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Minireview

Unusual dehydrations in anaerobic bacteria: considering ketyls (radical anions) as reactive intermediates in enzymatic reactions

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Abstract Dehydratases have been detected in anaerobic bacteria which use 2-, 4- or 5-hydroxyacyl-CoA as substrates and are involved in the removal of hydrogen atoms from the unactivated β- or γ-positions. In addition there are bacterial dehydratases acting on 1,2-diols which are substrates lacking any activating group. These enzymes contain either FAD, or flavins + iron-sulfur clusters or coenzyme B_{12}. It has been proposed that the overall dehydrations are actually reductions followed by oxidations or vice versa mediated by these prosthetic groups. Whereas the γ-hydrogen of 5-hydroxyvaleryl-CoA is activated by a transient two-electron α,β-oxidation, the other substrates are proposed to require either a transient one-electron reduction or an oxidation to a ketyl (radical anion).

Key words: Dehydratase; (*R*)-2-Hydroxyglutaryl-CoA; 4-Hydroxybutyryl-CoA; 5-Hydroxyvaleryl-CoA; 1,2-Propanediol; Iron-sulfur cluster; Flavin; Coenzyme B_{12}; Ketyl; Radical anion

1. Introduction

Dehydration is a very common reaction in many metabolic pathways. In the Krebs cycle two such reactions are found; the dehydration of L-malate to fumarate, catalysed by fumarase (EC 4.2.1.2), as well as the dehydration of citrate to *cis*-aconitate and its hydration to (2*R*,3*S*)-isocitrate, catalysed by aconitase (EC 4.2.1.3). In the Embden-Meyerhof pathway enolase (EC 4.2.1.11) catalyses the interconversion between D-2-phosphoglycerate and phosphoenolpyruvate. In the β-oxidation of fatty acids and their biosynthesis the hydration of 2-enoyl-CoA to L-β-hydroxyacyl-CoA (EC 4.2.1.17) and the dehydration of D-β-hydroxyacyl-acyl carrier protein to the 2-enoyl derivative (EC 4.2.1.58–61), respectively, are important reactions. All these β-eliminations of water – Enzyme Nomenclature (1992) lists about 70 *hydro-lyases* of this type [1] – have in common that the α-hydrogen to be removed as a proton is activated by the adjacent carboxylate, oxo group or thiol ester and the hydroxyl group leaves from the β-position. Remarkably, some of these dehydratases, e.g. the aconitases, the bacterial L-serine dehydratases and two of the three fumarases from *Escherichia coli* contain [4Fe-4S]$^{2+}$clusters, which are not involved in electron transfer but act as Lewis acids facilitating the leaving of the hydroxyl group (for a recent review see [2]).

In anaerobic bacteria, however, one finds a few enzymes catalysing the dehydrations of 2-, 4- or 5-hydroxyacyl-CoA derivatives, in which the hydrogen to be removed from the β- or γ-positions is too far away to be activated by the thiol-ester (for reviews see [3,4]). In addition, 1,2-diol dehydratases are known, the substrates of which contain no activating group at all. This review describes new developments in the field of these unusual dehydrations and postulates possible, but still speculative mechanisms involving ketyls (anion radicals) as intermediates.

