Coenzyme A to Crotonyl Coenzyme A**

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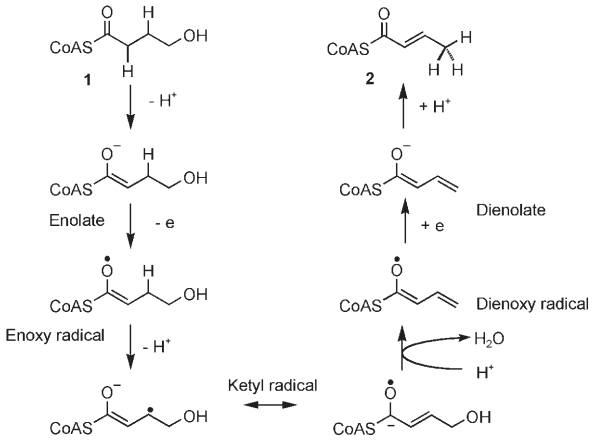
Dedicated to Professor Duilio Arigoni

4-Hydroxybutyryl coenzyme A (4-hydroxybutyryl-CoA, **1**, Scheme 1) contains three diastereotopic methylene groups which either lose or gain a hydrogen atom during the reversible dehydration to crotonyl-CoA (but-2-enoyl-CoA, **2**). 4-Hydroxybutyryl-CoA dehydratase from *Clostridium aminobutyricum*, which catalyzes this intriguing γ elimination of water,[1] has been shown to be also involved in the recently discovered fifth CO_2 fixation cycle.[2] Each 56-kDa subunit of the homotetrameric enzyme contains one flavin adenine dinucleotide (FAD) and a $[Fe_4S_4]^{2+}$ cluster.[1] The crystal structure reveals a cleft between the cluster and FAD in which the substrate can be bound.[3] The medium-chain acyl-CoA dehydrogenase, which also contains FAD but no $[Fe_4S_4]^{2+}$ cluster, has a related structure, which was determined with a bound octanoyl-CoA substrate.[4] Taking this structure as a paradigm, **1** could be modeled into the crystal structure of the dehydratase using hydrogen bonds from the NH group of alanine 460 and the 2′-OH group of FAD to the oxygen atom of the thioester carbonyl group as an anchor.[3] In this way the hydroxy group of the substrate becomes a ligand of the cluster iron atom that is coordinated to histidine 292 (extended Fe–N bond of 2.4 Å) instead of to cysteine residues like the other three iron atoms. It has been postulated that the liberated histidine base removes the 2*Re* proton[5] while the 3*Si* hydrogen atom can be abstracted by N5 of FAD (Scheme 1).[6,7] With the support of EPR measurements,[8] we proposed a one-electron oxidation by FAD and β-proton transfer to the cofactor to avoid the dead-end intermediate 4-hydroxycrotonyl-CoA. The resulting ketyl radical eliminates the hydroxy group, assisted by its coordination to the cluster, which leads to a dienoxy radical, which is reduced to a dienolate by the intermediate FAD semiquinone. The protonated histidine 292 residue could perform the final protonation and become recoordinated to the iron atom. Elimination of the hydroxy group in this way should occur with retention of configuration. Herein we confirm our stereochemical predictions by using all three hydrogen isotopes in combination with organic and enzymatic syntheses.



Scheme 1. Proposed mechanism for the dehydration of 4-hydroxybutyryl-CoA (**1**) to crotonyl-CoA (**2**).

To elucidate the stereochemistry at C2, we synthesized (2*R*)- and (2*S*)-4-hydroxy[2-$^2H_1$]butyryl-CoA ((2*R*)-**1** and (2*S*)-**1**, Scheme 2) and incubated each with 4-hydroxybutyryl-CoA dehydratase. The CoA thioesters that were produced were isolated and analyzed before and after incubation by MALDI-TOF mass spectrometry.[5] 3-Benzyloxy-1-propanol (**3**) was dideuterated by oxidation to 3-benzyloxypropionic acid, which was reduced with lithium aluminum deuteride. Reoxidation of the alcohol gave the deuterated aldehyde (**4**). A stereoselective reduction of the aldehyde with either (*R*)- or (*S*)-alpine borane gave (1*S*)- or (1*R*)-3-benzyloxy-1-[1-$^2H_1$]propanol ((*S*)-**3** and (*R*)-**3**), respectively.[9] Tosylation of the alcohol and subsequent reaction with sodium cyanide occurred with inversion of configuration,