2. Dehydration of 2-hydroxyacyl-CoA esters

The dehydration of 2-hydroxyacyl-CoA esters is found in amino acid fermenting anaerobic bacteria which all belong to the Gram-positive phylum [5]. Initially, the amino acid is converted by oxidation, amino transfer, or β-elimination to the corresponding 2-oxo acid; it is then reduced to the (*R*)-2-hydroxy acid. Activation to (*R*)-2-hydroxyacyl-CoA in a CoA-transferase reaction yields the substrate for the subsequent unusual *syn*-elimination of water to 2-enoyl-CoA, which generally is reduced to the saturated acyl-CoA. A final CoA transfer leads to the free fatty acid, which is excreted. The most intensive studied pathway of this type is the fermentation of glutamate via (*R*)-2-hydroxyglutarate to ammonia, carbon dioxide, acetate, butyrate and molecular hydrogen by the mesophilic anaerobe *Acidaminococcus fermentans* isolated from the intestine of mammals [6,7]. Glutamate is initially oxidized to 2-oxoglutarate and then reduced to (*R*)-2-hydroxyglutarate. The activation to the thiol ester level by acetyl-CoA is catalysed by glutaconate CoA-transferase [8,9]. The product (*R*)-2-hydroxyglutaryl-CoA is reversibly dehydrated to glutaconyl-CoA (Fig. 1). The oxygen-sensitive dehydratase (HgdAB), contains nonheme iron, inorganic sulfur, riboflavin and FMN [10,11]. The iron and sulfur contents as well a the kind of flavin varies among the enzymes from species (Table 1). A similar dehydratase from *Clostridium propionicum* catalyses the reversible dehydration of (*R*)-lactoyl-CoA and (*R*)-2-hydroxybutyryl-CoA to acryloyl-CoA and crotonyl-CoA, respectively [13–15]. The dehydratases have to be activated by ATP, Mg^{2+} and a reducing agent. In vitro, Ti(III) citrate is applied [11,12,15]; in vivo NADH and probably an additional enzyme serve for this purpose [16]. The activation is catalysed by a polypeptide (HgdC), forming either a third subunit of the dehydratase or an extra separable protein. The activator from *A. fermentans* could be characterized as a homodimer also containing nonheme iron and inorganic sulfur (Table 1). The genes from *A. fermentans* coding for the three polypeptides (α,β and γ) are clustered together in

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the order *hgdCAB*, which form the 3'-end of the 'hydroxyglutarate operon', comprising in addition *gctA* and *B* coding for glutaconate CoA-tranferase as well as *gcdA* coding for the hydrophilic carboxytransferase subunit of the biotin-dependent sodium ion pump glutaconyl-CoA decarboxylase, the consecutive enzyme of this pathway [7,17]. The extremely oxygen-sensitive activator HgdC (half-life under air at 25°C approx. 10 s), which is present in *A. fermentans* in tiny amounts (dehydratase:activator ≈ 100:1), could only be purified to homogeneity after overexpression of *hgdC* in *E. coli* [11,18]. Interestingly, genes sharing 35–37% identity in their deduced amino acid sequences with that of *hgdC* have been detected in *E. coli* [19], *Methanopyrus kandleri* (J. Vorholt and R. Thauer, personal communication) and in the benzoate degrading, phototrophic anaerobe *Rhodopseudomonas palustris* [20]; whereas no such gene was found in *Haemophilus influenzae*, the DNA of which has been completely sequenced [21]. In each of these HgdC-related amino acid sequences two cysteines are conserved which might be necessary for a [4Fe-4S]cluster formed by the two subunits of the homodimer. Whether these organisms are also able to dehydrate (*R*)-2-hydroxyglutaryl-CoA, or whether a more general function can be attributed to HgdC, remains to be established.

The prosthetic groups present in the (*R*)-2-hydroxyglutaryl-CoA dehydratases suggest that the overall dehydration is indeed a reduction followed by an oxidation. Since 2-hydroxyacyl-CoA derivatives can be regarded as α-hydroxyketones, a chemical model for the reductive part of the dehydration could be their easy reduction by one-electron donors such as metallic Zn in acetic acid via ketyls (radical anions) to the unsubstituted ketones [22]. Hence, the activation may actually comprise reduction by a single electron, which is energized by hydrolysis of ATP to ADP and P_i. This 'energy-rich' electron could be able to reduce the thiol ester of (*R*)-2-hydroxyglutaryl-CoA to a ketyl, which eliminates the adjacent hydroxyl group yielding an enoxy radical. The radical is deprotonated to the corresponding ketyl of glutaconyl-CoA followed by oxidation to the product [11,23]. Hence, the dehydration involves a one-electron cycle, which may last for many turnovers, explaining the catalytic rather than stoichiometric requirement for ATP. The transient inactivation caused by nitro compounds such as 1 μM nitrophenol or chloroamphenicol may be due to the trapping of this 'energy-rich' electron. The iron-sulfur clusters probably present in the dehydratase may have two functions, as electron carrier and as a Lewis acid, which coordinates the hydroxyl group and facilitates the elimination [11] (Fig. 1). It may be noted, however, that this appealing mechanism remains speculative until the enzymes can be obtained in sufficient quantities to characterize the postulated intermediates.

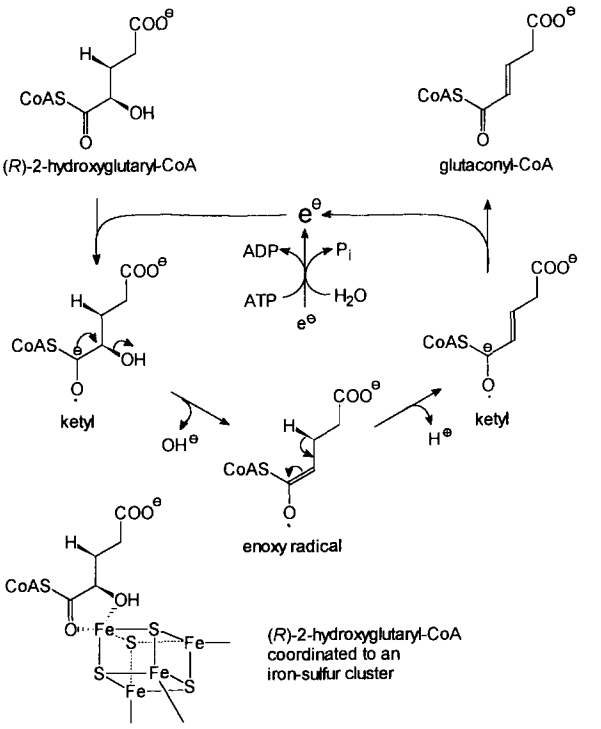

Fig. 1. Proposed mechanism for the reversible dehydration of (*R*)-2-hydroxyglutaryl-CoA to glutaconyl-CoA

3. 4-Hydroxybutyryl-CoA dehydratase

4-Hydroxybutyryl-CoA dehydratase catalyses the reversible

Table 1
Flavin and iron-sulfur cluster-containing dehydratases

Enzyme/source	Subunits	Mass (kDa)	Fe/S	Riboflavin	FMN	Spec. act. (U/mg)	Reference
(*R*)-2-Hydroxyglutaryl-CoA dehydratase							
A. fermentans	αβ	54+42	4/4	0.3	1.0	5.8	[10,11]
	γ_2	$(27)_2$	2×2/1.5	–	–	(2000)	
Clostridium symbiosum	αβ	52+41	7.5/6.5	–	1.0	30	[a]
	γ_2	?					
Fusobacterium nucleatum	αβγ	49+39+24	3.5/3.5	0.5	–	29	[12]
(*R*)-Lactoyl-CoA dehydratase							
C. propionicum	$(\alpha\beta)_3$	$(48+41)_3$	3×8/8	3×0.25	3×0.5	1.2	[13–15]
	γ	?					
4-Hydroxybutyryl-CoA dehydratase							
C. aminobutyricum	α_4	$(56)_4$	≈8/8	–	2 FAD	>13	[25]
C. kluyveri	α_4	$(59)_4$	≈8/8	–	2 FAD	>8	[26]

γ, activator which requires ATP and Mg^{2+} as well as Ti(III) citrate as an artificial reductant; the 4-hydroxybutyryl-CoA dehydratases need no activation.
[a]Müller, U. and Buckel, W., unpublished.