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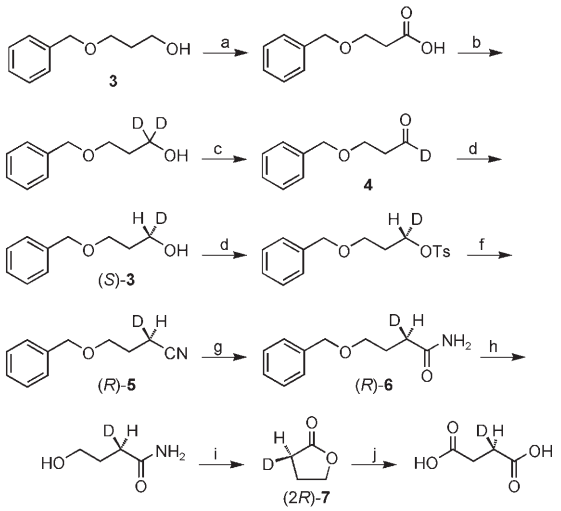


Scheme 2. Synthesis of (2*R*)-γ-[2-^{2}H$_1$]butyrolactone ((2*R*)-**7**). a) Jones reagent; b) $LiAlD_4$; c) oxalyl chloride, DMSO, N_2; d) (*R*)-alpine borane; e) *p*-TsCl, pyridine; f) NaCN; g) acetamide, $Pd(OAc)_2$; h) Pd/C, H_2; i) HCl. j) Samples of **7** were oxidized to succinic acid (TEMPO, NaClO, $NaClO_2$). (2*S*)-γ-[2-^{2}H$_1$]Butyrolactone ((2*S*)-**7**) was synthesized using (*S*)-alpine borane.

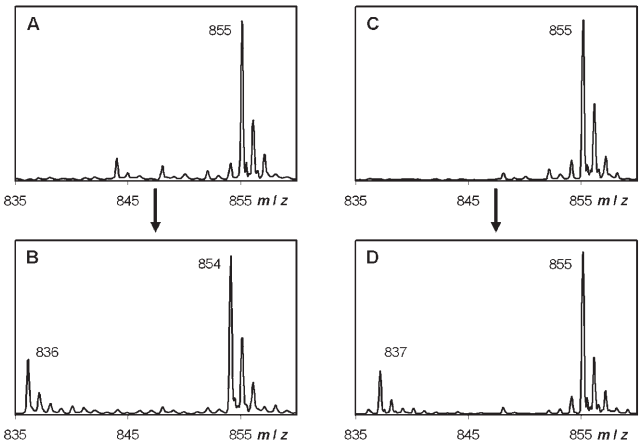


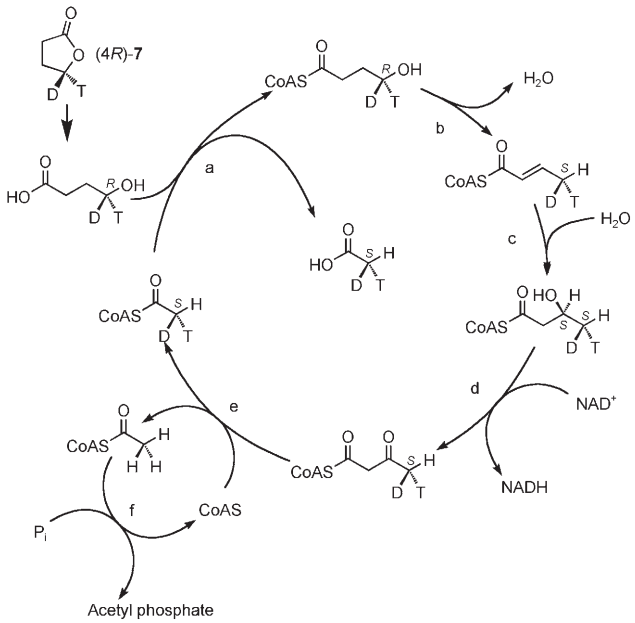
Figure 1. MALDI-TOF mass spectra. A) (2*R*)-**1** (855 Da); B) upon incubation of (2*R*)-**1** with dehydratase, loss of deuterium in the product crotonyl-CoA (**2**, 836 Da) and exchange by hydrogen in 4-hydroxybutyryl-CoA (**1**, 854 Da) are observed; C) (2*S*)-**1** (855 Da); D) upon equilibration with dehydratase, (2*S*)-**1** retains the deuterium, and [2-^{2}H]crotonyl-CoA (837 Da) is formed.