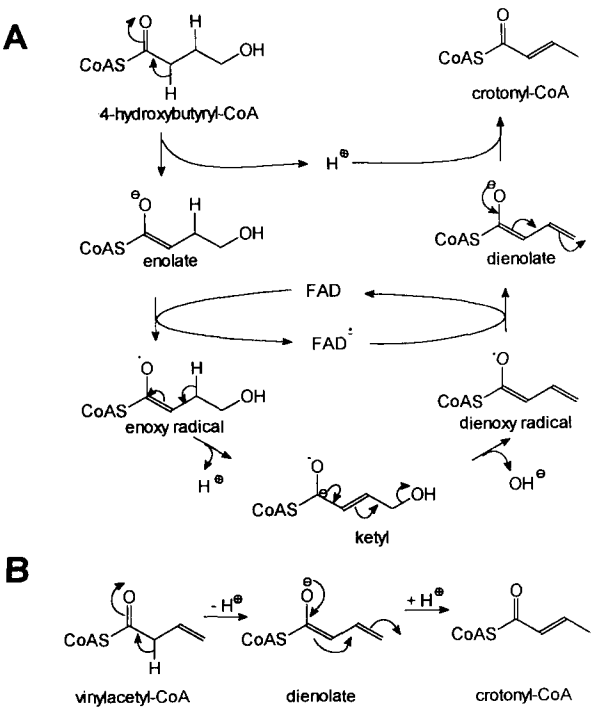

Fig. 2. (A) Proposed mechanism for the reversible dehydration of 4-hydroxybutyryl-CoA to crotonyl-CoA. (B) Isomerisation of vinylacetyl-CoA, also catalysed by 4-hydroxybutyryl-CoA dehydratase.

dehydration of 4-hydroxybutyryl-CoA to (*E*)-crotonyl-CoA, $K_{eq} = 4$, and the apparently irreversible isomerisation of vinylacetyl-CoA (3-butenoyl-CoA) to crotonyl-CoA (Fig. 2). The enzyme has been purified from *Clostridium aminobutyricum* fermenting 4-aminobutyrate via 4-hydroxybutyrate to butyrate and acetate [24,25], as well as from *C. kluyveri* able to reduce succinate via 4-hydroxybutyrate to butyrate [26]. In both organisms 4-hydroxybutyryl-CoA is generated from the free acid by a CoA transferase, which has been purified from *C. aminobutyricum* [27]. A very similar reaction is involved in the biosynthesis of ω-cyclohexyl fatty acids from shikimate by *Alicyclusbacillus acidocaldarius*, the dehydration of (1*S*,3*S*)-3-hydroxycyclohexanecarboxylate to cyclohex-1-encarboxylate probably also occurring at the CoA ester level [28] (Fig. 3).

4-Hydroxybutyryl-CoA dehydratase from *C. aminobutyricum* contains FAD as well as non-heme iron and inorganic sulfur [25,29] (Table 1). The amino acid sequence of the enzyme has been determined. It shares significant similarities with FAD-dependent hydroxylases (A. Gerhardt and W. Buckel, unpublished). The enzyme as isolated was EPR-silent. Only after extensive photoreduction could an EPR spectrum of a $[4Fe\text{-}4S]^{+}$cluster be obtained [29]. Mössbauer spectroscopy of a ^{57}Fe-enriched enzyme showed a completely symmetric $[4Fe\text{-}4S]^{2+}$cluster. Unexpectedly, addition of the substrate crotonyl-CoA did not change the spectrum. Prolonged exposure to air inactivated the enzyme which could not be

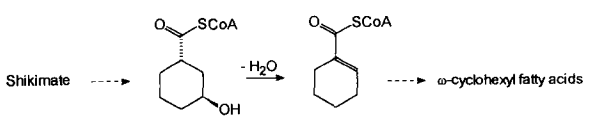

Fig. 3. A dehydration related to that of Fig. 2A in the biosynthesis of bacterial ω-cyclohexyl fatty acids.

reactivated by anaerobic incubation with Fe^{2+} as observed with aconitase [2]. Thereby, the symmetric $[4Fe\text{-}4S]^{2+}$cluster of 4-hydroxybutyryl-CoA dehydratase, probably coordinated to 4 cysteine residues, was irreversibly converted into a $[3Fe\text{-}4S]^{+}$cluster. Hence, this cluster may only be important for the structure of the enzyme and apparently does not participate in catalysis (U. Müh, W. Buckel and E. Bill, unpublished). Titration of 4-hydroxybutyryl-CoA dehydratase with dithionite reduces the dark-brown enzyme within seconds to a stable semiquinone, which can be detected by UV/visible spectroscopy (λ_{max} = 550 nm) and EPR (g = 2.0014). The further much slower reduction converts the enzyme into a pale yellow, inactive protein [29]. Reactivation is achieved by brief exposure to air (few seconds) or by the more controlled oxidation with hexacyanoferrate(III) [25].

In a possible mechanism, adapted from [11,23], deprotonation at the α-carbon yields the enolate which – in contrast to (*R*)-2-hydroxyglutaryl-CoA dehydratase – is *oxidized* by FAD to an enoxy radical. The now facilitated abstraction of a proton from the β-carbon gives a ketyl, from which the hydroxyl group is removed forming the dienoxy radical. Reduction to the dienolate and protonation leads to the product crotonyl-CoA. This mechanism is consistent with the easy reversible reduction of the prosthetic group FAD to a stable semiquinone (Fig. 2A). Remarkably, the dienolate is also an intermediate in the isomerisation of vinylacetyl-CoA to crotonyl-CoA [26] (Fig. 2B).

4. Dehydration of 5-hydroxyvalerate

C. viride is able to ferment 5-aminovalerate via 5-hydroxyvalerate to acetate, propionate and *n*-valerate [30]. Dehydration of 5-hydroxyvalerate is initially achieved by conversion of the acid to the CoA-derivative, which is catalysed by a specific CoA transferase [31]. The bifunctional dehydrogenase/dehydratase, a green FAD-containing homotetramer (170 kDa), oxidizes 5-hydroxyvaleryl-CoA to 5-hydroxy-2-pentenoyl-CoA. This is followed by dehydration to 2,4-pentadienoyl-CoA and a slow reduction to 4-pentenoyl-CoA (Fig. 4). This transient introduction of a double bond activates the γ-hydrogen to be easily removed as a proton [32]. In vivo 2,4-pentadienoyl-CoA is released from the enzyme and reduced in a different manner by 2,4-addition of a proton and a hydride to 3-pentenoyl-CoA, which is catalysed by a different also FAD-containing greenish reductase [33]. 3-Pentenoyl-CoA is isomerized to 2-pentenoyl-CoA, which disproportionates to acetate, propionate and valerate [30]. The amino acid sequence of 5-hydroxyvaleryl-CoA dehydrogenase/dehydratase

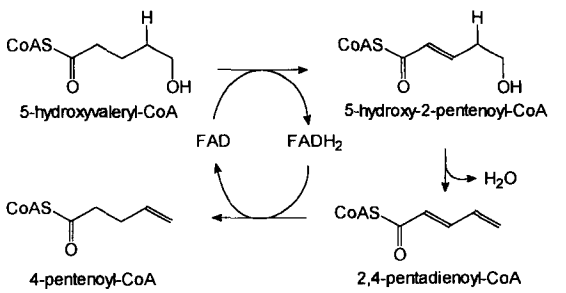

Fig. 4. Mechanism of the bifunctional 5-hydroxyvaleryl-CoA dehydrogenase/dehydratase.

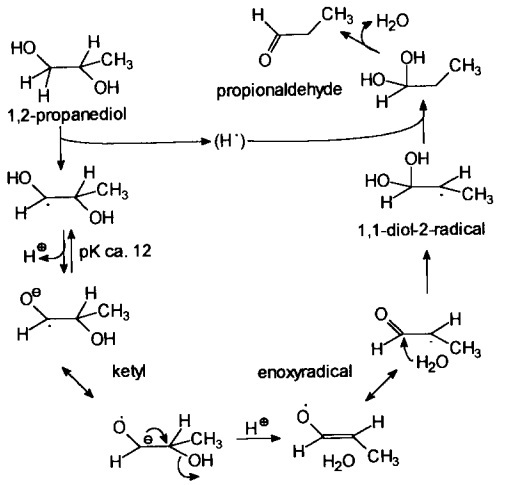

Fig. 5. Proposed mechanism for 1,2-propandiol dehydratase involving a ketyl as intermediate.

shows significant similarities to short and medium chain acyl-CoA dehydrogenases (U. Eikmanns, C. Buta and W. Buckel, unpublished). This enzyme and 2,4-pentadienoyl-CoA reductase have been crystallized, and their 3-dimensional structures are currently under investigation [32,34,35].