leading to nitrile **5**, which was hydrolyzed to the amide **6**.[10] Deprotection and acidification gave the stereoselectively deuterated lactones **7**. Aliquots of **7** were oxidized to succinic acid and analyzed by CD spectroscopy: (*R*)-[^{2}H$_1$]succinic acid, $[\Theta]_{206\,nm,24\,°C} = (-118 \pm 11)\ deg\,cm^3\,g^{-1}\,dm^{-1}$; (*S*)-[^{2}H$_1$]succinic acid, $[\Theta]_{206\,nm,24\,°C} = (134 \pm 13)\ deg\,cm^3\,g^{-1}\,dm^{-1}$. The latter value agrees with that published for the *S* enantiomer ($[\Theta]_{206\,nm,24\,°C} = +150\ deg\,cm^3\,g^{-1}\,dm^{-1}$).[11,12] The lactones (2*R*)-**7** and (2*S*)-**7** were hydrolyzed to the carboxylates and converted into the corresponding CoA thioesters ((2*R*)-**1** and (2*S*)-**1**) by incubation with acetyl-CoA and 4-hydroxybutyrate CoA-transferase.[13] MALDI-TOF mass spectrometry revealed that (2*R*)-**1** and (2*S*)-**1** (*m*/*z* 855) each contained less than 11% unlabeled material (Figure 1 A,C), which was mainly derived from the hydrogen content (8%) of $LiAlD_4$.

Incubation of (2*R*)-**1** with 4-hydroxybutyryl-CoA dehydratase led to the formation of an unlabeled mixture of **1** (*m*/*z* 854) and **2** (*m*/*z* 836), whose monoisotopic mass distributions corresponded to natural abundance distributions of nuclei (Figure 1 B). In contrast, when (2*S*)-**1** was used as a substrate, complete retention of the deuterium label in **1** (*m*/*z* 855) and **2** (*m*/*z* 837) was observed; the amount of unlabeled **1** remained constant (Figure 1 C, D). The results described above show that, as expected from crystal structure modeling, during dehydration of **1** to **2**, the 2*Re* hydrogen atom is selectively removed. Previous results have shown that loss of the 3*Si* hydrogen atom occurs.[5] Therefore, the stereochemical course at C2 and C3 can be described as *anti* elimination of the two hydrogen atoms, which is identical to that of acyl-CoA dehydrogenases.[14–18] It should be noted that the configuration of the 3*Si* hydrogen atom in 4-hydroxybutyryl-CoA corresponds to that of the 3*Re* hydrogen atom in unsubstituted acyl-CoA.

The question of inversion or retention at C4 during substitution of the hydroxy group by hydrogen was solved with (*R*)- and (*S*)-4-hydroxy[4-^{2}H$_1$,4-^{3}H]butyrates. For their syntheses, diethyl succinate was condensed with ethyl [^{2}H]formate to form diethyl [^{2}H]formylsuccinate, which was subsequently converted into γ-ethoxy[4-^{2}H]butyrolactone. Hydrolysis yielded 4-oxo[4-^{2}H$_1$]butanoic acid,[19] which was stereospecifically reduced with NAD^3H through catalysis either by 4-hydroxybutyrate dehydrogenase (4-HBDH) from *C. aminobutyricum* or horse liver alcohol dehydrogenase (ADH). NAD^3H was continuously formed with [^{3}H]formate and formate dehydrogenase. The product obtained with ADH was assigned as (*R*)-γ-[4-^{2}H$_1$,4-^{3}H]butyrolactone ((4*R*)-**7**), since it is known that ADH catalyzes the transfer of the hydride from NADH to the *Re* face of an aldehyde.[20] When we found that the much more active 4-HBDH acts by the same stereospecificity as ADH (see below), we used only this enzyme to generate the (*S*)-γ-[4-^{2}H$_1$,4-^{3}H]butyrolactone ((4*S*)-**7**) from 4-oxo[4-^{3}H]butanoic acid in the presence of [^{2}H]formate and formate dehydrogenase.

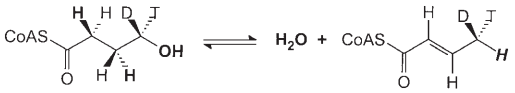
The synthesized (4*R*)-**7** and (4*S*)-**7** were hydrolyzed to carboxylates and enzymatically converted into acetates as indicated in Scheme 3. In this one-pot system, the primary product **2** was irreversibly hydrated to (3*S*)-3-hydroxybutyryl-CoA, with subsequent oxidation with excess NAD^+, cleavage to two equivalents acetyl-CoA, and final conversion into acetyl phosphate and acetic acid. After acetyl phosphate was hydrolyzed under acidic conditions, the acetic acid was isolated by steam distillation. The specific ^{3}H activities of the isolated acetates were determined enzymatically and by scintillation counting to be, as expected, (50 ± 5)% of the starting lactones, since each mole of substrate generates one labeled and one unlabeled mole of acetate. Hence, no significant ^{3}H exchange occurred during the incubation. The configuration of the acetates was analyzed enzymatically by using malate and fumarate.[21–23]