5. 1,2-Dioldehydratases

These remarkable enzymes catalyse the irreversible dehydrations of ethylene glycol, 1,2-propanediol or glycerol to acetaldehyde, propionaldehyde or 3-hydroxypropionaldehyde, respectively (for a review see [36]). As mentioned above all these substrates lack a functional, electron-withdrawing group facilitating the removal of the hydrogen as a proton. Whereas most of these enzymes are dependent on coenzyme B_{12} (adenosylcobalamin), an ethylene glycol dehydratase from *C. glycolicum* containing non-heme iron and acid labile sulfur but devoid of coenzyme B_{12} has also been described [37]. In the coenzyme B_{12}-dependent propanediol dehydratase from *Klebsiella pneumoniae* (EC 4.2.1.28) homolysis of the carbon-cobalt bond of the coenzyme generates a 5′-deoxyadenosine radical with abstracts the hydrogen at carbon-1, initiating the migration of the 2-hydroxy group to carbon-1. The hydrogen attached to 5′-deoxyadenosine is finally redonated to carbon-2 of the substrate. The resulting *gem*-1,1-diol loses H_2O to form the product propionaldehyde (for a review see [38]). Although the enzyme acts on (*R*)- as well as (*S*)-propanediol, the abstraction of the migrating hydrogen and the release of H_2O are stereospecific. (2*S*)-[1-^{18}O]Propanediol yields [(^{18}O]-propionaldehyde, whereas unlabelled product is generated from the correspondingly ^{18}O-labelled (*R*)-enantiomer (for a review see [39]).

Interestingly, the mechanism of the elimination might be related to that of the dehydration of (*R*)-2-hydroxyacyl-CoA. The initial hydrogen abstraction from carbon-1 yields a radical, which can be easily deprotonated to a ketyl, $pK \approx 12$ [40]. Hence, this ketyl is able to eliminate the hydroxy group at carbon-2 (see section 2) forming an enoxy radical, mesomeric with an aldehyde radical, to which the water is readded. The resulting 1,1-diol-2-radical is now reactive enough to reabstract the hydrogen atom from 5′-deoxyadenosine, whereby the initial coenzyme is regenerated. Water is finally removed from the 1,1-diol to yield the aldehyde (Fig. 5). The observed stereospecificity can be explained by the readdition of water derived from the (2*R*)-position at the *Si*-side of the aldehyde radical and vice versa. EPR-spectroscopy showed that cob(II)alamin is about 10 Å apart from the radical species [36]. Therefore, it appears likely that the 5′-deoxyadenosine radical derived from coenzyme B_{12} only acts as a radical starter, whereas a thiyl radical, as observed in the related ribonucleoside triphosphate reductase from *Lactobacillus leichmannii* [41], may propagate the reaction. In contrast, the carbon skeleton rearranging coenzyme B_{12}-dependent mutases appear to require coordination of the intermediate acrylate to cob(II)alamin [42,43].

6. Conclusions

The examples of unusual dehydrations described in this minireview show that unactivated hydrogens can be removed as protons after reductions or oxidations of the substrates. The dehydration of 5-hydroxyvalerate is most readily understood, since the transient introduction of a double bond converts the substrate into a vinylogous 3-hydroxyacl-CoA derivative. The other dehydrations require either reduction (2-hydroxyacyl-CoA) or oxidation (4-hydroxybutyryl-CoA and diols) to radical anions or ketyls able to eliminate the adjacent hydroxyl group. It should be noted that the removal of a hydrogen atom (proton+electron) from an alcohol is indeed an oxidation. Ketyls, which hitherto have not been considered as reactive species in biochemistry, may also play important roles in other enzymatic mechanisms such as ribonucleotide reductase, anaerobic degradation of aromatic compounds or pyruvate ferredoxin reductase (see [23]).

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