Scheme 3. Conversion of (*R*)-4-hydroxy[4-$^2H_1$,^3H]butyrate into (*S*)-[$^2H_1$,^3H]acetic acid. Under the conditions of isolation (H^+, 100 °C) acetylphosphate was hydrolyzed to acetic acid. The incubation mixture contained hydrolyzed lactone, potassium phosphate pH 7.4, NAD^+, acetyl-CoA, CoASH, 4-hydroxybutyrate CoA-transferase (a), 4-hydroxybutyryl-CoA dehydratase (b), crotonase (c), (*S*)-3-hydroxybutyryl-CoA dehydrogenase (d), thiolase (e), and phosphotransacetylase (f).

As seen in Table 1, malates derived from both (4*R*)-**7** species lost more than 50% tritium during the fumarase exchange assay (70 and 71%), thus indicating that the acetic acids (Scheme 3) had predominantly *S* configuration, whereas (4*S*)-**7** leads to acetic acid with predominantly *R* configuration (41% loss of tritium). The data show that the formation of the methyl group of **2** from the hydroxymethyl group of **1** (Scheme 3) occurs with retention of configuration. The result with (4*R*)-**7** also demonstrates that 4-HBDH and ADH share *Re* stereospecificity with respect to the side of the aldehyde substrate.

In summary, the course of the dehydration of 4-hydroxybutyryl-CoA to crotonyl-CoA, which involves three diastereotopic methylene groups, has been clarified by using six different stereospecifically labeled 4-hydroxybutyryl-CoA species (Scheme 4). Stereoselective organic synthesis is demonstrated to be a powerful tool to elucidate substrate–enzyme interactions. The observed retention of configuration supports the assumption of a planar dienolate intermediate (Scheme 1), which is formed by hydroxy group elimination by the Lewis acidic Fe_4S_4 cluster and is subsequently protonated



Scheme 4. Stereochemistry in the dehydration of 4-hydroxybutyryl-CoA to crotonyl-CoA.

from the same side of the molecule as the initial abstraction. The proton donor could be histidine 292, which initially abstracts the proton at C2. This scenario would infer a migration of the hydrogen atom from C2 to C4, which has not been observed (Figure 1), most likely owing to the long incubation times facilitating exchange with the solvent. Notably a similar reaction, the dehydration of (1*S*,3*S*)-3-hydroxycyclohexane carboxylate to cyclohex-1-ene carboxylate (most likely at the CoA ester level) in the biosynthesis of cyclohexane carboxylate from shikimic acid, occurs with the same stereochemistry.[24] Interestingly, in the most probable conformation of the cyclohexane ring, the two hydrogen atoms and the hydroxy group that get eliminated all occupy axial positions.

The stereochemically similar reactions of 4-hydroxybutyryl-CoA dehydratase and acyl-CoA dehydrogenase suggest a common mechanism for both enzymes. Whereas a pathway via radicals appears to be the only possibility for 4-hydroxybutyryl-CoA dehydratase, a hydride transfer from C3 to FAD is the favored mechanism for acyl-CoA dehydrogenase.[25] However, evidence for radical intermediates has also been obtained in experiments with acyl-CoA dehydrogenase.[26]

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Table 1: Analysis of the chiral acetates derived from (*R*)- and (*S*)-γ-[4-$^2H_1$,^3H]butyrolactone.[a]

Compound	(*R*)-γ-[4-$^2H_1$,^3H]butyrolactone ((4*R*)-**7**)								(*S*)-γ-[4-$^2H_1$,^3H]butyrolactone ((4*S*)-**7**)			
	generated with ADH [cpm]				generated with 4-HBDH [cpm]				generated with 4-HBDH [cpm]			
	^3H	^{14}C	$^3H/^{14}C$	^3H loss	^3H	^{14}C	$^3H/^{14}C$	^3H loss	^3H	^{14}C	$^3H/^{14}C$	^3H loss
malate	1239	914	1.36	–	4295	1377	3.12	–	1033	173	5.97	–
malate + fumarate	361	886	0.41	**70%**	961	1053	0.91	**71%**	881	250	3.52	**41%**

[a] [2-^{14}C]Acetic acid was added as an internal standard. ^{14}C cpm was adjusted to be 20–60% of ^3H cpm.

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